MASARYK UNIVERSITY

Faculty of Science

Regulation of the expression and activity of proteins involved in the inflammatory response by natural flavonoids

Habilitation thesis

Field: Genomics and Proteomics

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"The aim of education and wisdom is that one should see a clear path of life ahead of him, carefully move on, remember the past, know the present, and anticipate the future."

Jan Amos Komenský (1592 – 1670)

Acknowledgement

I would like to thank all my mentors who helped me to determine my direction, my colleagues who helped me to keep it, and my students who accompanied me.

A big thank you also goes to my girlfriend for her support.

Content

1. Introduction

Different illnesses and injuries have accompanied humanity since the very beginning, and the effort to heal them has lasted the same time. Initially, natural medicine, based purely on empiricism, was used. Since the 19th century, there has been a rapid development of the chemistry associated with the synthesis of new medicinal products. New synthetic drugs quickly displaced the original natural preparations. Recently, however, there has been a clear trend towards returning to natural medicine. Among the reasons are the low activity of synthetic substances and their serious undesirable side effects. The importance of natural medicines in modern times is evidenced by the awarding of the Nobel Prize in Physiology or Medicine in 2015 by prof. Youyou Tu for the isolation and identification of artemisinin from wormwood and its use as an anti-malarial agent. The field of pharmacy dealing with the isolation, identification, and description of natural compounds – pharmacognosy – is becoming more relevant as interest in alternative therapy grows.

Molecular biology is an incredibly versatile tool that, due to its methodical apparatus, finds uses in a number of biological and medical disciplines. In the past, there was no sharp boundary between these disciplines. On the contrary, an interdisciplinary approach was favoured. Molecular biology quickly settled among the pharmaceutical sciences. In the present "-omics" age, molecular biology helps to study the impact of new drugs on individual cells and complex organism – the whole complexity of biological and biochemical processes and interactions. All of this leads to revelation of the precise mechanism of action of the substance.

A new interdisciplinary field appeared in the 1990s at the interface of molecular biology with pharmacognosy, - molecular pharmacognosy. Bruhn and Bohlin redefined pharmacognosy as a molecular science as follows: "*Pharmacognosy is a molecular science that explores naturally occurring structure-activity relationships with a drug potential.*" [1]. Molecular pharmacognosy has become essential in the search and investigation of the effects of drugs isolated from medicinal plants, using all of the modern tools of genomics, proteomics, and metabolomics [2].

At the Department of Natural Drugs (DND) and later at the Department of Molecular Biology and Pharmaceutical Biotechnology (DMBPB) of the Faculty of Pharmacy of the University of Veterinary and Pharmaceutical Sciences Brno (FP UVPS), I started studying the biological activity of natural substances shortly after my arrival. The main direction of my research comprises the study of the anti-inflammatory activity of natural substances, especially flavonoids and their derivatives. Many of the substances I have examined were first isolated and identified at the FP UVPS, but after a few years of working on this topic, we managed to establish active cooperation with foreign research groups. Currently, we collaborate with universities in Austria, Bulgaria, Italy, and Slovenia. Thanks to this collaboration we can now get exotic materials that we would otherwise acquire only with difficulty. Testing a wide portfolio of substances and plant extracts makes it easier to understand their principles of action. Nowadays, the use of a phytotherapy is based not only on empiricism but also on scientific knowledge.

The habilitation thesis presented here comprises the aggregate of the author's publications accompanied by brief comments; and it is divided into three main chapters. The first briefly describes the physiological function of inflammation and its significance in the pathogenesis of some diseases. Considerable attention is also paid to the regulation of gene expression during inflammation. The second part briefly describes the current state of knowledge of the phytotherapy of inflammatory conditions. The last chapter represents the core of the work. It describes the flavonoids we have studied at FP UVPS and their effects on the expression and activity of genes and proteins associated with the inflammatory response *in vitro* and *in vivo*.

Most of the text of this thesis is adopted, modified and actualised from our current reviews – Appendixes No. 13, 14, and 17.

2. Inflammation and its role in immunity

Inflammation is phylogenetically and ontogenically the oldest animal defence mechanism and it belongs among the longest known (patho)physiological processes. It is a protective response of body tissues that occurs in reaction to any type of infectious agent, irritation, or injury, and is characterised by the activation of the innate nonspecific immune system [3]. The primary goal of inflammation is to establish homeostasis in tissues after injury or infection. The classical signs of inflammation, as defined by Cornelius Celsus in the 1st century AD and completed by Rudolf Virchow in the 19th century, are redness, heat, swelling, pain, and dysfunction of the organs [3]. These signs can be explained by increased blood flow, vasodilatation, release of the inflammatory mediators, elevated cellular metabolism, and the cellular influx of various immune-system cells. Migrated and activated immune cells synthetise and release a variety of mediators that control the progression and resolution of the inflammatory reaction. Among the numerous inflammatory mediators are cytokines and lipid mediators very important [4]. Under normal conditions, inflammation is self-limiting, but in some cases, it becomes continuous, and chronic inflammatory disease can develop subsequently [3]. Inflammation contributes to chronic diseases including diabetes, cardiovascular diseases, certain cancers and bowel diseases, arthritis, osteoporosis, and neurodegenerative diseases [5]. The inflammatory process has very specific characteristics, whether acute or chronic. The infiltration of innate immune system cells, specifically neutrophils and macrophages, characterises acute inflammation, whereas infiltrating T lymphocytes and plasma cells are features of chronic inflammation. Macrophages play an important role in both, contributing to the final consequence: the chronic inflammation that is represented by the loss of tissue function due to fibrosis [3]. The inflammation process involves a number of endogenous mediators, including the vasoactive amines histamine and serotonin, metabolites of arachidonic acid (AA), the complement system, the kinin system, the fibrinolytic system, platelet-activating factor (PAF), neuropeptides, hydrolytic enzymes, adhesion molecules, reactive oxygen species (ROS), and inflammatory cytokines. All of these mediators manifest a broad amount of overlapping actions, cross-reactions, redundancies, and synergistic effects [4, 5].

Inflammatory responses can be regulated at three levels: 1) the signal-specific level, when intracellular signal pathways are regulated; 2) the gen-specific level, when the

transcription of particular genes is regulated; and 3) the cell-specific level, when the cell differentiation and activation and the cell-cell signalling are regulated. However, the main control point lies on the transcription level [6]. The regulation of gene expression is a complex system of transcription factors, RNA-binding proteins, and chromatin-modifying proteins. A specific expression profile is created by their synchronisation and leads to fine tuning of the inflammatory response to the presence of a pathogen. With small modifications, this system is applicable to the autoimmune inflammatory response or tissue damage.

The key role in activation of the inflammatory response is played by the transcription nuclear factor (NF)-κB, which regulates the expression of more than 150 genes, including pro-inflammatory cytokines [e.g., tumour necrosis factor (TNF)-α (Appendix 1), interleukin (IL)-1β, etc.], and enzymes [e.g., cyclooxygenase (COX)-2, inducible NO synthase (iNOS), etc.] [7]. NF-κB is active in the form of a dimer, where the heterodimer p50/RelA(p65) is called "classic NF-κB". Other possible subunits are p52, c-Rel, and RelB [8]. Inactive NF-κB dimer is kept in the cytoplasm by its interaction with the inhibitor κB (IκB). After induction of the inflammatory response, IκB is phosphorylated by IκB kinase (IKK) and quickly ubiquitinated and degraded. NF-κB is released and translocates to the nucleus, where it binds to DNA and initiates transcription (Fig. 1).

Transcription factor NF-κB is called "the central mediator of the human immune system", or, more accurately, "the central mediator of the human stress response". Different combinations of monomers in NF-κB dimers are expressed in all cell types and are activated by tens of different activators [7]. The evolutionarily conserved function of NF-κB regulates the expression of inductors and effectors at many places in the huge metabolic network, thereby defining the immune response to an injury or the presence of a pathogen. It can also be active in ischemia, liver regeneration, or haemorrhagic shock [7, 9]. Apart from the above-mentioned function of modulating the inflammatory response, this transcription factor also regulates apoptosis, the mechanism by which it is involved in the development of cancer [7-9].
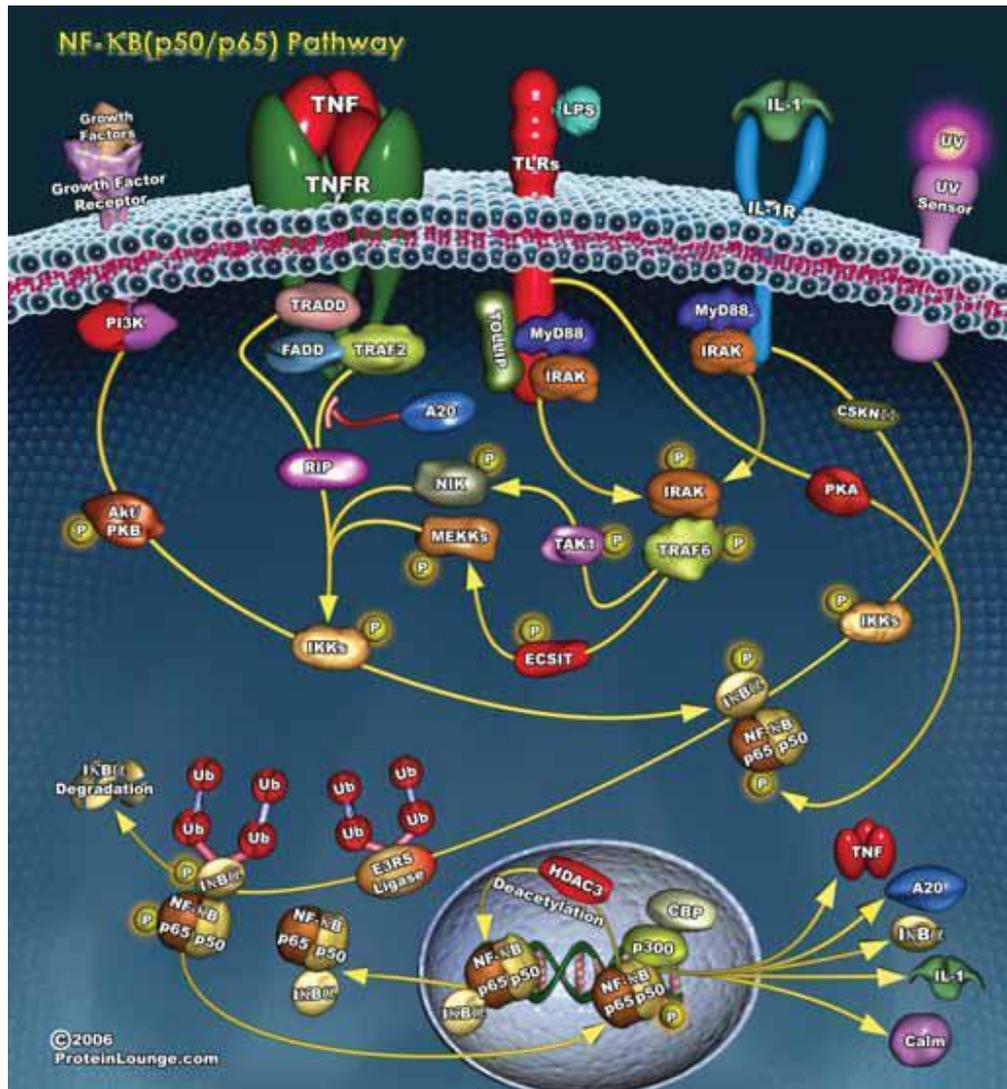
Fig. 1. NF-κB signalling pathway. Adopted from Sigma-Aldrich *BioFiles 2007, 2.4, 14.* (www.sigmaaldrich.com/technical-documents/articles/biofiles/nf-b-and-inflammation.html)

Another important pro-inflammatory transcription factor is the activating protein 1 (AP-1). AP-1 is the overall term for a family of dimeric transcription factors. These homo- or heterodimeric proteins contain the bZIP (basic region leucine zipper) domain and are composites of subunits belonging to the Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, FraI, Fra2), or activating transcription factor (ATF2, ATF3/LRF1, B-ATF) protein sub-families. Jun and Fos monomers can also interact with Maf (c-Maf, MafB, MafA, MafG/F/K) and Nrl proteins [10, 11]. The dimers that form bind in a specific DNA sequence, either 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements (5′-TGAG/CTCA-3′) or cAMP response elements (CRE, 5′-TGACGTCA-3′) in the regulation region of the target genes. Both binding DNA sequences are palindromic [10, 11].

The activity of AP-1 is induced by numerous stimuli, e.g., growth factors, pro-inflammatory cytokines, neurotransmitters, polypeptide hormones, bacterial and viral infectious agents, and plenty of physical and chemicals factors. These stimuli activate mitogen activated protein kinases (MAPKs) cascades, which modulate the activity of AP-1 by phosphorylation. The composition and character of the cell stimuli lead to phosphorylation of particular AP-1 subunits and thus contribute to fine tuning its activity [10, 11].

The regulation of AP-1 activity is complex and has multiple levels, including expression of individual monomers, dimer formation, and posttranslation modification. The AP-1 activity is also regulated by its interactions with other transcription factors [10-12].

The central point of all AP-1 complexes is the c-Jun subunit. The gene for c-Jun is expressed at a low level in many types of cell and its expression is elevated in response to stimulation. Exposure of a cell to UV irradiation, pro-inflammatory cytokines, or growth factors activates JNK (c-Jun N-terminal kinase) and p38 MAP kinases. Upon activation, JNK is translocated to the nucleus, where it phosphorylates c-Jun and ATF2 and augments their transcription activity, whereas p38 phosphorylates and activates ATF2, MEF2C (monocyte-specific enhancer binding factor 2c) and TCFs (ternary complex factors) [10, 11]. Serum and growth factors strongly induce the activation of AP-1 by ERK (extracellular-signal-regulated kinase), which is translocated to the nucleus and potentiates the transcription of genes with the *fos* promoter by phosphorylating of TCFs [11].

2.1 Author's publication related to Chapter 2

Appendix No.	Bibliographic record
[1]	**Zelová H., Hošek J.** 2013. TNF-a signalling and inflammation: interactions between old acquaintances. *Inflamm. Res*. **62**: 641-651.

3. Phytotherapy of inflammatory diseases

Natural bioactive compounds represent an important pool for the discovery of new drugs and especially novel classes of pharmaceutical lead structures. It has recently been estimated that natural products and their derivatives accounted for more than 25 % of all new drugs introduced between 1981 and 2010 [13]. Aside from being an important source for the development of modern drugs, herbal medicines represent an important option for alternative or supplementary treatment that is increasingly popular in the developed countries because the general public believes that such treatments are more "mild" and "natural".

Although several herbal medicines indeed exhibit clear efficacy, there are limitations connected with their use [14, 15]. The phytochemical compositions of herbal medications are variable, and it is difficult to know the effective dose administered to a patient. This problem arises mainly from the lack of knowledge of the active compounds that control or account for the quality or efficacy of herbal preparations. Knowledge of the relevant bioactive compounds and their mechanisms of action can help to overcome these problems and to achieve the effective quality control and standardization of herbal medicines that will ensure their safety and efficacy.

Experiments *in vitro* and *in vivo* have revealed many biological and pharmacological activities of natural compounds that serve as therapeutics or lead substances for many "small molecular drugs". The process of identifying an activity and its particular relationship to structure is complicated, especially *in vivo*, because many natural substances have pleiotropic effects that target many cellular proteins or mechanisms [16]. Moreover, these compounds are metabolized, and the metabolites may also contribute to the overall effect. Flavonoids have shown especially anti-oxidant and anti-inflammatory properties *in vitro* and *in vivo*, in addition to many other effects [17]. The metabolism of flavonoids has been studied [18, 19], but many questions are still unanswered, especially with respect to uncommon lipophilic flavonoids.

Medical procedures involving the use of medicinal plants have developed gradually from empirical procedures throughout history. Until the advent of chemical drugs, they constituted practically the only means to effect a cure. The first mention of plants and their

use in medicine is found in a Sumerian record on clay tablets dating back to approximately 4000 BC. Other valuable sources of information on the use of plants are from Ancient China, India, and the medicine of ancient Greece and Rome. We know especially the works of the physician Hippocrates (*Hippocratic Corpus*, 5th cent. BC.) and Dioscorides (*De Materia Medica*, 1st century AD.), wherein the authors described over 600 species of plants and their medicinal uses. Later studies showed that traditional European '*materia medica*' was based on a Dioscorides tradition that lasted through the 19th century with only little variation. The gradual development of science and technology accelerated the development of synthetic drugs and pushed herbal treatment aside. In recent years, however, plants have again gained considerable interest as potential sources of new drugs. Now that it is possible to obtain scientific confirmation of their therapeutic effects and mechanisms, the value of herbal medicines is increasing in popularity, and they are often used as effective alternatives to standard therapy or as a source of lead compounds. Nowadays, processed plant materials are often used as supplementary therapy in the treatment of many chronic diseases, including inflammatory bowel diseases (Appendix 2) [20, 21].

A number of compounds of plant origin have been experimentally proven to relieve pain in the human body, and it has been proposed that some of these may be the agents responsible for the action of the traditional herbal remedies associated with the reduction of pain, fever, and inflammation [22]. Probably the best known anti-inflammatory compound of plant origin is salicin, isolated from the bark of the white willow (*Salix alba* L.) back in 1828 [23]. Today, its synthetic derivative acetylsalicylic acid (aspirin) is widely used nonsteroidal anti-inflammatory drugs (NSAIDs) in the world, with an estimated 40 000 tons of it being consumed each year. The inhibition of arachidonic acid (AA) metabolism has been established as the main mechanism responsible for the anti-inflammatory action of aspirin [24]. Following this discovery, a number of studies have confirmed the significant inhibitory effect of various flavonoids, such as baicalein, quercetin, or rutin, on the pathways of AA metabolites [25].

However, the anti-inflammatory actions of medicinal plants are not limited to the inhibition of arachidonic acid metabolism. Compounds, including flavonoids, present in plant extracts possess redox potentials and are able to interact with intracellular signalling

pathways and thus modify gene expression. More details of this process will be described in Chapter 4.

3.1 Author's publication related to Chapter 3

Appendix No.	Bibliographic record
[2]	**Vochyánová Z., Sikorová K., Šmejkal K., Hošek J.** 2014. Rostliny v terapii idiopatických střevních zánětů. *Gastroent. Hepatol.* **68**: 248-254.

4. Regulation of inflammation-related gene expression and activity by flavonoids

Experimental *in vitro* and *in vivo* studies have revealed many biological and pharmacological activities of flavonoids. Their anti-oxidant and anti-inflammatory properties are the most studied, but these phytochemicals also show anti-bacterial, anti-viral, hypolipidemic, and even anti-cancer effects [17]. The process of identifying a particular structure-activity relationship is complicated, especially *in vivo*, because flavonoids can have pleiotropic effects that target many cellular proteins or mechanisms.

4.1 Chemical properties of flavonoids

The flavonoids are a group of plant secondary metabolites biosynthetically derived from shikimic acid and polyketide pathways. They are plant pigments containing benzopyran substituted with a phenyl ring at position carbon (C)2 or C3 and possess a wide spectrum of biological activities, including anti-oxidant and anti-inflammatory activities (Appendix 13, 14, 17) [26-28].

The technical term "flavonoid" arises from the basic skeleton of these plant pigments that are derived from benzo-γ-pyrone, a structure well known as chromone, modified by the addition of a phenyl ring at position C2 to yield a flavonoid or at C3 to form an isoflavonoid (Fig. 2). This three-ring skeleton may then be substituted with hydroxyl groups. Substitution of the A ring typically occurs at positions C5 and C7 (a *meta* arrangement characteristic for acylphloroglucinols). On the B ring positions C4´ or C3´ and C4´ (typical for shikimates) are preferred [17]. The hydroxyl groups of flavonoids are commonly substituted with one or more sugar units. The sugar can also be connected *via* a C-C bond. Glycosides of the flavonoids are relatively hydrophilic. The aglycones can be lipophilic and their lipophilicity can be further enhanced by methylation of the hydroxyl groups to form methoxyl groups, or by prenylation or geranylation at different positions on the skeleton. The prenyl or geranyl moiety may also be modified in different ways (oxidation, reduction, cyclisation, etc.) [29].
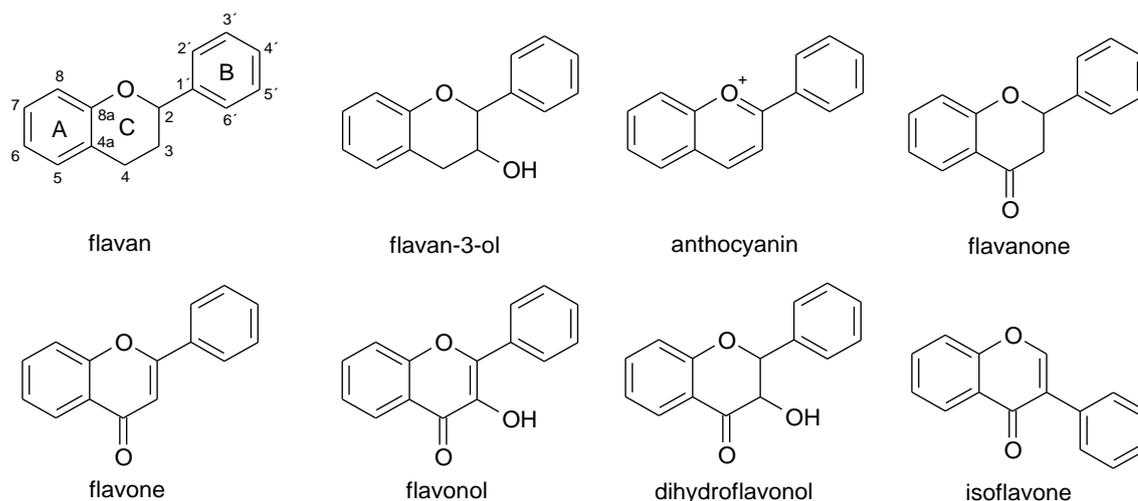
Fig. 2. Overview of the basic flavonoid skeletons. Adopted from Hošek & Šmejkal (2016) [26] – Appendix 13.

Diverse types of flavonoids predominante in different plants. The so-called dietary flavonoids present in common food are well known, but there are many other flavonoid species present in medicinal plants. Flavanones can be found in chick peas and cumin, but the major sources of flavanones are citrus fruits and juices. Flavones are not common in fruit, but they are often found in grains and herbs. The most common flavone aglycones are apigenin and luteolin. If they occur at high concentrations or are complexed with metal ions, flavones can contribute colour to the tissue of a plant and they also contribute to its taste.

The best known flavonols are quercetin and kaempferol. Quercetin is ubiquitous in fruits and vegetables. The most common quercetin glycoside is rutoside (also known as rutin). The isoflavonoids are a separate group of flavonoids, well known for their estrogenic properties. The group can be divided into isoflavanones, isoflavones, and isoflavonols. The isoflavonoids are found predominantly in legumes. Flavans, especially flavan-3-ols, can be found under different names: catechins, leucoanthocyanins, proanthocyanins, and tannins. They occur mainly as monoflavans, biflavans, and triflavans. Flavans are rarely glycosylated, but they may be esterified with gallic acid. Monoflavans are found in ripe fruits and fresh leaves. Anthocyanins are responsible for the blue and red pigmentation of berries, cherries, and plums, as well as eggplant, red cabbage, and radishes. The colour of anthocyanins is pH dependent. An anthocyanin is red at pH < 3 (the stable form); it turns blue or purple

when the pH is increased to 4 – 6. At higher pH values it becomes colourless and decomposes. Anthocyanins often occur in complex mixtures. In flowers, anthocyanins may form complexes with flavones or metal ions such as iron or magnesium. The anthocyanin content of a fruit usually increases as the fruit ripens [30, 31].

Furthermore, derivatives with a modified basic flavonoid skeleton can occur in many plants used medicinally. Prenylated flavonoids occur in several plant families, e.g., *Paulowniaceae*, *Leguminosae*, and *Cannabaceae*, but probably the largest number of prenylated flavonoids can be found in the *Moraceae* family. Prenylated flavonoids are a large group of compounds that combine a flavonoid skeleton with a lipophilic prenyl side-chain. As mentioned above, the side-chain can be modified, especially by oxidation and cyclisation, and this variation contributes many structural possibilities. Adding the lipophilicity of a prenyl side-chain to the flavonoid skeleton gives the resultant compound great potential for biological activity [29, 32].

4.2 Anti-oxidant activity of flavonoids

Oxidative stress is one of the main hallmarks of inflammation. Reactive oxygen species (ROS) are produced by immune cells, especially macrophages and neutrophils, to kill invading bacteria and viruses. However, excessive or prolonged oxidative stress can damage the surrounding molecules, e.g., the unsaturated lipids in plasma membranes, proteins, or DNA [17]. Anti-oxidant compounds could therefore be promising as anti-inflammatory agents. The anti-oxidant capabilities of flavonoids have been well described using many biochemical models, both *in vitro* (cell-based) and *in vivo* (Appendix 6, 10, 13, 14).

The main mechanisms of the anti-oxidant action of flavonoids are listed in Tab. 1. Flavonoids can scavenge ROS and reactive nitrogen species (RNS) directly, chelate metal ions, and inhibit oxidases. Moreover, they are involved in the regeneration of α-tocopherol and ascorbic acid radicals [33].
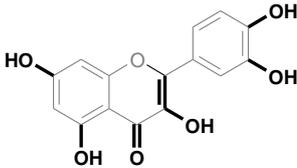
Chemical or biological effect	Responsible structural elements (shown in bold)	References
ROS scavenging	3′,4′-hydroxyl groups 2,3-double bond in conjugation with 4-carbonyl group 3,5,7-hydroxyl groups	[33, 34]
Peroxynitrite scavenging	3′,4′-hydroxyl groups 3-hydroxyl group	[33]
Metal chelating activity	3′,4′-hydroxyl groups 4-carbonyl group in conjugation with 3-hydroxyl group 4-carbonyl group with 5-hydroxyl group	[33, 34]
PKC inhibition	Coplanar flavone structure 3′,4′-hydroxyl groups 7-hydroxyl group	[33]

Table 1. Mechanisms of anti-oxidant activity of flavonoids. *PKC* protein kinase C; *ROS* reactive oxygen species. Adopted from Hošek & Šmejkal (2016) [26] – Appendix 13.

Flavonoids scavenge ROS directly by donating the hydrogen atom of a hydroxyl group. This radical scavenging activity relates to the oxidation-reduction potential of the flavonoids. Flavonoids interact with radicals, donating one hydrogen atom and forming a flavonoid phenoxyl radical (flavonoid-O$^\bullet$). This radical may then react with other radicals to form a stable quinoid structure. The number of hydroxyl groups and their positions on the flavonoid skeleton significantly affect the radical scavenging activity. Substituents on ring B (especially catechol and pyrogallol moieties) are more important than those on rings A and C. The anti-radical activity is diminished when only one hydroxyl group is present on ring B. The second structural element responsible for eliminating radicals is the C2,C3-double bond conjugated with the C4-oxo group on ring C. On the other hand, glycosylation attenuates the scavenging activity. Flavonoids are also able to trap the peroxynitrite radical, which can occur as a product of the reaction of superoxide with NO. This activity is attributed to the presence of the 3′ and 4′-hydroxyl groups along with the 3-hydroxyl group [17, 33, 34].

Another feature contributing to the anti-oxidant activity of flavonoids is their ability to chelate metal ions, especially Fe^{2+} and Cu^{2+}. Free metal ions can take part in Fenton's reaction and thus enhance the formation of ROS. Chelation of these ions neutralizes their ability to serve as redox catalysts. On the other hand, trace metal ions are essential cofactors in many biological systems, e.g., Fe^{2+} in haemoglobin or Cu^{2+} and Zn^{2+} in a superoxide dismutase (SOD), where the chelating ability of flavonoids would be detrimental. Metal ions can bind to the two hydroxyl groups of a catechol moiety of ring B, the 3-hydroxyl and 4-oxo groups of ring C, or the 4-oxo and 5-hydroxyl groups on rings A and C [33, 34].

The anti-oxidant action of flavonoids is also attributable to their ability to inhibit enzymes that produce radicals, such as xanthine oxidase (XO) or NADH oxidase (NOX). They also interact with protein kinase C (PKC), which is responsible for the activation of NOX, and thereby attenuate the functioning of NOX. It has been postulated that a coplanar flavone structure with free hydroxyl substituents at positions 3', 4', and 7 should diminish the activity of PKC [33, 34].

Fully evaluating the anti-oxidant activity of flavonoids *in vivo* is more complicated because of their relatively low bioavailability and destruction by metabolism. Flavonoid metabolites usually possess less anti-oxidative activity than their parent structures. It seems likely that flavonoids and flavonoid metabolites work in different ways, the flavonoids by scavenging up ROS and the flavonoid metabolites by preventing the formation of such radicals [33, 34].

4.3   Pro-oxidant activity of flavonoids

In addition to their anti-oxidant activity, flavonoids can also act as pro-oxidants. Their ability to trigger oxidative stress could explain some of the toxic effects of flavonoids observed *in vivo*. On the other hand, a mild pro-oxidant effect could also induce the expression of anti-oxidant enzymes, such as SOD or catalase (CAT), and thereby contribute to overall anti-oxidant cytoprotection [33].

The flavonoids can act as pro-oxidants under specific conditions, and they can therefore promote the oxidation of other compounds. As seen in the following text, the situation is

not clear. Studies of the structure-activity relationship carried out by Cao *et al*. [35] and Hanasaki *et al*. [36] have shown, that the pro-oxidant action of the flavonoid in Fenton's reaction is dependent on the presence of multiple hydroxyl groups, especially located on ring B of the skeleton. Studies of the structure-activity relation could thus depend strongly on the test conditions used. Especially the presence of high concentrations of transient metals in the environment of the cell or in the reaction mixture *in vitro* leads to pro-oxidative behaviour of flavonoids. They can reduce Fe^{3+} to Fe^{2+}, resulting in the further production of hydroxyl radicals by Fenton reactions [37]. The reduction of Cu^{2+} to Cu^+ has also been observed [35]. This process has been confirmed *in vitro* using different chemical and biochemical reactions and *in vitro* assays using cell-based models [38]. Under physiological conditions, transient metals are usually compartmentalized or secured against interaction with possible pro-oxidants by bonding with a special carrier or storage compound, but under pathological conditions they can escape and interact with flavonoids [34, 39, 40]. Whether flavonoids act as anti-oxidant or pro-oxidant depends on its concentration. A relatively high concentration of the flavonoid is needed to evoke the pro-oxidant effect, tens to hundreds of micromoles per liter [41].

Reactive oxygen species (ROS) play a dual role in inflammation – both beneficial and detrimental. Phagocyting cells produce increased amounts of ROS to kill captured bacteria or viruses. A low concentration of ROS serves as a signalling agent in cells. On the other hand, when the production of ROS exceeds the anti-oxidant capacity of the cells, different biomolecules, especially proteins, unsaturated lipids, and DNA can be damaged [42]. Oxidative stress itself could also trigger the inflammatory response in tissue and thus contribute accelerate some stages of the inflammation [43, 44]. However, it is still not clear whether greater production of ROS is a cause of inflammation or a consequence [45]. Although this dilemma has not been resolved, many therapeutic approaches based on eliminating of these potentially dangerous molecules are coming into use.

The response and fate of cells subjected to oxidative stress depend strongly on the concentration of ROS. A low level of ROS activates the Nrf2 (nuclear erythroid 2 p45-related factor 2) transcription factor, which positively regulates expression of the anti-oxidant enzymes that protect cells against further oxidative stress. Higher concentrations of ROS contribute to the activation of NF-κB (nuclear factor κB) and AP-1 (activator protein 1) and

thereby exacerbate the inflammation (Fig. 3). Very high concentrations of ROS cause the mitochondrial membrane potential collapse, irreversibly damaging cell structures and leading to apoptosis or necrosis [46].
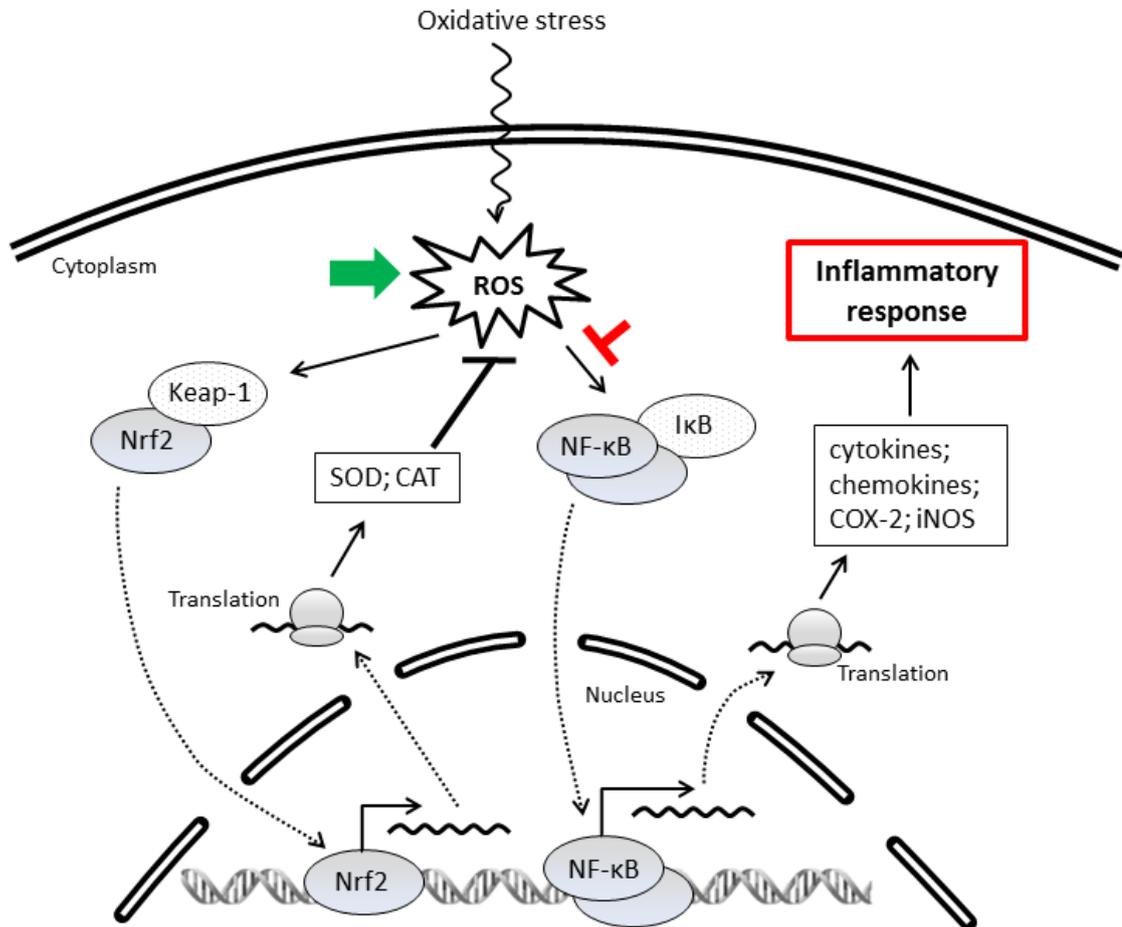

Figure 3. Mechanisms of redox action of flavonoids. Full arrows mean direct interaction, dashed arrows indicate translocation. The thick red **T** mark shows point, at which flavonoids cause inhibition; the thick green **arrow** indicates the pro-oxidant effect of flavonoids. *CAT* catalase; *COX* cyclooxygenase; *IκB* inhibitor of κB; *Keap-1* Kelch-like ECH-associated protein 1; *NF-κB* nuclear factor κB; *Nrf2* nuclear erythroid 2 p45-related factor 2; *ROS* reactive oxygen species; *SOD* superoxide dismutase. Adopted from Šmejkal *et al*. (2016) [27] and modified – Appendix 14.

Many experiments to describe the anti-oxidant potential of flavonoids connected with their anti-inflammatory effect have been performed, but only a limited number of studies have focused on the pro-oxidant role of flavonoids during modulation of the inflammatory response. The catechins in green tea have been studied the most from this perspective. The pro-oxidant effect of epigallocatechin gallate (EGCG) has long been known, but its influence on inflammation remains unclear. Ho *et al*. (2014) have shown that consumption

of green tea (a rich source of catechins) by healthy volunteers increased the quantity and activity of the anti-inflammatory protein haem oxygenase 1 (HO-1) [47]. The expression of this protein is regulated by changes in the cellular redox potential. Hence, the pro-oxidant effect of catechins could trigger production of HO-1 and thus contribute to the overall anti-oxidant, anti-inflammatory, and genoprotective capacities of cells. EGCG retains its pro-oxidant and anti-inflammatory activities even after biotransformation *in vivo* [48]. A pro-oxidant and a concurrent anti-inflammatory effect have also been shown by cudraflavone B and osajin, but that reduced activation of the NF-κB signalling pathway is caused directly by greater production of ROS has not been demonstrated [49] (Appendix 6).

The pro-oxidant effect of flavonoids on the attenuation of inflammation represents a big challenge for research. Flavonoids possess both anti-oxidant and pro-oxidant activities and both are concentration and time dependent [50]. The available results show that the mild oxidative stress caused by the pro-oxidant activity of flavonoids can be beneficial as a prophylactic treatment rather than as curative therapy. This justifies the long-term consumption of flavonoid-rich foods for the prevention of inflammatory disorders, but different anti-phlogistic mechanisms of flavonoids are involved in the resolution of acute and chronic inflammatory responses [50].

4.4   Anti-inflammatory activity of flavonoids

Inflammation is a multiple and complex response by the body to infection or injury. Flavonoids show pleiotropic effects and can modulate a broad spectrum of inflammatory regulatory nodes (Fig. 4). Their anti-phlogistic action combines many particular effects. The anti-phlogistic action of flavonoids can be mediated by several pathways (Tab. 2): *via* anti-oxidant and pro-oxidant effects (see above), by interacting directly with pro-inflammatory proteins, and by interacting with signal pathways and inhibiting the expression of inflammation-related genes.
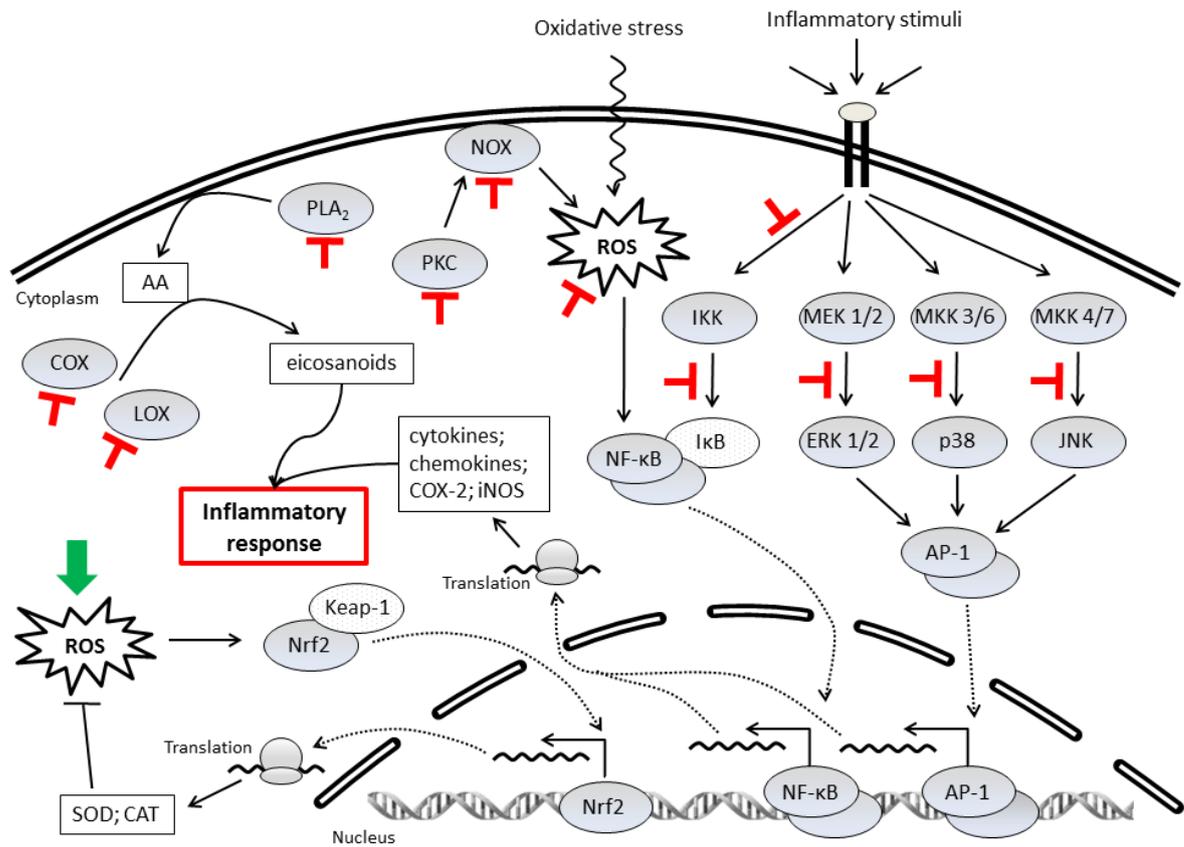
Figure 4. Mechanisms of anti-inflammatory action of flavonoids. Full arrows mean direct interaction, dashed arrows indicate translocation. The thick red **T** marks show points at which flavonoids inhibit; the thick green **arrow** indicates the pro-oxidant effect of flavonoids. *AA* arachidonic acid; *AP-1* activator protein 1; *CAT* catalase; *COX* cyclooxygenase; *ERK 1/2* extracellular signal-regulated kinase 1/2; *IκB* inhibitor of κB; *IKK* IκB kinase; *iNOS* inducible NO synthase; *JNK* c-Jun N-terminal kinase; *Keap-1* Kelch-like ECH-associated protein 1; *LOX* lipoxygenase; *MEK 1/2* MAPK/ERK kinase 1/2; *MKK* mitogen-activated protein kinase kinase; *NF-κB* nuclear factor κB; *NOX* NADH oxidase; *Nrf2* nuclear erythroid 2 p45-related factor 2; *PKC* protein kinase C; *PLA_2* phospholipase A_2; *ROS* reactive oxygen species; *SOD* superoxide dismutase. Adopted from Hošek & Šmejkal (2016) [26] – Appendix 13.
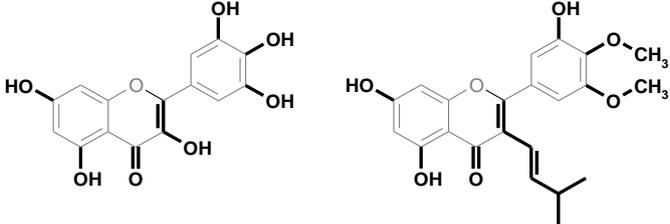
Biological effect	Responsible structural elements (shown in bold)	References
PLA$_2$ inhibition	2,3-double bond	[50]
COX-1 inhibition	2,3-double bond 6-prenyl group	[50, 51]
COX-2 inhibition	2,3-double bond 3-isoprenyl residue 6-prenyl group Galloyl moiety	[50, 51]
5-LOX, 12-LOX inhibition	2,3-double bond 3-hydroxyl group	[50]
Inhibition of inflammation-related gene expression (modification of signal transducing pathways)	5,7-hydroxyl groups 3´,4´-hydroxyl groups 2,3-double bond 4-carbonyl group Prenyl group	[33, 50, 52, 53]
In vivo anti-inflammatory activity	5,7-hydroxyl groups 3´,4´-hydroxyl or methoxyl groups 2,3-double bond	[50, 52]

Table 2. Mechanisms of anti-inflammatory action of some flavonoids. *COX* cyclooxygenase; *LOX* lipoxygenase; *PLA$_2$* phospholipase A$_2$. Adopted from Hošek & Šmejkal (2016) [26] – Appendix 13.

Modulation of the activity of pro-inflammatory enzymes is one of the most important mechanisms of flavonoid action. Pro-inflammatory enzymes, such as phospholipase A$_2$ (PLA$_2$), cyclooxygenase (COX), lipooxygenase (LOX), and NO synthase (NOS), produce very

potent inflammatory mediators, and inhibiting them can contribute to the overall anti-inflammatory potential of flavonoids. Special attention is focused on the enzymes metabolising arachidonic acid (AA). PLA_2 stands at the beginning of this AA process. Many studies have demonstrated the ability of flavonoids to inhibit different isoforms of this enzyme, with preference given to PLA_2-IIA. The most potent are flavonoids with a 2,3-double bond, and especially biflavonoids. Following its release, AA is oxidised to different eicosanoids in a process mediated by COX and LOX enzymes. Flavonoids diminish the activity of COX, but they inhibit COX-1 preferentially over COX-2 (Appendix 15). Only a limited number of observations of anti-COX-2 effects of flavonoids have been reported, experiments that have shown the positive influence of the 2,3-double bond and the presence of a prenyl group at C3 on this anti-inflammatory action. The presence of a galloyl moiety also favours the inhibition of COX-2 activity. Another way of using AA is *via* LOX enzymes. Whereas flavone derivatives have been described as demonstrating anti-COX activity, flavonol derivatives have shown anti-LOX effects. This confirms the importance of a hydroxyl group at C3. Furthermore, the 2,3-double bond has been found to be a very important factor in the inhibition of LOX activity. The majority of anti-LOX experiments have been performed using 5-LOX and 12-LOX, and information about any effects on other isoforms of LOX is limited (Appendix 15). Another enzyme that produces pro-inflammatory molecules is inducible NO synthase (iNOS), which produces NO in stimulated immune cells. Many studies have confirmed the ability of flavonoids to diminish the production of NO, but the effect was probably caused by reduced iNOS expression rather than direct inhibition [50, 54].

Another well-established mechanism for the anti-inflammatory activity of flavonoids comes from their ability to interact with intracellular signal transducing pathways and thereby regulate the expression of inflammation-related genes (Appendix 3-7, 9-12, 15). The most prominent position among inflammatory pathways belongs to the group of transcription factors NF-κB. Many studies have confirmed the ability of flavonoids to diminish the activity of NF-κB by inhibiting events upstream, e.g., reducing the phosphorylation of IKK (IκB kinase), which leads to less degradation of IκB (inhibitor of κB), or the attenuation of the DNA binding capability of NF-κB. Another important pro-inflammatory transcription factor is activator protein (AP)-1, the activity of which is

regulated by upstream mitogen-activated protein kinases (MAPKs). The three most frequently studied MAPKs are ERK 1/2 (extracellular signal-regulated kinase 1/2), p38, and JNK (c-Jun N-terminal kinase). These kinases not only influence AP-1, but also modulate the activity of NF-κB. Flavonoids are able to inhibit the phosphorylation of all of the above-mentioned MAPKs and thereby lower the transcription of the target genes. Analysis of the structure-activity relationship shows the importance of the 2,3-double bond, the carbonyl group at C4, and the pattern of hydroxylation for the ability of flavonoids to modulate signal pathways. Flavonoids are able to positively regulate the activity of several other signalling pathways. One of these is the signal pathway leading to the transcription factor Nrf2. Together with its negative regulator Keap-1 (Kelch-like ECH-associated protein 1), this protein is redox sensitive. Flavonoids possessing a redox potential are therefore able to trigger Nrf2, which leads, subsequently, to transcription of the genes that code the anti-oxidant enzymes that help to resolve the inflammatory response. Transcription factors from the PPAR (peroxisome proliferator-activated receptor) family are also activated by several flavonoids. The activation of these pathways, especially via PPARγ, attenuates the activity of NF-κB [50, 52, 54]. Because the transcription of inflammation-related genes is regulated by multiple transcription factors, the exact mechanism of flavonoid action remains unclear. It is safe to say that these phytochemicals disturb the intracellular signalling network at several different sites and thereby contribute to the overall anti-inflammatory effect.

In vivo tests have confirmed all of the effects of flavonoids previously observed in *in vitro* experiments. Various animal models using diverse ways to induce inflammation have shown the anti-inflammatory ability of flavonoids (Appendix 8, 16). The modulation of pro-inflammatory gene expression has been determined to be one of the major mechanisms of flavonoid action *in vivo*. However, because flavonoids are very quickly metabolised in the body, the particular types of cells or proteins that they target remain unclear. Nevertheless, it has been shown that a 2,3-double bond and increased levels of hydroxylation or methoxylation of the flavonoid skeleton enhance their action [50].

4.5   Author's publications related to Chapter 4

Appendix No.	Bibliographic record
[3]	**Hosek J., Zavalova V., Smejkal K., Bartos M.** 2010. Effect of diplacone on LPS-induced inflammatory gene expression in macrophages. *Folia Biol (Praha)*. **56**: 124-130.
[4]	**Hošek J., Bartoš M., Chudík S., Dall´Acqua S., Innocenti G., Kartal M., Kokoška L., Kollár P., Kutil Z., Landa P., Marek R., Závalová V., Žemlička M., Šmejkal K.** 2011. Natural Compound Cudraflavone B Shows Promising Anti-inflammatory Properties in Vitro. *J. Nat. Prod*. **74:** 614–619.
[5]	**Kollár P., Bárta T., Hošek J., Souček K., Müller Závalová V., Artinian S., Talhouk R., Šmejkal K., Suchý Jr. P., Hampl A**. 2013. Prenylated Flavonoids from Morus alba L. Cause Inhibition of G1/S Transition in THP-1 Human Leukemia Cells and Prevent the Lipopolysaccharide-Induced Inflammatory Response. *Evid.-based Complement Altern. Med.,* **2013**: Article ID 350519.
[6]	**Hošek J., Toniolo A., Neuwirth O., Bolego C**. 2013. Prenylated and Geranylated Flavonoids Increase Production of Reactive Oxygen Species in Mouse Macrophages but Inhibit the Inflammatory Response. *J. Nat. Prod*. **76**: 1586-1591.
[7]	**Zelová H., Hanáková Z., Čermáková Z., Šmejkal K., Dall'Acqua S., Babula P., Cvačka J., Hošek J.** 2014. Evaluation of Anti-inflammatory Activity of Prenylated Substances Isolated from *Morus alba* and *Morus nigra*. *J. Nat. Prod*. **77**: 1297–1303.
[8]	**Vochyánová Z., Bartošová L., Bujdáková V., Fictum P., Husník R., Suchý P., Šmejkal K., Hošek J.** 2015. Diplacone and Mimulone Ameliorate Dextran Sulfate Sodium-Induced Colitis in Rats. *Fitoterapia*. **101**: 201-207.
[9]	**Kollar P., Bárta T., Keltošová S., Trnová P., Müller Závalová V., Šmejkal K., Hošek J., Fedr R., Souček K., Hampl A.** 2015. Flavonoid 4´-O-methylkuwanon E from *Morus alba* Induces the Differentiation of THP-1 Human Leukemia Cells. *Evid.-based Complement Altern. Med.,* **2015**: Article ID 251895.
[10]	**Hanáková Z., Hošek J., Babula P., Dall'Acqua S., Václavík J., Šmejkal K.** 2015. *C*-Geranylated Flavanones from *Paulownia tomentosa* Fruits as Potential Anti-inflammatory Compounds Acting via Inhibition of TNF-α Production. *J. Nat. Prod*. **78**: 850-863.

[11] **Bahadır Acikara Ö., Hošek J., Babula P., Cvačka J., Budešínský M., Dračinský M., Saltan İşcan G., Kadlecová D., Ballová L., Šmejkal K.** 2015. Turkish *Scorzonera* Species Extracts Attenuate Cytokine Secretion via Inhibition of NF-κB Activation, Showing Anti-Inflammatory Effect *in Vitro*. *Molecules*. **21**: 43.

[12] **Khazneh E., Hřibová P., Hošek J., Suchý P., Kollár P., Pražanová G., Muselík J., Hanaková Z., Václavík J., Miłek M., Legáth J., Šmejkal K.** 2016. The Chemical Composition of *Achillea wilhelmsii* C. Koch and Its Desirable Effects on Hyperglycemia, Inflammatory Mediators and Hypercholesterolemia as Risk Factors for Cardiometabolic Disease. Molecules. **21**: 404.

[13] **Hošek J., Šmejkal K.** 2016. Flavonoids as anti-inflammatory agents. *In*: Parnham M.J. (ed.) Compendium of Inflammatory Diseases. Birkhäuser Springer, Basel. ISBN: 978-3-7643-8530-9.

[14] **Šmejkal K., Neuwirth O., Treml J., Hošek J.** 2016. Pro-oxidant Activity of Flavonoids and Their Possible Effects. *In*: Govil, J.N. and Pathak, M. (eds.) Recent Progress in Medicinal Plants Volume 40 - Flavonoids and Antioxidants. Studium Press LLC, USA. ISBN-13: 9781626990777

[15] **Hanáková Z., Hošek J., Kutil Z., Temml V., Landa P., Vaněk T., Schuster D., Dall'Acqua S., Cvačka J., Polanský O., Šmejkal K.** 2017. Anti-inflammatory Activity of Natural Geranylated Flavonoids: Cyclooxygenase and Lipoxygenase Inhibitory Properties and Proteomic Analysis. *J. Nat. Prod.* **80**: 999-1006.

[16] **Vochyánová Z., Pokorná M., Rotrekl D., Smékal V., Fictum P., Suchý P., Gajdziok J., Šmejkal K., Hošek J.** 2017. Prenylated flavonoid morusin protects against TNBS-induced colitis in rats s. *PLoS One*. **12**: e0182464.

[17] **Brezáni V., Šmejkal K., Hošek J., Tomášová V.** 2018. Anti-inflammatory Natural Prenylated Phenolic Compounds - Potential Lead Substances. *Curr. Med. Chem*. **24**: **24**: 1094-1159.

5. Conclusion

Plant remedies are becoming more popular nowadays. Plants contain a vast array of bioactive compounds and a considerable portion of these are flavonoids. The average intake of flavonoids in the normal human diet is estimated to be $1 - 2$ g per day. Such a high consumption of relatively unknown compounds is good reason to analyse research investigations into the mode of action of flavonoids in depth and possibly revise them.

Natural flavonoids represent an interesting group of compounds with plenty of biological effects, including anti-inflammatory effects. Because their effect is pleiotropic, it is difficult to determine the exact mechanism of action. However, the most studied effects are oxidative-reductive properties, interaction with intracellular signalling pathways leading to modification of gene expression, and direct inhibition of enzymes involved in the metabolism of the arachidonic acid.

Our research shows that prenylation of flavonoids significantly increases their activity. Other modifications of the basic structure influence one or more of the above-mentioned effects; sometimes the effects counteract each other. However, the overall effect is a positive attenuation of the inflammatory response.

Our research group focuses on the modification of gene expression caused by natural flavonoids, especially their interaction with transcription factors NF-κB and AP-1 and their intracellular signalling pathways. Our results confirm the importance of a side prenyl or geranyl group for the inhibitory effect of flavonoids on these transcription factors. These groups bring greater lipophilicity to such compounds and ease transport through the lipophilic cell membrane. In the intracellular space, they can interact directly with several proteins. In cell-based *in vitro* models, prenylated flavonoids effectively attenuate the activation of NF-κB and thus decrease the expression of pro-inflammatory genes and proteins, e.g., TNF-α, IL-1β and COX-2. *In vivo*, they ameliorate the symptoms of colitis in laboratory animals by the reducting the production of pro-inflammatory proteins, such as IL-1β, MMP2, or COX-2.

A promising therapeutic potential can be assigned to diplacone, which shows a relatively low cytotoxicity and a strong anti-inflammatory potential both *in vitro* and *in vivo*, but other natural flavonoids also possess interesting biological effects. Due to their pleiotropic action

and their effectiveness at relatively low concentrations, natural flavonoids have the potential to become leading compounds for new anti-inflammatory drugs.

6. List of references

1   Bruhn JG, Bohlin L. Molecular pharmacognosy: An explanatory model. *Drug Discov Today* 1997; **2**:243-6.

2   Schneider MJ, Lehotay SJ, Lightfield AR. Validation of a streamlined multiclass, multiresidue method for determination of veterinary drug residues in bovine muscle by liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 2015; **407**:4423-35.

3   Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. *Clin Exp Immunol* 2007; **147**:227-35.

4   Kang JX, Weylandt KH. Modulation of inflammatory cytokines by omega-3 fatty acids. *Subcell Biochem* 2008; **49**:133-43.

5   Libby P. Inflammatory mechanisms: the molecular basis of inflammation and disease. *Nutr Rev* 2007; **65**:S140-6.

6   Medzhitov R, Horng T. Transcriptional control of the inflammatory response. *Nat Rev Immunol* 2009; **9**:692-703.

7   Pahl HL. Activators and target genes of Rel/NF-kappa B transcription factors. *Oncogene* 1999; **18**:6853-66.

8   Perkins ND, Gilmore TD. Good cop, bad cop: the different faces of NF-kappa B. *Cell Death Differ* 2006; **13**:759-72.

9   Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. *Nat Rev Immunol* 2008; **8**:837-48.

10   Karin M, Liu Z, Zandi E. AP-1 function and regulation. *Curr Opin Cell Biol* 1997; **9**:240-6.

11   Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002; **4**:E131-6.

12   Vesely PW, Staber PB, Hoefler G, Kenner L. Translational regulation mechanisms of AP-1 proteins. *Mutat Res* 2009; **682**:7-12.

13   Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J Nat Prod* 2012; **75**:311-35.

14   Ernst E. The efficacy of herbal medicine--an overview. *Fundam Clin Pharmacol* 2005; **19**:405-9.

15 Li WF, Jiang JG, Chen J. Chinese medicine and its modernization demands. *Arch Med Res* 2008; **39**:246-51.

16 Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, et al. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol Adv* 2015; **33**:1582-614.

17 Havsteen BH. The biochemistry and medical significance of the flavonoids. *Pharmacol Ther* 2002; **96**:67-202.

18 Crozier A, Del Rio D, Clifford MN. Bioavailability of dietary flavonoids and phenolic compounds. *Mol Aspects Med* 2010; **31**:446-67.

19 Chen Z, Zheng S, Li L, Jiang H. Metabolism of flavonoids in human: a comprehensive review. *Curr Drug Metab* 2014; **15**:48-61.

20 Ghosh N, Ali A, Ghosh R, Das S, Mandal SC, Pal M. Chronic Inflammatory Diseases: Progress and Prospect with Herbal Medicine. *Curr Pharm Des* 2016; **22**:247-64.

21 Gilardi D, Fiorino G, Genua M, Allocca M, Danese S. Complementary and alternative medicine in inflammatory bowel diseases: what is the future in the field of herbal medicine? *Expert Rev Gastroenterol Hepatol* 2014; **8**:835-46.

22 Taylor JLS, Rabe T, McGaw LJ, Jager AK, van Staden J. Towards the scientific validation of traditional medicinal plants. *Plant Growth Regul* 2001; **34**:23-37.

23 Mahdi JG. Medicinal potential of willow: A chemical perspective of aspirin discovery. *J Saudi Chem Soc* 2010; **14**:317-22.

24 Warner TD, Mitchell JA. Cyclooxygenase-3 (COX-3): Filling in the gaps toward a COX continuum? *PNAS* 2002; **99**:13371-3.

25 Calixto JB, Otuki MF, Santos AR. Anti-inflammatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa B (NF-kappaB). *Planta Med* 2003; **69**:973-83.

26 Hošek J, Šmejkal K. Flavonoids as Anti-inflammatory Agents. In: Parnham MJ, ed. Compendium of Inflammatory Diseases. Basel, Switzerland: Springer Basel, 2016.

27 Šmejkal K, Neuwirth O, Treml J, Hošek J. Pro-oxidant Activity of Flavonoids and Their Possible Effects. In: Pathak M, Govil JN, eds. Recent Advances in Medicinal Plants. USA: Studium Press LLC, 2016.

28 Brezani V, Smejkal K, Hosek J, Tomasova V. Anti-inflammatory Natural Prenylated Phenolic Compounds - Potential Lead Substances. *Curr Med Chem* 2018; **25**:1094-159.

29 Šmejkal K. Cytotoxic potential of C-prenylated flavonoids. *Phytochem Rev* 2014; **13**:245-75.

30 Del Rio D, Rodriguez-Mateos A, Spencer JP, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxidants & redox signaling 2013; **18**:1818-92.

31 Peterson J, Dwyer J. Flavonoids: Dietary occurrence and biochemical activity. *Nutr Res* 1998; **18**:1995-2018.

32 Yazaki K, Sasaki K, Tsurumaru Y. Prenylation of aromatic compounds, a key diversification of plant secondary metabolites. *Phytochemistry* 2009; **70**:1739-45.

33 Prochazkova D, Bousova I, Wilhelmova N. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia* 2011; **82**:513-23.

34 Pietta PG. Flavonoids as antioxidants. *J Nat Prod* 2000; **63**:1035-42.

35 Cao GH, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: Structure-activity relationships. *Free Radical Bio Med* 1997; **22**:749-60.

36 Hanasaki Y, Ogawa S, Fukui S. The Correlation between Active Oxygens Scavenging and Antioxidative Effects of Flavonoids. *Free Radical Bio Med* 1994; **16**:845-50.

37 Rapta P, Misik V, Stasko A, Vrabel I. Redox Intermediates of Flavonoids and Caffeic Acid-Esters from Propolis - an Epr Spectroscopy and Cyclic Voltammetry Study. *Free Radical Bio Med* 1995; **18**:901-8.

38 Hadi SM, Bhat SH, Azmi AS, Hanif S, Shamim U, Ullah MF. Oxidative breakage of cellular DNA by plant polyphenols: A putative mechanism for anticancer properties. *Semin Cancer Biol* 2007; **17**:370-6.

39 Amic D, Davidovic-Amic D, Beslo D, Rastija V, Lucic B, Trinajstic N. SAR and QSAR of the antioxidant activity of flavonoids. *Curr Med Chem* 2007; **14**:827-45.

40 Jomova K, Valko M. Advances in metal-induced oxidative stress and human disease. *Toxicology* 2011; **283**:65-87.

41 Yen GC, Duh PD, Tsai HL, Huang SL. Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci Biotech Bioch* 2003; **67**:1215-22.

42 Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 2006; **160**:1-40.

43 Gutowski M, Kowalczyk S. A study of free radical chemistry: their role and pathophysiological significance. *Acta Biochim Pol* 2013; **60**:1-16.

44 Saeidnia S, Abdollahi M. Toxicological and pharmacological concerns on oxidative stress and related diseases. *Toxicol Appl Pharmacol* 2013; **273**:442-55.

45 Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell B* 2007; **39**:44-84.

46 Gloire G, Legrand-Poels S, Piette J. NF-kappa B activation by reactive oxygen species: Fifteen years later. *Biochem Pharmacol* 2006; **72**:1493-505.

47 Ho CK, Choi SW, Siu PM, Benzie IFF. Effects of single dose and regular intake of green tea (Camellia sinensis) on DNA damage, DNA repair, and heme oxygenase-1 expression in a randomized controlled human supplementation study. *Mol Nutr Food Res* 2014; **58**:1379-83.

48 Lambert JD, Sang SM, Hong J, Yang CS. Anticancer and Anti-inflammatory Effects of Cysteine Metabolites of the Green Tea Polyphenol, (-)-Epigallocatechin-3-gallate. *J Agr Food Chem* 2010; **58**:10016-9.

49 Hosek J, Toniolo A, Neuwirth O, Bolego C. Prenylated and Geranylated Flavonoids Increase Production of Reactive Oxygen Species in Mouse Macrophages but Inhibit the Inflammatory Response. *J Nat Prod* 2013; **76**:1586-91.

50 Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. *J Pharmacol Sci* 2004; **96**:229-45.

51 Hanakova Z, Hosek J, Kutil Z, Temml V, Landa P, Vanek T, et al. Anti-inflammatory Activity of Natural Geranylated Flavonoids: Cyclooxygenase and Lipoxygenase Inhibitory Properties and Proteomic Analysis. *J Nat Prod* 2017; **80**:999-1006.

52 Costa G, Francisco V, Lopes MC, Cruz MT, Batista MT. Intracellular Signaling Pathways Modulated by Phenolic Compounds: Application for New Anti-Inflammatory Drugs Discovery. *Curr Med Chem* 2012; **19**:2876-900.

53 Hanakova Z, Hosek J, Babula P, Dall'Acqua S, Vaclavik J, Smejkal K. C-Geranylated Flavanones from Paulownia tomentosa Fruits as Potential Anti-inflammatory Compounds Acting via Inhibition of TNF-alpha Production. *J Nat Prod* 2015; **78**:850-63.

54 Garcia-Lafuente A, Guillamon E, Villares A, Rostagno MA, Martinez JA. Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflamm Res* 2009; **58**:537-52.

7. List of abbreviations

AA – arachidonic acid

AP-1 – activating protein 1

ATF – activating transcription factor

bZIP - basic region leucine zipper

CAT – catalase

COX – cyclooxygenase

CRE – cAMP response elements

EGCG – epigallocatechin gallate

ERK – extracellular-signal-regulated kinase

HO-1 – haem oxygenase 1

IκB – inhibitor κB

IKK – IκB kinase

IL - interleukin

iNOS – inducible NO synthase

JNK - c-Jun N-terminal kinase

Keap-1 – Kelch-like ECH-associated protein 1

LOX – lipooxygenase

MAPK – mitogen activated protein kinase

MEF2C – monocyte-specific enhancer binding factor 2c

NADH – nicotinamide adenine dinukleotide

NF-κB – nuclear factor κB

NOX - NADH oxidase

Nrf2 - nuclear erythroid 2 p45-related factor 2

NSAIDs – nonsteroidal anti-inflammatory drugs

PAF – platelet-activating factor

PKC – protein kinase C

PLA_2 – phospholipase A_2

PPAR – peroxisome proliferator-activated receptor

ROS – reactive oxygen species

RNS – reactive nitrogen species

SOD – superoxide dismutase

TCFs – ternary complex factors

TNF-α – tumour necrosis factor α

TPA – 12-*O*-tetradecanoylphorbol-13-acetate

XO – xanthine oxidase

8. Appendix

1) **Zelová H., Hošek J.** 2013. TNF-a signalling and inflammation: interactions between old acquaintances. *Inflamm. Res*. **62**: 641-651.

2) **Vochyánová Z., Sikorová K., Šmejkal K., Hošek J.** 2014. Rostliny v terapii idiopatických střevních zánětů. *Gastroent. Hepatol.* **68**: 248-254.

3) **Hosek J., Zavalova V., Smejkal K., Bartos M.** 2010. Effect of diplacone on LPS-induced inflammatory gene expression in macrophages. *Folia Biol (Praha)*. **56**: 124-130.

4) **Hošek J., Bartoš M., Chudík S., Dall´Acqua S., Innocenti G., Kartal M., Kokoška L., Kollár P., Kutil Z., Landa P., Marek R., Závalová V., Žemlička M., Šmejkal K.** 2011. Natural Compound Cudraflavone B Shows Promising Anti-inflammatory Properties in Vitro. *J. Nat. Prod*. **74:** 614–619.

5) **Kollár P., Bárta T., Hošek J., Souček K., Müller Závalová V., Artinian S., Talhouk R., Šmejkal K., Suchý Jr. P., Hampl A**. 2013. Prenylated Flavonoids from Morus alba L. Cause Inhibition of G1/S Transition in THP-1 Human Leukemia Cells and Prevent the Lipopolysaccharide-Induced Inflammatory Response. *Evid.-based Complement Altern. Med*., **2013**: Article ID 350519.

6) **Hošek J., Toniolo A., Neuwirth O., Bolego C**. 2013. Prenylated and Geranylated Flavonoids Increase Production of Reactive Oxygen Species in Mouse Macrophages but Inhibit the Inflammatory Response. *J. Nat. Prod*. **76**: 1586-1591.

7) **Zelová H., Hanáková Z., Čermáková Z., Šmejkal K., Dall'Acqua S., Babula P., Cvačka J., Hošek J.** 2014. Evaluation of Anti-inflammatory Activity of Prenylated Substances Isolated from *Morus alba* and *Morus nigra*. *J. Nat. Prod*. **77**: 1297–1303.

8) **Vochyánová Z., Bartošová L., Bujdáková V., Fictum P., Husník R., Suchý P., Šmejkal K., Hošek J.** 2015. Diplacone and Mimulone Ameliorate Dextran Sulfate Sodium-Induced Colitis in Rats. *Fitoterapia*. **101**: 201-207.

9) **Kollar P., Bárta T., Keltošová S., Trnová P., Müller Závalová V., Šmejkal K., Hošek J., Fedr R., Souček K., Hampl A.** 2015. Flavonoid 4´-*O*-methylkuwanon E from *Morus alba* Induces the Differentiation of THP-1 Human Leukemia Cells. *Evid.-based Complement Altern. Med*., **2015**: Article ID 251895.

10) **Hanáková Z., Hošek J., Babula P., Dall'Acqua S., Václavík J., Šmejkal K.** 2015. *C*-Geranylated Flavanones from *Paulownia tomentosa* Fruits as Potential Anti-inflammatory Compounds Acting via Inhibition of TNF-α Production. *J. Nat. Prod*. **78**: 850-863.

11) **Bahadır Acikara Ö., Hošek J., Babula P., Cvačka J., Budešínský M., Dračinský M., Saltan İşcan G., Kadlecová D., Ballová L., Šmejkal K.** 2015. Turkish Scorzonera Species Extracts Attenuate Cytokine Secretion via Inhibition of NF-κB Activation, Showing Anti-Inflammatory Effect in Vitro. Molecules. **21**: 43.

12) **Khazneh E., Hřibová P., Hošek J., Suchý P., Kollár P., Pražanová G., Muselík J., Hanaková Z., Václavík J., Miłek M., Legáth J., Šmejkal K.** 2016. The Chemical Composition of *Achillea wilhelmsii* C. Koch and Its Desirable Effects on Hyperglycemia, Inflammatory Mediators and Hypercholesterolemia as Risk Factors for Cardiometabolic Disease. Molecules*. **21**: 404.

13) **Hošek J., Šmejkal K.** 2016. Flavonoids as anti-inflammatory agents. *In*: Parnham M.J. (ed.) Compendium of Inflammatory Diseases. Birkhäuser Springer, Basel. ISBN: 978-3-7643-8530-9.

14) **Šmejkal K., Neuwirth O., Treml J., Hošek J.** 2016. Pro-oxidant Activity of Flavonoids and Their Possible Effects. *In*: Govil, J.N. and Pathak, M. (eds.) Recent Progress in Medicinal Plants Volume 40 - Flavonoids and Antioxidants. Studium Press LLC, USA. ISBN-13: 9781626990777

15) **Hanáková Z., Hošek J., Kutil Z., Temml V., Landa P., Vaněk T., Schuster D., Dall'Acqua S., Cvačka J., Polanský O., Šmejkal K.** 2017. Anti-inflammatory Activity of Natural Geranylated Flavonoids: Cyclooxygenase and Lipoxygenase Inhibitory Properties and Proteomic Analysis. *J. Nat. Prod.* **80**: 999-1006.

16) **Vochyánová Z., Pokorná M., Rotrekl D., Smékal V., Fictum P., Suchý P., Gajdziok J., Šmejkal K., Hošek J.** 2017. Prenylated flavonoid morusin protects against TNBS-induced colitis in rats s. *PLoS One*. **12**: e0182464.

17) **Brezáni V., Šmejkal K., Hošek J., Tomášová V.** 2018. Anti-inflammatory Natural Prenylated Phenolic Compounds - Potential Lead Substances. *Curr. Med. Chem*. **25**: 1094-1159.

REVIEW

TNF-α signalling and inflammation: interactions between old acquaintances

Hana Zelová · Jan Hošek

Abstract

Introduction Inflammation is a very important part of innate immunity and is regulated in many steps. One such regulating step is the cytokine network, where tumor necrosis factor α (TNF-α) plays one of the most important roles.

Methods A PubMed and Web of Science databases search was performed for studies providing evidences on the role of TNF-α in inflammation, apoptosis, and cancer.

Results and Conclusion This review concisely summarizes the role of this pro-inflammatory cytokine during inflammation. It is focused mainly on TNF-α intracellular signaling and its influence on the typical inflammatory features in the organism. Being one of the most important pro-inflammatory cytokines, TNF-α participates in vasodilatation and edema formation, and leukocyte adhesion to epithelium through expression of adhesion molecules; it regulates blood coagulation, contributes to oxidative stress in sites of inflammation, and indirectly induces fever. The connection between TNF-α and cancer is mentioned as well.

Keywords TNF-α · TNFR · Inflammation · Apoptosis · Cancer

Introduction

Human immunity is a very complex apparatus which protects our body not only against external enemies, such as microbes and viruses, but also against our own damaged or malfunctioning tissues. One of the first protective barriers is inflammation, a part of innate immunity. An important component of immunity is the inflammatory response in which cytokine tumor necrosis factor α (TNF-α) plays a key role in. Its importance is attested by the fact that TNF-α is the target of drugs used for the treatment of some diseases, e.g., Infliximab—a monoclonal antibody against TNF-α—is used to cure Crohn's disease.

This short review is focused on describing the basic properties of TNF-α and its role during inflammation.

TNF-α and the TNF receptor superfamily

Tumor necrosis factor α (TNF-α, also known as cachectin) is a strong pro-inflammatory cytokine which plays an important role in the immune system during inflammation, cell proliferation, differentiation and apoptosis [1]. It was first described by Carwell et al., in 1975 as a cytokine which showed significant cytotoxic activity after stimulation of the immune system, and, thus, caused tumor necrosis. When the gene for TNF-α was cloned in 1984, a structure homology with lymphotoxin (LT)-α was found, and TNF-α was included in the group of cytokines known as the TNF ligand superfamily [2]. Its members are type II transmembrane proteins which can be expressed in both membrane-bound and secreted forms [3].

Receptors for TNF-α are part of TNF receptor (TNFR) superfamily (TNFRSF), whose members have a characteristic intracellular segment, a transmembrane domain, and an extracellular ligand-binding domain. They are type I transmembrane glycoproteins characterized by a cysteine-rich motif (up to 40 aminoacids) repeated 2–6 times in the extracellular N-terminal domain [4, 5].

The members of the TNFR superfamily can be divided into three groups on the basis of their structure: (1)

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receptors containing a death domain (2) receptors without a death domain, and (3) decoy receptors—soluble or membrane bound receptors which bind their ligand with high specificity and affinity but are not able to elicit an intracellular response (Table 1). Due to their unique structural features, the members of the TNFR superfamily participate in signal pathways leading to apoptosis, survival, proliferation, differentiation, or activation [6].

Gene and protein structure of TNF-α

The human gene for TNF-α is part of a major histocompatibility (MHC) gene cluster located on the short arm of chromosome 6 (6p21.32). These genes are divided into four main classes. The genes for TNF-α and two other members of the TNF super family—LT-α and LT-β—occur within the MHC class IV cluster. The gene for TNF-α is around 3 kb long and consists of four exons interrupted by three introns. More than 80 % of this cytokine is coded by the fourth exon; the first and second exons code only the leading sequence of the nascent peptide. Several regulating sites with sequences corresponding to the transcription factors AP-1, AP-2, NF-κB, and the cAMP responsive element (CRE) which are known from the regulatory regions of immunoglobulins and cytokines, on the 5′-end of the TNF-α gene are found there. These regulating sequences are sensitive to signals triggered not only by

Table 1 The TNFR superfamily

Receptor group	Receptor name	Alternative receptor name	CD number	Ligands
Receptors containing a death domain	TNFRSF1A	TNFR1	CD120a	TNF-α, LT-α
	TNFRSF6	FasR	CD95	FasL
	TNFRSF10A	DR4	CD261	TRAIL
	TNFRSF10B	DR5	CD262	TRAIL
	TNFRSF16	NGF receptor	CD271	NGF, BDNF, NT-3, NT-4
	TNFRSF21	DR6	CD358	N-APP
	TNFRSF25	DR3	–	TWEAK
Receptors without a death domain	TNFRSF1B	TNFR2	CD120b	TNF-α, LT-α
	TNFRSF3	LTβR	CD18	LT-αβ, LIGHT
	TNFRSF4	OX40	CD134	OX40L
	TNFRSF5	–	CD40	CD40L
	TNFRSF7	Tp55	CD27	CD27L
	TNFRSF8	Ki-1 antigen	CD30	CD30L
	TNFRSF9	4-1BB ligand receptor	CD137	4-1BBL
	TNFRSF11A	ODFR	CD265	RANKL
	TNFRSF12A	TWEAK receptor	CD266	TWEAK
	TNFRSF13B	TACI	CD267	BAFF, APRIL
	TNFRSF13C	BAFF receptor	CD268	BAFF
	TNFRSF14	TR2	CD270	BTLA, LIGHT, LT-α
	TNFRSF17	B cell maturation protein	CD269	BAFF, APRIL
	TNFRSF18	Glucocorticoid-induced TNFR-related protein	CD357	TNFSF18
	TNFRSF19	Toxicity and JNK inducer	–	Unknown
	TNFRSF19L	RELT	–	Unknown
	TNFRSF27	XEDAR	–	EDA-A2
Decoy receptors	TNFRSF6B	DcR3	–	LIGHT, TNFSF15, FasL
	TNFRSF10C	DcR1	CD263	TRAIL
	TNFRSF10D	DcR2	CD264	TRAIL
	TNFRSF11B	OPG	–	RANKL

APRIL a proliferation-inducing ligand, *BAFF* B-cell-activating factor, *BDNF* brain-derived neurotrophic factor, *BTLA* B- and T-lymphocyte attenuator, *CAML* calcium signal-modulating cyclophilin ligand, *CD* cluster differentiation, *DcR* decoy receptor, *DR* death receptor, *EDA-A2* ectodysplasin A2, *FasL* Fas ligand, *FasR* Fas receptor, *JNK* c-Jun N-terminal kinase, *LIGHT* herpes virus entry mediator L, *LT* lymphotoxin, *LTβR* lymphotoxin β receptor, *N-APP* N-terminal fragment of amyloid beta A4 protein, *NGF* nerve growth factor, *NT* neurotrophin, *ODFR* osteoclast differentiation factor receptor; *OPG* Osteoprotegerin, *RANKL* receptor activator of nuclear factor κB ligand, *RELT* receptor expressed in lymphoid tissues, *TNF* tumor necrosis factor, *TACI* transmembrane activator and calcium modulator and cyclophilin ligand interactor, *TNFR* TNF receptor, *TNFRSF* TNF receptor superfamily, *TR2* TNF receptor-like 2, *TRAIL* TNF-related apoptosis-inducing ligand, *TWEAK* TNF-related weak inducer of apoptosis, *XEDAR* X-linked ectodysplasin-A2 receptor

lipopolysaccharide (LPS) or TNF-α itself [7–9]. It is known that regulation also occurs on the post-transcription level, where the stability of TNF-α mRNA is regulated by the binding of tristetraprolin (TTP) to the AU-rich region of 3′-UTR (the untranslated region) [10].

TNF-α is produced in two forms—17 kDa soluble TNF-α (sTNF-α) and 26 kDa membrane-bound TNF-α (tmTNF-α) [11, 12]. The soluble form of TNF-α is created from tmTNF-α by the extracellular domain of the matrix metalloproteinase TNF-α converting enzyme (TACE; ADAM17) [13]. Membrane–bound TNF-α is able to serve as a ligand binding TNFR or as a receptor mediating the transfer of external signals back to the cell which has exprimed it on its surface [14]. Both cytokine forms—soluble and membrane–bound—are active as homotrimers with a characteristic cone-shape. Each monomer consists of two packed β-pleated sheets, which are created by eight anti-parallel β-strands arranged in a β-jellyroll topology [15]. The sTNF-α and tmTNF-α show different biological activities; the tmTNF-α form is more active than the sTNF-α form. TNF-α is not usually detectable in a healthy population, but increased serum and tissue levels are found under inflammatory and infectious conditions. The concentration of TNF-α in serum correlates with the severity of infection [2, 16].

TNF-α expression

Although macrophages and T-cells are thought to be the main producers of TNF-α, other cells can also produce this cytokine, e.g., B cells, NK-cells, neutrophils, mast cells, endothelial cells, smooth muscle cells, cardiomyocytes, fibroblasts, osteoclasts, osteoblasts, astrocytes, dendritic cells, microglial cells, keratinocytes, adipocytes, adrenocortical cells, and glomerular mesangial cells [16–18].

Bacterial lipopolysaccharide (LPS, endotoxin) has long been considered to be one of the main stimulants triggering TNF-α production. Beside LPS, viral, mycotic and parasitical antigens, enterotoxin, C5a anaphylotoxin, superantigen, immune complexes, IL-1, IFN-γ, GM-CSF, TGF-β, and TNF-α itself by autocrine mechanisms are able to induce the expression of TNF-α. Pathophysiological conditions that stimulate the production of TNF-α include the previously mentioned infection and inflammation, but burns, trauma, infarct, heart failure, unstable angina pectoris, apoplexy, brain injury, and asthma can also be listed in this tally [19]. Its production is attenuated by IL-4 and other agents that decrease the level of cAMP [20, 21].

It has been determined that cells never create reserves of TNF-α, but stimulation triggers de novo synthesis of this cytokine. Transcriptional, translational, and posttranslational regulation mechanisms are involved in TNF-α

expression. Chromatin modifications also influence the transcription of TNF-α mRNA. Histone acetylation in the *tnfa* promotor is connected with the decondensation of chromatin and greater transcriptional activity [22]. Negative feedback is another regulatory mechanism which has an important role during the biosynthesis of TNF-α. TNF-α itself increases the synthesis of anti-inflammatory factors, such as IL-10, corticosteroids, or prostanoids, that are able to negatively regulate its expression. On the whole, this system contributes to better control over the progress of inflammation, therewith to allow a quick immune reaction mediated by TNF-α and, subsequently, to inhibit its extent and duration [23, 24].

Receptors for TNF-α

The biological effects of TNF-α are mediated by binding on two different receptors—TNFR1 (CD120a, p55) and TNFR2 (CD120b, p75) (Table 1). The gene for TNFR1 is localized in the chromosome locus 12p13.31, has ten exons and codes for a 55/60 kDa transmembrane receptor, whereas the gene for TNFR2 is located in the chromosome locus 1p36.22, consists of ten exons, and codes for a 75/80 kDa transmembrane receptor [25–28].

The extracellular ligand-binding domains of these receptors have Cys-rich subdomains characteristic for TNFR superfamily members with 28 % homology. On the other hand, the intracellular domains of TNFR1 and TNFR2 have no sequence homology or intrinsic enzyme activity and are able to trigger different signal pathways due to their interaction with cytosolic proteins [29, 30].

Both receptors can occur in soluble form (sTNFR1 and sTNFR2), created from the membrane receptors by the TACE enzyme activity that is also responsible for generating sTNF-α. These free receptors are able to attenuate the effect of TNF-α by competing mechanisms or they can stabilize TNF-α and protect it against degradation [31–33].

While TNFR1 is expressed on the surface of almost all human cell types (except erythrocytes [34]) and can be activated by tmTNF-α and sTNF-α, TNFR2 is located mainly on immune and endothelial cells and tmTNF-α is required for its full activation. TNFR2 possesses a lower binding affinity for TNF-α and more easily dissociates cytokine from the receptor. This indicates that TNFR2 can momentarily bind and subsequently release TNF-α to increase its local concentration close to TNFR1 (this action is called ligand passing). Taken together, TNFR1 is the key mediator of TNF-α action for most cells, whereas TNFR2 plays the main role in cells of the lymphatic system or an accessory role by enhancing or synergizing with TNFR1 [2, 35, 36].

Although ligand-activated receptor trimerization was previously believed to signal inception, at present the

situation seems to be more complicated. Both TNFRs have specific Cys-rich pre-ligand binding assembly domains (PLAD), which form receptor trimers even in the absence of a ligand, keep them in a quiescent state, and prevent them from spontaneously autoactivating [2, 37].

TNF-α cell signaling

Signal transduction via TNFR1

Ligand induced activation of TNFR1 leads to the formation of signal complexes that activate intricate signal pathways directed in two basic ways—(a) stimulation of cell survival and expression of pro-inflammatory genes or (b) apoptosis and cell death. The balance between the two ways is regulated on several levels—signal strength, expression of signaling molecules and regulating proteins, and crosstalk with other cell signals can be mentioned as the predominant factors. The character of the signal pathway and its final effect depend on the kinds of protein adaptors which are involved in the formation of the signal complex [6].

TNFR1 belongs to a group of death receptors (DR) containing a characteristic death domain (DD). In the resting state, receptors are associated with a cytoplasmic silencer of the death domain (SODD), which prevents activation of the DD, and, thus, activation of the signal pathway, in the absence of TNF-α. After TNF-α activation, SODD is released from the receptor DDs and these domains can interact with the DDs of another adaptor proteins [16, 30].

Signaling complex I—TRADD-RIP1-TRAF2

When TNF-α binds the receptor and SODD dissociates from the DD, these domains interact with the C-terminal DD of TNFR type 1-associated death domain protein (TRADD). TRADD allows the binding of another two proteins—receptor interacting protein-1 (RIP-1) and TNF-receptor-associated factor-2 (TRAF-2). Both proteins mainly regulate pro-inflammatory and anti-apoptotic pathways. Transcription of pro-inflammatory genes is driven by many transcription factors, among these, NF-κB and AP-1 belong to the most important. They regulate the production of cytokines, adhesive molecules, growth factors, metalloproteinases, and many others proteins that contribute to prostaglandins, leucotrienes, and NO synthesis [16, 38–40].

The family of NF-κB transcription factors consists of five members—p65 (RelA), RelB, cRel, p50, and p52—which share an extensive (300 aminoacids) *Rel* homology domain. This domain allows subunit dimerization, nuclear translocation, and DNA binding. But only the p65, RelB

and cRel subunits are able to directly activate the transcription of target genes. The transcription abilities of p50 and p52, which are created from p105 and p100 precursors, depend on dimerization with p65, RelB, or cRel [41–43].

Most of the NF-κB dimers in cytoplasm are inactivated and are retained in unstimulated cells by the small inhibiting proteins IκBα, IκBβ, or IκBε. IκB proteins are able to mask the nuclear translocation sequences of NF-κB, and IκBα is also able to enter to the nucleus and block the NF-κB binding site on DNA [44].

One way to activate NF-κB is via the IκB kinase (IKK; an inhibitory protein of NF-κB kinase) complex. It has been determined that for IKK activation, both TRAF-2, which is sufficient to recruit this kinase, and RIP-1, which is responsible for its activation, are necessary [45]. It is supposed that RIP-1 indirectly activates IKK by phosphorylation via members of the serine/threonine kinase MAP3K (mitogen-activated protein kinase kinase kinase) [2]. There are two known ways of activating NF-κB via IKK—canonical and non-canonical.

Signal transduction after TNF-α stimulation occurs most often via the canonical pathway. The IKK complex consists of the two catalytic subunits IKKα and IKKβ and the regulating protein NEMO (NF-κB essential modulator; IKKγ). While NEMO serves as an adaptor protein, both catalytic subunits are able to phosphorylate specific serine residues on IκB and thus initiate its proteosomal degradation and allow NF-κB nuclear translocation. In the case of canonical activation, IκB is phosphorylated mainly by IKKβ, whereas in the non-canonical way, IKKα is very important and NEMO is not necessary in the IKK complex [43, 46–48]. Yamamoto et al. [49] have reported that IKKα is also an H-3 kinase which activates TNF-α-inducible genes by binding on target gene promotors.

As was mentioned above, mitogen-activated protein kinases (MAPK) have a significant position in TNF-α intracellular signaling. Their greater activity and participation in the production of pro-inflammatory mediators on the level of transcription and translation make them a potential target of anti-inflammatory therapy. Three MAPK families exist—extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPKs. They are activated by dual phosphorylation of Thr and Tyr in the Thr-X-Tyr protein motif by the following cascade: MAP3Ks (which are activated by a receptor-adaptor protein complex) ➡ MAP2Ks (mitogen-activated protein kinases kinases) ➡ MAPKs. Activated MAPKs phosphorylate and modulate the activity of other downstream enzymes, cytosolic proteins, and transcription factors which directly or indirectly regulate gene expression on the transcription level, the stability of mRNA and its cytoplasmic translocation, and protein synthesis. Possible consequences of MAPK cascade activation could be

inflammation, proliferation, differentiation, or apoptosis [50–54].

Signaling complex II—TRADD-RIP-1-TRAF-2-FADD-procaspase 8/10

Unlike signaling complex I, which triggers pathways resulting in survival, the formation of signaling complex II is directed towards apoptosis. Signaling complex I formation is transient and the TRADD-RIP1-TRAF-2 complex soon dissociates from TNFR1, leading to the formation of signaling complex II with FADD (Fas-associated death domain protein) and procaspase 8/10 in the cytosol. Complex II-activated apoptosis occurs under the conditions existing when NF-κB activation via complex I has failed [30].

FADD is a 26 kDa protein containing a death domain in the C-terminal part, which permits binding to TRADD, and a death effector domain (DED) in the N-terminal part responsible for caspase activation [55, 56]. Procaspase 8 is a proenzyme whose interaction with FADD creates a death inducing signaling complex (DISC). DISC ensures the cutting off a prodomain and the smaller 10 kDa subunits from procaspase 8 and, thus,creates active caspase 8. Then, caspase 8 contributes by autoactivation mechanisms to the amplification of the signal and activates effector caspases-3,-6, and -7. In the case of caspase 8, the signal pathways diverge and apoptosis can proceed by an extrinsic or intrinsic pathway, depending on the type of cell [57–59].

Apoptosis occurs in an extrinsic way in type I cells (e.g., thymocytes), where caspase 8 directly activates the effector caspase 3. Caspase 3 subsequently degrades the regulatory and structural proteins essential for cell integrity and activates specific caspase-activated DNase (CAD) [59].

Caspase 8 is able to activate an intrinsic (mitochondrial) apoptic pathway and, hence, intensify the effect caused by the extrinsic way in type II cells (e.g., hepatocytes). At the start of this process, caspase 8 digests the cytosolic protein Bid producing its truncated apoptogenic form (tBid). There is a balance between the anti-apoptogenic proteins Blc-2 or Bcl-XL and the pro-apopotogenic proteins Bax or Bak in living cells. tBid disrupts this balance and directly activates the pro-apoptogenic proteins Bax or Bak, which oligomerize in the mitochondrial outer membrane and disturbs this membrane, which leads to the releasing of cytochrome c. The released cytochrome c induces the formation of an apoptosome consisting of cytochrome c, apoptotic protease activating factor 1 (Apaf-1), dATP, and procaspase 9. Caspase 9 is autoactivated in the mature apoptosome and it then activates procaspase 3, where the extrinsic and intrinsic ways are joined [59].

Signal transduction via TNFR2

Signal pathways initiated by TNFR2 stimulation have been less investigated than those of TNFR1. It seems that whereas TNFR1 stimulation can lead to inflammatory or apoptotic responses, TNFR2 notably supports cell activation, migration, and proliferation [16].

In comparison with TNFR1, TNFR2 lacks an intracellular death domain, but it can interact with TRAF-2 and result in long-lasting NF-κB activation. It has been observed in several cell lines that TNFR2 stimulation by tmTNF-α is able to launch both canonical and non-canonical NF-κB activation [60].

After TNFR2 activation by tmTNF-α and its trimerization, TRAF-1 and TRAF-2 proteins can associate with a cytoplasmic TNFR2 domain as a heterocomplex, where only TRAF-2 contacts the receptor directly. In the cytoplasm of unstimulated cells, TRAF2 is already associated with other complex inhibitors of apoptosis proteins 1 and 2 (cIAP-1 and cIAP-2) [60]. cIAP inhibits caspases and some other proteins by ubiquitin-ligase activity [16].

It is interesting, that the canonical NF-κB activating pathway uses TRAF-2 as a signal inductor, whereas TRAF-2 and TRAF-3 show inhibitory features in a TNFR2 specific non-canonical NF-κB pathway. In unstimulated cells, TRAF-3 binds the complex TRAF-2-cIAP-1-cIAP-2 to NIK (NF-κB-inducing kinase), a MAP3K that plays an important role in non-canonical NF-κB activation. cIAP-1 and cIAP-2 ubiquitinate NIK in the complex formed, and, thereby, trigger its proteasomal degradation. Binding TNF-α to TNFR2 results in the translocation of the TRAF-2-cIAP-1-cIAP-2 complex from the cytoplasm to a receptor. Hence, NIK is not degrading. This enzyme is subsequently able to stimulate IKKα, which phosphorylates the precursor protein p100. This leads to its proteolytic digestion and the formation of the active dimer NF-κB-p52 [60–62]. In comparison with TNFR1, TNFR2 activates NF-κB more slowly, but it stays active for a longer time [60]. Some papers also indicate that TNFR2 can interact with proteins RIP-1 and TRADD, known from TNFR1 signaling, via TRAF-2 and contribute to activation of the transcription factors NF-κB and AP-1 through JNK and p38 pathways [63, 64].

TNFR2 has the specific ability to activate endothelial/epithelial tyrosine kinases (Etk), which participate in cell adhesion, migration, proliferation, and survival independently on TRAF-2. Direct interaction of Etk with TNFR2 leads to formation of the trimolecular complex TNFR2-Etk-VEGFR2 (vascular endothelial growth factor receptor 2), where coordinated reciprocal phosphorylation causes activation of PI3K/Akt kinase. Its activation by TNF-α in epithelial cells represents an anti-apoptotic and anti-necrotic pathway which could compensate pro-apoptic signals mediated by TNFR1 [65, 66].

Although TNFR2 lacks a death domain, its activation can result in apoptosis. Under conditions of stress in a cell, signaling complex II can be formed via TRAF-2, followed by the activation of caspase 8 and apoptotic cell death. The other theory supposes that prior TNFR2 activation could lead to exhaustion of the cIAP pool, which together with TRAF-2 is a part of the surviving complex, and this deficit could lead to apoptosis caused by TNFR1 activity [64].

Role of TNF-α signaling during inflammation and the response of an organism

Inflammation belongs to the main mechanisms of innate immunity [67] and is an organism's response to infection, antigen action, or tissue injury. TNF-α belongs to the pro-inflammatory cytokines. The aim of inflammation is to eradicate the irritating agent and accelerate the regeneration of tissue. On the other side, an inadequate inflammatory reaction can result in tissue damage and in severe cases it could even cause organ failure and death [68]. Optimally, the inflammatory reaction should be limited by eliminating the harmful agent, isolating the damaged tissue, and controlling this tissue [67].

Vasodilatation

Vasodilatation is a well described feature of acute inflammation and is clinically manifested as flare and higher temperature at the inflamed site. Its main purpose is to facilitate the penetration of inflammatory mediators and cells into the target tissue. In the case of severe systemic inflammation, e.g., in the case of sepsis, it can result in massive vasodilatation, which can end in systemic hypotension and shock. Vasodilatation is mediated primarily by nitric oxide (NO) and vasodilatation acting prostanoids (PGI$_2$, PGD$_2$, PGE$_2$, and PGF$_{2\alpha}$) [69–71].

NO is produced by three kinds of NO-synthases (NOS)—endothelial (eNOS), neuronal (nNOS), and inducible (iNOS) [70]. It has been found that TNF-α and some other pro-inflammatory cytokines stimulate iNOS expression in macrophages and some other leukocytes [72]. NO is created in the form of the reactive NO· radical during the conversion of L-arginine to L-citrulline. This radical activates guanylate cyclase leading to the generation of cGMP followed by relaxation of the smooth muscle cells of the vessel. The NO produced by iNOS simultaneously contributes to vascular dysfunction by inducing apoptosis in endothelial cells. TNF-α has the opposite effect on eNOS, suppressing expression. The NO produced by eNOS is applied during physiological functions of the endothelium [73, 74].

Via stimulation of cyclooxygenase 2 (COX-2), TNF-α increases the production of prostanoids PGE$_2$, PGF$_{2\alpha}$, and PGI$_2$, among them, PGI$_2$ has the highest vasodilatation activity [75].

Edema

One of the characteristic features of inflammation, as defined by Celsius in the first century, is *tumor*. This appellation comes from the ability of TNF-α and other mediators (e.g., histamine, bradykinine, substance P, leukotrienes, complement components, and PAF) to induce edema [16, 76, 77].

Edema develops as a consequence of higher vessel permeability, temporarily increased hydrostatic pressure in the capillaries and decreased oncotic blood plasma pressure. Synchronization of these three factors initiates the transfer of transvascular liquid and protein and thus facilitates the access of antibodies and acute phase proteins the site of the inflammation [68].

One possible mechanism, by which TNF-α could contribute to the creation of edema is by disrupting the structure of the endothelium of the vessel structure. Healthy endothelium is strengthened by a glycocalyx layer, which plays a key role in regulating inflammation and vessel permeability. TNF-α is able to induce degradation of this layer and, hence, augments permeability [78]. It also causes destabilization of the endothelial cell cytoskeleton, disturbs the integrity of the monolayer, and participates in the formation of intercellular gaps [79]. The adhesive molecule cadherin at endothelial cell junctions has a pivotal role in the integrity of the epithelium. TNF-α increases the tyrosine phosphorylation of this protein, which opens the paracellular passage for solutes and macromolecules. Up to 60 % of the disintegration of the endothelial barrier could be caused by this mechanisms [80].

TNF-α can also contribute indirectly to the occurrence of edema. It has been reported that this cytokine significantly increases the expression of bradykinin B$_1$ receptor and thus allows bradykinin to induce the formation of edema [81].

Facilitation of the adhesion of leukocytes

Vasodilatation and liquid exudation are accompanied by the migration of leukocytes to the inflamed site. This process consists of five partial steps: margination, rolling, adhesion of leukocytes to the endothelium, diapedesis, and chemotaxis to the target site. The interaction between leukocytes and endothelial cells is mediated by cell adhesion molecules (CAMs), where selectins and the immunoglobulin superfamily CAMs (IgSF CAM) are reckoned in.

Selectins are Ca^{2+}-dependent receptors that bind sialylated carbohydrate determinants. There are three known selectin members: P-selectin (platelet), E-selectin (endothelial), and L-selectin (leukocyte). The binding of selectins to ligands, together with the vascular shear force, promotes the rolling of leukocytes on the endothelial surface. TNF-α is able to stimulate the expression of E- and P-selectins [82]. E-selectin participates in the adhesion of neutrophils, monocytes, and memory T-leukocytes on stimulated platelets and endothelial cells. P-selectin is responsible for the binding of neutrophils and monocytes. The situation is different in the case of L-selectin. After neutrophil activation, L-selectin is cut off from the surface and forms sL-selectin. Demerging of L-selectin is necessary for the implementation of diapedesis and it has been proven that TNF-α decreases the L-selectin on the surface of neutrophils and increases its soluble form [83].

Selectin-mediated rolling of leukocytes does not lead to strong adhesion and transmigration until IgSF CAMs are involved. From this view, intracellular CAM (ICAM)-1, ICAM-2, vascular CAM (VCAM)-1, and platelet-endothelial CAM (PECAM)-1 represent the most important players. TNF-α stimulates the expression of ICAM-1 and VCAM-1 [82].

ICAM-1 is constitutively exprimed in small numbers on the endothelial cells of vessels, some lymphocytes, and monocytes. It contributes to leukocyte–leukocyte, leukocyte-endothelium, and leukocyte-epithelium interactions and transendothelial migration. The ligand for ICAM-1 binding is macrophage-1 antigen (MAC-1) which occurs on the surface of leukocytes [84]. VCAM-1 mediates the binding of lymphocytes, monocytes, basophiles, and eosinophiles to the endothelium, but it does not interact with neutrophils. It plays an important role in the creation of infiltrates rich in T-lymphocytes and, thus, contributes to forming special memory T-lymphocytes at the site of inflammation [85].

Regulation of blood coagulation during inflammation

Inflammation and blood coagulation are tightly associated. The coagulation cascade activated by injury of tissue or infection can be divided into extrinsic and intrinsic ways which connect together in the final state and result in thrombin activation following the processing of fibrinogen into insoluble fibrin. The intrinsic pathway is most commonly activated by direct damage of tissue, whereas the extrinsic pathway is characteristic for infection and system inflammation [68, 86].

Activation of the extrinsic pathway is initiated by the production of tissue factor (TF). This is a transmembrane protein exprimed on the surface of ranks of somatic cells (e.g., the smooth muscle cells surrounding vessels) which

do not occur in vessels under physiological conditions; intravascular cells (e.g., endothelial cells and blood elements) lack this protein. The situation changes during inflammation, when TNF-α and some other cytokines induce TF expression in monocytes and the endothelium. Thus activated, TF creates a complex with factor VII, which finally results in thrombin activation. Coagulation cascade activation is important not only from the view of fibrin clot formation, but it is also able to enhance the pro-inflammatory response. Specifically, factor Xa, thrombin, and the complex TF-factor VIIa can increase the production of TNF-α and other pro-inflammatory cytokines in mononuclear leukocytes and endothelial cells [68, 87].

Apart from favoring coagulation, TNF-α also attenuates fibrinolysis because of its ability to lower the expression of tissue plasminogen activator (tPA), which converts plasminogen to plasmin and, thus, initiate fibrinolysis. Experimental results indicate that TNF-α can stimulate the expression of tPA in the early state, but during long-lasting stimulation this effect changes to inhibition [88].

Changes in the production of reactive oxygen species

Many in vitro and some in vivo experiments confirm that reactive oxygen species (ROS) produced by NADPH oxidases (NOX) play an important role in inflammatory signal transduction by triggering some redox-dependent cell signal pathways [89]. NOX are strictly regulated membrane-bound enzymes whose main function is to catalyze electron transfer from NADPH to molecular oxygen and, thus, participate in creating superoxide radicals and hydrogen peroxide. Members of the NOX family (NOX1-5) consist of catalytic subunit gp91^{phox} and the regulatory subunits p40^{phox}, p47^{phox}, p67^{phox}, p22^{phox}, and the small GTPase RAC [90]. A study performed on isolated human coronary endothelial cells proved that TNF-α increases the expression of NOX4A, p47^{phox}, p67^{phox}, and p22^{phox}, which results in up to three-times more NOX activity [91]. ROS produced by endothelial cells after TNF-α stimulation activates some MAPKs and increases the synthesis of ICAM-1 and E-selectin [92, 93].

TNF-α is also a very potent stimulator of the respiratory burst in polymorphonuclear leukocytes allowing defusing of phagocytated foreign elements. But TNF-α uses ROS as secondary messengers to modulate the activity of NF-κB (for more information see the excellent reviews [89, 94]).

Fever

According to the humoral theory of the origin of fever, TNF-α serves as a pyrogenic cytokine which has the ability to infiltrate the hypothalamus through fenestrations in the hematoencephalic barrier or by active transport mediated

by the cells surrounding the hypothalamus. A key feature of this theory is that TNF-α, like other pyrogenic cytokines, activates fever indirectly by stimulating local endothelial cells or microglies to produce PGE$_2$. This prostaglandin subsequently initiates the response of the thermoregulatory neurons in the hypothalamus via PGE receptor 3 [95].

TNF-α and inflammation-related cancerogenesis

Increasing evidence from studies suggests that dysregulated inflammatory response plays a pivotal role in many chronic disorders, including cancer [96]. Current estimates suggest that about 25 % cancers are associated with chronic inflammation [97]. One of the key molecule mediating inflammatory processes to tumor promotion is TNF-α, although its role is paradoxical. According to its name, high-dose local administration of TNF-α has a powerful tumor-necrosis activity, while low-dose chronic production may act as endogenous tumor promoter participating in all steps of tumorigenesis, including cellular transformation, proliferation, growth, invasion, angiogenesis, and metastasis [98–100].

TNF-α may promote DNA damage by inhibition of DNA-repairing enzymes caused by an elevated production of NO. TNF-α acts as a paracrine or autocrine growth factor, which increases levels of positive cell cycle regulators, decreases levels of cyclin-dependent kinase (CDK)-inhibitors and stimulates production of other growth factors [100–103]. Its proangiogenic effect is mediated though VEGF, VEGFR2, fibroblast growth factor 2 (FGF2), IL-8, PAF, ephrin A, NO, E-selectin, ICAM-1 and thymidine phosphorylase [104–107]. TNF-α also promotes tissue remodeling via stimulation of matrix metalloproteinases (MMPs) as well as tumor dissemination and metastasis through expression of chemokine CXCR4 and its receptor CLCL12, up-regulation of migration-inhibitory factor (MIF) in macrophages, monocyte chemotactic protein 1 (MCP-1), IL-8 and ICAM-1 [108–110]. High circulating levels of TNF-α and its soluble receptors are associated with poor prognosis and resistance to cytotoxic drugs. Thus, novel strategies that neutralize systemic TNF-α may be useful in cancer treatment and prevention [96, 111].

Conclusion

The physiological and pathophysiological roles of TNF-α are very complex. Binding TNFR1 or TNFR2 triggers various intracellular signal pathways including activation of MAPKs, transcription factors or caspases in case of apoptosis. Being one of the most important pro-inflammatory cytokines, TNF-α participates in vasodilatation and edema formation, leukocyte adhesion to epithelium

through expression of adhesion molecules; it regulates blood coagulation, contributes to oxidative stress in sites of inflammation and indirectly induces fever. These effects can be mediated through induction of some enzymes producing extracellular signaling molecules like NO, prostanoids (PGE$_2$, PGF$_{2\alpha}$, PGI$_2$) or ROS. Interestingly, there has been observed a significant interplay between chronic inflammation and cancerogenesis. According to the dominant role of TNF-α in pathogenesis of inflammatory diseases, specific antibodies against TNF-α (e.g., infliximab, etanercept, adalimumab, golimumab, and certolizumab) have become invaluable in treatment of some autoimmune diseases like rheumatoid arthritis, inflammatory bowel diseases, asthma bronchiale, systemic lupus erythematosus, psoriasis, sarcoidosis and other diseases. Hence, the modulation of the level of TNF-α in tissue during inflammatory states has become a very promising target of therapy of the above mentioned diseases.

Although many actions of TNF-α has been well described nowadays, there still remains a lot unknown about the mechanisms by which TNF-α regulates the inflammatory reaction and tumorigenesis.

References

1. Baud V, Karin M. Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol. 2001;9:372–7.
2. Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ. 2003;1:45–65.
3. Zhou T, Mountz JD, Kimberly RP. Immunobiology of tumor necrosis factor receptor superfamily. Immunol Res. 2002;1–3:323–36.
4. Beutler B, Bazzoni F. TNF, apoptosis and autoimmunity: A common thread? Blood Cells Mol Dis. 1998;10:216–30.
5. Naismith JH, Devine TQ, Brandhuber BJ, Sprang SR. Crystallographic evidence for dimerization of unliganded tumor necrosis factor receptor. J Biol Chem. 1995;22:13303–7.
6. Duda E, Nagy T, Männel D, Eissner G. Signaling and reverse signaling in the tumor necrosis factor/TNF receptor system. In: Damjanovich S, editor. Biophysical aspects of transmembrane signaling. 8th ed. Berlin: Springer; 2005. p. 171–209.
7. Spriggs DR, Deutsch S, Kufe DW. Genomic structure, induction, and production of TNF-alpha. Immunol Ser. 1992;56:3–34.
8. Ruuls SR, Sedgwick JD. Unlinking tumor necrosis factor biology from the major histocompatibility complex: lessons from human genetics and animal models. Am J Hum Genet. 1999;2:294–301.
9. Idriss HT, Naismith JH. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). Microsc Res Tech. 2000;3:184–95.
10. Deleault KM, Skinner SJ, Brooks SA. Tristetraprolin regulates TNF TNF-alpha mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. Mol Immunol. 2008;1:13–24.
11. Kriegler M, Perez C, DeFay K, Albert I, Lu SD. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane

protein: ramifications for the complex physiology of TNF. Cell. 1988;1:45–53.

12. Luettig B, Decker T, Lohmann-Matthes ML. Evidence for the existence of two forms of membrane tumor necrosis factor: an integral protein and a molecule attached to its receptor. J Immunol. 1989;12:4034–8.

13. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature. 1997;6618:729–33.

14. Eissner G, Kolch W, Scheurich P. Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system. Cytokine Growth Factor Rev. 2004;5:353–66.

15. Eck MJ, Sprang SR. The structure of tumor necrosis factor-alpha at 2.6 A resolution. Implications for receptor binding. J Biol Chem. 1989;29:17595–605.

16. Bradley JR. TNF-mediated inflammatory disease. J Pathol. 2008;2:149–60.

17. Tracey KJ, Vlassara H, Cerami A. Cachectin/tumour necrosis factor. Lancet. 1989;8647:1122–6.

18. Lin E, Calvano SE, Lowry SF. Inflammatory cytokines and cell response in surgery. Surgery. 2000;2:117–26.

19. Cairns CB, Panacek EA, Harken AH, Banerjee A. Bench to bedside: tumor necrosis factor-alpha: from inflammation to resuscitation. Acad Emerg Med. 2000;8:930–41.

20. Camussi G, Albano E, Tetta C, Bussolino F. The molecular action of tumor necrosis factor-alpha. Eur J Biochem. 1991;1:3–14.

21. Sullivan KE. Regulation Of Inflammation. Immunol Res. 2003;2–3:529–38.

22. Lee JY, Kim NA, Sanford A, Sullivan KE. Histone acetylation and chromatin conformation are regulated separately at the TNF-alpha promoter in monocytes and macrophages. J Leukoc Biol. 2003;6:862–71.

23. Beutler B, Krochin N, Milsark IW, Luedke C, Cerami A. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. Science. 1986;4753:977–80.

24. Ksontini R, MacKay SL, Moldawer LL. Revisiting the role of tumor necrosis factor alpha and the response to surgical injury and inflammation. Arch Surg. 1998;5:558–67.

25. Loetscher H, Pan YC, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W. Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. Cell. 1990;2:351–9.

26. Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong GH, Gatanaga T, et al. Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell. 1990;2:361–70.

27. Fuchs P, Strehl S, Dworzak M, Himmler A, Ambros PF. Structure of the human TNF receptor 1 (p60) gene (TNFR1) and localization to chromosome 12p13 [corrected]. Genomics. 1992;1:219–24.

28. Santee SM, Owen-Schaub LB. Human tumor necrosis factor receptor p75/80 (CD120b) gene structure and promoter characterization. J Biol Chem. 1996;35:21151–9.

29. Ledgerwood EC, Pober JS, Bradley JR. Recent advances in the molecular basis of TNF signal transduction. Lab Invest. 1999;9:1041–50.

30. Ihnatko R, Kubes M. TNF signaling: early events and phosphorylation. Gen Physiol Biophys. 2007;3:159–67.

31. Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D. Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. J Exp Med. 1992;2:323–9.

32. Hajeer AH, Hutchinson IV. TNF-alpha gene polymorphism: clinical and biological implications. Microsc Res Tech. 2000;3:216–28.

33. Wang J, Al-Lamki RS, Zhang H, Kirkiles-Smith N, Gaeta ML, Thiru S, Pober JS, et al. Histamine antagonizes tumor necrosis factor (TNF) signaling by stimulating TNF receptor shedding from the cell surface and Golgi storage pool. J Biol Chem. 2003;24:21751–60.

34. Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. Pharmacol Ther. 2008;2:244–79.

35. Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, Georgopoulos S, et al. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell. 1995;5:793–802.

36. Grell M, Wajant H, Zimmermann G, Scheurich P. The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor. Proc Natl Acad Sci USA. 1998;2:570–5.

37. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science. 2000;5475:2351–4.

38. Hsu H, Shu HB, Pan MG, Goeddel DV. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell. 1996;2:299–308.

39. Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity. 1996;4:387–96.

40. Eder J. Tumour necrosis factor alpha and interleukin 1 signalling: do MAPKK kinases connect it all? Trends Pharmacol Sci. 1997;9:319–22.

41. Siebenlist U, Franzoso G, Brown K. Structure, regulation and function of NF-kappa B. Annu Rev Cell Biol. 1994;405–455.

42. Li Q, Verma IM. NF-kappaB regulation in the immune system. Nat Rev Immunol. 2002;10:725–34.

43. Dejardin E. The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development. Biochem Pharmacol. 2006;9:1161–79.

44. Siebenlist U, Brown K, Claudio E. Control of lymphocyte development by nuclear factor-kappaB. Nat Rev Immunol. 2005;6:435–45.

45. Devin A, Cook A, Lin Y, Rodriguez Y, Kelliher M, Liu Z. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. Immunity. 2000;4:419–29.

46. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell. 1997;2:243–52.

47. Li XH, Fang X, Gaynor RB. Role of IKKgamma/nemo in assembly of the Ikappa B kinase complex. J Biol Chem. 2001;6:4494–500.

48. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 2004;6:280–8.

49. Yamamoto Y, Verma UN, Prajapati S, Kwak YT, Gaynor RB. Histone H3 phosphorylation by IKK-alpha is critical for cytokine-induced gene expression. Nature. 2003;6940:655–9.

50. Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol Rev. 1999;1:143–80.

51. Kumar S, Blake SM, Emery JG. Intracellular signaling pathways as a target for the treatment of rheumatoid arthritis. Curr Opin Pharmacol. 2001;3:307–13.

52. Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev. 2001;2:807–69.

53. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy–from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta. 2005;1–2:253–62.

54. Schindler JF, Monahan JB, Smith WG. p38 pathway kinases as anti-inflammatory drug targets. J Dent Res. 2007;9:800–11.

55. Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, et al. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem. 1996;9:4961–5.

56. Tibbetts MD, Zheng L, Lenardo MJ. The death effector domain protein family: regulators of cellular homeostasis. Nat Immunol. 2003;5:404–9.

57. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 1995;22:5579–88.

58. Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM. An induced proximity model for caspase-8 activation. J Biol Chem. 1998;5:2926–30.

59. Stratford May Jr W, Deng X. Apoptosis. In: Runge MS, Patterson C, editors. Principles of Molecular Medicine, 2nd edition. Totowa: Humana Press; 2006. pp. 709–719.

60. Naude PJ, den Boer JA, Luiten PG, Eisel UL. Tumor necrosis factor receptor cross-talk. FEBS J. 2011;6:888–98.

61. Sun SCLSC. New insights into NF-kappaB regulation and function. Trends Immunol. 2008;10:469–78.

62. Rauert H, Wicovsky A, Muller N, Siegmund D, Spindler V, Waschke J, Kneitz C, et al. Membrane tumor necrosis factor (TNF) induces p100 processing via TNF receptor-2 (TNFR2). J Biol Chem. 2010;10:7394–404.

63. Haider S, Knofler M. Human tumour necrosis factor: physiological and pathological roles in placenta and endometrium. Placenta. 2009;2:111–23.

64. Faustman D, Davis M. TNF receptor 2 pathway: drug target for autoimmune diseases. Nat Rev Drug Discov. 2010;6:482–93.

65. Zhang R, Xu Y, Ekman N, Wu Z, Wu J, Alitalo K, Min W. Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway. J Biol Chem. 2003;51:51267–76.

66. Zhou Z, Gengaro P, Wang W, Wang XQ, Li C, Faubel S, Rivard C, et al. Role of NF-kappaB and PI 3-kinase/Akt in TNF-alpha-induced cytotoxicity in microvascular endothelial cells. Am J Physiol Renal Physiol. 2008;4:F932–41.

67. Cone JB. Inflammation. Am J Surg. 2001;6:558–62.

68. Sherwood ER, Toliver-Kinsky T. Mechanisms of the inflammatory response. Best Pract Res Clin Anaesthesiol. 2004;3:385–405.

69. Rees DD, Monkhouse JE, Cambridge D, Moncada S. Nitric oxide and the haemodynamic profile of endotoxin shock in the conscious mouse. Br J Pharmacol. 1998;3:540–6.

70. Vallance P, Chan N. Endothelial function and nitric oxide: clinical relevance. Heart. 2001;3:342–50.

71. Tabernero A, Schneider F, Potenza MA, Randriamboavonjy V, Chasserot S, Wolf P, Mitolo-Chieppa D, et al. Cyclooxygenase-2 and inducible nitric oxide synthase in omental arteries harvested from patients with severe liver diseases: immunolocalization and influence on vascular tone. Intensive Care Med. 2003;2:262–70.

72. Sanders DB, Larson DF, Hunter K, Gorman M, Yang B. Comparison of tumor necrosis factor-alpha effect on the expression of iNOS in macrophage and cardiac myocytes. Perfusion. 2001;1:67–74.

73. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994;249-58.

74. Neumann P, Gertzberg N, Johnson A. TNF-alpha induces a decrease in eNOS promoter activity. Am J Physiol Lung Cell Mol Physiol. 2004;2:L452–9.

75. Mark KS, Trickler WJ, Miller DW. Tumor necrosis factor-alpha induces cyclooxygenase-2 expression and prostaglandin release in brain microvessel endothelial cells. J Pharmacol Exp Ther. 2001;3:1051–8.

76. Denzlinger C, Rapp S, Hagmann W, Keppler D. Leukotrienes as mediators in tissue trauma. Science. 1985;4723:330–2.

77. Friedl HP, Till GO, Trentz O, Ward PA. Roles of histamine, complement and xanthine oxidase in thermal injury of skin. Am J Pathol. 1989;1:203–17.

78. Chappell D, Hofmann-Kiefer K, Jacob M, Rehm M, Briegel J, Welsch U, Conzen P, et al. TNF-alpha induced shedding of the endothelial glycocalyx is prevented by hydrocortisone and antithrombin. Basic Res Cardiol. 2009;1:78–89.

79. Goldblum SE, Sun WL. Tumor necrosis factor-alpha augments pulmonary arterial transendothelial albumin flux in vitro. Am J Physiol. 1990;2(Pt 1):L57–67.

80. Angelini DJ, Hyun SW, Grigoryev DN, Garg P, Gong P, Singh IS, Passaniti A, et al. TNF-alpha increases tyrosine phosphorylation of vascular endothelial cadherin and opens the paracellular pathway through fyn activation in human lung endothelia. Am J Physiol Lung Cell Mol Physiol. 2006;6:L1232–45.

81. Campos MM, Souza GE, Calixto JB. Modulation of kinin B1 but not B2 receptors-mediated rat paw edema by IL-1beta and TNFalpha. Peptides. 1998;7:1269–76.

82. Chandrasekharan UM, Siemionow M, Unsal M, Yang L, Poptic E, Bohn J, Ozer K, et al. Tumor necrosis factor alpha (TNF-alpha) receptor-II is required for TNF-alpha-induced leukocyte-endothelial interaction in vivo. Blood. 2007;5:1938–44.

83. Mommsen P, Barkhausen T, Hildebrand F, Zeckey C, Krettek C. van GM. Regulation of L-selectin expression by trauma-relevant cytokines. Pathol Res Pract. 2011;3:142–7.

84. Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. J Clin Invest. 1989;6:2008–17.

85. Briscoe DM, Schoen FJ, Rice GE, Bevilacqua MP, Ganz P, Pober JS. Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. Transplantation. 1991;2:537–9.

86. Riewald M, Ruf W. Science review: role of coagulation protease cascades in sepsis. Crit Care. 2003;2:123–9.

87. Pawlinski R, Pedersen B, Kehrle B, Aird WC, Frank RD, Guha M, Mackman N. Regulation of tissue factor and inflammatory mediators by Egr-1 in a mouse endotoxemia model. Blood. 2003;10:3940–7.

88. Ulfhammer E, Larsson P, Karlsson L, Hrafnkelsdóttir T, Bokarewa M, Tarkowski A, Jern S. TNF-alpha mediated suppression of tissue type plasminogen activator expression in vascular endothelial cells is NF-kappaB- and p38 MAPK-dependent. J Thromb Haemost. 2006;8:1781–9.

89. Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and NF-kappaB signaling. Cell Res. 2011;1:103–15.

90. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol. 2004;3:181–9.

91. Yoshida LS, Tsunawaki S. Expression of NADPH oxidases and enhanced H(2)O(2)-generating activity in human coronary artery endothelial cells upon induction with tumor necrosis factor-alpha. Int Immunopharmacol. 2008;10:1377–85.

92. Rahman A, Kefer J, Bando M, Niles WD, Malik AB. E-selectin expression in human endothelial cells by TNF-alpha-induced oxidant generation and NF-kappaB activation. Am J Physiol. 1998;3(Pt 1):L533–44.

93. Li JM, Fan LM, Christie MR, Shah AM. Acute tumor necrosis factor alpha signaling via NADPH oxidase in microvascular endothelial cells: role of p47phox phosphorylation and binding to TRAF4. Mol Cell Biol. 2005;6:2320–30.

94. Bubici C, Papa S, Dean K, Franzoso G. Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance. Oncogene. 2006;51:6731–48.

95. Steinman L. Modulation of postoperative cognitive decline via blockade of inflammatory cytokines outside the brain. Proc Natl Acad Sci USA. 2010;48:20595–6.

96. Sethi G, Shanmugam MK, Ramachandran L, Kumar AP, Tergaonkar V. Multifaced link between cancer and inflammation. Biosci Rep. 2012;1:1–15.

97. Balkwill FR, Mantovani A. Cancer-related inflammation: common themes and therapeutic opportunities. Semin Cancer Biol. 2012;1:33–40.

98. Szlosarek P, Charles KA, Balkwill FR. Tumour necrosis factor-α as a tumour promoter. Eur J Cancer. 2006;6:745–50.

99. Balkwill F. Tumor necrosis factor or tumor promoting factor? Cytokine Growth Factor Ref. 2002;2:135–41.

100. Aggarwal BB, Shishodia S, Sandur SK, Pandey MK, Sethi G. Inflammation andcancer: how hot is the link? Biochem Pharmacol. 2006;11:1605–21.

101. Gaiotti D, Chung J, Iglesias M, Nees M, Baker PD, Evans CH, Woodworth CD. Tumornecrosis fact-alpha promotes human papillomavirus (HPV) E6/E7 RNA expression and cyclin-dependent kinase activity in HPV-immortalized keratinocytes by a ras-dependent pathway. Mol Carcinog. 2000;2:97–109.

102. Wu S, Boyer CM, Whitaker RS, Berchuck A, Wiener JR, Weinberg JB, Bast RC Jr. Tumornecrosis factor alpha as an autocrine and paracrine growth factor for ovarian cancer: monokine inductionof tumor cell proliferation and tumor necrosis factor alpha expression. Cancer Res. 1993;8:1939–44.

103. Jaiswal M, LaRusso NF, Burgart LJ, Gores GJ. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependentmechanism. Cancer Res. 2000;1:184–90.

104. Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, Kuwano M. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol. 1997;7:4015–23.

105. Cheng N, Chen J. Tumor necrosis factor-alpha induction of endothelial ephrin A1 expression is mediated by a p38 MAPK-andSAPK/JNK-dependent but nuclear factor-kappa B-independent mechanism. J Biol Chem. 2001;17:13771–7.

106. De Cesaris P, Starace D, Starace G, Filippini A, Stefanini M, Ziparo E. Activation of Jun N-terminal kinase/stress-activated protein kinase pathway by tumor necrosis factor alpha leads to intercellular adhesion molecule-1 expression. J Biol Chem. 1999;41:28978–82.

107. Bussolino F, Camussi G, Baglioni C. Synthesis and release of platelet-activating factor by human vascular endothelial cells treated with tumor necrosis factor or interleukin 1 alpha. J Biol Chem. 1988;24:11856–61.

108. Wu Y, Zhou BP. TNF-alpha/NF-kappaB/Snail pathway in cancer cell migration and invasion. Br J Cancer. 2010;4:639–44.

109. Hagemann T, Wilson J, Kulbe H, Li NF, Leinster DA, Charles K, Klemm F, Pukrop T, Binder C, Balkwill FR. Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK. J Immunol. 2005;2:1197–205.

110. Kulbe H, Hagemann T, Szlosarek PW, Balkwill FR, Wilson JL. The inflammatory cytokine tumor necrosis factor-alpha regulates chemokine receptor expression on ovarian cancer cells. Cancer Res. 2005;22:10355–62.

111. Warzocha K, Salles G, Bienvenu J, Bastion Y, Dumontet C, Renard N, Neidhardt-Berard EM, Coiffier B. Tumor necrosis factor ligand-receptor system can predict treatment outcome in lymphoma patients. J Clin Oncol. 1997;2:499–508.

Rostliny v terapii idiopatických střevních zánětů

Plants in the therapy of inflammatory bowel disease

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Souhrn: Léčivé rostliny slouží již několik tisíc let jako prostředky v léčbě různých onemocnění. Přestože se jejich obliba v průběhu historie měnila, zájem o rostliny a jejich obsahové látky dnes opět vzrůstá. Výzkum se nyní zaměřuje na určení obsahových látek zodpovědných za účinek a na objasnění jejich mechanizmu působení. Rostliny mají své místo i v doplňkové terapii chronických zánětlivých onemocnění. Chronické střevní záněty, jako jsou Crohnova choroba a ulcerózní kolitida, jsou chronická onemocnění gastrointestinálního traktu. Jejich incidence v posledních letech neustále narůstá a onemocnění má těžko předvídatelný průběh. Vzhledem k nejasné etiologii střevních zánětů není dosavadní terapie zcela úspěšná. Z důvodů častých nežádoucích účinků a nedostatečného efektu standardní léčby je použití komplementární a alternativní medicíny u pacientů velmi rozšířené. Rostlinné produkty tak mohou nemocným poskytnout alternativní nebo doplňkovou možnost léčby. Toto review přináší přehled rostlin a případně jejich obsahových látek používaných v doplňkové terapii chronických střevních zánětů s ohledem na jejich hodnocení v klinických studiích.

Klíčová slova: experimentální modely – fytoterapie – idiopatické střevní záněty – klinické studie

Summary: Medicinal plants have been used to treat various diseases for thousands of years. Although their popularity has changed in the course of history, the interest in plants and in their active compounds is currently increasing. Today's research focuses on determining the active substances responsible for the effect and their mechanism of action. Plants have also their place in complementary therapy of chronic inflammatory diseases. Inflammatory bowel disease (IBD), such as Crohn's disease and ulcerative colitis, are chronic diseases of the gastrointestinal tract. Their incidence has been increasing in recent years and it is difficult to predict the progression of the disease. Due to the unclear aetiology of inflammatory bowel disease, current therapy is not completely successful. Complementary and alternative medicine is widely used by patients because of frequent side effects and lack of effectiveness of the standard therapy. Plant products may provide an alternative or supplementary treatment option to patients. This review brings a survey of plants and their content compounds used in complementary therapy of inflammatory bowel disease with special relevance to clinical studies.

Key words: experimental animal models – phytotherapy – inflammatory bowel disease – clinical trials

Crohnova choroba (Crohn's disease – CD) a ulcerózní kolitida (ulcerative colitis – UC) jsou chronické zánětlivé nemoci souhrnně označované jako idiopatické střevní záněty (inflammatory bowel disease – IBD). Postihují různé části gastrointestinálního traktu, především tenké a tlusté střevo. Liší se od sebe hloubkou postižení trávicí trubice a lokalizací zánětu. Příčina vzniku IBD není doposud zcela objasněna. Předpokládá se spolupůsobení několika faktorů: abnormální slizniční imunitní reakce, genetické predispozice a mikrobiální či environmentální vlivy [1]. V současnosti se v terapii IBD uplatňuje zejména farmakoterapie a chirurgické postupy. Hlavními skupinami používaných látek jsou aminosalicyláty, glukokortikoidy, imunosupresiva, antibiotika, chemoterapeutika a biologická léčiva. Současná léčebná strategie však není vždy zcela efektivní, a řada pacientů se tak uchyluje ke komplementární a alternativní medicíně (complementary and alternative medicine – CAM).

CAM je označením pro nekonvenční medicínu a zahrnuje širokou škálu diagnostických a léčebných postupů. Na rozdíl od klasické medicíny je přístup k péči o pacienta holistický, což je obzvláště důležité u chronických onemocnění. V řadě případů přináší alternativní metody možnost snížit dávku a počet užívaných léčiv nebo se vyhnout operaci [2]. Z různých typů CAM je u pacientů s IBD preferována homeopatie, akupunktura a v neposlední řadě také fytoterapie [3].

Byla již provedena celá řada studií hodnotících účinnost a mechanizmus působení rostlinných extraktů nebo čistých látek *in vivo* na laboratorních zvířatech či v klinických studiích u pacientů s IBD. Různé internetové stránky a diskuze určené pacientům doporučují každodenní užívání aloe gelu, zeleného čaje, kurkumy nebo pelyňku [4,5]. Některá doporučení jsou podložena relevantními klinickými studiemi, jiná pouze experimenty na laboratorních zvířatech. Dlouhodobé zkušenosti s alternativní léčbou, včetně fytoterapie, mají především asijské země. Tradiční léčba IBD v Iránu zahrnuje např. podávání pryskyřice z keře *Pistacia lenticus* a stromu *Boswellia serrata*, semen jitrocele a řeřichy nebo plodů fenyklu [6].

Toto review si klade za cíl shrnout dosud známé výsledky *in vivo* studií a závěry klinických hodnocení. Publikační podklady byly vyhledány v databázích PubMed a Web of Knowledge pod klíčovými slovy: „experimental colitis", „clinical trials", „Crohn's disease", „ulcerative colitis", „protective", „plant extracts".

In vivo experimenty
Studie na laboratorních zvířatech využívají několika možných způsobů navození střevního zánětu. Každý způsob má svou charakteristickou patogenezi a žádný zcela lidskou IBD nekopíruje, přesto přináší možnost otestovat aktivitu a zjistit mechanizmus účinku nových biologicky aktivních látek. Nejčastěji jsou používány modely kolitidy indukované chemickými látkami, např. podáním kyseliny 2,4,6-trinitrobenzensulfonové (TNBS), kyseliny octové, dextran sulfátu sodného (DSS) nebo indomethacinu. Tyto chemické látky vyvolávají u laboratorních zvířat imunitní reakci, ovlivňují hladinu ochranných prostaglandinů nebo způsobují přímé poškození střevní tkáně. Další modely vedou ke spontánnímu vývoji kolitidy využitím genových manipulací (interleukin (IL)-7 transgenní myši, myši s knock-outovaným genem pro IL-10).

Historicky nejstarší způsob navození střevního zánětu je založen na adoptivním buněčném transferu T lymfocytů do imunodeficientních zvířat, nejnovější modely využívají vlivu γ-záření [7].

Testované přírodní látky účinkují na experimentálních modelech kolitidy různými mechanizmy – působí antioxidačně, ovlivňují expresi zánětlivých cytokinů nebo zasahují do metabolizmu kyseliny arachidonové a signální dráhy vedoucí k aktivaci nukleárního faktoru (NF)-κB [8].

Jedním z etiologických faktorů Crohnovy choroby a ulcerózní kolitidy je abnormální slizniční imunitní reakce. V zánětlivě změněné sliznici gastrointestinálního traktu pacientů s IBD bylo nalezeno zvýšené množství T_H lymfocytů, makrofágů, neutrofilů a mastocytů. S tím souvisí i vyšší exprese prozánětlivých cytokinů jako např. IL-1ß, IL-6, IL-12, tumor nekrotizujícího faktoru – TNFα, interferonu (IFN)-γ [8]. Vliv na expresi těchto proteinů byl prokázán u celé řady rostlinných extraktů a izolovaných látek. Například kurkumin z rostliny *Curcuma longa* vykazoval tyto účinky v řadě *in vivo* studií [9–11] a na jejich základě byl zahrnut do klinických hodnocení, stejně jako látky získané z *Cannabis sativa* [12,13] či *Tripterygium wilfordii* [14,15].

Významnou roli by v etiologii střevních zánětů mohl hrát také oxidativní stres. Nedávné studie poukázaly na sníženou antioxidační kapacitu a zvýšené množství volných kyslíkových radikálů u pacientů s IBD [8]. U rostlin je v *in vivo* studiích testována schopnost redukovat peroxidaci lipidů a zvyšovat celkovou antioxidační kapacitu. Proto je sledována aktivita myeloperoxidázy, hladiny malondialdehydu, superoxiddismutáz, katalázy či glutathionu. Antioxidační efekt byl na experimentálních modelech kolitidy prokázán např. u rostlin *Polygonum multiflorum* [16], *Vitis vinifera* [17], *Camellia sinensis* [18,19] nebo *Zingiber officinale* [20].

V zánětlivém procesu hraje důležitou roli metabolizmus kyseliny arachidonové související s produkcí cyklooxygenáz (COX). COX-2 je inducibilní formou enzymu katalyzujícího syntézu prostaglandinů na základě působení prozánětlivých cytokinů. Snížit hladinu COX-2 u experimentálních modelů kolitidy dokázal resveratrol z *Vitis vinifera* [17,21,22], kurkumin z *Curcuma longa* [23–25], berberin z *Berberis vulgaris* [26] nebo glykoprotein z plodů rostliny *Gardenia jasminoides* [27]. Tyto přírodní látky také snižovaly hladinu oxidu dusnatého a inducibilní syntázy oxidu dusnatého (iNOS), která je rovněž zapojena do zánětlivých reakcí [9,17,22,25–27]. Nadprodukce dalšího metabolitu kyseliny arachidonové, leukotrienu (LT) B_4, byla pozorována u pacientů s různými chronickými zánětlivými chorobami, včetně IBD. Prozatím byl efekt redukce LT B_4 popsán u *Aloe vera* [28] a *Plantago ovata* [29].

U pacientů s chronickými střevními záněty byla dále zjištěna nadměrná aktivace NF-κB, transkripčního faktoru regulujícího expresi řady genů spojených se zánětlivou reakcí a apoptózou. Rostliny jako např. *Berberis vulgaris* [26], *Curcuma longa* [30], *Camellia sinensis* [31–33] a *Ginkgo biloba* [34] inhibují aktivaci nukleárního faktoru na různých úrovních signální dráhy.

Klinické studie
Klinické studie sledující účinnost fytoterapie jsou prováděny u pacientů s Crohnovou chorobou i ulcerózní kolitidou. Hodnotí se především změna aktivity onemocnění, endoskopický nález, doba setrvání v remisi nebo její dosažení a potřeba užití dalších léků. Vyšší výpovědní hodnotu mají studie randomizované, dvojitě zaslepené a s větším počtem pacientů. Ze studií s malým počtem pacientů, nekontrolovaných a nezaslepených nelze vyvodit spolehlivé závěry, mohou však sloužit jako podklady pro další hodnocení.

Jednou z přírodních látek, která by mohla mít do budoucna své využití v doplňkové terapii IBD, je kurkumin (v potravinářství označovaný jako E100), žluté přírodní barvivo extraho-

Tab. 1. Přehled klinických studií u pacientů s ulcerózní kolitidou.
Tab. 1. Summary of clinical trials of patients with ulcerative colitis.

Rostlina	Použitá část	Koncept studie	Počet pacientů	Výsledky studie	Studie
Aloe vera	gel	dvojitě zaslepená, randomizovaná, placebem kontrolovaná	44	remise u 30 % pacientů, v placebo skupině u 7 %; ↓SCCAI, histologické skóre	[45]
Andrographis paniculata	etanolický extrakt z rostliny HMPL-004	dvojitě zaslepená, randomizovaná, kontrolovaná mesalazinem	120	klinická remise u 21 % pacientů, ve skupině užívající mesalazin u 16 %; srovnatelný účinek s mesalazinem	[37]
	etanolický extrakt z rostliny HMPL-004	dvojitě zaslepená, randomizovaná, placebem kontrolovaná	224	remise u 34 % pacientů užívajících 1 200 mg, u 38 % užívajících 1 800 mg; v placebo skupině u 25 %	[38]
Angelica sinensis	ASI (léková forma pro injekční podání)	randomizovaná, kontrolovaná běžnou léčbou	94	inhibice aktivace trombocytů, zlepšení mikrocirkulace; ↓vaskulární poškození endotelu	[54]
Boswellia serrata	pryskyřice	kontrolovaná sulfasalazinem	neuvedeno	remise u 82 % pacientů, ve skupině užívající sulfasalazin u 75 %; ↓CAI	[40]
	pryskyřice	kontrolovaná sulfasalazinem	30	srovnatelný účinek se sulfasalazinem v dosažení remise; ↓CAI	[41]
Curcuma longa	kurkumin	pilotní	5	zlepšení počtu a kvality stolice; ↓CRP, rychlost sedimentace	[35]
	kurkumin	dvojitě zaslepená, randomizovaná, placebem kontrolovaná	89	relaps u 4,65 % pacientů , v placebo skupině u 20,51 %; ↓CAI a endoskopické hodnocení	[36]
Hordeum vulgare	naklíčený ječmen	otevřená	18	↓CAI, ↑fekální mikroflóra *Bifidobacterium spp.* a *Eubacterium limosum*	[47]
	naklíčený ječmen	otevřená	59	↓CAI	[48]
	naklíčený ječmen	otevřená	41	↓TNFα, IL-6, IL-8	[49]
Oenothera biennis	olej	randomizovaná, placebem kontrolovaná, srovnání s rybím a olivovým olejem	43	pupalkový olej významně zlepšil konzistenci stolice ve srovnání s rybím olejem a placebem	[53]
Plantago ovata	vláknina ze semen	randomizovaná, otevřená, kontrolovaná mesalazinem	105	srovnatelný účinek s mesalazinem	[50]
Triticum aestivum	šťáva z klíčků pšenice	dvojitě zaslepená, randomizovaná, placebem kontrolovaná	23	↓CAI a rektální krvácení	[46]

CAI – index aktivity kolitidy, CRP – C-reaktivní protein, IL – interleukin, SCCAI – zjednodušený index aktivity kolitidy, TNF – tumor nekrotizující faktor

vané z oddenku rostliny *Curcuma longa*. V řadě *in vivo* studií na různých modelech kolitidy se potvrdily jeho protizánětlivé i antioxidační účinky. Podávání kurkuminu zlepšilo makroskopické i histologické skóre, snížilo expresi prozánětlivých a zvýšilo expresi protizánětlivých cytokinů. Kurkumin potlačuje aktivaci NF-κB, snižuje expresi COX-2 a iNOS a neutrofilní infiltraci [9–11,23–25,30]. Výsledkem pilotní studie u pacientů s CD bylo snížení indexu aktivity onemocnění [35]. Pacientům s UC umožnilo přidání kurkuminu ke stávající terapii snížit dávky nebo úplně vysadit léčbu mesalazinem. Tato přírodní látka se rovněž osvědčila jako doplněk ke standardní terapii v udržovací léčbě UC. Randomizovaná, dvojitě zaslepená multicentrická studie vykázala nižší frekvenci relapsu onemocnění při kombinaci standardní terapie s kurkuminem ve srovnání s monoterapií aminosalicyláty [35,36].

Účinek srovnatelný s mesalazinem prokázal také extrakt z rostliny *Andrographis paniculata* u pacientů s různě aktivní UC. Rostlina je široce používaná k léčbě zánětlivých a infekčních onemocnění v asijských zemích. Za účinek extraktu jsou pravděpodobně zodpovědné diterpenické laktony andragrafolidy [37,38].

Další diskutovanou rostlinou je *Boswellia serrata* a její pryskyřice, která je testována ve formě různých extraktů. Efekt srovnatelný se standardní terapií vykázal pouze chloroform/metanolický extrakt s označením H15 u pacientů s aktivní CD [39] a čistá pryskyřice u pacientů s aktivní UC [40,41]. Větší frekvence remise u pacientů užívajících pryskyřici naznačuje, že by mohla být účinným lékem v terapii UC s minimálními vedlejšími účinky. Za působení je pravděpodobně zodpovědný derivát triterpenické kyseliny boswellové, acetyl-11-keto-ß-boswellová kyselina (AKBA), které je přiřazována řada biologických účinků [42]. To potvrzuje také *in vivo* experiment na modelu indomethacinové kolitidy. Extrakt H15 a AKBA prokázaly protizánětlivé i antioxidační působení a významně snížily poškození střevní tkáně [43]. Ostatní typy extraktů nevykazovaly účinek [44].

Vědci se v klinických studiích rovněž zaměřili na *Aloe vera*, rostlinu, která byla u pacientů s IBD roky používána i přes nedostatek důkazů o jejích účincích na střevní záněty. Nedávné *in vivo* studie na modelu DSS indukované kolitidy u potkanů prokázaly, že gel z aloe i jeho složky, aloin a aloesin, významně snižují hladinu prozánětlivých markerů jako TNFα, IL-1ß či aktivitu myeloperoxidázy [28]. Klinické studie hodnotily účinnost gelu u pacientů s aktivní ulcerózní kolitidou. Perorální léčba gelem z aloe dokázala snížit index aktivity onemocnění a zlepšit histologické skóre více než placebo [45].

Svými výsledky v klinickém testování zaujaly i obiloviny z čeledi Poaceae, ječmen (*Hordeum vulgare*) a pšenice (*Triticum aestivum*). Užívání pšeničné šťávy je pro léčbu různých gastrointestinálních obtíží a pro celkovou detoxikaci organizmu doporučováno po více než 30 let [46]. Randomizovaná dvojitě zaslepená kontrolovaná studie prokázala u pacientů s aktivní UC vý-

znamné snížení indexu aktivity onemocnění a snížení závažnosti rektálního krvácení [46]. Naklíčený ječmen se pak v různých studiích projevil jako vhodná doplňková léčba u aktivní UC [47] i u pacientů v remisi [48]. Ječmen snížil klinickou aktivitu choroby, snížil hladinu zánětlivých interleukinů a prodloužil dobu remise [49]. Za účinek naklíčeného ječmene jsou pravděpodobně zodpovědné kyselina máselná a propionová, které vznikají jeho fermentací ve střevě. Podobně je tomu u semen *Plantago ovata*. Randomizovaná klinická studie prokázala, že jejich přidání ke standardní terapii usnadňuje u UC dosažení remise [50]. *In vivo* studie na transgenních potkanech potvrdila, že právě mastné kyseliny s krátkým řetězcem inhibují produkci TNFα [29].

Léčebné účinky *Cannabis sativa* jsou již řadu let zkoumány v různých oborech medicíny. K popisu účinku konopí u pacientů s CD byla provedena retro-

Tab. 2. Přehled klinických studií u pacientů s Crohnovou chorobou.
Tab. 2. Summary of clinical trials of patients with Crohn's disease.

Rostlina	Použitá část	Koncept studie	Počet pacientů	Výsledky studie	Studie
Arthemisia absinthium	práškovaná droga	dvojitě zaslepená, placebem kontrolovaná	40	remise u 65 % pacientů, v placebo skupině u žádného; ↓Hamiltonovy stupnice deprese	[56]
Boswellia serrata	extrakt z pryskyřice H15	dvojitě zaslepená, randomizovaná, kontrolovaná mesalazinem	102	↓CDAI srovnatelné s mesalazinem	[39]
	etanolický extrakt z pryskyřice (Boswelan)	dvojitě zaslepená, randomizovaná, placebem kontrolovaná	108	v remisi zůstalo 59,9 % pacientů, v placebo skupině 55,3 %; žádné výhody u pacientů užívajících extrakt	[42]
Cannabis sativa	konopí pro lékařské účely	retrospektivní, observační	30	↓CDAI, ↓potřeba užívání dalších léků a operace	[51]
Curcuma longa	kurkumin	pilotní	5	↓CDAI, CRP, rychlost sedimentace	[35]
Pistacia lenticus	pryskyřice	pilotní	18	↓CDAI, CRP, IL-6; ↑celková antioxidační kapacita	[57]
Tripterygium wilfordii	chloroform/ metanolický extrakt T2	otevřená	20	↓CRP, TNFα a IL-1β; ↓CDAI	[59]
Vaccinium myrtillus	komerční preparát s vysokým obsahem anthokyaninů	pilotní	13	↓Mayo skóre, ↓kalprotektin ve stolici	[55]

CDAI – index aktivity Crohnovy choroby, CRP – C-reaktivní protein, IL – interleukin, TNF – tumor nekrotizující faktor

spektivní observační studie, která zjistila, že konopí snižuje index aktivity onemocnění a potřebu užívání dalších léků a operací. Přestože kouření bylo nejčastějším způsobem aplikace, protizánětlivý účinek mohl být stejně dobře dosažen i perorálním podáním [51]. *In vivo* studie potvrzují účinky na různých modelech kolitidy. Testovaný kanabidiol a kanabigerol, nepsychotropní kanabinoidy, snižují expresi iNOS, modulují hladiny cytokinů a vykazují antioxidační účinky [12,13,52].

Součástí řady doplňků stravy je olej ze semen pupalky, *Oenothera biennis*. Studie srovnávala účinky pupalkového oleje, olivového oleje a rybího tuku jako doplňku obvyklé terapie. Pupalkový olej jako jediný zlepšil konzistenci stolice, ale nepřinesl žádný jiný terapeutický efekt, stejně jako ostatní [53].

Klinické hodnocení rostliny *Angelica sinensis* proběhlo v návaznosti na zjištění, že u pacientů s UC jsou výrazně aktivovány trombocyty, což by mohlo souviset s vaskulárním poškozením endotelu. Andělika zpracovaná do lékové formy pro injekční podání inhibovala aktivaci trombocytů, snížila vaskulární poškození endotelu a zlepšila mikrocirkulaci. S účinkem je spojován ferulát sodný, hlavní složka injekcí [54].

Borůvky, plody *Vaccinium myrtillus*, jsou bohatým zdrojem fenolických látek, především anthokyaninů, u nichž byly prokázány antioxidační a protizánětlivé účinky. Pilotní studie ověřovala efekt vysokých dávek anthokyaninů u pacientů s ulcerózní kolitidou. Po šesti týdnech léčby došlo ke snížení aktivity onemocnění až ke kompletní remisi, která však nebyla udržitelná po vysazení testovaného preparátu [55].

Možnost vysazení terapie kortikoidy u pacientů s CD zjišťovala studie s bylinnou směsí obsahující prášek z pelyňku *Arthemisia absinthium*. I přes postupné snižování dávek kortikoidů se příznaky CD zmírňovaly a po několika týdnech došlo u pacientů užívajících bylinnou směs ke kompletní remisi onemocnění. Mimo jiné vyhodnocení Hamiltonovy stupnice deprese ukázalo, že pelyněk má vliv na náladu a kvalitu života pacientů [56].

V pilotní studii s pryskyřicí keře *Pistacia lenticus* u pacientů s aktivní CD bylo zjištěno snížení plazmatických hladin IL-6 a C-reaktivního proteinu (CRP). Významně se snížil i index aktivity choroby. Hlavní složkou pryskyřice, tzv. mastichy, je kyselina oleanolová, triterpen s protizánětlivými a protinádorovými účinky [57,58].

Extrakt z rostliny *Tripterygium wilfordii* byl také testován u pacientů s aktivní CD. Terapie extraktem vedla k poklesu plazmatických hladin CRP, TNFα a IL-1ß [59]. Účinek je přiřazován diterpenoidní látce triptolidu, jejíž aktivita byla potvrzena i na experimentálních modelech kolitidy. Podávání triptolidu vedlo u myší ke snížení exprese prozánětlivých cytokinů a k inhibici signální dráhy NF-κB [14,15].

Výsledky klinických studií na pacientech s UC a CD shrnují tab. 1 a 2.

Závěr

Rostlinná říše je zdrojem obrovského množství účinných látek, které by mohly přispět k zefektivnění terapie idiopatických střevních zánětů. Řada látek a extraktů je schopna snižovat index aktivity onemocnění, prodlužovat dobu klinické remise a snižovat poškození střevní tkáně a potřebu užívání dalších léků nebo nutnost operace. Rostliny, které pozitivně ovlivňují průběh onemocnění, jsou různorodou skupinou, ať už svým systematickým zařazením nebo obsahovými látkami. *In vivo* experimenty osvětlují mechanizmus účinku a klinická hodnocení sledují jejich působení na lidský organizmus. Výsledky studií poukazují na možnost využití některých přírodních látek v terapii chronických střevních zánětů samostatně či v kombinaci se standardní léčbou. Pro získání přesvědčivých důkazů o benefitu fytoterapie je však nutné provedení dalších, rozsáhlejších a zejména multicentrických, studií.

Literatura

1. Sartor RB. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. Nat Clin Pract Gastroenterol Hepatol 2006; 3(7): 390–407.
2. Bensoussan M, Jovenin N, Garcia B et al. Complementary and alternative medicine use by patients with inflammatory bowel disease: results from a postal survey. Gastroenterol Clin Biol 2006; 30(1): 14–23.
3. Langmead L, Rampton DS. Review article: complementary and alternative therapies for inflammatory bowel disease. Aliment Pharmacol Ther 2006; 23(3): 341–349. doi: 10.1111/j.1365-2036.2006.02761.x.
4. Life and IBD. Complementary and Alternative medicines (CAMs). [online]. Available from: http://www.lifeandibd.org/ulcerative-colitis-teenager/taking-control-of-your-ibd/complementary-and-alternative-medicines.html.
5. Mayo Clinic. Ulcerative Colitis – Alternative medicine. [online]. Available from: http://www.mayoclinic.com/health/ulcerative-colitis/DS00598/DSECTION=alternative-medicine.
6. Rahimi R, Shams-Ardekani MR, Abdollahi M. A review of the efficacy of traditional Iranian medicine for inflammatory bowel disease. World J Gastroenterol 2010; 16(36): 4504–4514.
7. Jurjus AR, Khoury NN, Reimund JM. Animal models of inflammatory bowel disease. J Pharmacol Toxicol Methods 2004; 50(2): 81–92.
8. Rahimi R, Mozaffari S, Abdollahi M. On the use of herbal medicines in management of inflammatory bowel diseases: a systematic review of animal and human studies. Dig Dis Sci 2009; 54(3): 471–480. doi: 10.1007/s10620-008-0368-x.
9. Ukil A, Maity S, Karmakar S et al. Curcumin, the major component of food flavour turmeric, reduces mucosal injury in trinitrobenzene sulphonic acid-induced colitis. Br J Pharmacol 2003; 139(2): 209–218.
10. Jian YT, Wang JD, Mai GF et al. Modulation of intestinal mucosal inflammatory factors by curcumin in rats wih colitis. Di Yi Jun Yi Da Xue Xue Bao 2004; 24(12): 1353–1358.
11. Zhang M, Deng CS, Zheng JJ et al. Curcumin regulated shift from Th1 to Th2 in trinitrobenzene sulphonic acid-induced chronic colitis. Acta Pharmacol Sin 2006; 27(8): 1071–1077.
12. Borrelli F, Aviello G, Romano B et al. Cannabidiol, a safe and non-psychotropic ingredient of the marijuana plant Cannabis sativa, is protective in a murine model of

colitis. J Mol Med 2009; 87(11): 1111–1121. doi: 10.1007/s00109-009-0512-x.

13. Borrelli F, Fasolino I, Romano B et al. Beneficial effect of the non-psychotropic plant cannabinoid cannabigerol on experimental inflammatory bowel disease. Biochem Pharmacol 2013; 85(9): 1306–1316. doi: 10.1016/j.bcp.2013.01.017.

14. Wei X, Gong J, Zhu J et al. Therapeutic effects of triptolide on interleukin-10 gene-deficient mice with colitis. Int Immunopharmacol 2008; 8(13–14): 1808–1812. doi: 10.1016/j.intimp.2008.08.019.

15. Wei X, Gong J, Zhu J et al. The suppressive effect of triptolide on chronic colitis and TNF-alpha/TNFR2 signal pathway in interleukin-10 deficient mice. Clin Immunol 2008; 129(2): 211–218. doi: 10.1016/j.clim.2008.07.018.

16. Wang X, Zhao L, Han T et al. Protective effects of 2,3,5,4'-tetrahydroxystilbene-2-O-beta-d-glucoside, an active component of Polygonum multiflorum Thunb, on experimental colitis in mice. Eur J Pharmacol 2008; 578(2–3): 339–348.

17. Wang YH, Yang XL, Wang L et al. Effects of proanthocyanidins from grape seed on treatment of recurrent ulcerative colitis in rats. Can J Physiol Pharmacol 2010; 88(9): 888–898. doi: 10.1139/y10-071.

18. Maity S, Ukil A, Karmakar S et al. Thearubigin, the major polyphenol of black tea, ameliorates mucosal injury in trinitrobenzene sulfonic acid-induced colitis. Eur J Pharmacol 2003; 470(1–2): 103–112.

19. Brückner M, Westphal S, Domschke W et al. Green tea polyphenol epigallocatechin-3-gallate shows therapeutic antioxidative effects in a murine model of colitis. J Crohns Colitis 2012; 6(2): 226–235. doi: 10.1016/j.crohns.2011.08.012.

20. El-Abhar HS, Hammad LN, Gawad HS et al. Modulating effect of ginger extract on rats with ulcerative colitis. J Ethnopharmacol 2008; 118(3): 367–372. doi: 10.1016/j.jep.2008.04.026.

21. Martín AR, Villegas I, La Casa C et al. Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. Biochem Pharmacol 2004; 67(7): 1399–1410.

22. Sánchez-Fidalgo S, Cárdeno A, Villegas I et al. Dietary supplementation of resveratrol attenuates chronic colonic inflammation in mice. Eur J Pharmacol 2010; 633(1–3): 78–84. doi: 10.1016/j.ejphar.2010.01.025.

23. Jiang H, Deng CS, Zhang M et al. Curcumin-attenuated trinitrobenzene sulphonic acid induces chronic colitis by inhibiting expression of cyclooxygenase-2. World J Gastroenterol 2006; 12(24): 3848–3853.

24. Zhang M, Deng C, Zheng J et al. Curcumin inhibits trinitrobenzene sulphonic acid-induced colitis in rats by activation of peroxisome proliferator-activated receptor gamma. Int Immunopharmacol 2006; 6(8): 1233–1242.

25. Camacho-Barquero L, Villegas I, Sánchez-Calvo JM et al. Curcumin, a Curcuma longa constituent, acts on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic experimental colitis. Int Immunopharmacol 2007; 7(3): 333–342.

26. Lee IA, Hyun YJ, Kim DH. Berberine ameliorates TNBS-induced colitis by inhibiting lipid peroxidation, enterobacterial growth and NF-κB activation. Eur J Pharmacol 2010; 648(1–3): 162–170. doi: 10.1016/j.ejphar.2010.08.046.

27. Oh PS, Lim KT. Plant originated glycoprotein has anti-oxidative and anti-inflammatory effects on dextran sulfate sodium-induced colitis in mouse. J Biomed Sci 2006; 13(4): 549–560.

28. Park MY, Kwon HJ, Sung MK. Dietary aloin, aloesin, or aloe-gel exerts anti-inflammatory activity in a rat colitis model. Life Sci 2011; 88(11–12): 486–492. doi: 10.1016/j.lfs.2011.01.010.

29. Rodríquez-Cabezas ME, Gálvez J, Camuesco D et al. Intestinal anti-inflammatory activity of dietary fiber (Plantago ovata seeds) in HLA-B27 transgenic rats. Clin Nutr 2003; 22(5): 463–471.

30. Jian YT, Mai GF, Wang JD et al. Preventive and therapeutic effects of NF-kappaB inhibitor curcumin in rats colitis induced by trinitrobenzene sulfonic acid. World J Gastroenterol 2005; 11(12): 1747–1752.

31. Ran ZH, Chen C, Xiao SD. Epigallocatechin-3-gallate ameliorates rats colitis induced by acetic acid. Biomed Pharmacother 2008; 62(3): 189–196. doi: 10.1016/j.biopha.2008.02.002.

32. Abboud PA, Hake PW, Burroughs TJ et al. Therapeutic effect of epigallocatechin-3-gallate in a mouse model of colitis. Eur J Pharmacol 2008; 579(1–3): 411–417.

33. Ukil A, Maity S, Das PK. Protection from experimental colitis by theaflavin-3,3´-digallate correlates with inhibition of IKK and NF-kappaB activation. Br J Pharmacol 2009; 149(1): 121–131.

34. Zhou YH, Yu JP, Liu YF et al. Effects of Ginkgo biloba extract on inflammatory mediators (SOD, MDA, TNF-alpha, NF-kappaBp65, IL-6) in TNBS-induced colitis in rats. Mediators Inflamm 2006; 2006(5): 92642.

35. Holt PR, Katz S, Kirshoff R. Curcumin therapy in inflammatory bowel disease: a pilot study. Dig Dis Sci 2005; 50(11): 2191–2193.

36. Hanai H, Iida T, Takeuchi K et al. Curcumin maintenance therapy for ulcerative colitis: randomized, multicenter, double-blind, placebo-controlled trial. Clin Gastroenterol Hepatol 2006; 4(12): 1502–1506.

37. Tang T, Targan SR, Li ZS et al. Randomised clinical trial: herbal extract HMPL-004 in active ulcerative colitis – a double-blind comparison with sustained release mesalazine. Aliment Pharmacol Ther 2011; 33(2): 194–202. doi: 10.1111/j.1365-2036.2010.04515.x.

38. Sandborn WJ, Targan SR, Byers VS et al. Andrographis paniculata extract (HMPL-004) for active ulcerative colitis. Am J Gastroenterol 2013; 108(1): 90–98. doi: 10.1038/ajg.2012.340.

39. Gerhardt H, Seifert F, Buvari P et al. Therapy of active Crohn disease with Boswellia serrata extract H 15. J Gastroenterol 2001; 39(1): 11–17.

40. Gupta I, Parihar A, Malhotra P et al. Effects of Boswellia serrata gum resin in patients with ulcerative colitis. Eur J Med Res 1997; 2(1): 37–43.

41. Gupta I, Parihar A, Malhotra P et al. Effects of gum resin of Boswellia serarata in patients with chronic colitis. Planta Med 2001; 67(5): 391–395.

42. Anthoni C, Laukoetter MG, Rijcken E et al. Mechanisms underlying the anti-inflammatory action of boswellic acid derivatives in experimental colitis. Am J Physiol Gastrointest Liver Physiol 2006; 290(6): G1131–G1137.

43. Krieglstein CF, Anthoni C, Rijcken EJ et al. Acetyl-11-keto-beta-boswellic acid, a constituent of a herbal medicine from Boswellia serrata resin, attenuates experimental ileitis. Int J Colorectal Dis 2001; 16(2): 88–95.

44. Holtmeier W, Zeuzem S, Preiss J et al. Randomized, placebo-controlled, double-blind trial of Boswellia serrata in maintaining remission of Crohn´s disease: good safety profile but lack of efficacy. Inflamm Bowel Dis 2011; 17(2): 573–582. doi: 10.1002/ibd.21345.

45. Langmead L, Feakins RM, Goldthorpe S et al. Randomized, double-blind, placebo-controlled trial of oral aloe vera gel for active ulcerative colitis. Aliment Pharmacol Ther 2004; 19(7): 739–747.

46. Ben-Arye E, Goldin E, Wengrower D et al. Wheat grass juice in the treatment of active distal ulcerative colitis: a randomized double-blind placebo-controlled trial. Scand J Gastroenterol 2002; 37(4): 444–449.

47. Kanauchi O, Suga T, Tochihara M et al. Treatment of ulcerative colitis by feeding with germinated barley foodstuff: first re-

port of a multicenter open control trial. J Gastroenterol 2002; 37 (Suppl 14): 67–72.

48. Hanai H, Kanauchi O, Mitsuyama K et al. Germinated barley foodstuff prolongs remission in patients with ulcerative colitis. Int J Mol Med 2004; 13(5): 643–647.

49. Faghfoori Z, Navai L, Shakerhosseini R et al. Effects of an oral supplementation of germinated barley foodstuff on serum tumour necrosis factor-alpha, interleukin-6 and -8 in patients with ulcerative colitis. Ann Clin Biochem 2011; 48(3): 233–237. doi: 10.1258/ /acb.2010.010093.

50. Fernández-Banares F, Hinojosa J, Sánchez-Lombrana JL et al. Randomized clinical trial of Plantago ovata seeds (dietary fiber) as compared with mesalamine in maintaining remission in ulcerative colitis. Spanish Group for the Study of Crohn's Disease and Ulcerative Colitis (GETECCU). Am J Gastroenterol 1999; 94(2): 427–433.

51. Naftali T, Lev LB, Yablecovitch D et al. Treatment of Crohn´s disease with cannabis: an observational study. Isr Med Assoc J 2011; 13(8): 455–458.

52. Schicho R, Storr M. Topical and systemic cannabidiol improves trinitrobenzene sulfonic acid colitis in mice. Pharmacology 2012; 89(3–4): 149–155. doi: 10.1159/000336871.

53. Greenfield SM, Green AT, Teare JP et al. A randomized controlled study of evening primrose oil and fish oil in ulcerative colitis. Aliment Pharmacol Ther 1993; 7(2): 159–166.

54. Dong WG, Liu SP, Zhu HH et al. Abnormal function of platelets and role of angelica sinensis in patients with ulcerative colitis. World J Gastroenterol 2004; 10(4): 606–609.

55. Biedermann L, Mwinyi J, Scharl M et al. Bilberry ingestion improves disease activity in mild to moderate ulcerative colitis – an open pilot study. J Crohns Colitis 2013; 7(4): 271–279. doi: 10.1016/j. crohns.2012.07.010.

56. Omer B, Krebs S, Omer H et al. Steroid--sparing effect of wormwood (Artemisia absinthium) in Crohn's disease: a double--blind placebo-controlled study. Phytomedicine 2007; 14(2–3): 87–95.

57. Kaliora AC, Stathopoulou MG, Triantafillidis JK et al. Chios mastic treatment of patients with active Crohn's disease. World J Gastroenterol 2007; 13(5): 748–753.

58. Liu J. Oleanolic acid and ursolic acid: research perspectives. J Ethnopharmacol 2005; 100(1–2): 92–94.

59. Ren J, Tao Q, Wang X et al. Efficacy of T2 in active Crohn's disease: a prospective study report. Dig Dis Sci 2007; 52(8): 1790–1797.

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Original Article

Effect of Diplacone on LPS-Induced Inflammatory Gene Expression in Macrophages

(geranyl flavanone / inflammation / MCP-1 / mRNA / TNF-α / ZFP36)

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Abstract. Flavonoids are commonly studied for their anti-inflammatory effects; however, this is the first paper describing the possible antiphlogistic activity of a geranylated flavanone. This study focused on the ability of diplacone to modulate the gene expression of pro-inflammatory tumour necrosis factor α and monocyte chemoattractant protein 1, and of anti-inflammatory zinc finger protein 36. The action of diplacone was also compared with that of conventional drug indomethacin. Human monocyte-derived macrophages of the human monocytic leukaemia cell line were pretreated with diplacone or indomethacin. Subsequently, inflammatory reaction was induced by lipopolysaccharide, and changes of tumour necrosis factor α, monocyte chemoattractant protein 1 and zinc finger protein 36 gene expression at the transcriptional level were measured. In this model, diplacone significantly down-regulated the expression of tumour necrosis factor α and monocyte chemoattractant protein 1 and up-regulated the zinc finger protein 36 expression. This makes diplacone a promising molecule for treatment of the inflammatory stage of diseases. The effect of diplacone in decreasing lipopolysaccharide-induced inflammatory gene expression is in many ways similar to that of the conventional drug indomethacin.

Introduction

Diplacone (also known as propoline C or nymphaeol A) is a naturally occurring geranyl flavanone that belongs to plant polyphenols (Fig. 1). Wu et al. (2008) demonstrated that adding an alkyl side chain can markedly change the features of the original natural compound. Plant polyphenols, especially flavonoids, have been studied intensively for their potential therapeutic applications, as was described in two excellent reviews (Havsteen, 2002; Dixon, 2004). Diplacone has been found in extracts of *Paulownia tomentosa* Steud. (*Scrophulariaceae*) (Smejkal et al., 2007), *Macaranga tanarius* (L.) Muell. Arg. (*Euphorbiaceae*) (Phommart et al., 2005), *Macaranga alnifolia* Baker (*Euphorbiaceae*) (Yoder et al., 2007), *Schizolaena hystrix* Capuron (*Sarcolaenaceae*) (Murphy et al., 2005), and *Mimulus clevelandii* Brandegee (*Scrophulariaceae*) (Phillips et al., 1996) and also in some kinds of propolis (Chen et al., 2004). Several biological activities of diplacone have been described. For example, cytotoxic and anti-cancer or anti-proliferation effects on various human carcinoma cells (Yoder et al., 2007; Smejkal et al., 2008a), probably caused by the ability of diplacone to induce apopto-

Abbreviations: AU – arbitrary unit, AUC – area under curve, DMSO – dimethylsulphoxide, FBS – foetal bovine serum, ERK – extracellular receptor kinase, iNOS – inducible isoform of nitric oxide synthase, LPS – lipopolysaccharide, MCP-1 – monocyte chemoattractant protein 1, PBS – phosphate-buffered saline, PCR – polymerase chain reaction, PMA – phorbol myristate acetate, RT-qPCR – reverse transcription quantitative polymerase chain reaction, THP-1 – human monocytic leukaemia cell line, TNF-α – tumour necrosis factor α, TTP – tristetraprolin, ZFP36 – zinc finger protein 36.
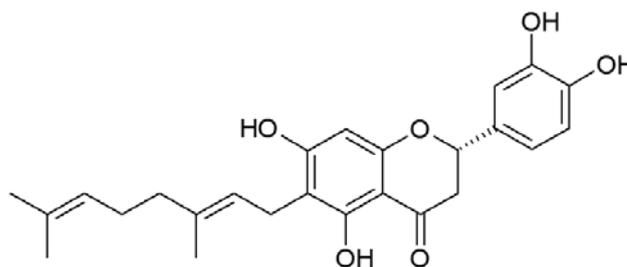
Fig. 1. Molecular structure of diplacone

sis (Chen et al., 2004), are well established. Diplacone also exhibits a strong anti-oxidative capacity (Chen et al., 2004; Smejkal et al., 2007). Antibacterial activity against Gram negative bacteria has also been described (Smejkal et al., 2008b).

The flavonoids, among which diplacone is classified, are commonly studied for their anti-inflammatory effects (Guardia et al., 2001). The favourite model used to study induced inflammation both *in vitro* and *in vivo* is stimulation of macrophages (or other cell types) by lipopolysaccharides (LPS) obtained from Gram-negative bacteria. Some previous papers have reported decreased production of pro-inflammatory cytokines (e.g., tumour necrosis factor α (TNF-α) or IL-1β) and enzymes (e.g., inducible isoform of nitric oxide synthase (iNOS)) after treatment of inflamed cells with a flavonoid (Hämäläinen et al., 2007; Bodet et al., 2008). This result is probably due to the modulating effects of NF-κB, ERK, STAT-1 or combination of any two or all three of them (Hämäläinen et al., 2007; Park et al., 2007). It has been suggested that the imbalance between pro-inflammatory and anti-inflammatory cytokines may contribute to the pathogenesis of autoimmune diseases (O'Shea et al., 2002), which are usually characterized by chronic inflammation. The effects of cytokines are clearly evident, especially for this kind of inflammation.

Therapeutic uses of different plant preparations as antiphlogistics are currently being resurrected and research on antiphlogistics is increasing in the global world (Plaeger, 2003). Many plant flavones and flavanones have been studied for potential application in the therapy of such chronic inflammatory diseases as chronic obstructive pulmonary disease (Weseler et al., 2009), type 2 diabetes (Weseler et al., 2009), and inflammatory bowel disease (Shin et al., 2009).

In this paper, we focused on the study how diplacone affects gene expression of proinflammatory cytokines tumour necrosis factor α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1), also known as CCL2, and regulatory protein zinc finger protein 36 (ZFP36), also known as tristetraprolin (TTP) at the transcription level. TNF-α is a typical hallmark of inflammation, MCP-1 chemotactically regulates movement of monocytes to the site of inflammation, and ZFP36 is an anti-inflammatory protein, which binds to AU-rich regions and destabilizes pro-inflammatory mRNA. The action of diplacone was compared with that of conventional drug indomethacin.

Material and Methods

Material

The RPMI 1640 medium, penicillin-streptomycin mixture, and trypsin 170 U/ml supplemented with EDTA 200 µg/ml were purchased from Lonza (Verviers, Belgium). Phosphate-buffered saline (PBS), foetal bovine serum (FBS), phorbol myristate acetate (PMA), indomethacin, Erythrosin B, and the lipopolysaccharide

(LPS) obtained from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich (Steinheim, Germany). Monoclonal antibody against F4/80-like receptor was obtained from BD Biosciences (San Jose, CA). Diplacone was isolated from *Paulownia tomentosa* fruits according to the procedure of Smejkal et al. (2007). A QuickGene RNA cultured cell HC kit S from FujiFilm (Tokyo, Japan) and an RNase-free DNase Set from Qiagen (Hilden, Germany) were used for isolation of RNA. Reverse transcription quantitative PCR (RT-qPCR) was accomplished with a TaqMan RNA-to-C_T 1-Step Kit from Applied Biosystems (Cheshire, UK) and TaqMan Gene Expression Assays from Applied Biosystems (Foster City, CA) were used for these reactions.

Maintenance of cell culture and differentiation to macrophages

Human monocytic leukaemia cell line THP-1 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). This cell line was used because it is the most similar to native immune cells (Auwerx, 1991). The cells were cultivated at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin in a humidified atmosphere containing 5% CO_2. The medium was changed twice a week when the cell concentration was $5–7 \times 10^5$ cells/ml. The cell number and viability were determined by staining with Erythrosin B. Cells were counted manually using a haemocytometer and a light microscope. Cells that remained unstained were considered viable, light red cells as non-viable.

Stabilized cells were split into 6-well plates to get a concentration of 150,000 cells/ml and cultivated for 72 h. To promote differentiation of monocytes to macrophages, PMA was added to make the final concentration 50 ng/ml and the cells were incubated for 24 h. In comparison with monocytes, differentiated macrophages tend to adhere to the bottoms of the cultivation plates. Maturation of macrophages was also confirmed by the immunohistochemical detection of surface glycoprotein marker F4/80. The F4/80 antigen is expressed on a wide range of mature macrophages. For the next 24 h the cells were incubated with fresh complete RPMI medium, i.e. containing antibiotics and FBS, without PMA. The medium was then aspirated, and the cells were washed with PBS and cultivated for another 24 hours in serum-free RPMI 1640 medium. These prepared macrophages were used for the follow-up experiments.

Drug treatment and induction of inflammation

Differentiated macrophages were pretreated for 1 h with 10 µM or 20 µM diplacone dissolved in dimethylsulphoxide (DMSO). Our previous study showed that these concentrations lack cytotoxic effect (data not showed). For comparison with a conventional drug, 10 µM indomethacin dissolved in DMSO was used. This concentration is commonly used for *in vitro* tests (Assreuy et al., 2003). Control cells contained a vehicle

(DMSO) only. The concentration of DMSO was 0.1 % in each well.

The effect of diplacone on the modulation of inflammatory gene expression was tested by adding 1 μg/ml LPS dissolved in water to drug-pretreated macrophages. LPS is able to trigger an inflammatory reaction through binding on TLR-4 and subsequently activates the NF-κB signalling pathway (Sharif et al., 2007).

Cell samples were harvested by trypsinization and scraping 1, 2, 4, 6, 10, and 24 h after the LPS treatment. Cells were spun down, frozen in liquid nitrogen, and stored at -80 °C for further processing.

RNA isolation and quantification of gene expression

In order to evaluate the expression of *TNF-α*, *MCP-1* and *ZFP36* mRNA, the total RNA was isolated from frozen samples using QuickGene RNA cultured cell HC kit S (FujiFilm) according to the manufacturer's instructions, and supplementing this with DNase treatment. The concentration and purity of the RNA was determined by using UV spectrophotometry.

The gene expression was quantified by using a one-step reverse-transcription quantitative (real-time) polymerase chain reaction (PCR) (RT-qPCR) with TaqMan Gene Expression Assays, which contain specific primers and a TaqMan probe that binds to an exon-exon junction to avoid DNA contamination. Assay number Hs00174128_m1 was used for *TNFα*, Hs00234140_m1 for *MCP-1* and Hs00185658_m1 for *ZFP36* gene expression quantification. β-Actin, assay number 4326315E, served as an internal control for gene expression. A total of 1 μg of isolated RNA was added to 25 μl of the PCR reaction mixture containing both reverse transcriptase and DNA polymerase. The parameters for the qPCR work with the TaqMan RNA-to-C_T 1-Step Kit were set up according to the manufacturer's recommendations:

48 °C for 15 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. RT-qPCR reactions were designed to be duplex, expressions of both β-actin and the gene of interest were evaluated in one tube. Results were normalized to the amount of β-actin and the change in gene expression was determined by the $\Delta\Delta C_T$ method using StepOne Software, version 2.1 (Applied Biosystems).

Statistical analysis

All experiments were performed in triplicate and the results are presented as the mean values with error bars representing the standard error (SE) of the mean. A one-way ANOVA test was used for statistical analysis, followed by Tukey's test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant. Unistat 5.1 (Unistat Ltd., London, UK) was used to perform the analysis.

Results

The expression peaks of *TNF-α* and *ZFP36* were observed between 1 and 2 h after LPS stimulation, and the mRNA level then rapidly decreased, whereas the peak of *MCP-1* was achieved 10 hours after LPS stimulation and the decrease was much slower (Figs. 2–4).

In Table 1, the relative changes in the gene expression of *TNF-α* for LPS-stimulated cells are compared to those for vehicle-treated cells. Two hours after the LPS-induced inflammation, both concentrations of diplacone had significantly decreased the *TNF-α* expression by a factor of ~1.7 ($P < 0.001$). A similar effect was observed for the indomethacin-treated cells (Fig. 2). The *TNF-α* expression rapidly decreased after reaching a maximum, when cells were stimulated with LPS alone. Pre-treatment with diplacone led to a more moderate decline of the *TNF-α* expression. Ten hours after LPS induction,
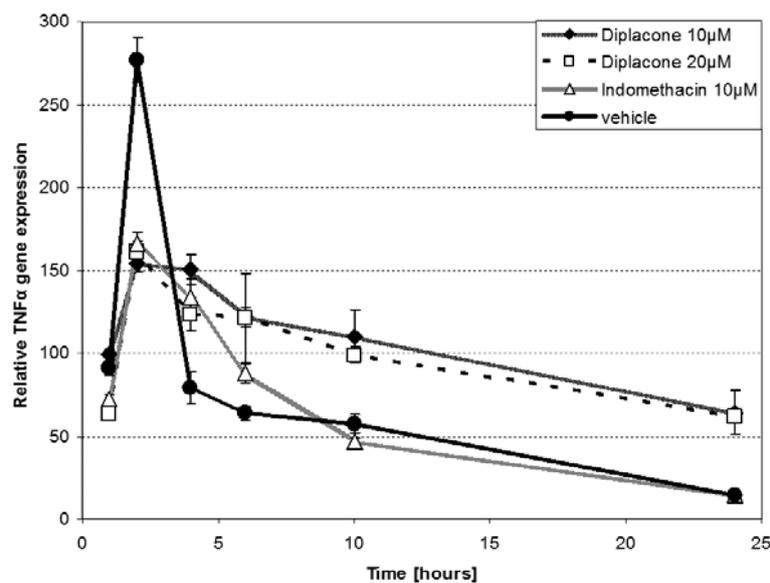
Fig. 2. Effect of diplacone and indomethacin on LPS-induced TNF-α gene expression. Cells were pre-treated with diplacone, indomethacin or vehicle only. After one hour of incubation, inflammation was induced by LPS.
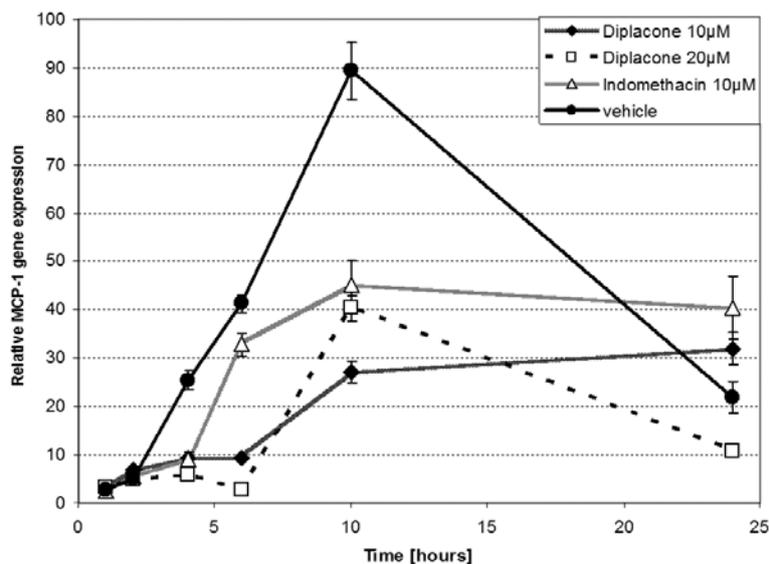
Fig. 3. Effect of diplacone and indomethacin on LPS-induced MCP-1 gene expression. Cells were pre-treated with diplacone, indomethacin or vehicle only. After one hour of incubation, inflammation was induced by LPS.
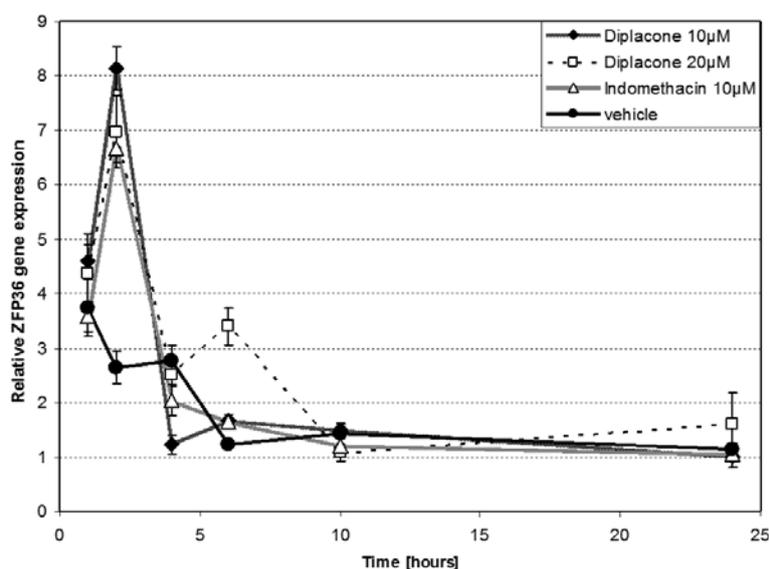
Fig. 4. Effect of diplacone and indomethacin on LPS-induced ZFP36 gene expression. Cells were pre-treated with diplacone, indomethacin or vehicle only. After one hour of incubation, inflammation was induced by LPS.

1.8 times as much mRNA for *TNF-α* was presented in pre-treated cells as in cells without pre-treatment. After 24 h there was 4.4 times as much mRNA in the pre-treated as in the untreated cells. Indomethacin also showed slower decrease of *TNF-α* mRNA, but in this case the *TNF-α* mRNA reached a level similar to that of the mRNA treated by the vehicle 10 hours after LPS induction (Fig. 2).

The expression of *MCP-1*, another pro-inflammatory gene, was also studied. The influence of diplacone on this expression is summarized in Table 2. Four hours after LPS stimulation, cells influenced by 10 μM diplacone and by indomethacin showed expression of *MCP-1* lower by a factor of 2.8 (P = 0.0005) than was found for cells treated with vehicle alone; for those treated with 20 μM diplacone the factor was 4.3 (P = 0.0001) (Fig. 3).

Six hours after LPS stimulation, statistically significant lower expressions of *MCP-1* mRNA were detected only for the diplacone treatments at both concentrations (P < 0.0001); indomethacin decreased the level of *MCP-1* mRNA by only a factor of 1.3, which was not statistically significant (P = 0.063). However, when the peak of expression was observed, 10 hours after LPS induction, both diplacone and indomethacin significantly diminished the *MCP-1* expression; the lowest expression was then found for cells treated with 10 μM diplacone (lower by a factor of 3.3 than for cells treated with the vehicle (P = 0.0001)).

The only anti-inflammatory gene for which the expression was studied was *ZFP36*. Diplacone and indomethacin significantly increased the expression of mRNA for *ZFP36* two hours after LPS stimulation (Ta-

Table 1. Relative changes in LPS-induced TNF-α expression

	Time after LPS stimulation [hours]					
	1	2	4	6	10	24
Control			1.12 ± 0.20			
Vehicle	90.97 ± 4.64	276.67 ± 13.83	78.84 ± 9.95	63.78 ± 4.23	57.23 ± 5.72	14.13 ± 1.18
Diplacone 10 μM	99.26 ± 3.02	153.83 ± 5.08**	150.07 ± 9.04‡	121.74 ± 6.03	109.92 ± 16.16‡	64.07 ± 13.08‡
Diplacone 20 μM	63.14 ± 3.35*	160.83 ± 7.16**	123.53 ± 10.05	120.70 ± 27.73	98.63 ± 4.84	61.54 ± 2.87‡
Indomethacin 10 μM	72.34 ± 3.09	165.85 ± 6.94**	133.52 ± 11.83	87.72 ± 6.14	46.36 ± 2.64	13.91 ± 1.96

Results are means ± SE for three independent experiments. * indicates significant decrease in TNF-α expression relatively to vehicle-treated cells ($P < 0.05$), ** indicates significant decrease in TNF-α expression relatively to vehicle-treated cells ($P < 0.005$), ‡ indicates significant increase in TNF-α expression relatively to vehicle-treated cells ($P < 0.05$).

ble 3). The expression of *ZFP36* was otherwise comparable for all samples at all other times (Fig. 4). For the same period, 2 h after LPS induction, a significant decrease of mRNA was detected for TNF-α (Fig. 2). Cells pretreated with 20 μM diplacone exhibited significantly higher (a factor of 2.7, $P = 0.0006$) expression of *ZFP36* 6 h after LPS stimulation than cells without diplacone treatment. However, this value does not differ to a statistically significant degree from values obtained 4 h ($P = 0.91$) and 10 h ($P = 0.18$) after LPS induction.

From Fig. 2–4 it is apparent that diplacone has almost the same effect on the expression of selected genes at a concentration of 10 μM as at a concentration of 20 μM. This suggests that the maximal biological effect might be achieved with concentrations lower than 10 μM.

To obtain the overall picture for the total production of mRNA, the area under curve (AUC) of the mean values was calculated. Both concentrations of diplacone had almost twice higher AUC (2384 AU for 10 μM and 2200 AU for 20 μM diplacone) than indomethacin and vehicle-treated cells (1330 AU for indomethacin and 1423 AU for vehicle) in the case of the *TNF-α* gene expression. The opposite effect was observed for the *MCP-1* gene expression; diplacone decreased the total

production of this cytokine mRNA to one half compared to the vehicle (525.3 AU for 10 μM and 466 AU for 20 μM diplacone vs. 1140 AU for the vehicle). Indomethacin had a moderate effect – 814.9 AU. In the case of the *ZFP36* gene expression, the differences were not so noticeable. Diplacone and indomethacin slightly increased total production of mRNA for ZFP36 compared to the vehicle (42.49 AU for 10 μM, 48.69 AU for 20 μM diplacone and 39.17 AU for indomethacin vs. 35.94 AU for the vehicle).

Discussion

The anti-inflammatory effects of various flavonoids have been studied using different models. For example, sigmoidins A and B attenuate 12-*O*-tetradecanoylphorbol 13-acetate and phospholipase-A$_2$-induced mouse paw oedema (Njamen et al., 2004), naringenin inhibits inflammatory neuronal injury by reducing the LPS/INF-γ-induced glial cell activation (Vafeiadou et al., 2009), and fisetin eliminates pulmonary LPS-induced inflammation (Geraets et al., 2009). *In vitro* studies on cell cultures have also been carried out (Manna et al., 2007; Matsuda et al., 2008). It is difficult to base comparison of

Table 2. Relative changes in LPS-induced MCP-1 expression

	Time after LPS stimulation [hours]					
	1	2	4	6	10	24
Control			0.99 ± 0.24			
Vehicle	2.85 ± 0.26	4.92 ± 0.55	25.36 ± 1.85	41.24 ± 1.91	89.39 ± 5.81	21.75 ± 3.32
Diplacone 10 μM	2.99 ± 0.19	6.87 ± 0.46	9.22 ± 0.58**	9.23 ± 0.25**	27.08 ± 2.33**	31.95 ± 3.32
Diplacone 20 μM	3.34 ± 0.24	4.66 ± 0.13	5.86 ± 1.03**	2.87 ± 0.94**	40.26 ± 2.55**	10.67 ± 1.05
Indomethacin 10 μM	2.53 ± 0.48	5.61 ± 0.48	9.10 ± 1.49**	32.77 ± 2.40	45.16 ± 5.05**	40.33 ± 6.45

Results are means ± SE for three independent experiments. ** indicates significant decrease in MCP-1 expression relatively to vehicle-treated cells ($P < 0.005$).

Table 3. Relative changes in LPS-induced ZFP36 expression

	Time after LPS stimulation [hours]					
	1	2	4	6	10	24
Control			1.09 ± 0.08			
Vehicle	3.74 ± 0.53	2.65 ± 0.30	2.77 ± 0.29	1.24 ± 0.08	1.42 ±0.19	1.15 ± 0.11
Diplacone 10 μM	4.61 ± 0.31	8.14 ± 0.39 ‡‡	1.24 ±0.18 *	1.67 ± 0.11	1.49 ± 0.14	1.01 ± 0.19
Diplacone 20 μM	4.37 ± 0.72	6.97 ± 0.66 ‡‡	2.51 ± 0.21	3.40 ± 0.34 ‡‡	1.07 ± 0.14	1.60 ± 0.59
Indomethacin 10 μM	3.58 ± 0.27	6.66 ± 0.26 ‡‡	2.05 ± 0.28	1.66 ± 0.04	1.21 ± 0.02	1.06 ± 0.14

Results are means ± SE for three independent experiments. * indicates significant decrease in ZFP36 expression relatively to vehicle-treated cells ($P < 0.05$), ‡‡ indicates significant increase in ZFP36 expression relatively to vehicle-treated cells ($P < 0.005$).

the anti-inflammatory potentials of flavonoids directly on previous evaluations. In this paper we focused on the human macrophages derived from monocytic leukaemia cell line THP-1, which behave very similarly to native monocyte-derived macrophages (Auwerx, 1991), to test inflammatory gene expression modulation *in vitro*.

Diminished expression and production of TNF-α have been described for various flavones (Lin et al., 2003; Geraets et al., 2009; Vafeiadou et al., 2009) and are ascribed to the inhibition of NF-κB activity (Manna et al., 2007), which controls the transcription of this cytokine (Pahl, 1999). We have not been able to explain the slower decrease of *TNF-α* mRNA after diplacone treatment. The *MCP-1* gene is also under the transcriptional control of NF-κB (Pahl, 1999), so the inhibition of this transcription factor, likely caused by diplacone, has a similar influence on the expression of *MCP-1* and *TNF-α*.

Deleault et al. (2008) found that the function of ZFP36 is controlled by the activity of ERK and p38 kinases. LPS activates these kinases, which subsequently inhibit the function of ZFP36. Inhibition of ERK by isoflavones, which can display activities such as antioxidant activity or oestrogen receptor-binding ability, comparable to flavones in some cases (Dixon, 2004), has been observed (Park et al., 2007). Diplacone could have a similar effect – decreasing the activity of extracellular receptor kinase (ERK), and thereby stabilizing ZFP36 protein and subsequently reducing the mRNA level of TNF-α and elevating the level of ZFP36. Significantly higher expression of *ZFP36* was observed six hours after LPS induction, when 20 μM diplacone was used. On the other hand, the measured value did not statically differ from values obtained 4 and 10 h after LPS induction. However, the effect of 20 μM diplacone on *ZFP36* gene expression is opposite to the effect of 10 μM diplacone or indomethacin, which did not affect this expression at that time point. We have yet to satisfactorily explain the biological relevance of this observation.

Total production of selected mRNAs was calculated from AUC of the mean values. Diplacone increased almost twice the total production of TNF-α mRNA, but on the other hand decreased twice production of mRNA of another pro-inflammatory cytokine, MCP-1. The total production of mRNA of anti-inflammatory protein ZFP36 was slightly higher when the cells were pretreated with diplacone. Both TNF-α and MCP-1 are target molecules of ZFP36 and their production is modulated by this protein (Sauer et al., 2006). It is possible that ZPF36 is more active in down-regulation of MCP-1 than TNF-α in this case. There may also be some unknown mechanism of diplacone that prolongs the half-life of TNF-α mRNA in the cells. In any case, the diplacone's biological effect of higher total production of TNF-α mRNA and lower production of MCP-1 mRNA should be elucidated on an *in vivo* model. Although indomethacin is primarily used to inhibit cyclooxygenase activity, in our model it was able to moderately decrease the total production of TNF-α and MCP-1 mRNA and slightly increase the total production of ZFP36 mRNA.

Conclusion

This paper is the first to describe the effects of geranylated flavanone, diplacone, on transcription of pro-inflammatory and anti-inflammatory genes. We have found that diplacone is able to down-regulate the expression of pro-inflammatory genes for TNF-α and MCP-1 and up-regulate that of anti-inflammatory genes for ZFP36 at the transcriptional level. It thus represents a promising drug candidate for the treatment of inflammation. The exact mechanisms of its action should be elucidated in detail in order to better understand its biological function *in vivo*. The effect of diplacone in decreasing LPS-induced inflammatory gene expression is in many ways similar to that of the conventional drug indomethacin in this model of inflammation.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Assreuy, A. M. S., Alencar, N. M. N., Cavada, B. S., Rocha, D. R., Feitosa, R. F. G., Cunha, F. Q., Calvete, J. J., Ribeiro, R. A. (2003) Porcine spermadhesin PSP-I/PSP-II stimulates macrophages to release a neutrophil chemotactic substance: Modulation by mast cells. *Biol. Reprod.* **68**, 1836-1841.

Auwerx, J. (1991) The human leukemia-cell line, Thp-1 – a multifaceted model for the study of monocyte-macrophage differentiation. *Experientia* **47**, 22-31.

Bodet, C., La, V. D., Epifano, F., Grenier, D. (2008) Naringenin has anti-inflammatory properties in macrophage and ex vivo human whole-blood models. *J. Periodont. Res.* **43**, 400-407.

Chen, C. N., Wu, C. L., Lin, J. K. (2004) Propolin C from propolis induces apoptosis through activating caspases, Bid and cytochrome c release in human melanoma cells. *Biochem. Pharmacol.* **67**, 53-66.

Deleault, K. M., Skinner, S. J., Brooks, S. A. (2008) Tristetraprolin regulates TNF TNF-α mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways. *Mol. Immunol.* **45**, 13-24.

Dixon, R. A. (2004) Phytoestrogens. *Annu. Rev. Plant. Biol.* **55**, 225-261.

Geraets, L., Haegens, A., Brauers, K., Haydock, J. A., Vernooy, J. H., Wouters, E. F., Bast, A., Hageman, G. J. (2009) Inhibition of LPS-induced pulmonary inflammation by

specific flavonoids. *Biochem. Biophys. Res. Commun.* **382**, 598-603.

Guardia, T., Rotelli, A. E., Juarez, A. O., Pelzer, L. E. (2001) Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco* **56**, 683-687.

Hämäläinen, M., Nieminen, R., Vuorela, P., Heinonen, M., Moilanen, E. (2007) Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediat. Inflamm.* **2007**, 45673.

Havsteen, B. H. (2002) The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.* **96**, 67-202.

Lin, N., Sato, T., Takayama, Y., Mimaki, Y., Sashida, Y., Yano, M., Ito, A. (2003) Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. *Biochem. Pharmacol.* **65**, 2065-2071.

Manna, S. K., Aggarwal, R. S., Sethi, G., Aggarwal, B. B., Ramesh, G. T. (2007) Morin (3,5,7,2',4'-Pentahydroxyflavone) abolishes nuclear factor-κB activation induced by various carcinogens and inflammatory stimuli, leading to suppression of nuclear factor-κB-regulated gene expression and up-regulation of apoptosis. *Clin. Cancer Res.* **13**, 2290-2297.

Matsuda, H., Wang, Q., Matsuhira, K., Nakamura, S., Yuan, D., Yoshikawa, M. (2008) Inhibitory effects of thunberginols A and B isolated from Hydrangeae Dulcis Folium on mRNA expression of cytokines and on activation of activator protein-1 in RBL-2H3 cells. *Phytomedicine* **15**, 177-184.

Murphy, B. T., Cao, S., Norris, A., Miller, J. S., Ratovoson, F., Andriantsiferana, R., Rasamison, V. E., Kingston, D. G. (2005) Cytotoxic flavanones of Schizolaena hystrix from the Madagascar rainforest. *J. Nat. Prod.* **68**, 417-419.

Njamen, D., Mbafor, J. T., Fomum, Z. T., Kamanyi, A., Mbanya, J. C., Recio, M. C., Giner, R. M., Manez, S., Rios, J. L. (2004) Anti-inflammatory activities of two flavanones, sigmoidin A and sigmoidin B, from Erythrina sigmoidea. *Planta Med.* **70**, 104-107.

O'Shea, J. J., Ma, A., Lipsky, P. (2002) Cytokines and autoimmunity. *Nat. Rev. Immunol.* **2**, 37-45.

Pahl, H. L. (1999) Activators and target genes of Rel/NF-κB transcription factors. *Oncogene* **18**, 6853-6866.

Park, J. S., Woo, M. S., Kim, D. H., Hyun, J. W., Kim, W. K., Lee, J. C., Kim, H. S. (2007) Anti-inflammatory mechanisms of isoflavone metabolites in lipopolysaccharide-stimulated microglial cells. *J. Pharmacol. Exp. Ther.* **320**, 1237-1245.

Phillips, W. R., Baj, N. J., Gunatilaka, A. A., Kingston, D. G. (1996) C-geranyl compounds from Mimulus clevelandii. *J. Nat. Prod.* **59**, 495-497.

Phommart, S., Sutthivaiyakit, P., Chimnoi, N., Ruchirawat, S., Sutthivaiyakit, S. (2005) Constituents of the leaves of Macaranga tanarius. *J. Nat. Prod.* **68**, 927-930.

Plaeger, S. F. (2003) Clinical immunology and traditional herbal medicines. *Clin. Diagn. Lab. Immunol.* **10**, 337-338.

Sauer, I., Schaljo, B., Vogl, C., Gattermeier, I., Kolbe, T., Muller, M., Blackshear, P. J., Kovarik, P. (2006) Interferons limit inflammatory responses by induction of tristetraprolin. *Blood* **107**, 4790-4797.

Sharif, O., Bolshakov, V. N., Raines, S., Newham, P., Perkins, N. D. (2007) Transcriptional profiling of the LPS induced NF-κB response in macrophages. *BMC Immunol.* **8**, 1.

Shin, E. K., Kwon, H. S., Kim, Y. H., Shin, H. K., Kim, J. K. (2009) Chrysin, a natural flavone, improves murine inflammatory bowel diseases. *Biochem. Biophys. Res. Commun.* **381**, 502-507.

Smejkal, K., Grycova, L., Marek, R., Lemiere, F., Jankovska, D., Forejtnikova, H., Vanco, J., Suchy, V. (2007) C-Geranyl compounds from Paulownia tomentosa fruits. *J. Nat. Prod.* **70**, 1244-1248.

Smejkal, K., Babula, P., Slapetova, T., Brognara, E., Dall'acqua, S., Zemlicka, M., Innocenti, G., Cvacka, J. (2008a) Cytotoxic activity of C-geranyl compounds from Paulownia tomentosa fruits. *Planta Med.* **74**, 1488-1491.

Smejkal, K., Chudik, S., Kloucek, P., Marek, R., Cvacka, J., Urbanova, M., Julinek, O., Kokoska, L., Slapetova, T., Holubova, P., Zima, A., Dvorska, M. (2008b) Antibacterial C-geranylflavonoids from Paulownia tomentosa fruits. *J. Nat. Prod.* **71**, 706-709.

Vafeiadou, K., Vauzour, D., Lee, H. Y., Rodriguez-Mateos, A., Williams, R. J., Spencer, J. P. (2009) The citrus flavanone naringenin inhibits inflammatory signalling in glial cells and protects against neuroinflammatory injury. *Arch. Biochem. Biophys.* **484**, 100-109.

Weseler, A. R., Geraets, L., Moonen, H. J. J., Manders, R. J. F., van Loon, L. J. C., Pennings, H. J., Wouters, E. E. M., Bast, A., Hageman, G. J. (2009) Poly (ADP-ribose) polymerase-1-inhibiting flavonoids attenuate cytokine release in blood from male patients with chronic obstructive pulmonary disease or type 2 diabetes. *J. Nutr.* **139**, 952-957.

Wu, J. Y., Chung, K. T., Liu, Y. W., Lu, F. J., Tsai, R. S., Chen, C. H., Chen, C. H. (2008) Synthesis and biological evaluation of novel C(6) modified baicalein derivatives as antioxidative agents. *J. Agric. Food Chem.* **56**, 2838-2845.

Yoder, B. J., Cao, S., Norris, A., Miller, J. S., Ratovoson, F., Razafitsalama, J., Andriantsiferana, R., Rasamison, V. E., Kingston, D. G. (2007) Antiproliferative prenylated stilbenes and flavonoids from Macaranga alnifolia from the Madagascar rainforest. *J. Nat. Prod.* **70**, 342-346.

Natural Compound Cudraflavone B Shows Promising Anti-inflammatory Properties in Vitro

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ABSTRACT: Cudraflavone B (**1**) is a prenylated flavonoid found in large amounts in the roots of *Morus alba*, a plant used as a herbal remedy for its reputed anti-inflammatory properties. The present study shows that this compound causes a significant inhibition of inflammatory mediators in selected in vitro models. Thus, **1** was identified as a potent inhibitor of tumor necrosis factor α (TNFα) gene expression and secretion by blocking the translocation of nuclear factor κB (NF-κB) from the cytoplasm to the nucleus in macrophages derived from a THP-1 human monocyte cell line. The NF-κB activity reduction resulted in the inhibition of cyclooxygenase 2 (COX-2) gene

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expression. Compound **1** acts as a COX-2 and COX-1 inhibitor with higher selectivity toward COX-2 than indomethacin. Pretreatment of cells by **1** shifted the peak in an regulatory gene zinc-finger protein 36 (ZFP36) expression assay. This natural product has noticeable anti-inflammatory properties, suggesting that **1** potentially could be used for development as a nonsteroidal anti-inflammatory drug lead.
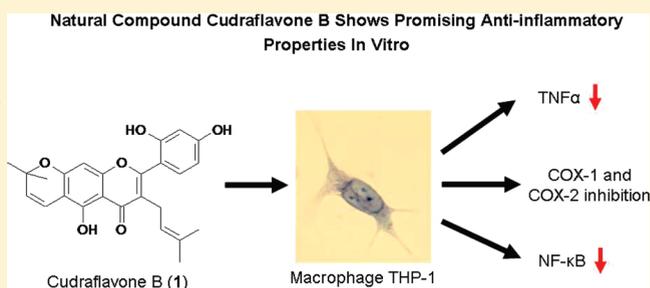
White mulberry (*Morus alba* L., Moraceae) is well-known as a feedstock for silkworms. In an addition, extracts of its fruits, leaves, and twigs are used widely in traditional Asian medicine for their anti-inflammatory properties, for their ability to regulate the level of blood sugar, and for diuretic, antitussive, and antipyretic properties.[1,2] The antioxidant activities of extracts and compounds isolated from the white mulberry have also been well documented.[3,4] These extracts showed an ability to decrease the production of NO and prostaglandin E$_2$ (PGE$_2$) in lipopolysaccharide (LPS)-stimulated mouse macrophages.[5,6] Compounds isolated from *M. alba* roots exhibit anti-inflammatory properties.[7,8] One such compound is cudraflavone B (**1**), first obtained from the root bark of *Cudrania tricuspidata* (Moraceae),[9] but later from *Morus* and *Artocarpus* spp.[10,11] This prenylated flavone has shown hepatoprotective activity,[10,12] cytotoxicity against the human gastric carcinoma cell line BGC-823[13] and mouse melanoma cells B16,[14] moderate

inhibitory effects on mouse brain monoamine oxidase (MAO),[15] and antiproliferative activity caused by the downregulation of pRb phosphorylation.[16] On the other hand, **1** has been shown to lack antioxidant activity.[10]

Macrophages are cells that play an important role in the regulation of inflammation. Their activation leads to the production of various growth factors, cytokines, and other substances, which participate in the inflammatory process. This process is controlled mainly on the level of transcription, as has been reviewed by Medzhitov and Horng.[17] The rapid action of the pro-inflammatory impulse is followed by a negative regulation reaction that returns the affected tissue to homeostasis. One such feedback regulatory protein is ZPF36. This protein binds to the AU-rich regions of some pro-inflammatory mRNAs (such as

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TNFα), destabilizes them, and consequently decreases their production.[18]

Nuclear factor κB (NF-κB) (in the present paper "NF-κB" represents a p50/p65 heterodimer) plays an important role in activating the inflammatory response by releasing the production of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) or CCL2, and enzymes, such as cyclooxygenase 2 (COX-2) and inducible NO-synthase (iNOS or NOS2).[19,20] NF-κB is also called "the central mediator of the human immune system", or more precisely "the central mediator of the human stress response".[21] Along with the modulation of the inflammatory response described, this factor is also involved in the regulation of apoptosis and can be linked to cancer development.[22]

The aim of the present report is to describe the anti-inflammatory properties of **1** and to introduce it as a new potential nonsteroidal anti-inflammatory drug lead.

1
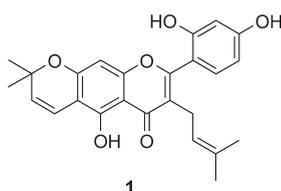
■ RESULTS AND DISCUSSION

Cudraflavone B (**1**) was isolated from the root of *M. alba* as a brownish, amorphous powder. An ethanol extract of the root was apportioned in a mixture of immiscible solvents, as described in the Experimental Section. The chloroform-soluble portion was separated by reverse-phase high-performance liquid chromatography to obtain pure **1** in a single separation step. The identity of the isolated sample as **1** was confirmed by comparing the measured data obtained from ^{1}H and ^{13}C NMR spectroscopy and HRMS with values in the literature.[23]

To assess the effect of **1** initially on the viability and growth of cells, THP-1 cells were exposed to increasing concentrations (1−50 μM) of this compound for 24 h, stained for viability, and counted. The final LD$_{50}$ value for **1** was determined to be 47.6 μM. To compare the cytotoxicity of **1** with a clinically used anti-inflammatory agent, increasing concentrations of indomethacin (1−100 μM) were added to THP-1 cells. The LD$_{50}$ value for indomethacin derived from this data was assessed as >100 μM.

An initial screening of prenyl and geranyl flavonoids isolated from the *M. alba* roots and *Paulownia tomentosa* (Paulowniaceae) fruits (some of these flavonoids have been described in a previous paper[24]) showed that **1** is a potent inhibitor of TNFα gene transcription after LPS stimulation (data not shown). Subsequent detailed time-course experiments confirmed this observation (Figure 1). Cudraflavone B (**1**), at a concentration of 10 μM, decreased significantly the transcription of TNFα at almost every point in the time of measurement. The greatest inhibitory effect was found 2 h after LPS stimulation, when **1** reduced the mRNA of TNFα by a factor of 5.8 in comparison with vehicle-only treated cells. Cudraflavone B (**1**) decreased TNFα transcription more effectively than indomethacin (Figure 1). The inhibition of TNFα transcription caused by **1** was in concert with the inhibited TNFα secretion into the medium (Figure 2), for which the production of this cytokine by cells pretreated with **1** was reduced by a factor of 20.
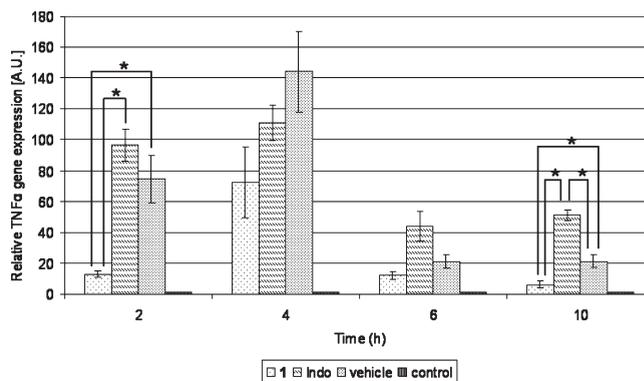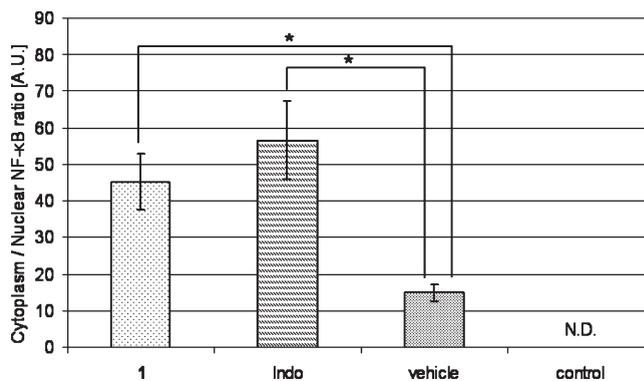
Figure 1. Effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced TNFα gene expression. Cells were pretreated with **1** (10 μM), indomethacin (10 μM), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means ± SE for three independent experiments. A.U. = arbitrary unit. *$p < 0.05$.

Figure 2. Effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced TNFα protein synthesis. Cells were pretreated with **1** (10 μM), indomethacin (10 μM), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are means ± SE for three independent experiments. N.D. = not detected. *$p < 0.05$.
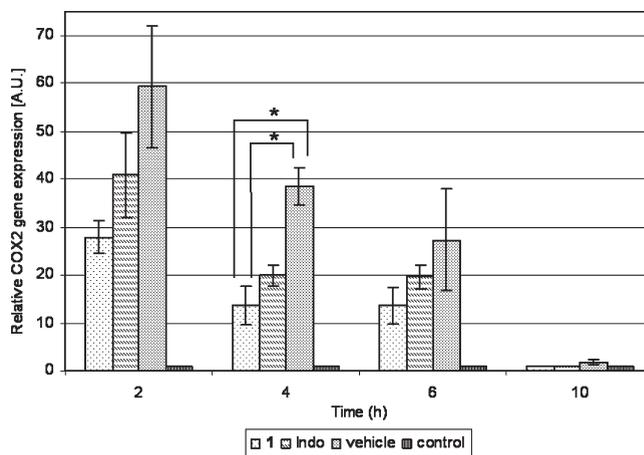
Figure 3. Comparison of the effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced COX-2 gene expression. Cells were pretreated with **1** (10 μM), indomethacin (10 μM), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are means ± SE for three independent experiments. A.U. = arbitrary unit. *$p < 0.05$.

Figure 4. Comparison of the effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced ZFP36 gene expression. Cells were pretreated with **1** ($10\,\mu$M), indomethacin ($10\,\mu$M), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means $\pm$ SE for three independent experiments. A.U. = arbitrary unit. *$p < 0.05$.

Figure 5. Effect of cudraflavone B (**1**) and indomethacin on LPS-induced NF-κB nuclear translocation. Cells were pretreated with **1** (10 μM), indomethacin ($10\,\mu$M), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means $\pm$ SE for three independent experiments and three independent measurements. A.U. = arbitrary unit; N.D. = not detected. *$p < 0.05$.

It was found that **1** diminished significantly the gene transcription of COX-2 4 h after LPS stimulation (Figure 3). The ability of **1** to inhibit COX-2 function was also measured. This flavone gave a COX-2 IC$_{50}$ value of $2.5 \pm 0.89\,\mu$M (mean $\pm$ SE for four independent experiments with at least two replicates). A similar value was obtained for indomethacin, which is a known COX inhibitor. Its COX-2 IC$_{50}$ value was found to be $1.9 \pm 0.61\,\mu$M (mean $\pm$ SE for four independent experiments with at least two replicates). Many of the adverse side-effects of NSAIDs are caused by COX-1 inhibition. Therefore, the COX-1 IC$_{50}$ value was determined for **1**, which was $1.5 \pm 0.65\,\mu$M (mean $\pm$ SE for four independent experiments with at least two replicates). For indomethacin, the COX-1 IC$_{50}$ value was equal to $0.3 \pm 0.14\,\mu$M (mean $\pm$ SE for four independent experiments with at least two replicates). The in vitro selectivity of both **1** and indomethacin was then calculated as COX-2/COX-1 inhibitory ratios. The values calculated for **1** and indomethacin were 1.70 and 6.45, respectively. Thus, **1** acts as a COX-2 and COX-1 inhibitor with higher selectivity toward COX-2 than indomethacin.

The maximum ZFP36 transcription was observed 2 h after LPS induction (Figure 4). At this point in time, cells pretreated with **1** showed a gene transcription effect of ZFP36 5.8 times lower than cells treated with the vehicle only. On the other hand, 4 h after LPS stimulation, **1** was able to increase the transcription of ZFP36 by a factor of 2. Only a weak effect of indomethacin in comparison to the vehicle was observed.

When cudraflavone B (**1**) affected the expression of proinflammatory genes, its influence on the activity of the transcription factor NF-κB, which controls the production of the relevant mRNA, was evaluated. The inactive form of NF-κB is kept in the cytoplasm, whereas the active form is located in the nucleus.[21] The ratio between the cytoplasmic and the nuclear NF-κB contents was measured to evaluate the ability of **1** to keep this protein in the cytoplasm (Figure 5). The results obtained are in concert with the foregoing observations, where **1** down-regulated the transcription of pro-inflammatory genes. Cells pretreated with **1** showed an occurrence of NF-κB in their nuclei 3.2 times lower than in vehicle-only treated cells. Similar results were observed for indomethacin-treated cells.

Several flavonoids have previously shown an ability to combat inflammation using in vivo models. The inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) represents routine assays to reveal new compounds with potential impact on inflammatory-active metabolites of arachidonic acid. Many other tests, including assays in vivo, have been used to indicate new antiphlogistic agents, and some flavonoids have relatively strong activity. It should be mentioned that more lipophilic flavonoids have displayed better anti-inflammatory potential.[25] Flavonoids with one or more prenyl substituents belong to a group of lipophilic secondary metabolites are often present on the plant surface as defending agents. The lipophilic character of prenylated flavonoids allows these compounds to penetrate the membranes of animal cells and readily enter into cells and affect metabolism, better than nonprenylated analogues. As secondary metabolites combining two biosynthetic pathways, these compounds have attracted the attention of phytochemists because their structural parameters can display many possibilities for biological activity. The anti-inflammatory potential of these compounds is known, for example, from the study of Chi et al.,[26] which revealed the anti-COX and anti-LOX activities of some prenyl flavonoids. However, morusin, a prenyl flavonoid compound structurally similar to **1**, had IC$_{50}$ values for COX-1, COX-2, 5-LOX, and 12-LOX of 100 μM or higher in this study. Thus, the position of the cyclic prenyl side chain at the flavonoid skeleton could be important for anti-COX activity. In contrast, Wei et al.[27] discovered that cudraflavone A (unlike **1**, cudraflavone A has additional functionality of the B- and C-rings by a prenyl in the B-ring and a hydroxyl in the C-ring) is able to trigger a moderate production of superoxide anion and thus displays a pro-inflammatory effect, but concentrations higher (30 and 90 μM) than were used here (10 μM) were employed in their study. According to the literature, the only study dealing with an assay of the anti-inflammatory activity of **1** was an analysis of iNOS gene expression and NO production in RAW 264.7 cells, which showed activity in a concentration-dependent manner.[28]

In the present work, we have found that cudraflavone B (**1**) isolated from white mulberry roots decreases the inflammatory response in LPS-stimulated macrophages. The expression of the typical pro-inflammatory cytokine TNFα was decreased

significantly by **1** on both transcriptional and translational levels. Previously, a decrease in the level of TNFα transcription has been observed for extracts obtained from white mulberry bark[29] and leaves,[6] but these studies lacked any identification of compounds present in the extracts. Therefore, the anti-inflammatory constituents of *Morus* species are still unknown, and as a starting point it was considered that these compounds could be represented by cudraflavone B (**1**), which is present in large amounts in *M. alba* root extracts.

It was found that cudraflavone B (**1**) inhibits the nuclear translocation of NF-κB at micromolar levels. Cudraflavone B (**1**) decreased the transcription of the chemokine CCL2 and inducible NO-synthase (iNOS), which are also under the transcription control of NF-κB, but without any statistical significance (data not shown). It has been observed in previous studies that *M. alba* aqueous leaf[30] and bark[29] extracts, which could also contain **1**, are able to ameliorate NF-κB activity.

A further demonstration of the anti-inflammatory potential of **1** was shown by the transcription of a key enzyme of the prostaglandin biosynthesis pathway, COX-2, which was attenuated by **1**. This compound has a noticeable ability to inhibit the in vitro synthesis of prostaglandin E_2 (PGE$_2$), a COX-2 product. This effect is comparable to that of the typical COX inhibitor indomethacin (COX-2 IC$_{50}$ = 2.5 ± 0.89 μM for **1** and 1.9 ± 0.61 μM for indomethacin), showing that **1** is a strong COX-2 inhibitor, but **1** also exhibited COX-1 inhibitory activity (COX-1 IC$_{50}$ = 1.5 ± 0.65 μM). In addition to **1**, several other compounds with COX-2 inhibitory activity have been found in mulberry root bark, but their IC$_{50}$ values were around 40−50 μM.[8] The COX-2 pathway is one of the major targets for the nonsteroidal anti-inflammatory drugs (NSAIDs), whereas constitutive COX-1 produces housekeeping prostaglandins, and its inhibition is undesirable. Since some anti-inflammatory drugs have adverse side-effects, e.g., gastrointestinal toxicity, caused by COX-1 inhibition, anti-inflammatory drugs with preferential COX-2 inhibitory activity are desirable. The only protein tested with an ability to modulate mRNA level is ZFP36. Cells pretreated with **1** showed only a shift in the peak of transcription, while cells treated with indomethacin or only the vehicle reached a maximum at 2 h after LPS stimulation. Cells pretreated with **1** showed a peak at 4 h after LPS treatment. This shift in the gene transcription of ZFP36 could not be elucidated well, but it may have influence on some pro-inflammatory genes expression, such as TNFα.

This study has shown that cudraflavone B (**1**) has some potential as a new anti-inflammatory drug lead, but further analysis and in vivo tests are needed for better understanding of the exact mechanisms of its action.

■ EXPERIMENTAL SECTION

General Experimental Procedures. NMR spectra were recorded using a Bruker Avance 300 spectrometer operating at frequencies of 300.13 MHz (^{1}H) and 75.48 MHz (^{13}C). The spectra were measured in DMSO-d_6 or CDCl$_3$ at 303 K. The ^{1}H and ^{13}C NMR chemical shifts (δ in ppm) were referenced to the signal of the solvent [2.50 (^{1}H) and 39.43 (^{13}C) for DMSO-d_6 and 7.26 (^{1}H) and 77.00 (^{13}C) for CDCl$_3$]. 2D NMR, gs-COSY, gs-HSQC, and gs-HMBC were used to assign the individual ^{1}H and ^{13}C resonances. The HSQC experiment was adjusted for the coupling $^1J_{HC}$ = 150 Hz and the HMBC experiment for long-range couplings of 7.5 Hz. A Mariner PE Biosystem workstation with APITOF was used to collect the HRMS. These spectra were collected in the negative mode. Preparative HPLC was carried out on a LCP 4100

instrument, with loop injection of 100 μL, column block LCO 101, and UV detector LCD 2084 (Ecom, CR). An Agilent 1100 apparatus equipped with a diode-array detector was used for the chromatographic purity determination (Supelcosil ABZ+Plus, 150 mm × 4.6 mm i.d., particle size 3 μm).

RPMI 1640 medium, penicillin−streptomycin mixture, and trypsin 170 U/mL supplemented with EDTA 200 μg/mL were purchased from Lonza (Verviers, Belgium). Human recombinant COX-2 enzyme was obtained from Sigma-Aldrich (St. Louis, MO), and COX-1 enzyme isolated from ram seminal vesicles was from Cayman Chemical (Ann Arbor, MI). Phosphate-buffered saline (PBS), porcine hematin, fetal bovine serum (FBS), phorbol myristate acetate (PMA), indomethacin (99%), erythrosin B, L-epinephrine, sodium EDTA, and *Escherichia coli* 0111:B4 lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Steinheim, Germany). Two-step reverse transcription quantitative PCR (RT-qPCR) was accomplished with a TaqMan Gene Expression Cell-to-Ct kit (Ambion, Austin, TX) and TaqMan Gene Expression Master Mix from Applied Biosystems (Foster City, CA). Specific primers and probes (assays) were lyophilized in TaqMan array plates (Applied Biosystems). The following assays were chosen for the quantification of gene expression: Hs00174128_m1 for TNFα, Hs00185658_m1 for ZFP36, Hs01573471_m1 for COX-2, and Hs99999903_m1 for β-actin, which served as an internal control of gene expression. A PARIS kit (Ambion, Austin, TX) was used to isolate the nuclear proteins. An EZ-Detect NF-κB p65 transcription factor kit (Thermo Scientific, Rockford, IL) was utilized to detect NF-κB. An AlphaLISA TNFα kit (PerkinElmer, Boston, MA) was used to evaluate the production of TNFα. The EIA kit used to measure the concentration of prostaglandin E_2 was purchased from Assay Designs (Ann Arbor, MI).

Extraction and Isolation. A 537 g amount of dried *M. alba* roots was processed according to the literature.[24] The CHCl$_3$ extract was separated using reversed-phase preparative HPLC (Supelcosil ABZ+Plus, 250 × 21.2 mm i.d., particle size 5 μm). Gradient elution employed 0.2% HCOOH and a mixture of MeCN and MeOH, 8:2 (v/v) (A), in the gradient: initial composition 20% A, final composition 100% A in the 40th min; flow rate 25 mL/min. Fractions were acquired according to the detector response at λ = 280 nm. After removal of the organic solvent and precipitation, the fraction with a HPLC t_R of 24−25 min yielded cudraflavone B (**1**) (680 mg). The identity of **1** was confirmed by comparing the spectroscopic data with those reported previously.[23] The purity of compound **1** was established to be more than 98% using HPLC DAD analysis.

Maintenance and Preparation of Macrophages. The THP-1 human monocytic leukemia cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were cultivated at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO$_2$. The medium was changed twice a week, when cells had reached a concentration of 5−7 × 10^5 cells/mL. The viability of cells was greater than 94% throughout the experiment. Stabilized cells were split into 96-well plates to afford a concentration of 300 000 cells/mL, and differentiation into macrophages was induced by phorbol myristate acetate (PMA), as described previously.[31]

Cytotoxicity Test. Cudraflavone B (**1**) and indomethacin were dissolved in dimethylsulfoxide (DMSO) at five increasing concentrations and added to the monocyte suspension in culture medium. The final concentration of DMSO in culture medium was 0.1%. Incubation for 24 h at 37 °C with 5% CO$_2$ followed. The cell number and viability were determined following staining with erythrosin B. The stain (0.1% erythrosin B (w/v)) in phosphate-buffered saline (PBS), pH 7.2−7.4, was mixed with an equal amount of the cell suspension, and the numbers of viable and nonviable cells were counted manually using a hemocytometer. Cells that remained unstained were considered viable and light

red cells as nonviable. Each compound was characterized by an LD_{50} value, the concentration of the compound lethal for 50% of the cells, as calculated from the dose–response curves obtained. The cytotoxic LD_{50} concentrations of the compounds tested were determined by combining the data from the equation generated by KURV+ version 4.4b software (Conrad Button Software, Arlington, WA), with statistical analysis using GraphPad Prism 5.02 software (GraphPad Software, San Diego, CA), which was used for the LD_{50} data derived from the values plotted on the graph.

Drug Treatment and Induction of Inflammation. Differentiated macrophages were pretreated for 1 h with 10 μM **1** dissolved in DMSO. For comparison with a conventional drug, 10 μM indomethacin dissolved in DMSO was used. These concentrations lacked any cytotoxic effects, and the cell viability was found to be over 90%. Vehicle-treated cells contained a vehicle (DMSO) only, and control cells were without LPS treatment. The concentration of DMSO was 0.1% in each well.

The effect of **1** on the modulation of inflammatory gene expression was tested by adding 1 μg/mL LPS dissolved in sterile water to macrophages pretreated with the drug. LPS is able to trigger an inflammatory reaction through binding on TLR-4 and subsequently activates the NF-κB signaling pathway.[32] The cultivation medium was aspirated at 2, 4, 6, and 10 h after the LPS treatment. Adherent cells were then directly lysed in the cultivation wells, and cell lysates were collected. Samples were frozen in liquid nitrogen and stored at -80 °C for the next processing.

Isolation of RNA and Evaluation of Gene Expression. In the order to evaluate the expression of TNFα, COX-2, ZFP36, and β-actin mRNA, the total RNA was isolated directly from cells in cultivation plates using a TaqMan Gene Expression Cell-to-Ct kit, according to the manufacturer's instructions. The concentration and purity of the RNA were determined by using UV spectrophotometry.

The gene expression was quantified by two-step reverse-transcription quantitative (real-time) PCR (RT-qPCR) with a TaqMan Gene Expression Cell-to-Ct kit and TaqMan array plates. These contain specific primers and TaqMan probes that bind to an exon–exon junction to avoid DNA contamination. The parameters for the qPCR work with the TaqMan Gene Expression Cell-to-Ct kit were adjusted according to the manufacturer's recommendations: (a) reverse transcription step – 37 °C for 1 h and then 95 °C for 5 min, (b) polymerase step –50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The results were normalized to the amount of ROX reference dye, and the change in gene expression was determined by the $\Delta\Delta C_T$ method.[33] Transcription of the control cells was set as 1, and other experimental groups were multiples of this value.

Determination of Nuclear NF-κB Translocation. Pretreated LPS-stimulated macrophages were harvested by trypsinization and scraping 1 h after the LPS treatment. Cells were spun down, frozen in liquid nitrogen, and stored at -80 °C for the next processing. Cytoplasmatic and nuclear protein fractions were isolated from collected cells using a PARIS kit. The presence of NF-κB subunit p65 was measured in both fractions by an EZ-Detect NF-κB p65 transcription factor kit, and the cytoplasm/nuclear ratio was calculated. The lower detection limit was 25 pg/mL.

Evaluation of TNFα Secretion. Macrophages that had been pretreated for 1 h with the test compounds were incubated with LPS for the next 24 h. After this period, the medium was collected and the concentration of TNFα was measured using an AlphaLISA TNFα kit on the EnSpire device (PerkinElmer, Boston, MA). The excitation time was 80 ms, and emission was measured for 120 ms. The lower detection limit was 3.2 pg/mL.

Determination of COX-1 and COX-2 Inhibitory Effects. The assay was performed according to the procedure described previously by Reininger and Bauer[34] with human recombinant COX-2 or COX-1 from ram seminal vesicles. In brief, COX-2 (0.5 unit/reaction) or COX-1 (1 unit/reaction) was added to the incubation mixture consisting of 0.1 M Tris/HCl buffer (pH 8.0), 5 μM porcine hematin, 18 mM L-epinephrine, and 50 μM sodium EDTA. The test substances dissolved in DMSO or pure DMSO (in the case of the blank) were added to the reaction and preincubated for 5 min at room temperature. The reaction was started by adding arachidonic acid and then incubated for 20 min at 37 °C. The concentration of PGE_2, the main product of the reaction, was determined by using a PGE_2 EIA kit based on a competitive enzyme immunoassay. The intensity of the yellow color generated, which is inversely proportional to the concentration of PGE_2 in the sample, was measured on a Tecan Infinite M200 microplate reader (Tecan Group, Männedorf, Switzerland) at 405 nm. IC_{50} values were determined by regression analysis from at least three concentrations.

Statistical Analysis. All experiments were performed in triplicate, and results are presented as mean values, with error bars representing the standard error (SE) of the mean. A one-way ANOVA test was used for statistical analysis, followed by a Newman–Keuls posthoc test for multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant. GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA) was used to perform the analysis.

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■ REFERENCES

(1) Chen, C. C.; Liu, L. K.; Hsu, J. D.; Huang, H. P.; Yang, M. Y.; Wang, C. J. *Food Chem.* **2005**, *91*, 601–607.

(2) Kang, T. H.; Hur, J. Y.; Kim, H. B.; Ryu, J. H.; Kim, S. Y. *Neurosci. Lett.* **2006**, *391*, 122–126.

(3) Zhishen, J.; Mengcheng, T.; Jianming, W. *Food Chem.* **1999**, *64*, 555–559.

(4) Butt, M. S.; Nazir, A.; Sultan, M. T.; Schroen, K. *Trends Food Sci. Technol.* **2008**, *19*, 505–512.

(5) Hong, C. H.; Hur, S. K.; Oh, O. J.; Kim, S. S.; Nam, K. A.; Lee, S. K. *J. Ethnopharmacol.* **2002**, *83*, 153–159.

(6) Choi, E. M.; Hwang, J. K. *Fitoterapia* **2005**, *76*, 608–613.

(7) Kimura, Y.; Okuda, H.; Nomura, T.; Fukai, T.; Arichi, S. *J. Nat. Prod.* **1986**, *49*, 639–644.

(8) Rollinger, J. M.; Bodensieck, A.; Seger, C.; Ellmerer, E. P.; Bauer, R.; Langer, T.; Stuppner, H. *Planta Med.* **2005**, *71*, 399–405.

(9) Fujimoto, T.; Hano, Y.; Nomura, T.; Uzawa, J. *Planta Med.* **1984**, *50*, 161–163.

(10) Oh, H.; Ko, E. K.; Jun, J. Y.; Oh, M. H.; Park, S. U.; Kang, K. H.; Lee, H. S.; Kim, Y. C. *Planta Med.* **2002**, *68*, 932–934.

(11) Zheng, Z. P.; Cheng, K. W.; To, J. T. K.; Li, H. T.; Wang, M. F. *Mol. Nutr. Food Res.* **2008**, *52*, 1530–1538.

(12) An, R. B.; Sohn, D. H.; Kim, Y. C. *Biol. Pharm. Bull.* **2006**, *29*, 838–840.

(13) Zou, Y. S.; Hou, A. J.; Zhu, G. F.; Chen, Y. F.; Sun, H. D.; Zhao, Q. S. *Bioorg. Med. Chem.* **2004**, *12*, 1947–1953.

(14) Arung, E. T.; Yoshikawa, K.; Shimizu, K.; Kondo, R. *Fitoterapia* **2010**, *81*, 120–123.

(15) Hwang, J. H.; Hong, S. S.; Han, X. H.; Hwang, J. S.; Lee, D. H.; Lee, H. S.; Yun, Y. P.; Kim, Y. S.; Ro, J. S.; Hwang, B. Y. *J. Nat. Prod.* **2007**, *70*, 1207–1209.

(16) Kim, T. J.; Han, H. J.; Lim, Y.; Song, M. C.; Kim, J.; Hong, J. T.; Yoo, H. S.; Pyo, M. Y.; Hwang, B. Y.; Lee, M. K.; Yun, Y. P. *J. Cardiovasc. Pharmacol.* **2009**, *53*, 341–348.

(17) Medzhitov, R.; Horng, T. *Nat. Rev. Immunol.* **2009**, *9*, 692–703.

(18) Lai, W. S.; Carballo, E.; Thorn, J. M.; Kennington, E. A.; Blackshear, P. J. *J. Biol. Chem.* **2000**, *275*, 17827–17837.

(19) Sun, Y.; Li, Y. H.; Wu, X. X.; Zheng, W.; Guo, Z. H.; Li, Y.; Chen, T.; Hua, Z. C.; Xu, Q. *Int. J. Mol. Med.* **2006**, *17*, 957–962.

(20) Shoelson, S. E.; Lee, J.; Goldfine, A. B. *J. Clin. Invest.* **2006**, *116*, 1793–1801.

(21) Pahl, H. L. *Oncogene* **1999**, *18*, 6853–6866.

(22) Perkins, N. D.; Gilmore, T. D. *Cell Death Differ.* **2006**, *13*, 759–772.

(23) Hano, Y.; Matsumoto, Y.; Shinohara, K.; Sun, J. Y.; Nomura, T. *Heterocycles* **1990**, *31*, 1339–1344.

(24) Smejkal, K.; Svacinova, J.; Slapetova, T.; Schneiderova, K.; Dall'acqua, S.; Innocenti, G.; Zavalova, V.; Kollar, P.; Chudik, S.; Marek, R.; Julinek, O.; Urbanova, M.; Kartal, M.; Csollei, M.; Dolezal, K. *J. Nat. Prod.* **2010**, *73*, 568–572.

(25) Harborne, J. B.; Williams, C. A. *Phytochemistry* **2000**, *55*, 481–504.

(26) Chi, Y. S.; Jong, H. G.; Son, K. H.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Biochem. Pharmacol.* **2001**, *62*, 1185–1191.

(27) Wei, B. L.; Weng, J. R.; Chiu, P. H.; Hung, C. F.; Wang, J. P.; Lin, C. N. *J. Agric. Food Chem.* **2005**, *53*, 3867–3871.

(28) Park, K. H.; Park, Y. D.; Han, J. M.; Im, K. R.; Lee, B. W.; Jeong, I. Y.; Jeong, T. S.; Lee, W. S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 5580–5583.

(29) Lee, K. H.; Yeh, M. H.; Kao, S. T.; Hung, C. M.; Chen, B. C.; Liu, C. J.; Yeh, C. C. *J. Ethnopharmacol.* **2009**, *124*, 530–538.

(30) Shibata, Y.; Kume, N.; Arai, H.; Hayashida, K.; Inui-Hayashida, A.; Minami, M.; Mukai, E.; Toyohara, M.; Harauma, A.; Murayama, T.; Kita, T.; Hara, S.; Kamei, K.; Yokode, M. *Atherosclerosis* **2007**, *193*, 20–27.

(31) Hošek, J.; Závalová, V.; Šmejkal, K.; Bartoš, M. *Folia Biol. (Praha)* **2010**, *56*, 124–130.

(32) Sharif, O.; Bolshakov, V. N.; Raines, S.; Newham, P.; Perkins, N. D. *BMC Immunol.* **2007**, *8*, 1.

(33) Livak, K. J.; Schmittgen, T. D. *Methods* **2001**, *25*, 402–408.

(34) Reininger, E. A.; Bauer, R. *Phytomedicine* **2006**, *13*, 164–169.

Research Article

Prenylated Flavonoids from *Morus alba* L. Cause Inhibition of G1/S Transition in THP-1 Human Leukemia Cells and Prevent the Lipopolysaccharide-Induced Inflammatory Response

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Morus alba L. (MA) is a natural source of many compounds with different biological effects. It has been described to possess anti-inflammatory, antioxidant, and hepatoprotective activities. The aim of this study was to evaluate cytotoxicity of three flavonoids isolated from MA (kuwanon E, cudraflavone B, and $4'$-*O*-methylkuwanon E) and to determine their effects on proliferation of THP-1 cells, and on cell cycle progression of cancer cells. Anti-inflammatory effects were also determined for all three given flavonoids. Methods used in the study included quantification of cells by hemocytometer and WST-1 assays, flow cytometry, western blotting, ELISA, and zymography. From the three compounds tested, cudraflavone B showed the strongest effects on cell cycle progression and viability of tumor and/or immortalized cells and also on inflammatory response of macrophage-like cells. Kuwanon E and $4'$-*O*-methylkuwanon E exerted more sophisticated rather than direct toxic effect on used cell types. Our data indicate that mechanisms different from stress-related or apoptotic signaling pathways are involved in the action of these compounds. Although further studies are required to precisely define the mechanisms of MA flavonoid action in human cancer and macrophage-like cells, here we demonstrate their effects combining antiproliferative and anti-inflammatory activities, respectively.

1. Introduction

The root bark of *Morus alba* L. (Moraceae; white mulberry) is used for its diuretic, antitussive, antidiabetic, and antipyretic effects in world traditional medicine [1]. Therefore, *Morus* species plants have been intensively studied from phytochemical point of view, and bioactive compounds of flavonoid character have been isolated. Within the flavonoid class of natural products, the prenylated subclass is quite rich in structural variety and pharmacological activities. Compounds obtained from *M. alba* L. possess anti-inflammatory, antibacterial, antiviral, antioxidant, and hepatoprotective activities [2–6]. Extracts obtained from *M. alba* L. were evaluated for their cytotoxicity against various tumor cells, such as K-562, B380 human leukemia cells, and B16 mouse melanoma cells [7]. Several studies have been published in

which bioactive compounds isolated from white mulberry exerted potent effect on human cancer cell lines. Morusin, one of the most efficient substances, showed strong activity against cervical carcinoma HeLa, breast carcinoma MCF-7, and hepatocarcinoma Hep3B cells [8]. Anticancer mechanism of morusin action in colorectal HT-29 cells is proposed to be mediated by induction of apoptosis and by suppression of NF-κB activity [9]. Another mulberry constituent, albanol A, induces apoptotic cell death in HL60 leukemia cell line via both the cell death receptor pathway by stimulation of death receptor and the mitochondrial pathway by topoisomerase II inhibition through caspase 2 activation [10].

The connection between inflammation and cancer can be thought of as consisting of two pathways: an extrinsic mechanism, where a constant inflammatory state contributes to increased cancer risk (such as in an inflammatory bowel disease), and an intrinsic mechanism, where acquired genetic alterations (such as activation of oncogenes) trigger tumor development [11]. The NF-κB signaling plays crucial roles in both precancerous chronic inflammation as well as cancer induced inflammation. An activation of this pathway induces expression of inflammatory cytokines, adhesion molecules, enzymes involved in the prostaglandin-synthesis pathway (such as COX-2), inducible nitric oxide synthase (iNOS), angiogenic factors, and antiapoptotic genes (such as Bcl-2) [12]. Proinflammatory cytokines implicated in carcinogenesis include, for instance, IL-1, IL-6, IL-15, colony stimulating factors (CSF), or TNF-α [13].

We have previously identified prenylated and geranylated flavanone compounds from plants of Moraceae and Paulowniaceae families with cytostatic activity in normal human fibroblasts and five human cancer cell lines [14]. Furthermore, we clarified the underlying molecular mechanisms mediating the effects of geranylated flavanone tomentodiplacone B on cell growth [15]. We showed that tomentodiplacone B induced accumulation of human monocytic leukaemia (THP-1) cells in G1 phase of cell cycle, which was in concert with downregulation of the cyclin E1 isoform and cyclin A2 levels, reduced CDK2 activity, and reduced pRb phosphorylation [15]. Our most recent work focusing on prenyl flavonoid cudraflavone B, which is contained in large amounts in the roots of white mulberry, showed unusually pronounced anti-inflammatory properties of this compound [2]. Moreover, throughout the course of experiments, we found that cudraflavone B had a strong effect on proliferation of human macrophage-like cells. It was therefore interesting to evaluate its effect on cell cycle progression and to elucidate the mechanisms of its cell proliferation inhibitory action. However, besides cudraflavone B (given designation **2**) we also isolated and characterized two other prenylated flavanones from *M. alba* L., which we have identified as kuwanon E (**1**), and 4′-*O*-methylkuwanon E (**3**), a new compound detected and described in our laboratory. Structures of all three tested *M. alba* L. prenylated (geranylated) flavonoids are shown in Figure 1(a). Based on our preliminary pilot data and the literature search (structure-effect relationship) we expected cytotoxic effect via targeting the cell cycle kinetics and viability.

The aim of our work was to evaluate effect of prenylated and geranylated flavonoids isolated from *M. alba* L. on proliferation of THP-1 cells and also to determine cell cycle profiles in several human cancer cells treated with *M. alba* L. flavonoids. As the role of inflammation in cancer is recently intensively discussed, we have also assessed anti-inflammatory effects of the previously mentioned flavonoids.

2. Methods

2.1. Test Compounds and Reagents. All three tested compounds (**1**, **2**, and **3**) were isolated and supplied by the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. The identification of substances was carried out using HRMS, ^{1}H, and ^{13}C NMR analyses, and their purity exceeded 95% according to the HPLC analysis [2, 14]. These compounds are poorly soluble in water; therefore, fresh 10 mM stock solutions in dimethylsulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) were prepared 1 day prior to experiments and stored at −20°C. These solutions were further diluted in the culture media to the desired final concentrations. RPMI 1640, DMEM, and IMDM culture media, phosphate buffered saline (PBS), and antibiotics (penicillin and streptomycin) were purchased from Lonza (Verviers, Belgium). Foetal bovine serum (FBS), phorbol myristate acetate (PMA), prednisone (purity ≥ 98%), and the lipopolysaccharide (LPS) obtained from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich. Instant ELISA Kits (eBioscience, Vienna, Austria) were used to evaluate the production of TNFα and IL-1β. Cytoscreen Kit (BioSource Europe S.A., Nivelles, Belgium) was used to detect IL-1RA cytokine by ELISA method. Mouse monoclonal antibody against cyclin B1 (MS-868) was purchased from Neomarkers (Fremont, CA, USA). Mouse monoclonal antibody against cyclin A (sc-53228) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against poly(adenosine diphosphate (ADP) ribose) polymerase (PARP), caspase 3, and phospho histone H3 were purchased from Cell Signaling Technologies (Beverly, MA, USA). Mouse monoclonal antibody against γ-H2AX [pS139] (05-636) was purchased from Millipore (Billerica, MA, USA). Mouse monoclonal antibodies against pRb (554136) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit polyclonal antibody against pRb [pT821] (44-582G) was purchased from BioSource (Carlsbad, CA, USA). Parthenolide (PTL), oxaliplatin, cisplatin and camptothecin, and all other reagents were purchased from Sigma-Aldrich.

2.2. Cell Culture. The human monocytic leukemia THP-1 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK; methods of characterization: DNA fingerprinting (multilocus probes) and isoenzyme analysis). Cells were cultured in RPMI 1640 medium supplemented with antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), 10% FBS, and 2 mM L-glutamine. Cultures were kept in an incubator at 37°C in a water-saturated 5% CO$_2$ atmosphere in air. Cells were passaged at approximately 1-week intervals. Cells were routinely tested for the absence of mycoplasma infection (Hoechst 33258 staining method). Mouse mammary epithelial cell line, SCp2 cells
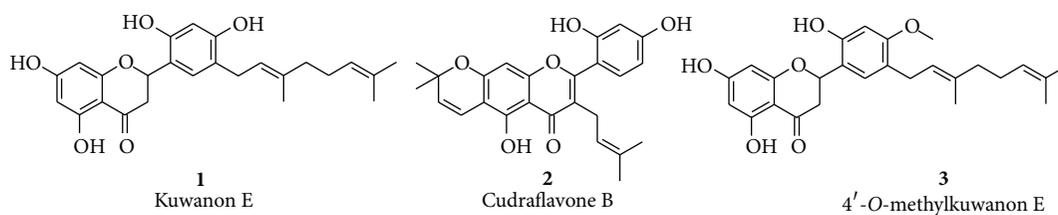
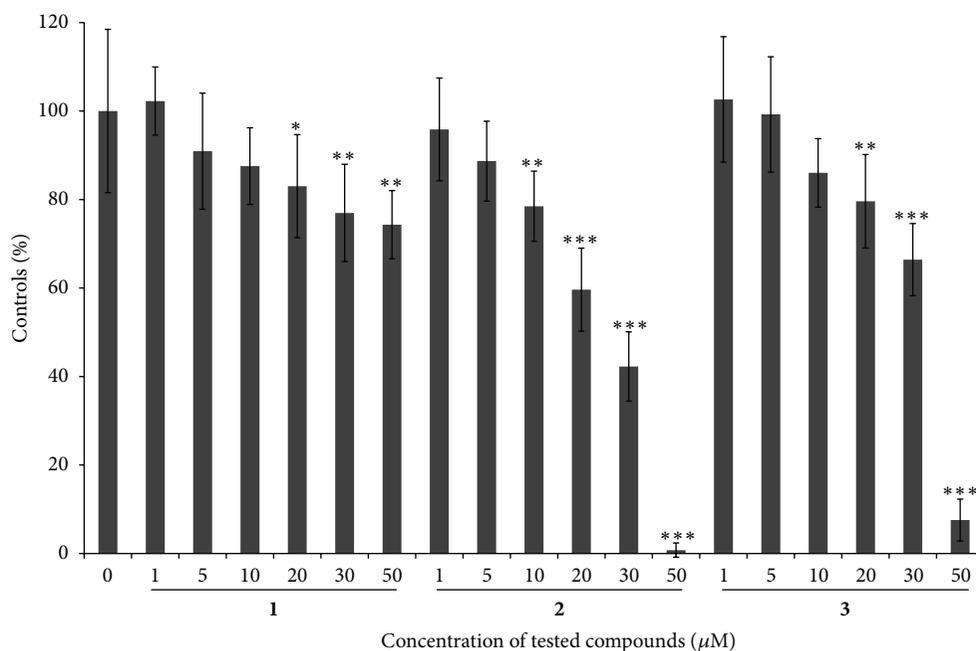| Tested substance	LD$_{50}$ (μM ± SD)
1	>50
2	24.3 ± 2.41
3	45.7 ± 3.72
Oxaliplatin	1.7 ± 0.64
Camptothecin	0.2 ± 0.07

(b)

(c)

FIGURE 1: Toxicity and inhibitory effects of tested MA compounds on THP-1 leukemia cell proliferation. (a) Structure of *M. alba* L. prenylated flavonoids. (b) THP-1 cells were seeded (2×10^5 cells/mL), treated with the indicated concentrations of **1**, **2**, and **3** for 24 h, cell numbers were counted, and viability was determined by erythrosin B exclusion. Toxicity was expressed as the LD$_{50}$ values. (c) THP-1 cells were seeded (5 $\times 10^4$ cells/well) in 96-well plates. Proliferation of cells was determined using WST-1 assays. Bars represent the proliferation of cells cultured in the presence of increasing concentrations of MA compounds as a percentage of controls at 24 h. The results shown are expressed as the means ± S.D. of three independent experiments, with each condition tested in triplicate (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

(kindly provided by P. Y. Desprez, Geraldine Brush Cancer Research Institute, California Pacific Medical Center, San Francisco, CA, USA), was cultured in DMEM supplemented with insulin 5 μg/mL (Sigma, St. Louis), 1% penicillin/streptomycin mixture (Lonza Walkersville, Inc., USA), and 5% heat inactivated FBS (Sigma-Aldrich), in a humidified incubator (95% air, 5% CO$_2$) at 37°C [16]. THP-1 cells were split into 24-well plates to achieve concentration of 100 000 cells/mL and were differentiated to macrophages by a phorbol myristate acetate (PMA) as described previously [17]. PC3 and DU-145 cells were obtained from the American Type Culture Collection (ATCC). PC3 and DU-145 were cultured in RPMI-1640, Ham's F12, or McCoy's media, respectively (Gibco Invitrogen Corporation, Carlsbad, CA, USA) with 2 mM L-glutamine, streptomycin (0.1 mg/mL), and penicillin (100 U/mL), and supplemented with 10% fetal bovine serum. LAPC-4 cells [18], a generous gift of Dr. R. Reiter (UCLA, Los Angeles, CA, USA), were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen) supplemented with NaHCO$_3$, penicillin/streptomycin, 10% FBS, and 1 nM R1881 (PerkinElmer). Benign prostatic hyperplasia (BPH) epithelial cells BPH-1 [19] were obtained from the German Collection

of Microorganisms and Cell Cultures. The cells are androgen unresponsive and were cultured in RPMI 1640 (Invitrogen), supplemented with 20% bovine fetal serum (PAA Laboratories, Pasching, Austria), 5 μg/mL transferrin, 5 ng/mL sodium selenite, 5 μg/mL insulin (Invitrogen), streptomycin (0.1 mg/mL), and penicillin (100 U/mL) (PAA). Cells were cultured at 37°C in a humidified 5% CO_2 incubator.

2.3. In Vitro Analysis of Cytotoxicity and Cell Proliferation. THP-1 cells were seeded (2×10^5 cells/mL) and incubated for 24 h at 37°C with 5% CO_2 with tested compounds dissolved in DMSO (Sigma-Aldrich) in concentrations ranging from 1 to 50 μM in RPMI 1640 medium. The maximum concentration of DMSO in the assays never exceeded 0.1%. Numbers and viabilities of the cells were determined by counting with a hemocytometer as we previously described [15]. Cell proliferation was determined using a WST-1 assay kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. For WST-1 assays, cells were seeded into 96-well plates (5×10^4 cells/well in 100 μL culture medium) in triplicates in complete RPMI 1640 medium, and measurements were taken 24 h after adding the tested MA compounds. All data were evaluated using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com/).

2.4. Cell Cycle Analysis. Cancer THP-1, LAPC-4, DU-145, PC3 cells, and human nontumorigenic benign prostatic hyperplasia BPH-1 cells were incubated with increasing concentrations of tested MA compounds for 24 h, washed in PBS (pH 7.4), and fixed for 30 min in an ice-cold 70% ethanol. Fixed cells were washed three times in PBS (pH 7.4) and incubated with RNase A (0.02 mg/mL) (Boehringer, Ingelheim, Germany) for 30 min at 37°C. Nuclei were stained with propidium iodide (40 μg/mL) and analysed by flow cytometry using a Beckman Coulter Cytomics FC500 flow cytometer (Beckman Coulter, Brea, CA, USA). Cell cycle distribution was analysed using FlowJo software (http://www.flowjo.com/).

2.5. Western Blotting. Cells were washed three times with PBS (pH 7.4) and lysed in 100 mM Tris-HCl (pH 6.8) containing 20% glycerol and 1% SDS. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Lysates were supplemented with bromophenol blue (0.01%) and β-mercaptoethanol (1%). Equal amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA), immunodetected using the appropriate primary and secondary antibodies, and visualised with ECL Plus reagent (Amersham, Aylesbury, UK) according to the manufacturer's instructions.

2.6. Treatment of THP-1 Cells with Drugs, Induction of Inflammatory Response, and Determination of Cytokines Production. Macrophages differentiated from THP-1 cells were pretreated for 1 h with tested compounds dissolved in DMSO to obtain

final concentrations of 10 μM (this concentration lacked cytotoxic effect). For comparison with conventional drugs, 1 μM prednisone dissolved in DMSO was used. Vehicle-treated cells contained a vehicle (DMSO) only. The concentration of DMSO was 0.1% in each well. The inflammatory response was triggered by adding LPS dissolved in water (1 μg/mL) to drug-pretreated macrophages, and cells were incubated for another 24 h. After this time period, medium was collected and the concentration of cytokines was measured by ELISA assay. The lowest detection limit was 7.8 pg/mL for TNF-α and 31.3 pg/mL for both IL-1β and IL-1RA. LPS-untreated cell served as controls.

2.7. Treatment of SCp2 Cells with Drugs, Induction of Inflammatory Response, and Zymography. SCp2 cells were plated in a 24-well plate in density of 4×10^4 cells/mL. After 24 h of incubation in medium containing 5% FBS, the medium was replaced, the cells were washed by PBS and fresh media supplemented with 1% FBS, and tested compounds were added. Final concentrations of tested compounds were 5 μM (this concentration lacked cytotoxic effect (data not shown)). Vehicle-treated and control cells were prepared using the same protocol as for THP-1 macrophages. For comparison with conventional drugs, parthenolide (5 μM dissolved in DMSO) was used, because of its usual use as a control for this type of cells and experiments and its well-known ability to inhibit the expression of matrix metalloprotease (MMP)-9 [20]. The inflammatory response was triggered by adding a nontoxic dose of LPS (10 μg/mL) to the drug-pretreated SCp2 cells, which were then incubated at 37°C for another 24 h [21]. After this time period, medium was collected and the pro-MMP-2 and MMP-2 activity was measured by zymography as described previously by Talhouk et al. [22]. Briefly, 20 μL of collected medium was loaded into polyacrylamide gel impregnated by 0.1% gelatin. After electrophoresis, SDS from gels was washed out by 2.5% Triton X-100, and gels were incubated for 30 min at room temperature (~23°C) and overnight (16–20 h) at 37°C in developing buffer (50 mM Tris (pH 8.8), 5 mM $CaCl_2$, 3 mM NaN_3, and 0.5% Triton X-100). Gels were then stained by Coomassie blue [22]. Intensity of digested regions was determined by densitometry followed by calculation using AlphaEaseFC 4.0.0 software (Alpha Innotech, USA). It should be noted that the conditioned medium contained active MMP-2, which represented 75.3% of measured activity. Therefore, this value was subtracted from all obtained results of MMP-2 activity.

2.8. Statistical Analysis. Statistical significance was tested using the one-way ANOVA with Dunnett's test and Tukey post test for comparisons between the means, and differences between two conditions were retained for $P < 0.05$. Statistical significance was determined at levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3. Results

3.1. Cytotoxic and Growth Inhibitory Effects of 1, 2, and 3 on THP-1 Cells. To determine the effects of all three tested

substances obtained from *M. alba* on the viability and growth of human leukemia cells, the THP-1 cells were exposed for 24 h to increasing concentrations (1, 2.5, 5, 10, 20, and 50 μM) of **1**, **2**, and **3**, respectively, stained for viability, and counted by hemocytometer. From this data, the LD$_{50}$ values for each MA compound were calculated (Figure 1(b)). Toxicity expressed as LD$_{50}$ increased as follows: **1** (>50 μM), **3** (45.7 $\pm$ 3.72), and **2** (24.3 $\pm$ 2.41). To compare toxicity of MA compounds with already known chemical or natural substances, we assessed LD$_{50}$ of oxaliplatin (1.7 $\pm$ 0.64) and camptothecin (0.2 $\pm$ 0.07), and, in both, it showed much lower toxic concentration values. Subsequent WST-1 assay, determining cell number using metabolic activity as a readout following exposure to MA compounds for 24 h, revealed that proliferation of THP-1 cells was inhibited by all three tested substances. As shown in Figure 1(c), substance **2** exhibited the strongest effect, as 10 μM and higher doses caused dose-dependent inhibition of THP-1 cell growth. The significant reduction of metabolic activity ($P < 0.05$) was though observed in cells treated with each of the three flavonoids at concentrations of 20 μM or higher. Based on cytotoxicity and proliferation data, the concentration range of MA compounds from 5 to 30 μM was selected for all subsequent experiments.

3.2. Effects of 1, 2, and 3 on Distribution of Cells in Cell Cycle Phases. In order to investigate the effect of tested substances on the cell cycle progression, we performed cell cycle analysis based on DNA content using flow cytometry of THP-1 cells. The data shown in Figures 2(a)–2(c) demonstrate that all compounds tested (**1**, **2**, and **3**) accumulate human leukemia cells in G1 phase dose-dependently after 24 h treatment. While the percentage of S phase cells decreased, the percentage of cells with 4N DNA content, representing G2/M phase, was unchanged upon treatment with tested compounds. This effect was dominant in substance **3**, lasting even after 72 h (data not shown).

Since compound **2** exerted the strongest impact on viability and proliferation, together with impact on the cell cycle profile of THP-1 cells (observed already from 10 μM concentration), we have expanded our analysis with this substance to further 3 human cancer cell lines (Figure 2(e)). The inhibition of G1/S transition, accompanied by the decreased proliferation caused by **2**, was observed in all cancer cell lines used in this experiment (LAPC-4—metastatic prostate, established from lymph nodes in SCID mice; PC3—androgen receptor null, p53-null, metastatic (bone) prostate cancer; and DU145—androgen receptor, p53-mutated, metastatic (brain) prostate cancer). To assess whether **2** affects also the cell cycle of human nontumorigenic cell line, we exposed the prostate epithelial BPH-1 cells, derived from the benign prostatic hyperplasia, to this compound. Interestingly, the distribution of BPH-1 cells in all three cell cycle phases remained unchanged even after the treatment with high concentrations of **2** used in the study (20 and 30 μM) (Figure 2(e)).

Although a G1 subpeak in a DNA histogram detected by flow cytometry cannot be considered as specific hallmark of apoptosis, it represents besides cellular debris also the apoptotic population of cells [23, 24]. The appearance of

the G1 subpeak was increased at 24 h after beginning the treatment with MA compounds, although with a different intensity of this effect (Figure 2(d)). While **1** exerted no G1 subpeak increase, the strongest induction of apoptosis was found in 30 μM **2**-treated THP-1 cells (~15-fold higher compared to control). Significant increase of G1 subpeak (~8-fold higher compared to control) was caused also by 30 μM **3** compound. Nevertheless, even the highest concentration of **2** used did not cause such massive apoptosis that we found in 5 or 10 μg/mL cisplatin, included as a model compound (~36- and ~60-fold higher, resp., compared to control).

3.3. Expression of Cell Cycle Regulators in MA-Treated Cells. Based on the fact that all tested compounds cause accumulation of cells in G1 phase, we determined the expression and phosphorylation status of key cell cycle and stress-related proteins. Phosphorylated Rb protein is the key regulatory molecule, which coordinates processes critical for G1/S progression. We therefore examined whether pRb phosphorylation is suppressed in THP-1 cells treated by MA compounds. As shown in Figures 3(a) and 3(c), 24 h exposure to 20 μM **1** or **3** results in reduced phosphorylation of Rb protein on serine 780. For **2** this effect was even more pronounced (Figure 3(b)). Phosphorylation on serines 807/811 was also decreased in THP-1 cells exposed to MA compounds, in clearly dose-dependent manner (Figures 3(a)–3(c)). It is highly probable that MA-induced Rb dephosphorylation corresponds to the accumulation of cells in G1 phase.

Another protein involved in cell cycle machinery, which we analyzed in MA-treated cells, was proliferating cell nuclear antigen (PCNA). This protein is well known as a DNA sliding clamp for DNA polymerase delta and as an essential component for eukaryotic chromosomal DNA replication and repair [25]. All flavonoids tested downregulated the expression of PCNA in THP-1 cells (Figures 3(a)–3(c)), again correspondingly to the observed decrease of cells in S phase of the cell cycle.

Cyclins A and B are members of the cyclin family, expression of which fluctuates during cell cycle progression peaking in S and G2 phases, respectively. We found that none of the tested MA compounds affects the quantity of these cyclins, when measured in asynchronously growing cells. Moreover, phosphorylation of histone H3 at threonine 11, which normally peaks at M phase, remained unaffected even after 24 h treatment with MA compounds. Unchanged phosphorylation of histone H3 with normal expression of cyclins A and B suggests that MA compounds do not influence progression through G2 and M phases of cell cycle.

Caspase 3-mediated PARP cleavage has been considered as a hallmark of apoptosis. It is also known that PARP activation is induced by DNA strand breaks [26]. Neither **1** nor **3** did cause PARP cleavage, and so its activation in THP-1 cells. However, increased histone γ-H2AX phosphorylation together with cleavage of both caspase 3 and PARP in **2**-treated THP-1 cells indicates the activation of the stress signaling apoptotic pathways caused by the highest concentration used (20 μM).
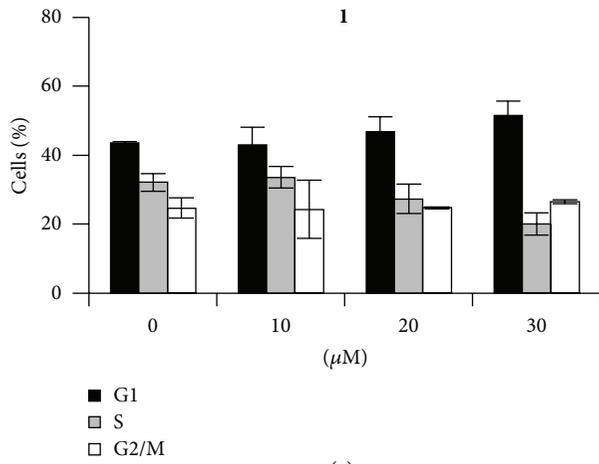
(a)

(b)

(c)

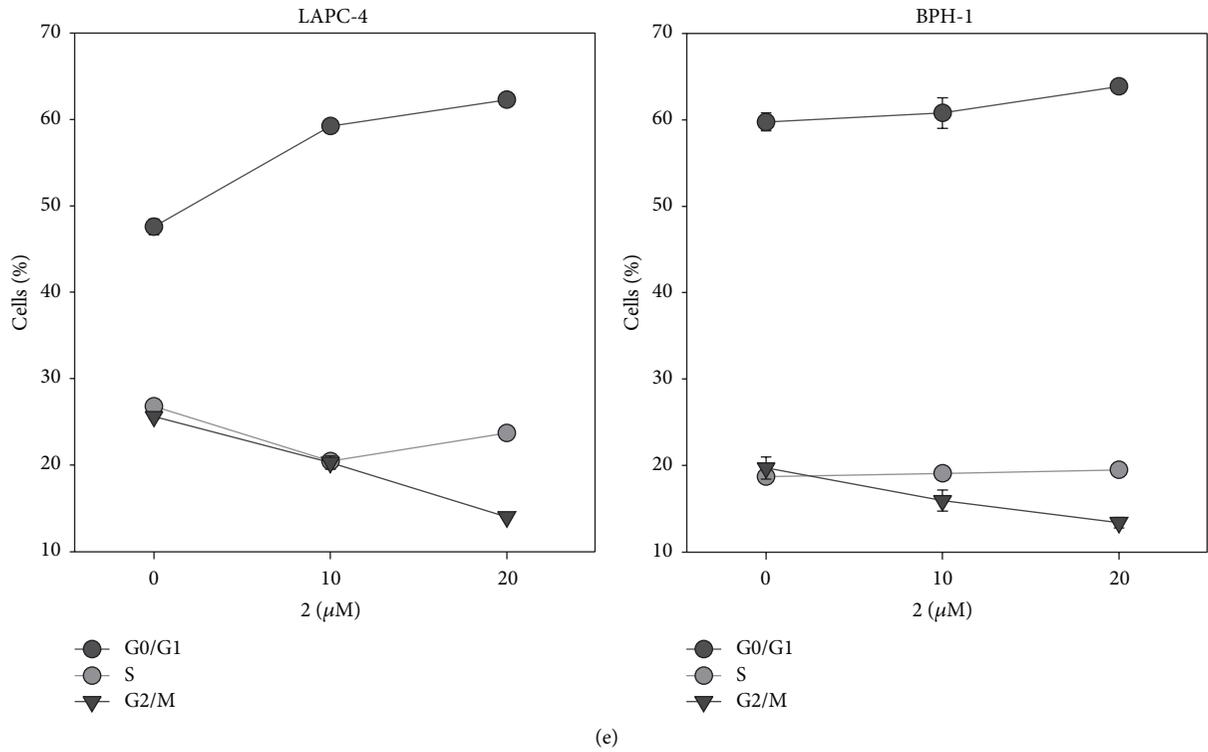(d)
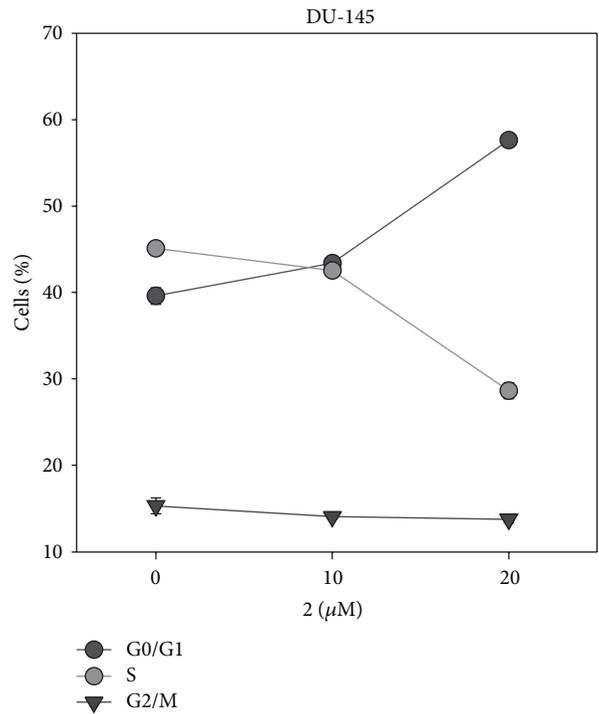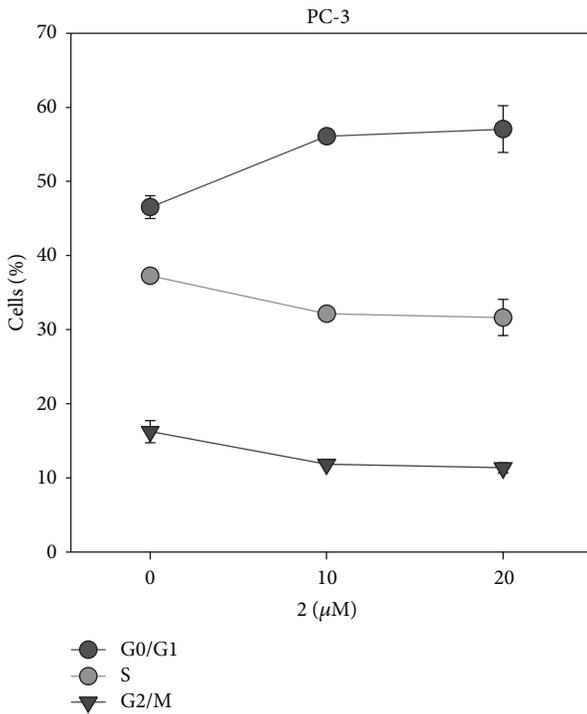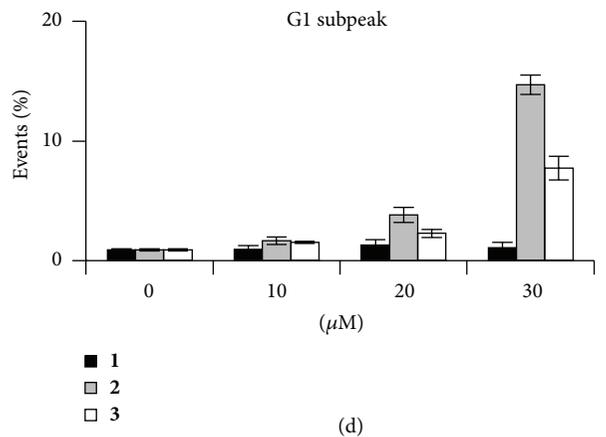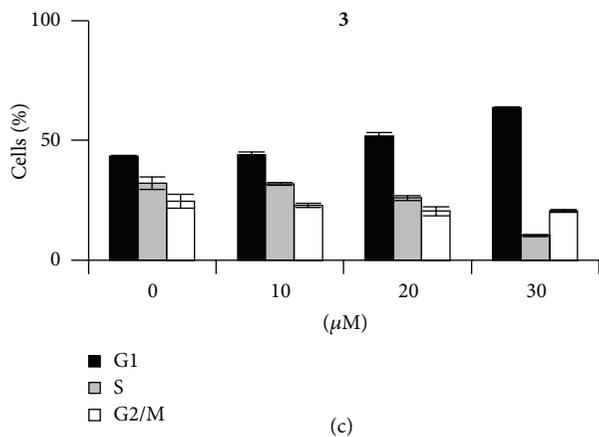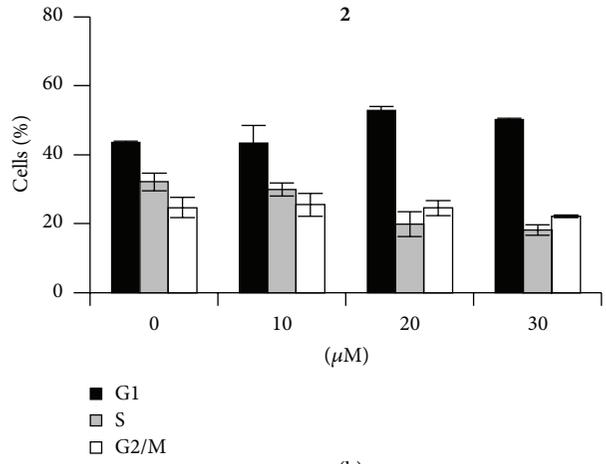
FIGURE 2: Continued.

(e)

FIGURE 2: Treatment with *M. alba* L. prenylated flavonoids causes accumulation of several cancer cells in G1 phase. (a) Cell cycle distribution at 24 h upon treatment of THP-1 cells with **1** as determined by flow cytometry. (b) Cell cycle distribution at 24 h upon treatment of THP-1 cells with **2** as determined by flow cytometry. (c) Cell cycle distribution at 24 h upon treatment of THP-1 cells with **3** as determined by flow cytometry. (d) Quantification of G1 subpeak in MA flavonoids treated THP-1 cells. (e) Cell cycle distribution at 24 h upon treatment of cancer PC3, DU-145, LAPC-4, and immortalized BPH-1 cells with **2** as determined by flow cytometry.
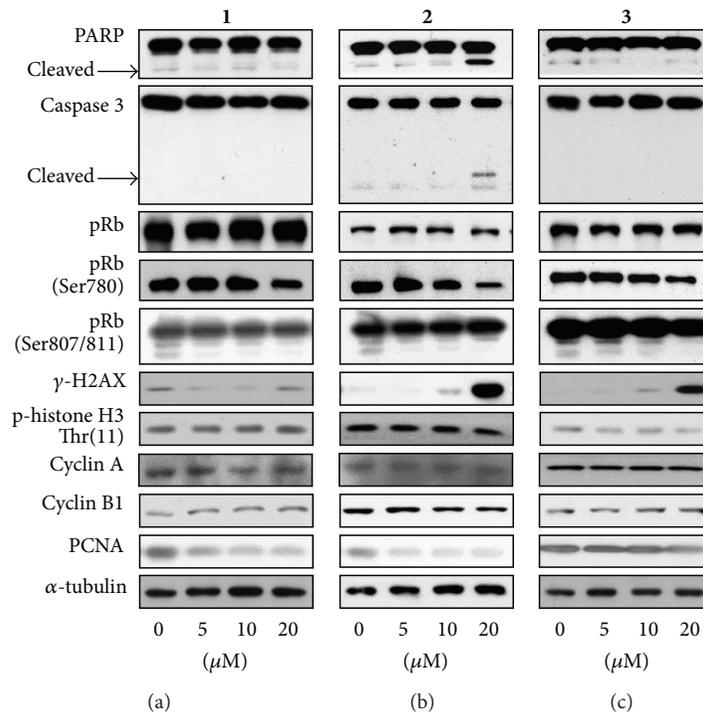
FIGURE 3: Expression of cell cycle regulators and stress response proteins after 24 h of (a) **1**, (b) **2**, and (c) **3** treatment.

3.4. Behaviour of Inflammatory Response Markers in MA-Treated Cells. Protein TNF-α together with other cytokines, such as interleukins, not only plays crucial role in the inflammatory response but also is involved in carcinogenesis [13]. To investigate whether antiproliferative effects of MA compounds are accompanied by anti-inflammatory activity, we assessed levels of selected inflammatory response markers secreted into the culture medium by LPS-activated macrophages derived from THP-1 cell line. As evident from Figure 4(a), LPS-induced TNF-α secretion by macrophages was reduced upon the treatment with MA compounds, similarly to prednisone used as the reference anti-inflammatory drug. Notably, all three compounds tested were significantly more effective than prednisone. Levels of IL-1β, the most studied member of the IL-1 family [27], produced by THP-1-derived macrophages were slightly decreased by tested substances, except for **3** (Figure 4(b)). Treatment with this compound (10 μM) led to significant ($P < 0.01$) increase of IL-1β secreted to cell culture medium. The natural antagonist of IL-1β is IL-1RA, and their mutual ratio is crucial for a progression of inflammation and maintaining a homeostasis. All tested flavonoids, similarly to prednisone, significantly decreased the secretion of IL-1RA (Figure 4(c)). This secretion attenuation affected the IL-1β/IL-1RA ratio (Figure 4(d)). This increase was nonsignificant for compounds **1**, **2**, and prednisone. On the other hand, $4'$-O-methylkuwanon E (**3**) increased the IL-1β/IL-1RA ratio by the factor of 5.33. It is caused by enormously elevated secretion of IL-1β. Matrix metalloproteinase 2 (MMP2) is involved in the tissue development and remodelling, but it also contributes to inflammation progression. It is secreted as inactive pro-MMP2 form, which is extracellularly cleaved to its active form. The amount of (pro-)MMP2 was significantly decreased only by **2** and the control drug parthenolide (PTL) (Figure 5(a)) in SCp2 cell line. Whereas PTL inhibits proteinase activity to the level typical for unstimulated cells, **2** was able to reduce the (pro-)MMP2 activity below these control cells. **2** uniquely and significantly decreased the pro-MMP2/MMP2 ratio (Figure 5(b)).

4. Discussion and Conclusions

Relevance of the crosstalk between components of the immune system and cancer cells is widely discussed. During the last decade the clear evidence that inflammation plays a critical role in tumorigenesis has been obtained, and some of underlying molecular mechanisms have been elucidated [28]. A role of inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumors, including some in which a direct causal relationship with inflammation is not yet proven [11].

In the present study, we assessed cytotoxicity and the effects of three prenylated (geranylated) flavonoids from *M. alba* L., kuwanon E (**1**), cudraflavone B (**2**), and $4'$-O-methylkuwanon E (**3**) on cell cycle progression and selected cell cycle regulatory proteins. We have also extended our study with the aim of evaluating the effect of these substances

on proinflammatory markers, because we recently reported that **2** has potent anti-inflammatory properties in human macrophages [2]. Compounds are poorly soluble in water; therefore, we used DMSO as a solvent. The final DMSO concentration of 0.5–1% is frequently employed in *in vitro* studies to solubilize/deliver bioactive compounds to cells. However, it has been shown that DMSO exhibits a myriad of biological actions, such as reported effects on cell cycle, differentiation, inflammatory response, and apoptosis studies [29–31]. Since our intention was focused on evaluation of these types of effects, it was necessary to take into account the effects of DMSO in arrangement of all conducted experiments. In particular, the concentration of DMSO in experiments never exceeded 0.1%. Moreover, to minimize misinterpretations of our results due to biological effects of DMSO, we employed DMSO-only-treated THP-1 cells as controls in each experiment setting. Based on our previously published results [14, 15, 17] we used human monocytic leukaemia cells THP-1 as a model system to detect cytotoxic and cytostatic effects of newly isolated natural compounds and THP-1-derived macrophages for studies on inflammatory response. We found strong antiproliferative effects of all three tested MA compounds in concentrations ranging from 10 to 50 μM. When comparing these data with the LD$_{50}$ values, we may conclude that unlike **2**, both **1** and **3** at concentrations of 20 μM and 30 μM had significant growth inhibitory effect without being cytotoxic to the cells. As regards substance **2**, we speculate that the observed reduction of metabolic activity is more likely a sign of cell dying rather than growth inhibition.

To reveal whether antiproliferative effects seen in THP-1 cells after 24 h treatment with MA flavonoids reflect inhibition of cell transition between specific cycle phases, we conducted the cell cycle analysis. Our results showed that all tested compounds caused accumulation of THP-1 human leukemia cells in G1 phase of cell cycle (and inhibited their entry into the S phase) in a dose-dependent manner. Taking into account the strength of **2** effect on viability, proliferation, and the cell cycle profile (showed from the concentration of 10 μM, in contrast to other MA substances), we exposed three other human cancer as well as nontumorigenic cell lines to **2**. While in all tumor cells **2** exhibited inhibitory effect on the G1/S transition, in nontumor line (prostate epithelial BPH-1 cells) such activity was not observed. This might indicate a partially selective effect of this substance on tumor versus nontumor cells. Nevertheless, such selectivity of **2** would have to be verified by more detailed analysis.

The cell cycle analysis allowed us to study the percentage of THP-1 cells in specific phase, including determining sub-G1 peak, which covers also cells undergoing the process of apoptosis. One of the characteristic events of apoptosis is the proteolytic cleavage of poly(ADP-ribose)polymerase (PARP), a nuclear enzyme involved in DNA repair, DNA stability, and transcriptional regulation. Caspases, in particular caspases 3 and 7, cleave the 116-kDa form of PARP at the DEVD site to generate an 85- and a 24-kDa fragment [26]. PARP is inactivated by caspase 3 cleavage (in a specific domain of the enzyme) during programmed cell death. One-day treatment with **1** had no effect on induction of apoptosis as determined
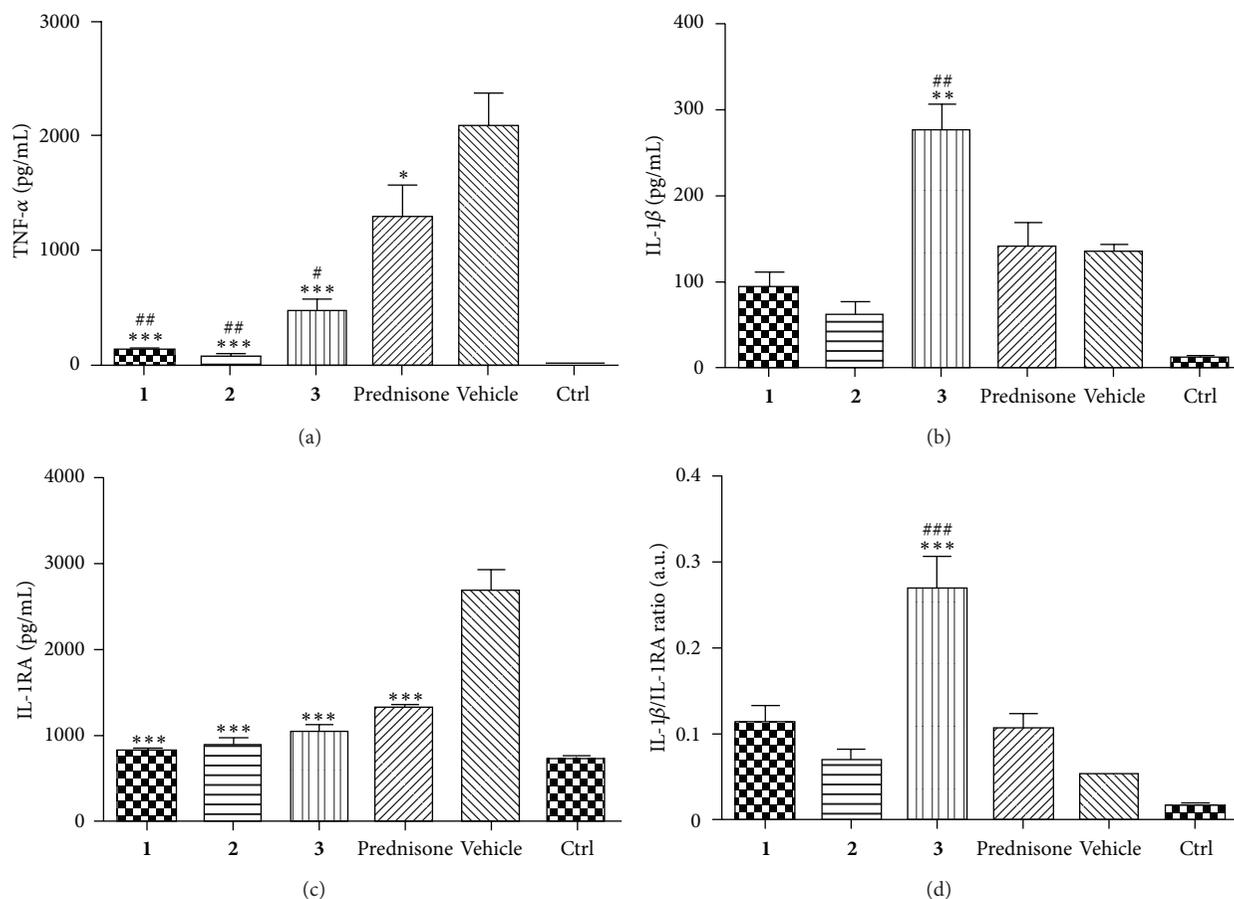
(a)

(b)

(c)

(d)

Figure 4: (a) Effects of *M. alba* L. prenylated flavonoids and the reference drug prednisone on LPS-induced TNF-α secretion at macrophages derived from THP-1 cell line. Cells were pretreated with given compounds (10 μM), prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means ± S.E. for three independent experiments. *Significant difference in comparison to vehicle-treated cells ($P < 0.05$), ***significant difference in comparison to vehicle-treated cells ($P < 0.001$), #significant difference in comparison to prednisone-treated cells ($P < 0.05$), and ##significant difference in comparison to prednisone-treated cells ($P < 0.01$). (b) Effects of *M. alba* L. prenylated flavonoids and the reference drug prednisone on LPS-induced IL-1β secretion at macrophages derived from THP-1 cell line. Cells were pretreated with given compounds (10 μM), prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means ± S.E. for three independent experiments. **Significant difference in comparison to vehicle-treated cells ($P < 0.01$); ##significant difference in comparison to prednisone-treated cells ($P < 0.01$). (c) Effects of *M. alba* L. prenylated flavonoids and the reference drug prednisone on LPS-induced IL-1RA secretion at macrophages derived from THP-1 cell line. Cells were pretreated with given compounds (10 μM), prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are expressed as means ± S.E. for three independent experiments. ***Significant difference in comparison to vehicle-treated cells ($P < 0.001$). (d) Ratio IL-1β/IL-1RA production calculated for macrophages derived from THP-1 cell line. Values were obtained from ELISA measurements of individual cytokines as it is described in Figures 2 and 3. Results are expressed as means ± S.E. for three independent experiments. A.U. = arbitrary unit. ***Significant difference in comparison to vehicle-treated cells ($P < 0.001$); ###significant difference in comparison to prednisone-treated cells ($P < 0.001$).

by flow cytometry assessment of G1 subpeak and western blot analysis of PARP and caspase 3 cleavage. Significant increase of G1 subpeak (~8-fold higher compared to control) was caused by 30 μM **3** compound; however, no cleavage of PARP and caspase 3 was observed (Figure 3(c)). Conversely, massive increase in G1 subpeak (~15-fold higher compared to control), together with occurrence of both apoptotic markers (cleaved PARP and subsequently caspase 3), was observed in cells exposed to **2** for 24 h. However, effects of **2** on THP-1 cells are not comparable with those of cisplatin, added as

a model anticancer drug. Cisplatin caused considerably more substantial changes in both G1 subpeak accumulation and caspase 3 cleavage (see Figure S1 in Supplementary Material Available online at http://dx.doi.org/10.1155/2013/350519), suggesting that **2** mechanism of action is not similar to that of platinum derivatives. These results prompted us to experimentally address molecular mechanisms underlying the effects of MA compounds on cell growth.

Cyclins A and B are members of the cyclin family, with the maximum of their expression during S and G2 phases of

(a)

(b)

pro-MMP-2 $\longrightarrow$
MMP-2 $\longrightarrow$

(c)
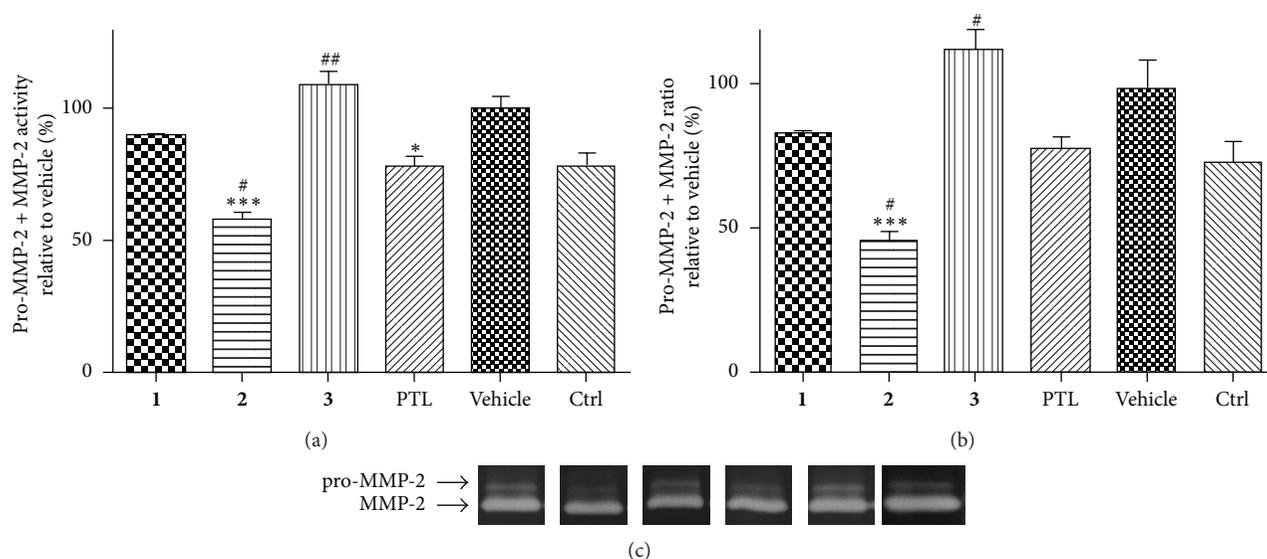
FIGURE 5: Effects of *M. alba* L. prenylated flavonoids and the reference drug parthenolide (PTL) on LPS-induced (pro-)MMP-2 activity at SCp2 cells. Cells were pretreated with given compounds (5 μM), parthenolide (5 μM), or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Activity of (pro-)MMP-2 was detected by zymography (a). Intensity of digested bands was analyzed by densitometry. (b) shows pro-MMP-2/MMP-2 ratio. Showed gels represent results of three independent experiments (c). Results are expressed as means ± S.E. for three independent experiments. ***Significant difference in comparison to vehicle-treated cells ($P < 0.001$), #significant difference in comparison to parthenolide-treated cells ($P < 0.05$), and ##significant difference in comparison to parthenolide-treated cells ($P < 0.01$).

a cell cycle. Cyclin A is required for cell to progress through the S phase, and cyclin B is necessary for cells to enter mitosis and divide into two daughter cells [32]. It is also known that activation of tumor suppressor retinoblastoma protein (pRb) permits transcription of key S-phase-promoting genes, including some that are required for DNA replication. In contrast, dephosphorylation of pRb slows the progression of cells into S phase [33]. None of the MA compounds tested were found to reduce, after 24 h exposure, the expression of cyclins A and B. This fact, together with the reduced pRb phosphorylation caused by all MA compounds, possibly indicates that these substances affect rather the G1/S than G2/M transition. Flow cytometry data further support this hypothesis, since significant accumulation of cells in G1 at the expense of S phase was observed upon the treatment with MA compounds. Since such cell cycle distortion could be mediated by stress response signaling pathways, their activation was evaluated in THP-1 cells treated by all three MA flavonoids. In THP-1 cells stress-associated regulators such p21, p27, and p53 proteins are not detectable [15]. Therefore, we focused on histones γ-H2AX (becomes phosphorylated on damaged DNA) and H3 (its phosphorylation on Thr11 correlates with mitotic/meiotic chromosome condensation). Cells treated with any MA compound displayed no changes in phosphorylation of histone H3 on the given residue. For γH2AX we observed increased phosphorylation only in cells treated with **2** at concentration of 20 μM, which is the same as such causing cleavage of caspase 3 and PARP. Collectively, we speculate that **2** exerts mode of action that is different from that of **1** and **3**. Compound **2** seems to inhibit proliferation via triggering the stress-related pathway leading

to Rb dephosphorylation and apoptosis with typical cleavage of PARP and caspase 3. On the other hand, no induction of the stress-related proteins occurs in **1**- and **3**-treated cells, and **1** in all tested concentrations clearly affects PCNA, which facilitates and controls DNA replication, and is at the very heart of cell-cycle progression.

As mentioned at the beginning, our previous study on effects of cudraflavone B (**2**) in human macrophages showed an interesting anti-inflammatory activity of this flavonoid. Since newly characterized compounds **1** and **3** were also isolated from *M. alba* L., and chemically belong to the same category, we expected similar effects. Yet, except secretion of TNF-α and IL-1RA, we found different results after application of MA substances to macrophages. Therefore, only relatively little structural differences between compounds tested (presence of 2,3 double bond at **2**, presence and position of prenyl or geranyl side chains, or substitution of flavonoid *B*-ring) strongly affect the mechanism of action and play a role in final effect of compound.

Importantly, our results pointed to huge differences among the tested compounds. **1** and **2** showed similar inhibition effect on TNF-α, IL-1β, and IL-1RA expression. On the other hand, **3**, which differs from **1** by substitution of one hydroxyl group on the *C*-ring for methoxy group, attenuated only TNF-α and IL-1RA expression, but less effectively than **1** or **2**, and secretion of IL-1β was strongly elevated. It is obvious that all three compounds are able to downregulate expression of genes that are under transcriptional control of NF-κB. In comparison with other two cytokines, IL-1β is synthetized as proprotein, and it is cleaved into active form by caspase-1-containing inflammasome [34]. Increased IL-1β

production in the presence of LPS in cells was observed following incubation with doxorubicin and daunorubicin [35] or Cu(II) complexes [36]. We cannot exclude the possibility that **3** activates an inflammasome, and, thus, augments IL-1β secretion. It should be noted that although **1** and **2** inhibited IL-1β secretion, the effect is much smaller than in the case of TNF-α. This may indicate that all three compounds are able to positively regulate inflammasome action. The low ability of tested compounds to downregulate proinflammatory IL-1β and significant downregulation of anti-inflammatory IL-1RA are showed in higher IL-1β/IL-1RA ratio. The MMP-2 activity is in agreement with TNF-α and IL-1β expression— **2** significantly decreased its level, **1** inhibited its activity only slightly, and **3** moderately raised its level. According to these results, the highest antiphlogistic potential has **2** followed by **1**. Depending on conditions, flavonoids can act both as prooxidants and antioxidants. The ability to cause dysfunction of mitochondria by prooxidant effect is connected with possible mechanisms of anticancer action, which may lead to apoptosis of tumor cells. Their antioxidant activity is connected with direct scavenging effect of excessive reactive oxygen/nitrogen species or with interaction with enzymes involved in their production or elimination. Interaction with enzymes responsible for carcinogen activation can lead to prevention of tumor formation [37]. Only a few reports on anti/prooxidative activity of compounds analysed in this study have been published. In general, these compounds do not fulfil the structural requirements needed for direct scavenging effect *in vitro* [38], which was confirmed for compound **2** [6, 39]. Park et al. [40] showed only weak activity of **2** in protecting LDL particles against oxidation (TBARS assay), but the inhibition of NO formation mediated via inhibition of iNOS was proved using RAW 264.7 cells. Protective effects of prenylated compounds (**1** and **3**) against oxidative stress-induced damage of human neuroblastoma SH-SY5Y cells were observed, showing their potential antioxidant activity [41]. Compound **1** showed inhibitory activity on NO production in RAW 264.7 cells [42]. Possible pro/antioxidant activity of tested compounds and its interconnection with their anticancer effects should be clarified in further experiments.

In conclusion, the reported active agents isolated from *M. alba* L. have an interesting impact on human cells, which are involved in both tumor and inflammation. Of the three compounds tested, **2** showed the strongest effects on cell cycle progression and viability of tumor cells and on inflammatory response of macrophage-like cells. In addition, substances **1** and **3** exerted more sophisticated rather than direct toxic effect on used cell types. Our data indicate that mechanisms different from stress-related or apoptotic signaling pathways are involved in the action of these compounds. Although further studies are required to precisely define the mechanisms of MA flavonoid actions, here we clearly demonstrate their effects combing antiproliferative and anti-inflammatory activities in human cancer and macrophage-like cells, respectively. Confirmed dual activity of tested prenylated flavonoids could be an inspiration for chemical modifications of their structures or isolation of similar substances in order to get more potent agents usable for clinical practice in future.

Abbreviations

MA: *Morus alba* L.
1: Kuwanon E
2: Cudraflavone B
3: 4′-*O*-methylkuwanon E
PBS: Phosphate buffered saline
FBS: Fetal bovine serum
DMSO: Dimethylsulfoxide
PMA: Phorbol myristate acetate
LPS: Lipopolysaccharide
PTL: Parthenolide.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

References

[1] S. Ercisli and E. Orhan, "Chemical composition of white (*Morus alba*), red (*Morus rubra*) and black (*Morus nigra*) mulberry fruits," *Food Chemistry*, vol. 103, no. 4, pp. 1380–1384, 2007.

[2] J. Hošek, M. Bartos, S. Chudík et al., "Natural compound cudraflavone B shows promising anti-inflammatory properties *in vitro*," *Journal of Natural Products*, vol. 74, no. 4, pp. 614–619, 2011.

[3] H. Y. Sohn, K. H. Son, C. S. Kwon, G. S. Kwon, and S. S. Kang, "Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussnetia papyrifera* (L.) Vent, *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai," *Phytomedicine*, vol. 11, no. 7-8, pp. 666–672, 2004.

[4] J. Du, Z. D. He, R. W. Jiang et al., "Antiviral flavonoids from the root bark of *Morus alba* L.," *Phytochemistry*, vol. 62, no. 8, pp. 1235–1238, 2003.

[5] H. A. El-Beshbishy, A. N. B. Singab, J. Sinkkonen et al., "Hypolipidemic and antioxidant effects of *Morus alba* L. (Egyptian mulberry) root bark fractions supplementation in cholesterol-fed rats," *Life Sciences*, vol. 78, no. 23, pp. 2724–2733, 2006.

[6] H. Oh, E. K. Ko, J. Y. Jun et al., "Hepatoprotective and free radical scavenging activities of prenylflavonoids, coumarin, and stilbene from *Morus alba*," *Planta Medica*, vol. 68, no. 10, pp. 932–934, 2002.

[7] S. Y. Nam, H. K. Yi, J. C. Lee et al., "Cortex mori extract induces cancer cell apoptosis through inhibition of microtubule assembly," *Archives of Pharmacal Research*, vol. 25, no. 2, pp. 191–196, 2002.

[8] N. T. Dat, P. T. X. Binh, L. T. P. Quynh et al., "Cytotoxic prenylated flavonoids from *Morus alba*," *Fitoterapia*, vol. 81, no. 8, pp. 1224–1227, 2010.

[9] J. C. Lee, S. J. Won, C. L. Chao et al., "Morusin induces apoptosis and suppresses NF-κB activity in human colorectal cancer HT-29 cells," *Biochemical and Biophysical Research Communications*, vol. 372, no. 1, pp. 236–242, 2008.

[10] T. Kikuchi, M. Nihei, H. Nagai et al., "Albanol A from the root bark of *Morus alba* L. induces apoptotic cell death in HL60 human leukemia cell line," *Chemical & Pharmaceutical Bulletin*, vol. 58, no. 4, pp. 568–571, 2010.

[11] A. Mantovani, P. Allavena, A. Sica, and F. Balkwill, "Cancer-related inflammation," *Nature*, vol. 454, no. 7203, pp. 436–444, 2008.

[12] Y. Wu and B. P. Zhou, "TNF-α/NFκB/Snail pathway in cancer cell migration and invasion," *British Journal of Cancer*, vol. 102, no. 4, pp. 639–644, 2010.

[13] S. P. Hussain and C. C. Harris, "Inflammation and cancer: an ancient link with novel potentials," *International Journal of Cancer*, vol. 121, no. 11, pp. 2373–2380, 2007.

[14] K. Šmejkal, J. Svačinová, T. Šlapetová et al., "Cytotoxic activities of several geranyl-substituted flavanones," *Journal of Natural Products*, vol. 73, no. 4, pp. 568–572, 2010.

[15] P. Kollár, T. Bárta, V. Závalová, K. Šmejkal, and A. Hampl, "Geranylated flavanone tomentodiplacone B inhibits proliferation of human monocytic leukaemia (THP-1) cells," *British Journal of Pharmacology*, vol. 162, no. 7, pp. 1534–1541, 2011.

[16] B. Safieh-Garabedian, G. M. Mouneimne, W. El-Jouni, M. Khattar, and R. Talhouk, "The effect of endotoxin on functional parameters of mammary CID-9 cells," *Reproduction*, vol. 127, no. 3, pp. 397–406, 2004.

[17] K. Pencikova, P. Kollar, V. Muller Zavalova, E. Taborska, J. Urbanova et al., "Investigation of sanguinarine and chelerythrine effects on LPS-induced inflammatory gene expression in THP-1 cell line," *Phytomedicine*, vol. 19, pp. 890–895, 2012.

[18] K. A. Klein, R. E. Reiter, J. Redula et al., "Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice," *Nature Medicine*, vol. 3, no. 4, pp. 402–408, 1997.

[19] S. W. Hayward, R. Dahiya, G. R. Cunha, J. Bartek, N. Deshpande, and P. Narayan, "Establishment and characterization of an immortalized but non-transformed human prostate epithelial cell line: BPH-1," *In Vitro Cellular & Developmental Biology*, vol. 31, no. 1, pp. 14–24, 1995.

[20] A. Ghantous, H. Gali-Muhtasib, H. Vuorela, N. A. Saliba, and N. Darwiche, "What made sesquiterpene lactones reach cancer clinical trials?" *Drug Discovery Today*, vol. 15, no. 15-16, pp. 668–678, 2010.

[21] S. W. Maalouf, R. S. Talhouk, and F. L. Schanbacher, "Inflammatory responses in epithelia: endotoxin-induced IL-6 secretion and iNOS/NO production are differentially regulated in mouse mammary epithelial cells," *Journal of Inflammation*, vol. 7, article 58, 2010.

[22] R. S. Talhouk, J. R. Chin, E. N. Unemori, Z. Werb, and M. J. Bissell, "Proteinases of the mammary gland: developmental regulation in vivo and vectorial secretion in culture," *Development*, vol. 112, no. 2, pp. 439–449, 1991.

[23] W. L. Xia, S. Spector, L. Hardy et al., "Tumor selective G_2/M cell cycle arrest and apoptosis of epithelial and hematological malignancies by BBL22, a benzazepine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 13, pp. 7494–7499, 2000.

[24] M. Kajstura, H. D. Halicka, J. Pryjma, and Z. Darzynkiewicz, "Discontinuous fragmentation of nuclear DNA during apoptosis revealed by discrete "Sub-G_1" peaks on DNA content histograms," *Cytometry A*, vol. 71, no. 3, pp. 125–131, 2007.

[25] S. N. Naryzhny, "Proliferating cell nuclear antigen: a proteomics view," *Cellular and Molecular Life Sciences*, vol. 65, no. 23, pp. 3789–3808, 2008.

[26] M. Los, M. Mozoluk, D. Ferrari et al., "Activation and caspase-mediated inhibition of PARP: a molecular switch between fibroblast necrosis and apoptosis in death receptor signaling," *Molecular Biology of the Cell*, vol. 13, no. 3, pp. 978–988, 2002.

[27] C. A. Dinarello, "Immunological and inflammatory functions of the interleukin-1 family," *Annual Review of Immunology*, vol. 27, pp. 519–550, 2009.

[28] M. Karin, "Nuclear factor-κB in cancer development and progression," *Nature*, vol. 441, no. 7092, pp. 431–436, 2006.

[29] S. Hollebeeck, T. Raas, N. Piront, Y. J. Schneider, O. Toussaint et al., "Dimethyl sulfoxide (DMSO) attenuates the inflammatory response in the *in vitro* intestinal Caco-2 cell model," *Toxicology Letters*, vol. 206, no. 3, pp. 268–275, 2011.

[30] S. W. Jacob and R. Herschler, "Pharmacology of DMSO," *Cryobiology*, vol. 23, no. 1, pp. 14–27, 1986.

[31] N. C. Santos, J. Figueira-Coelho, J. Martins-Silva, and C. Saldanha, "Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects," *Biochemical Pharmacology*, vol. 65, no. 7, pp. 1035–1041, 2003.

[32] U. Galderisi, F. P. Jori, and A. Giordano, "Cell cycle regulation and neural differentiation," *Oncogene*, vol. 22, no. 33, pp. 5208–5219, 2003.

[33] A. A. Romani, S. Desenzani, M. M. Morganti, S. La Monica, A. F. Borghetti, and P. Soliani, "Zoledronic acid determines S-phase arrest but fails to induce apoptosis in cholangiocarcinoma cells," *Biochemical Pharmacology*, vol. 78, no. 2, pp. 133–141, 2009.

[34] O. Gross, C. J. Thomas, G. Guarda, and J. Tschopp, "The inflammasome: an integrated view," *Immunological Reviews*, vol. 243, no. 1, pp. 136–151, 2011.

[35] K. A. D. Sauter, L. J. Wood, J. Wong, M. Iordanov, and B. E. Magun, "Doxorubicin and daunorubicin induce processing and release of interleukin-1β through activation of the NLRP3 inflammasome," *Cancer Biology and Therapy*, vol. 11, no. 12, pp. 1008–1016, 2011.

[36] Z. Travnicek, J. Vanco, J. Hosek, R. Buchtik, and Z. Dvorak, "Cellular responses induced by Cu(II) quinolinonato complexes in human tumor and hepatic cells," *Chemistry Central Journal*, vol. 6, article 160, 2012.

[37] G. Galati and P. J. O'Brien, "Potential toxicity of flavonoids and other dietary phenolics: significance for their chemopreventive and anticancer properties," *Free Radical Biology and Medicine*, vol. 37, no. 3, pp. 287–303, 2004.

[38] B. H. Havsteen, "The biochemistry and medical significance of the flavonoids," *Pharmacology & Therapeutics*, vol. 96, no. 2-3, pp. 67–202, 2002.

[39] S. Maneechai, W. De-Eknamkul, K. Umehara, H. Noguchi, and K. Likhitwitayawuid, "Flavonoid and stilbenoid production in callus cultures of *Artocarpus lakoocha*," *Phytochemistry*, vol. 81, pp. 42–49, 2012.

[40] K. H. Park, Y. D. Park, J. M. Han et al., "Anti-atherosclerotic and anti-inflammatory activities of catecholic xanthones and flavonoids isolated from *Cudrania tricuspidata*," *Bioorganic & Medicinal Chemistry Letters*, vol. 16, no. 21, pp. 5580–5583, 2006.

[41] H. J. Lee, D. H. Lyu, U. Koo, K. W. Nam, S. S. Hong et al., "Protection of prenylated flavonoids from mori cortex radicis (Moraceae) against nitric oxide-induced cell death in neuroblastoma SH-SY5Y Cells," *Archives of Pharmacal Research*, vol. 35, no. 1, pp. 163–170, 2012.

[42] Z. G. Yang, K. Matsuzaki, S. Takamatsu, and S. Kitanaka, "Inhibitory effects of constituents from *Morus alba* var. multicaulis on differentiation of 3T3-L1 cells and nitric oxide production in RAW264.7 cells," *Molecules*, vol. 16, no. 7, pp. 6010–6022, 2011.

Prenylated and Geranylated Flavonoids Increase Production of Reactive Oxygen Species in Mouse Macrophages but Inhibit the Inflammatory Response

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ABSTRACT: In this study, four prenylated and geranylated flavonoids, cudraflavone B (**1**), pomiferin (**2**), osajin (**3**), and diplacone (**4**), were tested for their antioxidant and anti-inflammatory effects and to identify any potential relationships between chemical structure and antioxidant or anti-inflammatory properties. The selected flavonoids were examined in cell-free models to prove their ability to scavenge superoxide radicals, hydrogen peroxide, and hypochlorous acid. Further, the ability of the flavonoids to influence the formation of reactive oxygen species in the murine macrophage cell line J774.A1 was tested in the presence and absence of lipopolysaccharide (LPS). The ability of flavonoids to inhibit LPS-induced IκB-α degradation and COX-2 expression was used as a model for the inflammatory response. The present results indicated that the antioxidant activity was dependent on the chemical structure, where the catechol moiety is especially crucial for this effect. The most potent antioxidant activities in cell-free models were observed for diplacone (**4**), whereas cudraflavone B (**1**) and osajin (**3**) showed a pro-oxidant effect in J774.A1 cells. All flavonoids tested were able to inhibit IκB-α degradation, but only diplacone (**4**) also down-regulated COX-2 expression.
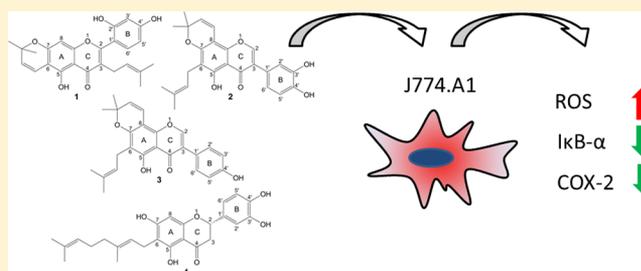
It is well known that the intake of flavonoids (and of many other natural polyphenols) is associated with a lower risk of oxidative-stress-related diseases such as cardiovascular diseases and cancer.[1,2] However, it should be noted that some flavonoids also induce pro-oxidant effects, which may account for their beneficial effects.[3−5] Flavonoids are also very often studied for their anti-inflammatory properties.[6] On the other hand, the antioxidant activity of flavonoids can be dissociated from their anti-inflammatory actions, probably because of contributions of different moieties of the molecule.[7]

Higher oxidative stress very often results in inflammation. The contribution of reactive oxygen species (ROS) to the activation of the nuclear factor (NF)-κB pathway is well known.[8,9] NF-κB is a transcription factor that regulates the expression of inductors and effectors in many steps of the wide network defining the immune response to a pathogenic stimulation. In addition to the described modulation of the inflammatory response, this factor is also involved in the regulation of apoptosis and can be connected with the development of cancer.[10,11] On the other hand, a low oxidative stress activates the nuclear factor erythroid 2-related factor 2 (NRF-2). The transcription factor NFR-2 triggers transcription of hundreds of genes, including antioxidant genes, e.g., heme oxygenase 1 (HO-1).[12] The antioxidant action of NRF-2 leads to attenuation of the lipopolysaccharide (LPS)-induced inflammatory response.[13]

Bacterial LPS is a well-established NF-κB inducer. This unit of the cell walls of Gram-negative bacteria directly activates the NF-κB pathway and simultaneously induces the production of ROS, as described in a review by Gloire et al.[14] Thus, LPS represents a widely used model for the inflammatory response and oxidative stress.

This work was focused on four flavonoids, three prenylated and one geranylated, cudraflavone B (**1**, a flavone), pomiferin (**2**, an isoflavone), osajin (**3**, an isoflavone), and diplacone (**4**, a flavanone). Previous experiments have indicated an anti-inflammatory effect of cudraflavone B (**1**),[15] but this compound lacks antioxidant activity.[16] On the other hand, diplacone (**4**) showed strong antioxidant, cytoprotective,[17,18] and immuno-modulating activities.[19] In the case of two structurally very similar isoflavones (**2** and **3**), it has been demonstrated that pomiferin (**2**) is a potent antioxidant, while osajin (**3**) has almost no activity.[20,21] The aim of this study was to determine the potential relationships between the chemical structure and the antioxidant or anti-inflammatory properties of these four flavonoids.
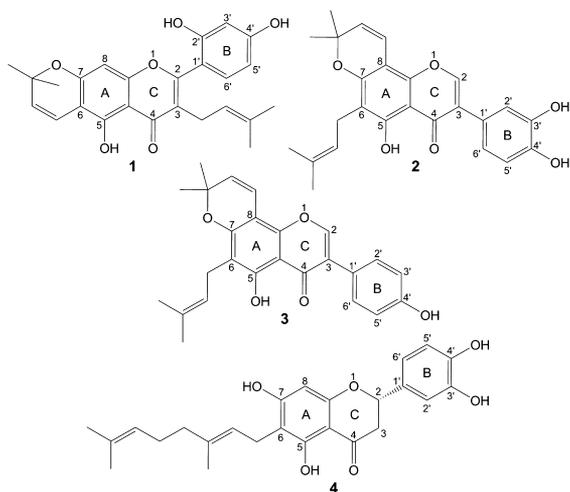
RESULTS AND DISCUSSION

Four flavonoids were tested for their scavenging activity against three reactive oxygen species ($O_2^{\bullet-}$, H_2O_2, and HClO) (Table 1). Diplacone (**4**) showed the highest level of scavenging

Table 1. $O_2^{\bullet-}$, H_2O_2, and HClO Scavenging Activity of Test Flavonoids (IC$_{50}$, mean $\pm$ SE)

	IC$_{50}$ (μM)		
compound	$O_2^{\bullet-}$	H_2O_2	HClO
cudraflavone B (**1**)	219.0 ± 15.0	641.2 ± 31.4	1.8 ± 0.1
pomiferin (**2**)	73.3 ± 4.8	899.3 ± 40.1	9.1 ± 0.2
osajin (**3**)	$>1000^a$	$>1000^b$	31.0 ± 1.7
diplacone (**4**)	41.2 ± 3.7	554.0 ± 45.8	2.3 ± 0.1

^{a}39% scavenging activity at 1 mM. ^{b}17% scavenging activity at 1 mM.

activity against superoxide radicals and hydrogen peroxide with IC$_{50}$ values of 41.2 ± 3.7 and 554.0 ± 45.8 μM, respectively. As described previously, **4** exhibited the most potent DPPH antioxidant activity of the compounds isolated from *Paulownia tomentosa* (Thunb.) Steud. (Paulowniaceae) fruits.[17] Zima et al. tested geranylated flavonoids obtained from this same plant and found **4** to be one of the most effective scavenging agents against superoxide, hydroxyl, ABTS, and DPPH radicals.[18] The antioxidant effect of **4** corresponds to two hydroxyl group substituents, present at positions C-3′ and C-4′ on the B-ring, which is one of the most important criteria for antioxidant activity.[2] A similar catechol moiety on the B-ring is found in pomiferin (**2**), which also showed good antioxidant activity against superoxide radicals. However, translocation of the B-ring from C-2 to C-3 reduces the resultant antioxidant activity. Of the isoflavonoids tested, **2** was a more potent scavenger than **3**, with an IC$_{50}$ value of 9.1 ± 0.2 μM for **2** and 31.0 ± 1.7 μM for **3** in the case of the HClO assay. This could be caused by a greater number of hydroxyl group substituents on the B-ring. This is consistent with the studies of Veselá et al. and Diopan et al., where **3** showed little or no antioxidant activity.[20,21] Compound **3** exhibited $O_2^{\bullet-}$-scavenging activity of 39% and H_2O_2-scavenging activity of only 17% at the highest concentration tested (1 mM). Cudraflavone B (**1**) showed the best activity against hypochlorous acid (IC$_{50}$ 1.8 ± 0.1 μM). Oh et al. tested **1** with DPPH and found its antioxidant activity to be 10-fold lower than that of oxyresveratrol.[16]

Some compounds induce the production of ROS in cells, which in turn may be responsible for beneficial properties.[22,23]

The ability of the test flavonoids to generate ROS in J774A.1 cells was measured in a short-term (30 min) culture experiment. Compounds **2**−**4** slightly increased the levels of ROS by a factor of 0.95 to 1.67 compared with unstimulated cells (Figure 1), whereas compound **1** augmented the amount
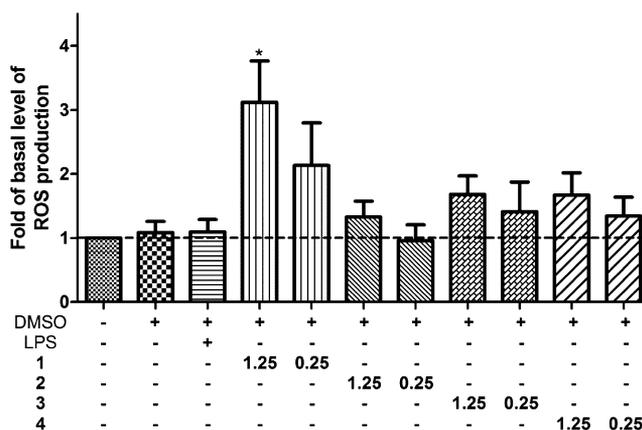
Figure 1. Effect of flavonoids on short-term (30 min) production of ROS. J774A.1 cells were incubated with cudraflavone B (**1**), pomiferin (**2**), osajin (**3**), diplacone (**4**) (1.25 or 0.25 μM; concentrations are indicated below the graph), LPS (1 μg/mL), or only the vehicle [dimethyl sulfoxide (DMSO)]. After 30 min, the production of ROS was measured. The results are expressed as means $\pm$ SE for four independent experiments. (* indicates a significant difference ($p < 0.05$) in comparison to cells treated only with the vehicle. The dashed line indicates the basal level of ROS production in J774.A1 cells.)

of ROS significantly (3.12-fold at a concentration of 1.25 μM and 2.13-fold at 0.25 μM). The effects of these flavonoids on the generation of ROS were found to be concentration-dependent. The pro-oxidant effect of natural flavonoids has been well described for catechins, especially for (−)-epigallo-catechin-3-gallate (EGCG), isolated from green tea leaves.[22] According to this new research line, our group has also demonstrated that a short-term exposure of HUVEC (human umbilical vein endothelial cells) to EGCG increases the production of ROS,[24] whereas a long-term incubation is associated with antioxidant properties (unpublished data). Some other flavonoids, e.g., fisetin, also show pro-oxidant activity.[23] The high pro-oxidant potential of **1** could account partially for its low antioxidant activity, as determined in cell-free models. Interestingly, compound **3**, which shows a markedly less potent antioxidant effect than **1**, exhibited a very low production of ROS in the cell culture during a short-term (30 min) exposure. This could result from the presence of only one hydroxy group on the B-ring. The pro-oxidant features of natural flavonoids are often connected with their potential anticancer action.[3,22]

The next experiment was focused on the ability of the test flavonoids to affect the generation of ROS in LPS-activated macrophages after a 24 h exposure. Bacterial LPS induces the production of ROS in immune cells via NADPH oxidase (NOX)-4[25] and thus participates in the activation of NF-κB.[9,14] The presence of LPS in the culture medium significantly increased the level of ROS in J774A.1 macrophages (Figure 2). Co-incubation of the cells with LPS and **4** had no effect on the production of ROS, and co-incubation with **2** reduced the level of ROS only slightly. On the other hand, at a concentration of 1.25 μM, **1** and **3** significantly increased the production of ROS
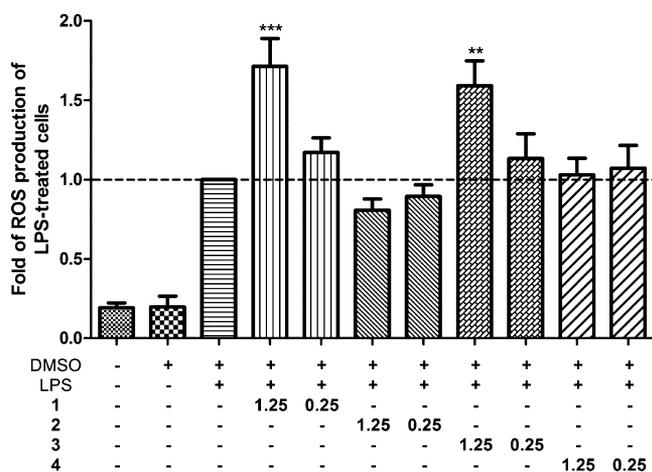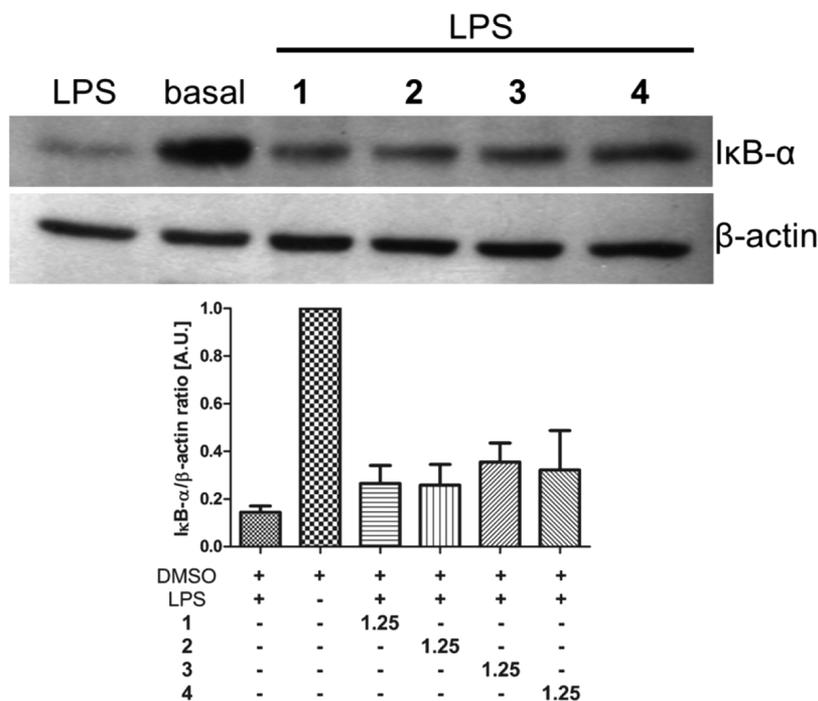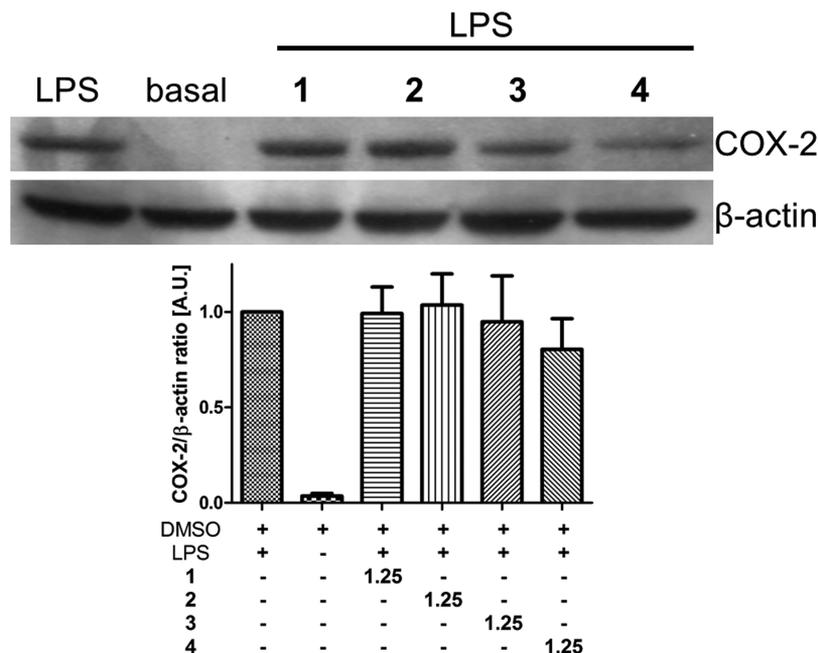
Figure 2. Effect of flavonoids on the LPS-induced production of ROS. J774A.1 cells were pretreated for 1 h with cudraflavone B (**1**), pomiferin (**2**), osajin (**3**), diplacone (**4**) (1.25 or 0.25 μM; concentrations are indicated below the graph), or only the vehicle (DMSO). Subsequently, LPS (1 μg/mL) was added and the level of ROS was measured after incubation for 24 h. (** indicates a significant difference ($p < 0.01$) in comparison with cells treated only with the vehicle and LPS, *** indicates a significant difference ($p < 0.001$) in comparison with cells treated only with the vehicle and LPS. The dashed line indicates a basal level of ROS production of LPS-treated cells.)

in the presence of LPS, by factors of 1.71 and 1.59, respectively. To determine whether this effect was related to the pro-oxidant properties of the chosen flavonoids that were detected in a short-term experiment (30 min), the level of ROS was also measured after a 24-h incubation (Figure 3). The present data
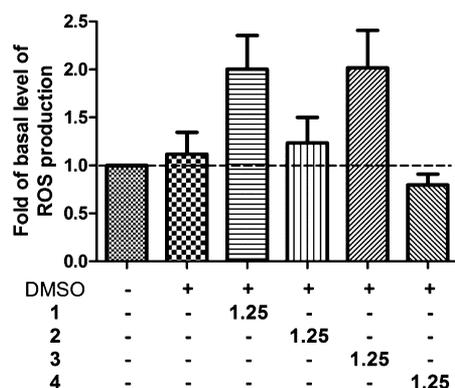
Figure 3. Effect of flavonoids on the 24 h production of ROS. J774A.1 cells were incubated with cudraflavone B (**1**), pomiferin (**2**), osajin (**3**), diplacone (**4**) (1.25 μM), or only the vehicle (DMSO). After 24 h, the production of ROS was measured. The results are expressed as the means $\pm$ SE for four independent experiments. The dashed line indicates a basal level of ROS production in J774.A1 cells.

indicated that **1** and **3** still failed to change the level of ROS at this time point (24 h). Taken together, cudraflavone B (**1**) and osajin (**3**) enhanced the LPS-induced production of ROS. These effects correlate well with the low antioxidant properties of **1** and **3**. From a comparison of Figure 1 with Figure 3 it is evident that the level of ROS for compound **2** was similar after 30 min and 24 h incubations. On the other hand, **3** increased the generation of ROS during longer incubations. In the presence of diplacone (**4**), the production of ROS was

increased after a 30 min incubation and was attenuated after a 24 h incubation (a 1.67-fold higher level of ROS in comparison to the basal concentration of ROS after 30 min and a 1.26-fold lower level of ROS in comparison to the basal concentration of ROS after 24 h, respectively). The level of ROS decreased in the presence of **1**, when the short- and long-term incubations were compared (a 3.12-fold higher level of ROS in comparison to the basal concentration of ROS after 30 min and a 2.00-fold lower level of ROS in comparison to the basal concentration of ROS after 24 h). However, the ROS concentration was still twice as high as the basal level after this long-term incubation.

To determine whether the anti- or pro-oxidative properties of the test flavonoids are connected to their anti-inflammatory action, the activation of the NF-κB signaling pathway was investigated. One of the crucial steps in the activation of the canonical (classical) NF-κB pathway is the degradation of the NF-κB inhibitor IκB-α.[26] Pretreatment of the cells with tested flavonoids led to reduced IκB-α degradation (Figure 4), where compounds **3** and **4** showed greater activity than **1** and **2**. These results support previous observations made of an immunomodulating effect for compounds **1** and **4**.[15,19] An apparent discrepancy was observed between the pro-oxidant effect of **1** and its protective action against LPS-induced IκB-α degradation. However, it should be noted that LPS alone did not induce the production of ROS in a 30 min challenge test (Figure 1); hence the anti-inflammatory effect of the flavonoids tested must derive from some mechanism other than the antioxidant property.

A higher level of IκB-α had a downstream effect on gene expression, as was determined by measuring the synthesis of COX-2 (Figure 5), which is regulated by the transcription factor NF-κB. The level of COX-2 correlates well with the level of IκB-α; that is, the greater the amount of IκB-α, the lower the amount of COX-2. From this viewpoint, only diplacone (**4**) showed a trend to reduce the expression of COX-2. Surprisingly, cudraflavone B (**1**), which had significantly attenuated the transcription of the *COX-2* gene in our previous work,[15] had no effect on the translation of COX-2 in this study. One reason for this could be that the concentration of **1** used in the present study was eight times lower than in the previous study.

The present results have confirmed the ability of the test flavonoids to act as antioxidants in cell-free models in the decreasing order diplacone (**4**) > cudraflavone B (**1**) $\approx$ pomiferin (**2**) $\gg$ osajin (**3**). This antioxidant potential results from the number and position of the hydroxy groups on the B-ring; the catechol moiety is especially crucial for this effect. The low antioxidant action in cell-free models correlates with the pro-oxidant properties of **1** and **3** in J774A.1 cells during 24 h of incubation. Compounds **1** and **3** also showed enhanced production of ROS in LPS-stimulated cells. On the other hand, no relationship was found between chemical structure and anti-inflammatory effects. The most promising potential agent for the treatment of inflammatory diseases is diplacone (**4**), which has demonstrated beneficial effects in different settings including alloxan-induced diabetes mellitus in vivo.[18] Cudra-flavone B (**1**) might be useful as a potential anticancer agent because of its pro-oxidant action. However, detailed in vivo studies will be required to elucidate the efficacy and exact mechanisms of action of these flavonoids.

Figure 4. Effect of flavonoids on the LPS-induced degradation of IκB-α. J774A.1 cells were pretreated for 15 min with cudraflavone B (**1**), pomiferin (**2**), osajin (**3**), diplacone (**4**) (1.25 or 0.25 μM), or only the vehicle (DMSO). Subsequently, LPS (1 μg/mL) was added and the levels of IκB-α and β-actin were measured after 30 min. The basal level of expression (without LPS stimulation) of IκB-α is indicated, as well. The blots shown are representative results from four independent experiments. The graph below the blots indicates the IκB-α/β-actin ratio.

Figure 5. Effect of flavonoids on the LPS-induced expression of COX-2. J774A.1 cells were pretreated for 1 h with cudraflavone B (**1**), pomiferin (**2**), osajin (**3**), diplacone (**4**) (1.25 or 0.25 μM), or only the vehicle (DMSO). Subsequently, LPS (1 μg/mL) was added, and the levels of COX-2 and β-actin were measured after 24 h. The basal level of expression (without LPS stimulation) of COX-2 is indicated, as well. The blots shown are representative results from four independent experiments. The graph below the blots indicates the COX-2/β-actin ratio.

■ EXPERIMENTAL SECTION

Test Compounds. All four of the flavonoids were generously donated by Dr. Karel Šmejkal of the Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic. They were isolated and characterized as previously described [i.e., cudraflavone B (**1**),[15] pomiferin (**2**) and osajin (**3**),[20] and

diplacone (**4**)[17]]. The purity of all compounds tested was checked via HPLC analysis and exceeded 95%.

Superoxide Scavenging Assay. The superoxide radical ($O_2^{\bullet-}$) was generated by the NADH/phenazine methosulfate (PMS) system, following the methodology described by Valantao et al.[27] The final volume of 300 μL of reaction mixture in each sample well contained the following reagents at the indicated final concentrations: 19 mM

phosphate buffer, pH 7.4; test compound dissolved in dimethyl sulfoxide (DMSO) at concentrations ranging from 12.5 to 1000 μM; 166 μM NADH; 43.3 μM nitrotetrazolium blue (NBT); and 2.7 μM PMS (all compounds were purchased from Sigma-Aldrich, Steinheim, Germany). The assays were performed on a Biotek microplate reader. The absorbance was measured at 560 nm immediately after mixing all reagents for 2 min at room temperature. The absorbance was increased by the $O_2^{\bullet-}$-reduction of NBT to a diformazan. Each compound was measured in five independent experiments, performed in triplicate.

Hydrogen Peroxide Scavenging Assay. The H_2O_2 scavenging assay was performed according to the chemiluminescence method described by Gomes et al.[28] The light emitted as lucigenin disintegrated to form methylacridine was monitored as an indication of the H_2O_2-induced oxidation. In a final volume of 250 μL, each reaction mixture contained 50 mM Tris-HCl buffer, pH 7.4; a test compound dissolved in DMSO at concentrations ranging from 125 to 1000 μM; 800 μM lucigenin (Sigma-Aldrich); and 1% (v/v) H_2O_2. The assays were performed on a Biotek microplate reader in the luminescent mode. Measurements were taken at 37 °C immediately after all of the reagents were mixed and added to the plate. Each compound was measured in five independent experiments, performed in triplicate.

Hypochlorous Acid Scavenging Assay. The scavenging activity with HClO was measured using a modified version of the fluorescence methodology described by Gomes et al.[29] The nonfluorescent agent dihydrorhodamine 123 (DHR) was oxidized to the fluorescent rhodamine by hypochlorous acid. The final volume of 300 μL of each reaction mixture in its sample well contained the following reagents at the specified final concentrations: 100 mM phosphate buffer, pH 7.4; a test compound dissolved in methanol at concentrations ranging from 0.625 to 100 μM; 5 μM DHR; and 5 μM HClO (all compounds were purchased from Sigma-Aldrich). The assays were measured on a Biotek microplate reader in the fluorescent mode. Measurements using excitation at 485 ± 20 nm and emission at 528 ± 20 nm were taken at 37 °C immediately after mixing all of the reagents and adding them into the plate. Each compound was measured in five independent experiments, performed in triplicate.

Maintenance and Subcultivation of the Cell Line J774.A1. The murine macrophage cell line J774A.1 (Lonza, Verveirs, Belgium) was cultivated in Dulbecco's modified Eagle medium (DMEM) containing 0.45% (w/v) glucose and 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (Life Technologies, Monza, Italy) with the addition of 10% (v/v) fetal bovine serum (FBS) (Euroclone, Milano, Italy), 1% (w/v) penicillin/streptomycin, and 1% (w/v) L-glutamine (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cells were subcultivated when the confluence reached 80−90%. The culture medium was aspirated, and the cells were washed with phosphate-buffered saline (PBS) (Life Technologies). Adherent cells were detached by adding 1.5 mL of PBS containing 8 mg/mL lidocaine (Sigma-Aldrich) and 5 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) for each 10 mm culture plate. Culture plates were then returned to the incubator for 2 min. A further 3 mL of PBS was then added to each plate, and the cells were scraped into sterile centrifuge tubes. After centrifugation at $1000g$ for 5 min, the supernatant was removed, and the cells were resuspended in fresh DMEM and transferred into new culture plates.

In Vitro Measurement of Reactive Oxygen Species. J774A.1 cells were split into 30 mm dishes at a cell density of 250 000 cells/mL in a total volume of 2 mL. When the confluence had reached 80−90% after 36−48 h, the culture medium was removed and the cells were washed with PBS. Fresh serum-free DMEM was then added to the cells. The cells prepared in this manner were used for subsequent ROS measurements.

To measure the ability of the test compounds to induce the generation of reactive oxygen species during a short-term (30 min) exposure, the cells were incubated for 1 h with 10 μM 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH$_2$-DA) (Sigma-Aldrich) dissolved in methanol [the final concentration of methanol in the medium was 2% (v/v)]. The test compound was dissolved in DMSO

[the final concentration of DMSO in the medium was 0.1% (v/v)] and added 30 min before the end of the DCFH$_2$-DA incubation at concentrations of 0.25 and 1.25 μM. At these levels, none of the compounds induced a toxic effect (data not shown). The medium was then aspirated, and the cells were washed with PBS. PBS with lidocaine and a scraper were used to harvest the cells. The level of oxidized 2′,7′-dichlorfluorescein (DCF) was measured by flow cytometry at 488 nm for excitation and 525 nm for emission. The measurements were performed on a Beckman Coulter Epics XL flow cytometer.

To detect the potential ability of the test flavonoids to inhibit the production of ROS in the presence of lipopolysaccharide obtained from *Escherichia coli* 0111:B4 (Sigma-Aldrich), the cells were pretreated with test compounds at concentrations of 0.25 and 1.25 μM for 1 h. Subsequently, 1 μg/mL LPS dissolved in water was added, and the cells were incubated for a further 24 h. DCFH$_2$-DA was added 30 min before the end of the LPS incubation. Next, the cells were collected and the production of ROS was measured as described above.

IκB-α Detection. The J774.A1 cells were prepared as described above and pretreated with the tested compounds at a concentration of 1.25 μM for 15 min. Then, 1 μg/mL LPS was added and the incubation was continued for a further 30 min. The cells were washed with PBS and lysed in lysis buffer [50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% (v/v) Nonidet P-40; 25 mM NaF; 0.5% (w/v) sodium deoxycholate; 10% (w/v) SDS; 1 mM EGTA; 1 mM phenyl-methylsulfonyl fluoride; 1 mM orthovanadate; and 10 mM sodium pyrophosphate, supplemented with Complete protease inhibitor mixture obtained from Roche, Mannheim, Germany] with sonication. The protein concentration was measured by the Lowry method.[30] Cell lysates were denatured in the presence of β-mercaptoethanol at 100 °C for 5 min, and 40 μg of the denatured protein was loaded onto a 12% polyacrylamide gel. After electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF) membranes, which were subsequently blocked with 5% (w/v) skimmed milk (Sigma-Aldrich) dissolved in TBST buffer [150 mM NaCl, 10 mM Tris base, 0.1% (v/v) Tween-20]. The membranes were incubated with either a primary anti-IκB-α antibody (Cell Signaling, Danvers, MA, USA) at 1:1000 dilution and 4 °C for 16 h or a primary anti-β-actin antibody (Sigma-Aldrich) at 1:5000 dilution and room temperature for 1 h. After washing, the secondary antibody [anti-mouse IgG; anti-rabbit IgG (Vector, Peterborough, UK)] diluted 1:5000 was applied on the membranes and incubated for 1 h at room temperature (~23 °C). The secondary antibodies were detected using a chemiluminiscent ECL substrate (GE Healthcare, Little Chalfont, UK). The intensity of protein bands was calculated by AlphaEasy FC 4.0.0 software (Alpha Innotech, San Leandro, CA, USA) for densitometric analysis.

COX-2 Detection. J774A.1 macrophages prepared as described above were pretreated with each of the test compounds at a concentration of 1.25 μM for 1 h. The inflammatory response was then triggered using 1 μg/mL LPS, and the cells were incubated for 24 h. The cells were lysed, and their protein concentrations were measured as described above. Forty micrograms of the denatured protein was loaded into a 12% polyacrylamide gel and blotted onto a PVDF membrane. The primary anti-COX-2 antibody (Cayman Chemicals, Ann Arbor, MI, USA) was diluted 1:500. β-Actin detection and membrane development was performed as described above.

Statistical Analysis. All of the experiments were performed in at least three independent replications; the results are presented as mean values, with error bars representing the standard deviation (SE) of the average value. A one-way ANOVA test was used for statistical analysis, followed by Tukey's post hoc test for multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant. GraphPad Prism 5.02 (GraphPad Software Inc., La Jolla, CA, USA) was used to perform the analysis.

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■ REFERENCES

(1) Han, R. M.; Zhang, J. P.; Skibsted, L. H. *Molecules* **2012**, *17*, 2140−2160.

(2) Gulcin, I. *Arch. Toxicol.* **2012**, *86*, 345−391.

(3) Galati, G.; O'Brien, P. J. *Free Radical Biol. Med.* **2004**, *37*, 287−303.

(4) Kweon, M. H.; Adhami, V. M.; Lee, J. S.; Mukhtar, H. *J. Biol. Chem.* **2006**, *281*, 33761−33772.

(5) Pullikotil, P.; Chen, H.; Muniyappa, R.; Greenberg, C. C.; Yang, S.; Reiter, C. E.; Lee, J. W.; Chung, J. H.; Quon, M. J. *J. Nutr. Biochem.* **2012**, *23*, 1134−1145.

(6) Gonzalez, R.; Ballester, I.; Lopez-Posadas, R.; Suarez, M. D.; Zarzuelo, A.; Martinez-Augustin, O.; Sanchez de, M. F. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 331−362.

(7) Loke, W. M.; Proudfoot, J. M.; Stewart, S.; McKinley, A. J.; Needs, P. W.; Kroon, P. A.; Hodgson, J. M.; Croft, K. D. *Biochem. Pharmacol.* **2008**, *75*, 1045−1053.

(8) Bubici, C.; Papa, S.; Dean, K.; Franzoso, G. *Oncogene* **2006**, *25*, 6731−6748.

(9) Morgan, M. J.; Liu, Z. G. *Cell Res.* **2011**, *21*, 103−115.

(10) Pahl, H. L. *Oncogene* **1999**, *18*, 6853−6866.

(11) Ghosh, S.; Hayden, M. S. *Nat. Rev. Immunol.* **2008**, *8*, 837−848.

(12) Hybertson, B. M.; Gao, B.; Bose, S. K.; McCord, J. M. *Mol. Aspects Med.* **2011**, *32*, 234−246.

(13) Kuhn, A. M.; Tzieply, N.; Schmidt, M. V.; von Knethen, A.; Namgaladze, D.; Yamamoto, M.; Brune, B. *Free Radical Biol. Med.* **2011**, *50*, 1382−1391.

(14) Gloire, G.; Legrand-Poels, S.; Piette, J. *Biochem. Pharmacol.* **2006**, *72*, 1493−1505.

(15) Hošek, J.; Bartoš, M.; Chudík, S.; Dall'acqua, S.; Innocenti, G.; Kartal, M.; Kokoška, L.; Kollár, P.; Kutil, Z.; Landa, P.; Marek, R.; Závalová, V.; Žemlička, M.; Šmejkal, K. *J. Nat. Prod.* **2011**, *74*, 614−619.

(16) Oh, H.; Ko, E. K.; Jun, J. Y.; Oh, M. H.; Park, S. U.; Kang, K. H.; Lee, H. S.; Kim, Y. C. *Planta Med.* **2002**, *68*, 932−934.

(17) Šmejkal, K.; Grycová, L.; Marek, R.; Lemiere, F.; Jankovská, D.; Forejtníková, H.; Vančo, J.; Suchý, V. *J. Nat. Prod.* **2007**, *70*, 1244−1248.

(18) Zima, A.; Hošek, J.; Treml, J.; Muselík, J.; Suchý, P.; Pražanová, G.; Lopes, A.; Žemlička, M. *Molecules* **2010**, *15*, 6035−6049.

(19) Hošek, J.; Závalová, V.; Šmejkal, K.; Bartoš, M. *Folia Biol. (Praha)* **2010**, *56*, 124−130.

(20) Veselá, D.; Kubínová, R.; Muselík, J.; Žemlička, M.; Suchý, V. *Fitoterapia* **2004**, *75*, 209−211.

(21) Diopan, V.; Babula, P.; Shestivska, V.; Adam, V.; Zemlicka, M.; Dvorska, M.; Hubalek, J.; Trnkova, L.; Havel, L.; Kizek, R. *J. Pharm. Biomed. Anal.* **2008**, *48*, 127−133.

(22) Lambert, J. D.; Elias, R. J. *Arch. Biochem. Biophys.* **2010**, *501*, 65−72.

(23) Constantin, R. P.; Constantin, J.; Pagadigorria, C. L.; Ishii-Iwamoto, E. L.; Bracht, A.; de Castro, C. V.; Yamamoto, N. S. *J. Biochem. Mol. Toxicol.* **2011**, *25*, 117−126.

(24) Toniolo, A.; Buccellati, C.; Pinna, C.; Gaion, R. M.; Sala, A.; Bolego, C. *PLoS One* **2013**, *8*, e56683.

(25) Park, H. S.; Jung, H. Y.; Park, E. Y.; Kim, J.; Lee, W. J.; Bae, Y. S. *J. Immunol.* **2004**, *173*, 3589−3593.

(26) Perkins, N. D.; Gilmore, T. D. *Cell Death Differ.* **2006**, *13*, 759−772.

(27) Valentao, P.; Fernandes, E.; Carvalho, F.; Andrade, P. B.; Seabra, R. M.; Bastos, M. L. *J. Agric. Food Chem.* **2002**, *50*, 4989−4993.

(28) Gomes, A.; Costa, D.; Lima, J. L.; Fernandes, E. *Bioorg. Med. Chem.* **2006**, *14*, 4568−4577.

(29) Gomes, A.; Fernandes, E.; Silva, A. M.; Santos, C. M.; Pinto, D. C.; Cavaleiro, J. A.; Lima, J. L. *Bioorg. Med. Chem.* **2007**, *15*, 6027−6036.

(30) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265−275.

Evaluation of Anti-Inflammatory Activity of Prenylated Substances Isolated from *Morus alba* and *Morus nigra*

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ABSTRACT: Chromatographic separation of root extracts of *Morus alba* and *M. nigra* led to the identification of the 2-arylbenzofurans moracin C (**1**), mulberrofuran Y (**2**), and mulberrofuran H (**3**), and the prenylated flavonoids kuwanon E (**4**), kuwanon C (**5**), sanggenon H (**6**), cudraflavone B (**7**), and morusinol (**8**), and the Diels−Alder adducts soroceal (**9**), and sanggenon E (**10**). The cytotoxicity and their antiphlogistic activity, determined as the attenuation of the secretion of TNF-α and IL-1β and the inhibition of NF-κB nuclear translocation in LPS-stimulated macrophages, were evaluated for compounds **1−10**.
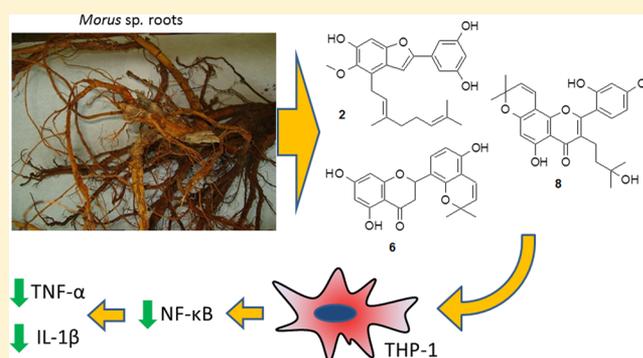
Morus alba L. and *M. nigra* L.(both known as mulberry) are deciduous trees belonging to the family Moraceae. They are found in mainland China, Japan, and Korea and often cultivated for different purposes. Their leaves are used to feed silkworms (*Bombyx mori* L.). Mulberry plants have edible and tasty fruits, while various plant parts have been used in traditional Chinese medicine for centuries. The root bark of the *M. alba* is used traditionally as an antipyretic, antitussic, diuretic, and expectorant agent.[1,2] Previous studies have shown that extracts from *M. alba* root bark contain mainly flavonoids and prenylated flavonoids, alkaloids, 2-arylbenzofurans, coumarins, Diels−Alder type adducts, dihydrofuran derivatives, stilbenes, and terpenes.[3,4] The root bark of *M. alba* and the compounds it contains possess antiallergic, anti-inflammatory, antimicrobial, antioxidant, antiviral, cytotoxic, hypoglycemic, hypolipidemic, and neuroprotective activities.[5] In contrast to *M. alba*, less is known about the root bark of *M. nigra*, with extracts showing tyrosinase inhibitory and antinociceptive activities.[6,7]

Inflammation is a complex of defensive reactions that an organism makes to various pathophysiological stimuli, seeking to eliminate the irritating stimuli and repair tissue. However, the increasing incidence of chronic inflammatory diseases connected with the destruction of tissue presents a growing health problem.[8] The anti-inflammatory drugs used clinically have unfavorable side effects and are expensive (especially in the case of biologics). Traditional medicines and natural products may provide an alternative to these drugs because bioactive constituents may lead to the development of drugs for the treatment of inflammatory diseases.[9]

Owing to its successful use in traditional medicine, white mulberry root bark (*Cortex Mori Radicis*, *sang bai pi*) is considered to be a promising source of compounds with anti-inflammatory activity. Beside to constituents used in this paper, the antiphlogistic activity of many other compounds from *M. alba* has been described. For example, the constituent oxyresveratrol inhibits the lipopolysaccharide (LPS)-stimulated expression of inducible NO synthase (iNOS), the nuclear translocation of nuclear factor (NF)-κB, and the activity of cyclooxygenase (COX)-2 in RAW264.7 cells.[10] The inhibition of NF-κB has also been observed for two chalcone-derived Diels−Alder type adducts, namely, kuwanon J 2,4,10″-trimethyl ether and kuwanon R.[11] Nonselective inhibition of COX-1 and COX-2 has been reported for sanggenon C, sanggenon E, and sanggenon O, whereas the prenylated flavonoids morusin and kuwanon C inhibit COX-1, COX-2, 5-lipoxygenase (LOX), and 12-LOX, to varying degrees.[12,13] Previous studies have confirmed the significant inhibition of NO production by arylbenzofurans and prenylated flavonoids obtained from *M. alba* root bark, and sanggenon C, sanggenon D, sanggenon O, morusin, and kuwanon C are able to suppress the expression of inducible NO synthase.[4,14,15] Mornigrol D and norartocarpetin obtained from *M. nigra* root bark have shown potent anti-inflammatory activity by inhibiting the release of β-glucuronidase from rat polymorphonuclear leucocytes.[16]

In the present report, the isolation of the 2-arylbenzofuran derivatives moracin C (**1**), mulberrofuran Y (**2**), and mulberrofuran H (**3**), the prenylated flavonoids kuwanon E (**4**), kuwanon C (**5**), sanggenon H (**6**), cudraflavone B (**7**), and morusinol (**8**), from *M. alba* root bark is described. In addition, the Diels−Alder type adducts soroceal (**9**) and sanggenon E (**10**) were obtained from the root bark of *M. nigra*. These compounds were identified on the basis of NMR spectroscopy, mass spectrometry, and optical methods (circular dichrism and specific optical rotation), and the data were compared with those previously published in the literature.[4,17−22] The cytotoxicity and anti-inflammatory activities of these ten compounds were evaluated for their ability to decrease the secretion of the pro-inflammatory cytokines TNF-α and IL-1β in LPS-stimulated macrophages. The effects of the isolated compounds on the nuclear translocation of transcription factor NF-κB were also evaluated.

RESULTS AND DISCUSSION

The cytotoxicity of compounds **1−10** were determined using a THP-1 human monocytic leukemic cell line, primarily in order to determine the safe and nontoxic concentrations for the subsequent analysis of the TNF-α secretion. On the basis of the results of the tests, the compounds were divided into two groups according to their estimated IC$_{50}$ values: nontoxic substances, with IC$_{50}$ values of >10 μM, and toxic compounds, with IC$_{50}$ values of <10 μM (see Table 1). All of the compounds, except **6** and **7**, were found to be toxic, with IC$_{50}$ values of <5 μM. Sanggenon H (**6**) together with cudraflavone B (**7**), the cytotoxicity of which has been tested in previous studies,[17,23] both showed an IC$_{50}$ values of >10 μM and were assigned as nontoxic. On the basis of these results, a relatively nontoxic concentration of 1 μM was selected for measuring the secretion of cytokines.

The cytotoxic activity of some compounds or extracts from the root bark of *M. alba* against different cell lines has been demonstrated previously.[24,25] The greatest activity among the group of prenylated flavonoids was shown by kuwanon C (**5**) (IC$_{50}$ 1.7 ± 0.03 μM), the antiproliferative activity of which has also been shown against B16 melanoma cells.[26] The greater toxicity of kuwanon C (**5**) in comparison to kuwanon E (**4**) could be caused by the presence of two prenyl moieties in **5** in contrast to only one geranyl group in **4**, as well as by the positions at which these are attached to the flavonoid skeleton. It has been shown previously that the cytotoxicity of flavonoids increases not only with the number of prenyl moieties (augmentation of the lipophility and possible penetration into cells) but also with changes in their location. Prenyl substitution of ring A increases the cytotoxicity more than substitution of the same group on ring B. Comparison of the activities of kuwanon C (**5**) and kuwanon E (**4**) with sanggenon H (**6**) and morusinol (**8**) indicated that cyclization of a prenyl group with a hydroxy group can lead to a small decrease in the effect. The greater activity of **8** compared with **6** seems to be caused by double prenyl substitution, of both ring A and ring C, in comparison to a single cyclic prenyl group substituted on ring B of **6**.[26,27] All of the three 2-arylbenzofuran derivatives (**1−3**) that were tested could be designated as cytotoxic compounds according to their effects on THP-1. Moracin C (**1**) has also previously demonstrated cytotoxic activity against murine leukemia P-388 cells and MCF-7 human breast cancer cells.[28,29] Zelefack et al. found that prenylation of the arylbenzofuran ring at position 4′ increased the

Table 1. IC$_{50}$ Values Calculated for Compounds 1−10[a]
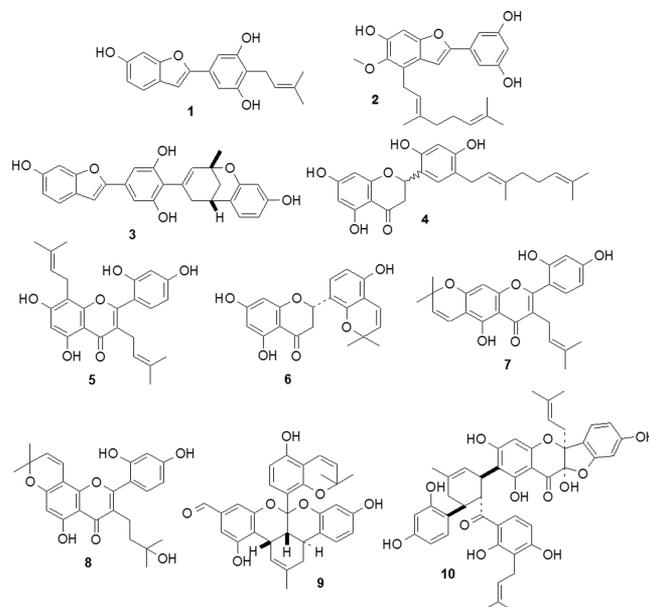
compound	IC$_{50}$ (mean ± S.E.)
moracin C (**1**)	3.2 ± 0.13 μM
mulberrofuran Y (**2**)	4.8 ± 0.19 μM
mulberrofuran H (**3**)	3.2 ± 0.13 μM
kuwanon E (**4**)	4.0 ± 0.08 μM
kuwanon C (**5**)	1.7 ± 0.03 μM
sanggenon H (**6**)	>10 μM
cudraflavone B (**7**)	>10 μM[17,23]
morusinol (**8**)	4.3 ± 0.09 μM
soroceal (**9**)	4.7 ± 0.19 μM
sanggenon E (**10**)	4.0 ± 0.12 μM

[a]Mean ± S.E.

cytotoxicity.[29] In addition to its toxic effects on cancer cells, **1** also inhibits the breast cancer resistance protein (BCRP/ABCG2).[30] In contrast to the well-known cytotoxicity of **1**, this is the first report of cytotoxic activity for mulberrofuran Y (**2**) and for mulberrofuran H (**3**), which is a Diels−Alder adduct of chalcone and dehydroprenyl-2-arylbenzofuran. The Diels−Alder adducts soroceal (**9**) and sanggenon E (**10**) are also among the compounds assigned as toxic (IC$_{50}$ < 10 μM) because of their effects on THP-1 cells. To date, no information about the cytotoxic activity of these compounds has been reported in the literature. On the other hand, known Diels−Alder adducts isolated from *Morus* species, have shown little or no cytotoxic activity against several cancer cell lines.[31,32] The only exception is mulberrofuran F, which showed a significant cytotoxic effect against the A2780 human ovarian cancer cell line (IC$_{50}$ 1.2 μM).[32]

The next part of this study focused on testing the anti-inflammatory activities of compounds **1−10**. Owing to the dominant role of TNF-α in the pathogenesis of inflammation, the ability of each of the isolated compounds to reduce the secretion of TNF-α in LPS-stimulated macrophages was tested (Figure 1A), as was their ability to diminish the production of IL-1β, another pro-inflammatory cytokine (Figure 1B). The prenylated flavonoids (**4−8**) were most efficient in significantly reducing the secretion of TNF-α ($p < 0.01$), but the production of this cytokine was in no case significantly lower than that of prednisone used as a positive control. The most potent
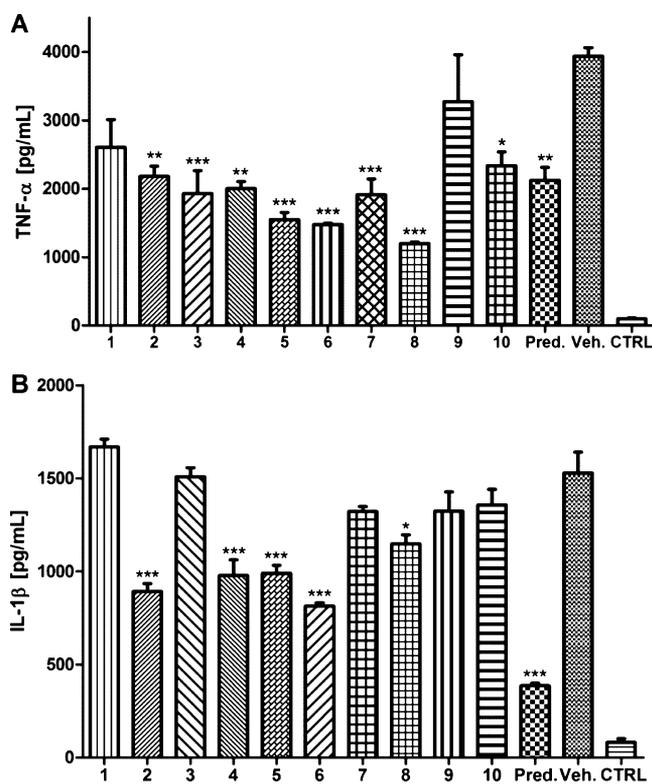
Figure 1. Effects of compounds isolated from mulberry root bark and prednisone on the LPS-induced secretion of TNF-α (A) and IL-1β (B). The cells were pretreated with compounds **1−10** (1 μM), prednisone (**Pred.**, 1 μM), or the vehicle (**Veh.**, DMSO) only. After 1 h of incubation, the inflammatory response was induced by using LPS [except for the control cells (**CTRL**)]. The secretion was measured 24 h after the addition of LPS. The results are expressed as the mean ± SE for each of three independent experiments. Significant differences are shown in comparison, with * vehicle-treated cells ($p < 0.05$), ** vehicle-treated cells ($p < 0.01$), and *** vehicle-treated cells ($p < 0.001$).

compound found was morusinol (**8**), with an effect nearly twice that of prednisone. On the other hand, only compounds **4−6** greatly reduced ($p < 0.001$) the level of IL-1β. Morusinol (**8**) had only a moderate effect ($p < 0.05$) and cudraflavone B (**7**) did not significantly diminish the production of IL-1β.

In general, flavones (with double bond between C-2 and C-3) are reported to be more effective in diminishing the secretion of TNF-α than flavanones (without double bond between C-2 and C-3).[33] In contrast, herein, the compound **6**, a flavanone, showed more activity than the flavones **5** and **7**. The keto group at position C-4 of the flavonoid skeleton and the hydroxy groups at positions C-5, C-7, and C-4′ are additional structural elements that are probably needed to reduce the secretion of TNF-α, and all of the flavonoids (**4−8**) tested in this study met these structural requirements. The results show that the cyclization of the prenyl moiety in **6−8** did not reduce the anti-inflammatory activities of these compounds although it reduced their cytotoxicity. Previous studies have indicated that substitution of ring B strongly influences the activity of flavonoids. It has been reported that flavonoids with a 3′,4′-dihydroxy substitution are more active than those with only a single hydroxy group on ring B.[33] The present work showed that the secretion of TNF-α is also influenced by flavonoids with the 2′,4′ oxidation pattern that is typical for such compounds from the family Moraceae. On the other hand,

cyclization of the prenyl group on ring C (**7**, **8**) dramatically decreased the ability of flavonoids to attenuate the secretion of IL-1β. This is in agreement with a previuos report, that cudraflavone B (**7**) gave a greater effect than kuwanon E (**4**) on the secretion of IL-1β in THP-1 cells at a concentration of 10 μM.[23] Although the present study used a lower concentration (1 μM), the level of IL-1β was affected only moderately.

In addition to the inhibition of the secretion of TNF-α and IL-1β, other mechanisms for testing the anti-inflammatory activity of flavonoids have been reported in the literature. Kuwanon C (**5**) influences the metabolism of arachidonate by inhibiting COX-1, COX-2, 5-LOX, and 12-LOX. It also inhibits the production of NO and platelet-activating factor (PAF) and arachidonic acid-induced aggregation of platelets.[4,13,15,34] Kuwanon E (**4**) significantly inhibits the production of IL-6 in the A549 lung epithelial cell line and the production of NO in lung macrophages (MH-S) in a model of airway inflammation.[35] The anti-inflammatory effects of cudraflavone B (**7**) include the inhibition of COX-1, COX-2, and MMP-2 and antiatherogenic activity caused by inhibiting the proliferation of aortic smooth muscle cells.[17,23,36]

Although all of the 2-arylbenzofurans tested (**1−3**) reduced the secretion of TNF-α, these compounds showed a different efficacies. Mulberrofuran H (**3**) was the most effective compound ($p < 0.001$), with activity more potent than prednisone, while moracin C (**1**) showed the least activity, which was statistically insignificant. Clearly, the substitution pattern of the common skeleton of 2-(3,5-dihydroxyphenyl)-benzofuran greatly influences its ability to suppress the production of TNF-α. The effects of **1-3** on IL-1β did not match the inhibition of TNF-α, since only mulberrofuran Y (**2**) was able to reduce the secretion of this cytokine. Neither moracin C (**1**) nor mulberrofuran H (**3**) diminished the production of IL-1β, possibly because of the presence of a bulky substituent at the para position of the 2-aryl ring. The reduction of the expression of TNF-α is not the only mechanism of anti-inflammatory activity of **1**. Moracin C (**1**) has been proven to diminish the release of β-glucuronidase from PAF-stimulated rat polymorphonuclear (PMN) cells, to reduce the production of NO in LPS-stimulated macrophages, and to inhibit phosphodiesterase-4, a promising target for the treatment of asthma.[4,37,38] The antioxidative potential of **1** may possibly contribute to its anti-inflammatory activity.[38] Little is known about the anti-inflammatory effects of mulberrofuran Y (**2**), although Yang et al. have found that it inhibits the production of NO.[4] Significant inhibition of the secretion of TNF-α and IL-1β was demonstrated in the present investigation. There is no prior literature information on the potential anti-inflammatory activity of mulberrofuran H (**3**).

Soroceal (**9**) did not affect the production of TNF-α, but the efficacy of sanggenon E (**10**) was statistically significant ($p < 0.05$), even though it was lower than that of prednisone. Neither of these Diels−Alder adducts influenced the secretion of IL-1β. In contrast to the dearth of information about the activity of **10** (an inhibitor of COX-1 and COX-2),[18] there have been several reports about the anti-inflammatory effects of structurally related sanggenons.[13−15,18] The antioxidative effect of the structurally similar sorocein A suggests the need to test **9** for such biological activity.[38]

On the basis of previous results, it was hypothesized that the mechanism of action of the compounds tested might involve inhibition of the transcriptional factor NF-κB, which plays a key role in the LPS-stimulated expression of TNF-α and IL-1β. To

prove this, the inhibitory effects of **3**, **6**, **7**, and **8** on the nuclear translocation of NF-κB following LPS-stimulation were determined. As seen in Figure 2, all of these compounds (**3**,
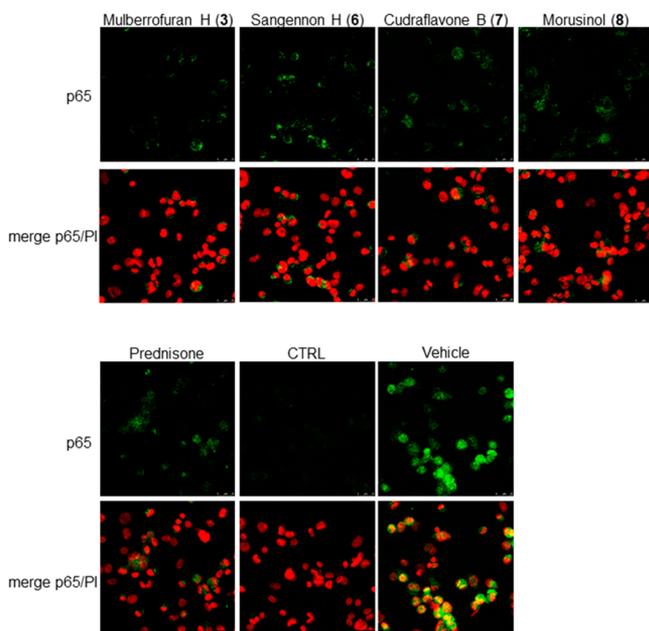
Figure 2. Effect of compounds **3**, **6**, **7**, and **8** on the nuclear translocation of NF-κB (p65) as determined by immunohistochemistry (green color). The cells were counterstained with propidium iodide (PI; red color) to visualize the nuclei. The colocalization of the fluorescence-conjugated antibody and the nuclear stain in the vehicle and their almost total lack of colocalization in the other experimental variants should be noted, especially for sangennon H (**6**). Representative immunofluorescence images of three independent experiments yielding identical results for the nuclear translocation of NF-κB are shown.

6−8) reduced the activation of this transcription factor. The precondition that the compounds act by suppressing NF-κB was supported by the fact that the suppression of NF-κB has been observed previously for extracts of the leaves and roots of *M. alba*.[39,40] Moreover, the inhibition of NF-κB by cudraflavone B (**7**) and kuwanon E (**4**) has also been proven previously.[17,23] To suppress the NF-κB pathway, natural products may influence the stability of the NF-κB/IκB complex, the activity of the IKK complex, the phosphorylation and proteolytic degradation of IκB, or nuclear translocation or the DNA binding activity of NF-κB.[41−43] Compounds **1−10** showed different effects on the secretion of TNF-α and IL-1β. These compounds may affect different post-translation regulation points for the two different cytokines and, for example, could have different mechanisms for regulation of their secretion or release. Another possible mechanism of action may be the inhibition of kinases of the MAPK family, especially ERK, JNK, and p38, which participate in the production of pro-inflammatory mediators in LPS-stimulated cells. Inhibition of the secretion of TNF-α and IL-1β on this level has been demonstrated by many flavonoids, with differences in the type of MAPK inhibited. The inhibition of Akt kinase has been observed less frequently.[43−46] It is also possible that the compounds tested could inhibit the secretion of cytokines by inhibiting other transcriptional factors, such as AP-1 or STAT-1, as is the case with other natural compounds.[47−49]

Therefore, in the present work, the isolation of several 2-arylbenzofuran derivatives, prenylated flavonoids, and Diels−Alder adducts from the roots of both *M. alba* and *M. nigra* has been described. Their cytotoxicity and anti-inflammatory activity against the THP-1 cell line has been evaluated. The cytotoxicity of the 2-arylbenzofurans **2** and **3**, kuwanon C (**5**), and sanggenon E (**10**) has been observed. It would be worthwhile to test these phytochemicals against further cell lines and noncancer cells in order to elucidate their anticancer potential. The noticeable anti-inflammatory activity of eight of the compounds tested has been demonstrated. Compounds **2**, **4**, **5**, and **8** significantly inhibited the secretion of both cytokines due, at least in part, to inhibition of the NF-κB signaling pathway. However, further detailed testing will be required to elucidate the exact mechanism of action and any potential impact on other pro-inflammatory mediators of these compounds.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-2000 digital polarimeter, and circular dichroism (CD) spectra were obtained using JASCO J-815 instruments (CD spectra were measured twice, independently on two different spectrometers). NMR (1D and 2D) spectra were obtained using a Bruker Avance 400 spectrometer with TMS as the internal standard. HRMS spectra were measured with an Orbitrap spectrometer (Thermo Scientific) using ESI in the positive mode. Analytical HPLC measurements were obtained using an Agilent 1100 chromatographic system with a DAD (Agilent). Preparative HPLC was performed using a YL 9100 HPLC System (Young Lin) with a Foxy R2 Fraction Collector (Teledyne Isco). Column chromatography was performed on silica gel with a particle size of 40−63 μm (Merck). Silica gel 100 (Fluka) was used for flash chromatography. TLC plates [silica gel 60 F$_{254}$ 20 × 20 cm, 200 μm (Merck)] and an analytical HPLC column [Ascentis Express RP-Amide, 10 cm × 2.1 mm, particle size 2.7 μm (Supelco)] were used for analytical purposes. Final compound purifications were performed using preparative TLC plates [Uniplate, silica gel GF, 20 × 20 cm, with fluorescent indicator F$_{254}$, 500 μm (Analtech)] and a semipreparative HPLC column [Ascentis RP-amide, 25 cm × 10 mm, particle size 5 μm (Supelco)].

RPMI 1640 medium and phosphate-buffered saline (PBS) were purchased from PAA (Pasching, Austria). The penicillin−streptomycin mixture, fetal bovine serum (FBS), phorbol myristate acetate (PMA), erythrosin B, *Escherichia coli* 0111:B4 lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO), and prednisone (purity >98%) were obtained from Sigma-Aldrich (Steinheim, Germany). Cytotoxicity was tested by using a Cell Proliferation Reagent WST-1 kit from Roche Applied Science (Mannheim, Germany) and the productions of TNF-α and IL-1β were evaluated by using a Human Instant ELISA kit from eBioscience (Vienna, Austria).

Plant Material. The roots of *M. alba* were collected in Konya, Turkey in April 2007 (for compounds **3**, **4**, **6**, and **7**) and in the area of the University of Veterinary and Pharmaceutical Sciences Brno (UVPS Brno), Brno, Czech Republic in April 2011 (for compounds **1**, **2**, **5**, and **8**). The roots of *M. nigra* were collected in Konya, Turkey in April 2007 (for compounds **9** and **10**). The botanical identifications were confirmed by Professor Murat Kartal (Faculty of Pharmacy, Ankara University, Turkey) and Associate Professor Petr

Babula (UVPS Brno). Voucher specimens were deposited in the herbarium of the Department of Natural Drugs, UVPS Brno (No. MA-07A, MA-11A, and MN-07A).

Extraction and Isolation. The procedures used to isolate kuwanon E (**4**) and cudraflavone B (**7**) have been reported previously.[17,27] Mulberrofuran H (**3**) and sanggenon H (**6**) were obtained from the CHCl$_3$ extract of *M. alba*,[17,28] and separated using reversed-phase preparative HPLC (Supelcosil ABZ+Plus, 250 × 21.2 mm i.d., particle size 5 μm). The gradient elution employed 0.2% HCOOH and a mixture of MeCN and MeOH, 8:2 (v/v) (A). The initial composition of 20% A increased to a final composition of 100% A after 40 min, with a flow rate of 25 mL/min. Fractions were acquired using a detector response at λ = 280 nm. After removal of the organic solvent and precipitation, the fraction with an HPLC t_R value of 14−15 min yielded **3** (22 mg), whereas the fraction with a t_R value of 15−16 min was further purified using preparative TLC [(C$_6$H$_6$−acetone, 95:5 (v/v), R_f 0.55)] to yield **6** (7 mg).

Moracin C (**1**), mulberrofuran Y (**2**), kuwanon C (**5**), and morusinol (**8**) were isolated from *M. alba* root bark collected in the area of the UVPS campus. The liquid−liquid extraction was performed in the same way as for the Turkish *M. alba* roots.[27] Twenty-two kilograms of chopped root bark yielded 218 g of solid material from the CHCl$_3$-soluble portion. A 90 g aliquot of this material was separated using column chromatography. The mobile phase was composed of CHCl$_3$−C$_6$H$_6$−MeOH (45:48:7), and 150 mL fractions were collected. Fractions 52−59 were subsequently combined and separated using column chromatography with a mobile phase of C$_6$H$_6$−acetone (7.5:2.5). Subfractions 21 and 22 from this separation were combined and subjected to preparative HPLC (gradient elution with 0.2% HCOOH and MeCN). Compounds **1** (61 mg), **2** (58 mg), and **5** (545 mg) were isolated. Fractions 60−69 were combined and separated using column chromatography, with a mobile phase of C$_6$H$_6$−acetone (7:3), and subfractions 11 and 12 yielded compound **8** (256 mg).

The same extraction procedure was applied to *M. nigra* root bark.[17,28] A total of 216 g of chopped root bark yielded 9 g of solid material from the CHCl$_3$-soluble portion. This was separated using flash chromatography with a mobile phase of CHCl$_3$:C$_6$H$_6$:MeOH (8:1:1) and a flow rate of 40 mL/min. Fractions of 320 mL each were collected. Fraction 2 was separated using preparative HPLC (gradient elution with 0.2% HCOOH and MeCN), and subfractions 4 and 5 were then purified using preparative TLC with a mobile phase of CHCl$_3$−C$_6$H$_6$−MeOH (7.5:1.5:1) to yield compounds **9** (1.5 mg) and **10** (15 mg), respectively.

The purity of all compounds exceeded 95%, as checked via analytical HPLC.

Maintenance and Preparation of Macrophages. The THP-1 human monocytic leukemia cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, U. K.). The cells were cultivated at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere containing 5% CO$_2$. The medium was changed twice a week, when cells had reached a concentration of 5−7 × 10^5 cells/mL. The cell number and viability were determined following staining with erythrosin B. Cells were counted manually using a hemocytometer and a light microscope. Cells that remained unstained were considered viable and light red cells as nonviable. Stabilized cells were split into multititration plates to afford a concentration of 5 × 10^5 cells/mL, and

differentiation into macrophages was induced by phorbol myristate acetate (PMA), as described previously.[50]

Cytotoxicity Assay. Compounds **1−6** and **8−10** were dissolved in DMSO at concentrations decreasing from 30 to 0.37 μM and added to the monocyte suspension in the culture medium. The final concentration of DMSO in the culture medium was 0.1%. Incubation for 24 h at 37 °C with 5% CO$_2$ followed. After incubation, the cytotoxicity was measured by using a Cell Proliferation Reagent WST-1 kit, according the manufacturer's instructions. The cytotoxicity of cudraflavone B (**7**) on the THP-1 cell line has been described previously.[17,23] The IC$_{50}$ values were calculated from viability curves, and the results are presented as arithmetic means ± SE.

Drug Treatment and Induction of Inflammation. Differentiated macrophages were pretreated for 1 h with compounds **1−10** at 1 μM dissolved in DMSO. According to the cytotoxicity assays, these concentrations lacked a cytotoxic effect. For comparison with a conventional drug, 1 μM prednisone dissolved in DMSO was used. Vehicle-treated cells contained only the vehicle (DMSO), and control cells were not treated with LPS. The concentration of DMSO in each well was 0.1%.

The modulating effect of each compound on the secretion of TNF-α was tested by adding 1 μg/mL of LPS dissolved in sterile water to macrophages pretreated with these compounds. After being treated with LPS, the cultivation medium was aspirated for 24 h, the cell residue was eliminated by centrifugation, and the samples were stored at −80 °C to await further processing.

Evaluation of Cytokine Secretion. Pretreated macrophages were incubated with LPS for the next 24 h. After this period, the medium was collected and the concentrations of the secreted TNF-α and IL-1β were measured using a Human Instant ELISA kit.

Detection of NF-κB Nuclear Translocation. Macrophage-like THP-1 cells were prepared in serum-free RPMI 1640 medium at a concentration of 5 × 10^5 cells/mL and seeded into 35 mm dishes in 1.5 mL aliquots. Cells were pretreated with either compound **3**, **6−8**, or prednisone, all at a concentration of 1 μM for 1 h. After preincubation with the compounds being tested, LPS at a final concentration of 1 μg/ mL was added to activate the NF-κB pathway, and the cells were cultivated for the next 3 h. The cultivation medium was then removed, and the cells were washed three times with PBS (pH = 7.4) at room temperature (2 min per washing). After fixation (ice-cold acetone, for 3 min) they were washed twice with cold PBS. The cells were then incubated in PBS containing 1% BSA (w/v) for 30 min and then overnight with a primary antibody (rabbit polyclonal anti-NF-κB p65 antibody, Abcam, U. K.) at 4 °C. After this incubation, the cells were washed three times with PBS (5 min per washing) and incubated with a secondary antibody (antirabbit IgG − FITC conjugate, PBS containing 1% BSA, w/v) for 1 h at room temperature. Finally, the liquid was decanted from the mixture, the cells were washed three times with PBS (5 min per washing in darkness), incubated with propidium iodide (0.5 μg/mL, PBS) for 5 min and washed again with PBS. The cells were then observed under a fluorescence microscope (Axioskop 40, Carl Zeiss, Germany) equipped with an appropriate set of filters (Carl Zeiss). Photographs were taken using a digital microscope camera (ProgRess MF, Jenoptik, Germany). The NIS-element program (Czech Republic) was used to process images− convert concentration images to a color scale, perform analysis,

and evaluate the intensity of emission. Unless otherwise specified, all chemicals used in this part of the investigation were purchased from Sigma-Aldrich.

Statistical Analysis. All experiments were performed in triplicate, and the results are presented as mean values with error bars representing the standard error (S.E.) of the mean. A one-way ANOVA test was used for statistical analysis, followed by a Tukey's post hoc test for multiple comparisons. A value of $p < 0.05$ was considered to be statistically significant. The program GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA, U. S. A.) was used to perform the analysis.

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Notes
The authors declare no competing financial interest.

■ REFERENCES

(1) Ma, X.; Iwanaka, N.; Masuda, S.; Karaike, K.; Egawa, T.; Hamada, T.; Toyoda, T.; Miyamoto, L.; Nakao, K.; Hayashi, T. *J. Ethnopharmacol.* **2009**, *122*, 54−59.

(2) Fukai, T.; Satoh, K.; Nomura, T.; Sakagami, H. *Fitoterapia* **2003**, *74*, 720−724.

(3) Piao, S. J.; Chen, L. X.; Kang, N.; Qiu, F. *Phytochem. Anal.* **2011**, *22*, 230−235.

(4) Yang, Z. G.; Matsuzaki, K.; Takamatsu, S.; Kitanaka, S. *Molecules* **2011**, *16*, 6010−6022.

(5) Venkatesh, K. P.; Chauhan, S. *J. Med. Plants Res.* **2008**, *2*, 271−278.

(6) de Souza, M. M.; Bittar, M.; Cechinel, V.; Yunes, R. A.; Messana, I.; Delle Monache, F.; Ferrari, F. *Z. Naturforsch. C: J. Biosci.* **2000**, *55*, 256−260.

(7) Zheng, Z. P.; Cheng, K. W.; Zhu, Q.; Wang, X. C.; Lin, Z. X.; Wang, M. F. *J. Agric. Food Chem.* **2010**, *58*, 5368−5373.

(8) Molodecky, N. A.; Soon, I. S.; Rabi, D. M.; Ghali, W. A.; Ferris, M.; Chernoff, G.; Benchimol, E. I.; Panaccione, R.; Ghosh, S.; Barkema, H. W.; Kaplan, G. G. *Gastroenterology* **2012**, *142*, 46−54.

(9) Gautam, R.; Jachak, S. M. *Med. Res. Rev.* **2009**, *29*, 767−820.

(10) Chung, K. O.; Kim, B. Y.; Lee, M. H.; Kim, Y. R.; Chung, H. Y.; Park, J. H.; Moon, J. O. *J. Pharm. Pharmacol.* **2003**, *55*, 1695−1700.

(11) Phung, T. X.; Tran, T. H.; Dan, T. T.; Chau, V. M.; Hoang, T. H.; Nguyen, T. D. *J. Asian Nat. Prod. Res.* **2012**, *14*, 596−600.

(12) Schneider, I.; Bucar, F. *Phytother. Res.* **2005**, *19*, 81−102.

(13) Chi, Y. S.; Jong, H. G.; Son, K. H.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Biochem. Pharmacol.* **2001**, *62*, 1185−1191.

(14) Dat, N. T.; Phung, T. X. B.; Le, T. P. Q.; Huong, H. T.; Minh, C. V. *Immunopharmacol. Immunotoxicol.* **2012**, *34*, 84−88.

(15) Cheon, B. S.; Kim, Y. H.; Son, K. S.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Planta Med.* **2000**, *66*, 596−600.

(16) Wang, L.; Yang, Y.; Liu, C.; Chen, R. Y. *J. Asian Nat. Prod. Res.* **2010**, *12*, 431−437.

(17) Hošek, J.; Bartoš, M.; Chudík, S.; Dall'Acqua, S.; Innocenti, G.; Kartal, M.; Kokoška, L.; Kollár, P.; Kutil, Z.; Landa, P.; Marek, R.; Závalová, V.; Žemlička, M.; Šmejkal, K. *J. Nat. Prod.* **2011**, *74*, 614−619.

(18) Rollinger, J. M.; Bodensieck, A.; Seger, C.; Ellmerer, E. P.; Bauer, R.; Langer, T.; Stuppner, H. *Planta Med.* **2005**, *71*, 399−405.

(19) Fukai, T.; Hano, Y.; Hirakura, K.; Nomura, T.; Uzawa, J. *Chem. Pharm. Bull.* **1984**, *32*, 808−811.

(20) Hano, Y.; Nomura, T. *Heterocycles* **1983**, *20*, 1071−1076.

(21) Nomura, T.; Fukai, T.; Katayanagi, M. *Chem. Pharm. Bull.* **1977**, *25*, 529−532.

(22) Messana, I.; Ferrari, F.; Delle Monache, F.; Yunes, R. A.; Calixto, J. B.; Bisognin, T. *Heterocycles* **1991**, *32*, 1287−1296.

(23) Kollár, P.; Bárta, T.; Hošek, J.; Souček, K.; Závalová, V. M.; Artinian, S.; Talhouk, R.; Šmejkal, K.; Suchý, P.; Hampl, A. *Evid.-Based Complement. Altern. Med.* **2013**, *2013*, 350519.

(24) Zhang, M.; Wang, R.-R.; Chen, M.; Zhang, H.-Q.; Sun, S.; Zhang, L.-Y. *Chin. J. Nat. Med.* **2009**, *7*, 105−107.

(25) Nam, S. Y.; Yi, H. K.; Lee, J. C.; Kim, J. C.; Song, C. H.; Park, J. W.; Lee, D. Y.; Kim, J. S.; Hwang, P. H. *Arch. Pharmacal Res.* **2002**, *25*, 191−196.

(26) Arung, E. T.; Yoshikawa, K.; Shimizu, K.; Kondo, R. *Fitoterapia* **2010**, *81*, 120−123.

(27) Šmejkal, K.; Svačinová, J.; Šlapetová, T.; Schneiderová, K.; Dall'Acqua, S.; Innocenti, G.; Závalová, V.; Kollár, P.; Chudík, S.; Marek, R.; Julinek, O.; Urbanová, M.; Kartal, M.; Csollei, M.; Doležal, K. *J. Nat. Prod.* **2010**, *73*, 568−572.

(28) Ferlinahayati; Syah, Y. M.; Juliawaty, L. D.; Achmad, S. A.; Hakim, E. H.; Takayama, H.; Said, I. M.; Latip, J. *Z. Naturforsch. C: J. Biosci.* **2008**, *63*, 35−39.

(29) Zelefack, F.; Guilet, D.; Valentin, A.; Fongang, R. C. S.; Kom, B.; Chevalley, S.; Ngouela, S. A.; Tsamo, E.; Fabre, N.; Dijoux-Franca, M. G. *Greener J. Biol. Sci.* **2012**, *2*, 5.

(30) Nicolle, E.; Boccard, J.; Guilet, D.; Dijoux-Franca, M. G.; Zelefac, F.; Macalou, S.; Grosselin, J.; Schmidt, J.; Carrupt, P. A.; Di Pietro, A.; Boumendjel, A. *Eur. J. Pharm. Sci.* **2009**, *38*, 39−46.

(31) Shi, Y. Q.; Fukai, T.; Sakagami, H.; Chang, W. J.; Yang, F. Q.; Wang, F. P.; Nomura, T. *J. Nat. Prod.* **2001**, *64*, 181−188.

(32) Tan, Y. X.; Liu, C.; Zhang, T.; Chen, R. Y.; Yu, D. Q. *Phytochem. Lett.* **2010**, *3*, 57−61.

(33) Costa, G.; Francisco, V.; Lopes, M. C.; Cruz, M. T.; Batista, M. T. *Curr. Med. Chem.* **2012**, *19*, 2876−2900.

(34) Ko, H. H.; Yu, S. M.; Ko, F. N.; Teng, C. M.; Lin, C. N. *J. Nat. Prod.* **1997**, *60*, 1008−1011.

(35) Lim, H. J.; Jin, H. G.; Woo, E. R.; Lee, S. K.; Kim, H. P. *J. Ethnopharmacol.* **2013**, *149*, 169−175.

(36) Kim, T. J.; Han, H. J.; Lim, Y.; Song, M. C.; Kim, J.; Hong, J. T.; Yoo, H. S.; Pyo, M. Y.; Hwang, B. Y.; Lee, M. K.; Yun, Y. P. *J. Cardiovasc. Pharmacol.* **2009**, *53*, 341−348.

(37) Chen, S. K.; Zhao, P.; Shao, Y. X.; Li, Z.; Zhang, C. X.; Liu, P. Q.; He, X. X.; Luo, H. B.; Hu, X. P. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3261−3264.

(38) Tan, Y. X.; Yang, Y.; Zhang, T.; Chen, R. Y.; Yu, D. Q. *Fitoterapia* **2010**, *81*, 742−746.

(39) Lee, K. H.; Yeh, M. H.; Kao, S. T.; Hung, C. M.; Chen, B. C.; Liu, C. J.; Yeh, C. C. *J. Ethnopharmacol.* **2009**, *124*, 530−538.

(40) Shibata, Y.; Kume, N.; Arai, H.; Hayashida, K.; Inui-Hayashida, A.; Minami, M.; Mukai, E.; Toyohara, M.; Harauma, A.; Murayama, T.; Kita, T.; Hara, S.; Kamei, K.; Yokode, M. *Atherosclerosis* **2007**, *193*, 20−27.

(41) Indra, M. R.; Karyono, S.; Ratnawati, R.; Malik, S. G. *BMC Res. Notes* **2013**, *6*, 275.

(42) Choi, E. J.; Lee, S.; Chae, J. R.; Lee, H. S.; Jun, C. D.; Kim, S. H. *Life Sci.* **2011**, *88*, 1121−1126.

(43) Han, J. M.; Jin, Y. Y.; Kim, H. Y.; Park, K. H.; Lee, W. S.; Jeong, T. S. *Biol. Pharm. Bull.* **2010**, *33*, 1019−1023.

(44) Soromou, L. W.; Zhang, Z.; Li, R.; Chen, N.; Guo, W.; Huo, M.; Guan, S.; Lu, J.; Deng, X. *Molecules* **2012**, *17*, 3574−3585.

(45) Kim, S. C.; Kang, S. H.; Jeong, S. J.; Kim, S. H.; Ko, H. S.; Kim, S. H. *Immunopharmacol. Immunotoxicol.* **2012**, *34*, 645−650.

(46) Lee, J. K. *Arch. Pharm. Res.* **2011**, *34*, 671−679.

(47) Laavola, M.; Nieminen, R.; Yam, M. F.; Sadikun, A.; Asmawi, M. Z.; Basir, R.; Jukka, W.; Vapaatalo, H.; Korhonen, R.; Moilanen, E. *Planta Med.* **2012**, *78*, 779–786.

(48) Kim, H. S.; Kim, A. R.; Park, H. J.; Park, D. K.; Kim do, K.; Ko, N. Y.; Kim, B.; Choi, D. K.; Won, H. S.; Shin, W. S.; Kim, Y. M.; Choi, W. S. *J. Ethnopharmacol.* **2011**, *136*, 392–398.

(49) Choi, E. M.; Lee, Y. S. *Food Chem. Toxicol.* **2010**, *48*, 2607–2611.

(50) Pěnčíková, K.; Kollár, P.; Závalová, V. M.; Táborská, E.; Urbanová, J.; Hošek, J. *Phytomedicine* **2012**, *19*, 890–895.

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Diplacone and mimulone ameliorate dextran sulfate sodium-induced colitis in rats

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ABSTRACT

Diplacone (**1**) and mimulone (**2**), two geranylated flavanones, have previously shown anti-inflammatory and antiradical activity in vitro. The present study aimed to evaluate their activity in vivo on a model of colitis induced in Wistar rats by an oral administration of dextran sulfate sodium (DSS).

Diplacone (**1**) and mimulone (**2**) were administered at a bolus dose of 25 mg/kg by gastric gavage 48 and 24 h prior to the induction of colitis by DSS and every 24 h on the following days of the experiment. The effect of the treatment was assessed by monitoring the disease activity index (DAI), histopathological examination, evaluation of the weight and length of the colon and by analysis of the levels and activities of cyclooxygenase-2 (COX-2), matrix metalloproteinase-2 (MMP2), superoxide dismutase-2 (SOD2), and catalase (CAT) in the inflamed tissue.

Administration of the test compounds prior and after induction of colitis ameliorated the symptoms of colitis (diarrhea, presence of the blood in the stool) and delayed their onset. The ability of compounds **1** and **2** to reduce the levels of COX-2 and to increase the ratio of pro-MMP2/MMP2 activity correlates with the values of the DAI. The lowering of the levels of the antioxidant enzymes SOD2 and CAT reflects the ability of the test compounds to scavenge reactive oxygen species.

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1. Introduction

Inflammatory bowel diseases (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are chronic inflammatory illnesses of the gastrointestinal tract with intermittent periods of relapse and with the progression of the disease difficult to predict. Their incidence in Western populations has been increasing in recent years. The etiology of chronic inflammatory illnesses of the gastrointestinal tract remains unclear and there is therefore no causal treatment. Current therapy is not completely successful and it is frequently connected with adverse effects [1]. Plant products may provide an option for the alternative or supplementary treatment of the patients, and their structures can serve to provide leads or inspiration for the synthesis of new anti-inflammatory agents.

In vitro and *in vivo* studies have shown that flavonoids are appropriate candidates for new anti-inflammatory drugs. Their ability to inhibit the activity and expression of phospholipase A2, cyclooxygenases, lipoxygenases, and inducible synthase of nitric oxide leads to diminished production of the mediators of inflammation [2]. Scavenging the reactive oxygen species (ROS) moderates the damage to tissue and affects the signal transduction pathways [3]. Furthermore, the intervention in the signal cascade of nuclear factor κB (NF-κB) changes the expression of a series of genes connected with the inflammatory response and apoptosis [4].

This study is focused on two geranylated flavanones diplacone (**1**) and mimulone (**2**). Compounds **1** and **2** have shown anti-inflammatory [5,6], antiradical, cytoprotective [7,8], and antibacterial activities [9] in previous studies. The aim of the present study was to determine the biological activity of these compounds *in vivo* in the model of dextran sulfate sodium (DSS)-induced colitis in rats. The efficacy of compounds **1** and **2** was compared to sulfasalazine (SAS), a conventional drug used in IBD therapy. Besides the histological and physical examinations, the levels of the antioxidant enzymes superoxide dismutase-2 (SOD2) and catalase (CAT), the levels of cyclooxygenase-2 (COX-2), and the activity of matrix metalloproteinase-2 (MMP2) were investigated to elucidate the anti-inflammatory effects of these compounds.

2. Materials and methods

2.1. Test compounds

Diplacone (**1**) and mimulone (**2**) were isolated from the unripe fruits of *Paulownia tomentosa* (Thunb.) Steud. (Paulowniaceae) and characterized as previously described [7]. The identification was carried out using ESIMS, ^{1}H and ^{13}C NMR analyses (see Supplemental Data Fig. S1–S7); the purity was checked *via* HPLC analysis and exceeded 95% [7]. The chemical structures are shown in Fig. 1. Sulfasalazine was purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Experimental animals

Male Wistar rats (180–220 g) were supplied by AnLab, Ltd. (Prague, Czech Republic). The animals were kept under standard conditions (temperature 22 ± 2 °C, relative humidity $50 \pm 10\%$, alternating 12 hour light/dark cycle). They were fed a standard diet and given water *ad libitum*. The experimental protocol was approved by the Expert Committee for the Welfare of Experimental Animals of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (Approval No. 26-2013). To minimize the suffering of the laboratory animals, the number of pharmacological interventions was limited to the necessary minimum.

2.3. Induction of colitis

Rats were randomly divided into five groups ($n = 8$ for each group) after a one week period of acclimation. Colitis was induced by using 10% (*w/v*) DSS (MP Biomedicals, Illkirch-Graffenstaden, France; molecular weight 36,000–50,000 Da) in the drinking water, as previously described [10], with some modifications. The rats in the intact group received drinking water only instead of DSS solution.

2.4. Experimental design

The tested compounds **1** and **2**, and the positive control sulfasalazine were administrated prophylactically, 48 and 24 h before the induction of colitis and in the following days concomitantly by gastric gavage under light isoflurane anesthesia. The substances were suspended in a 4% gel of PVP K90 (polyvinylpyrrolidone K90, Sigma-Aldrich) and were given at a bolus dose of 25 mg/kg of diplacone (**1**, 589 mmol/kg), mimulone (**2**, 612 mmol/kg), or sulfasalazine (SAS, 628 mmol/kg). Animals in the untreated group (DSS group) and the intact group were treated with the vehicle only (4% gel of PVP). On day five of the experiment, the animals were sacrificed by using an overdose of T61 (Intervet International B. V., Boxmeer, Netherlands), a veterinary euthanasia drug. Immediately after the euthanasia, the entire colon was removed, including the caecum; it was opened and cleaned with physiological saline, measured and weighed. Samples for histological evaluation were obtained and the remaining parts of the colons were frozen in liquid nitrogen and stored at −80 °C for the next processing.

2.5. Disease activity index

The rats were monitored daily for loss of body weight, stool consistency, and rectal bleeding in order to calculate the disease activity index (DAI) for each animal. The DAI, which reflects the severity of colitis, was modified according to Oh *et al.* (2006). The loss of body weight was assigned 0–2 points; it did not exceed 10% by the end of the experiment. The change in stool consistency was 0 points for normally formed stool, 1 point for loose stool, and up to 4 points for diarrhea according to the intensity of the changes. The presence of blood in the feces was assigned 0, 2, or 4 points (Table S1). The total DAI was then the sum of the points obtained from all of the parameters monitored. In the case of death during the experiment, the animal was assigned 10 points to enable the statistical evaluation.

2.6. Histological evaluation

Samples about 1.5 cm long were taken from the proximal and distal colon and fixed in 10% neutral buffered formalin then embedded in paraffin. Sections 3-μm-thick were stained with hematoxylin and eosin (H&E) and examined microscopically by a veterinary pathologist. The loss of epithelial surface, destruction of crypts and inflammatory cell infiltration into mucosa was assessed according to the criteria by Araki *et al.* (2000). Each parameter was assigned as follows: 0 = no change, 1 = localized and mild, 2 = localized and moderate, 3 = extensive and moderate, and 4 = extensive and severe. Total histological score is the sum of the points from all of the parameters [11].

2.7. Preparation of protein for immunodetection

Using a bench blender, approximately 2 g of colonic tissue was homogenized in lysis buffer [50 mM Tris–HCl (pH 7.5),

1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M saccharose] at a ratio of 3 mL of buffer per gram of tissue. After centrifugation at 9000 rpm for 15 min at 4 °C, the supernatant was stored. The protein concentration was measured using a Bradford-method protein assay kit (Amresco, Solon, USA) according to the manufacturer's instructions.

2.8. Western blot analysis

Protein samples were denatured in the presence of β-mercaptoethanol and SDS at 70 °C for 5 min. The denatured proteins (80 μg for SOD2 and CAT, 120 μg for COX-2) were loaded onto 12% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (0.2 μm, Bio-Rad, Hercules, USA), which was subsequently blocked with 5% BSA (Sigma-Aldrich) in TBST buffer [10 mM Tris–HCl (pH 7.5), 150 mM sodium chloride, 0.1% (v/v) Tween-20] for 1 h. The membrane was then incubated with the primary mouse anti-COX-2 antibody at a dilution of 1:200 (Abcam, Cambridge, UK), the rabbit anti-SOD2 antibody at a dilution of 1:1000 (Sigma-Aldrich), the mouse anti-CAT antibody at a dilution of 1:1000 (Sigma-Aldrich), or the mouse anti-β-actin antibody at a dilution of 1:5000 (Abcam) overnight at 4 °C. After washing with distilled water, the membrane was incubated with the secondary antibody (anti-mouse IgG or anti-rabbit IgG, Sigma-Aldrich) at a dilution of 1:2000 for 1 h at room temperature. The secondary antibody was detected by colorimetric analysis using an Opti-CN™ Substrate Kit (Bio-Rad). The intensity of the protein bands was evaluated by densitometric analysis using AlphaEase FC 4.0.0 software (Alpha Innotech Corporation, San Leandro, USA).

2.9. MMP2 activity

MMP2 activity (pro-MMP2, MMP2) was evaluated using gelatin zymography, as described previously [12]. Twenty micrograms of the native proteins (without denaturation in the presence of β-mercaptoethanol) was loaded onto 10% SDS-polyacrylamide gel impregnated with 0.1% gelatin. After electrophoresis the gel was washed twice for 15 min in 2.5% (v/v) Triton X-100 to remove SDS. The gel was then incubated for 15 min at room temperature and subsequently overnight (about 16 h) at 37 °C in the development buffer [50 mM Tris–HCl (pH 8.8), 5 mM calcium chloride, 3 mM sodium azide, 0.5% (v/v) Triton X-100]. After that, the gel was stained with Coomassie blue for 2 h and destained until the bands were clearly visible. The intensity of the digested bands was determined by densitometric analysis using AlphaEase FC 4.0.0 software (Alpha Innotech Corporation). The ratio between the pro-MMP2 form and the active MMP2 form was calculated.
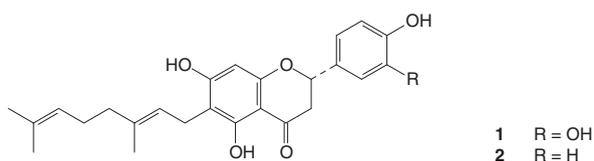
Fig. 1. Chemical structures of diplacone (**1**) and mimulone (**2**).

2.10. Statistical analysis

The results are expressed as the mean values with error bars representing the standard error (SE) of the mean. One-way ANOVA followed by Tukey's *post hoc* test, two-way ANOVA followed by Bonferroni's *post hoc* test, and Kaplan–Meier analysis were used for the statistical evaluation. The specific analyses are referenced with the particular results. Values of $p < 0.05$ were considered to be statistically significant. GraphPad Prism 5.01 (GraphPad Software Inc., San Diego, USA) was used for the analysis.

3. Results

3.1. Disease activity index

Administration of 10% DSS in the drinking water led to the loss of body weight, changes in the consistency of the stool, and rectal bleeding in the treated animals. The loss of body weight was comparable in all experimental groups. Changes in the consistency of the stool were observed in the untreated DSS group on day 3 of the experiment, on day 4 in the group treated with **1** and **2**, and on day 3 or 4 in the SAS group. Blood appeared in the stool of all experimental groups treated with DSS one day after changes in the consistency of the stool. None of the described changes were observed in the intact group. The test compounds ameliorated the symptoms of colitis (diarrhea, presence of blood in the stool) and delayed their onset. The groups treated with diplacone (**1**) and mimulone (**2**) showed the lowest DAI on the last day of the experiment ($p > 0.001$ compared to the DSS-only group). Fig. 2A shows the development of the disease activity index for each group in the course of experiment. Data are also presented in Table S2.

Four animals in the DSS group, one in the group treated with **1** and **2**, and two in the SAS group died during the last day of the experiment. No mortality was observed in the intact group (Fig. 2B).

3.2. Weight/length ratio of the colon

The administration of 10% DSS for five days caused the weight/length ratio of the colon to increase by 30.4% compared to the intact group. Although the ratio increased less in the treated groups (20.4% for treatment with **1**, 16.2% for treatment with **2**, and 17.7% for treatment with SAS, as compared to the intact group), the differences were not statistically significant. The increase in the weight/length ratio of the colon was caused by shortening of the colon (Fig. S8).

3.3. Histopathological examination

Administration of DSS for five days induced pathological changes involving shortening or entire loss of the crypts, erosion of the epithelium, atrophy of the mucosa, infiltration of the lamina propria with neutrophils and macrophages, fibroplasia, and ulcerations. These changes were less severe in the proximal part of the colon. They were not observed in the intact group of animals. In the treated groups of animals, the intensity of the changes was similar to that observed in the untreated DSS group. Only two samples showed less severe lesions compared to the DSS group: one from the diplacone (**1**)
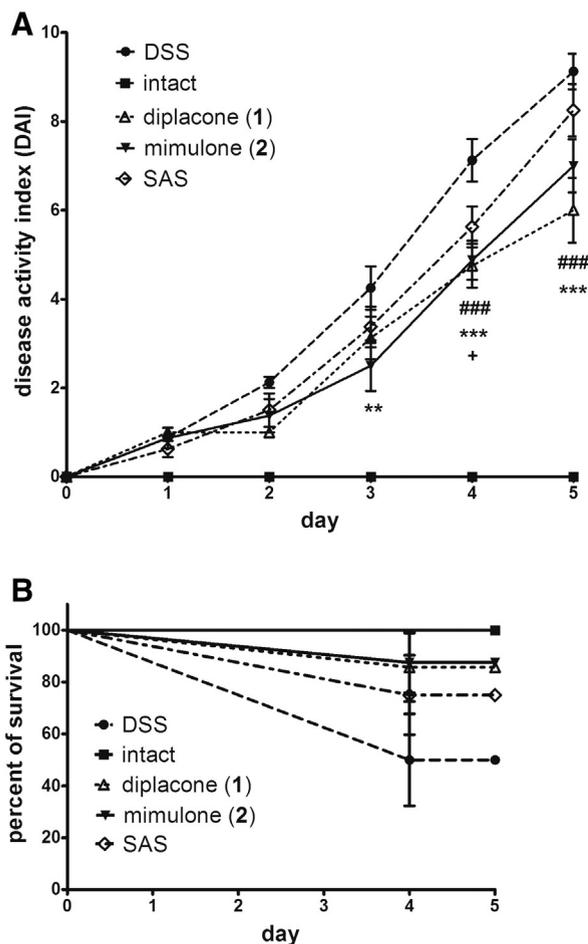
Fig. 2. Effect of the test compounds on DAI and survival in DSS-induced rat colitis. Colitis was induced by administration of 10% DSS in the drinking water for five days. Administration of diplacone (**1**), mimulone (**2**), and sulfasalazine (SAS) at a dose of 25 mg/kg or the vehicle (the DSS group and the intact group) was started two days before the induction of colitis. Colitis was not induced in the intact group. (A) Changes in the disease activity index (DAI), statistical evaluation: two-way ANOVA followed by Bonferroni's *post hoc* test. Diplacone (**1**) vs. DSS: ### $p < 0.001$, mimulone (**2**) vs. DSS: ** $p < 0.01$, *** $p < 0.001$, sulfasalazine vs. DSS: + $p < 0.05$. (B) Kaplan–Meier survival analysis.

group and one from the SAS. Evaluation of colonic damage is shown in Fig. S9. The differences were not statistically significant. Representative samples are shown in Fig. 3.

3.4. Effect of treatment on protein production

The levels of SOD2 and CAT were not significantly modified by the induction of colitis. Treatment with compound **1** reduced the levels of the tested enzymes the most (Fig. 4).

The administration of DSS led to a significant increase in the expression of COX-2 as compared to the intact group ($p < 0.05$). All of the test compounds lowered the levels of COX-2 in comparison to the untreated DSS group (Fig. 5). The levels of COX-2 were reduced in the SAS group the most (by 69.8% compared to the DSS group). The administration of compounds **1** and **2** reduced the expression of COX-2 by 55.9% and 48.7%, respectively.

3.5. Effect of treatment on MMP2 activity

A marked decrease in the ratio of pro-MMP2/MMP2 was observed in the untreated DSS group compared to the intact group ($p < 0.01$). As shown in Fig. 6, the test compounds increased this ratio (by 50.7% in the case of **1**, by 43.3% in the case of **2**, and by 37.6% for SAS, as compared to the DSS group).

4. Discussion

Pharmacotherapy (aminosalicylates, glucocorticoids, immunosuppressives) and surgical procedures are currently used as the main instruments in the therapy of inflammatory bowel diseases. Contemporary approaches are not always completely effective; they are associated with a number of adverse effects, and they can negatively affect the quality of the patient's life. Therefore, there is an urgent need for a new strategy, either alternative or supplementary, for treating IBD. The beneficial properties of flavonoids (the ability to scavenge reactive oxygen species and to reduce the expression of pro-inflammatory markers) can also be utilized in the treatment of chronic inflammatory diseases, such as CD and UC. Diplacone (**1**) and mimulone (**2**) belong to the group of geranylated flavonoids. The lipophilic chain formed by the attachment of a geranyl unit at *C*-6 of the flavanone skeleton modifies their observed biological activities, as compared to their non-geranylated analogs [9,13,14]. This observation is connected with the increased affinity of such compounds for different biological membranes, with possible higher bioavailability and therefore greater therapeutic potential or potential toxicity than the parent non-geranylated compounds [15]. Nonetheless, only one study describes the anti-inflammatory activity of flavonoids substituted with a prenyl chain in animal models of colitis [16]. However, naringenin, the parent compound of **2**, without a geranyl side chain, has been shown to ameliorate DSS-induced colitis in mice [17] and acetic acid-induced colitis in rats [18]. DSS-induced colitis was chosen as the optimal model for determining the benefits of prophylactic and curative administration of the geranylated flavanones diplacone (**1**) and mimulone (**2**).

The administration of 10% DSS in drinking water caused acute colitis, localized especially in the distal part of the colon. In the pilot study preceding this experiment, the administration of lower concentrations of DSS (5% and 7%) was not effective—apart from the loss of body weight, no symptoms of colitis were observed (unpublished data), in contrast to the published studies carried out using Wistar rats [10]. Colitis induced by the administration of 10% DSS for five days was characterized by the loss of body weight, diarrhea, bloody stool and histologically observable damage (shortening or entire loss of the crypts, erosion of the epithelium, atrophy of the mucosa, infiltration of the lamina propria with neutrophils and macrophages, fibroplasia, and ulcerations). Other studies have also described the shortening of the colon in DSS-induced colitis [16,19]. We confirmed this fact, but the shortening was not significant to be compared to the intact group, probably because of the short-term administration of DSS. All of the compounds tested ameliorated and delayed changes in the consistency of the stool and rectal bleeding. Diplacone (**1**) showed the greatest therapeutic effect (i.e. the lowest DAI on the last day of experiment) when all of the compounds tested
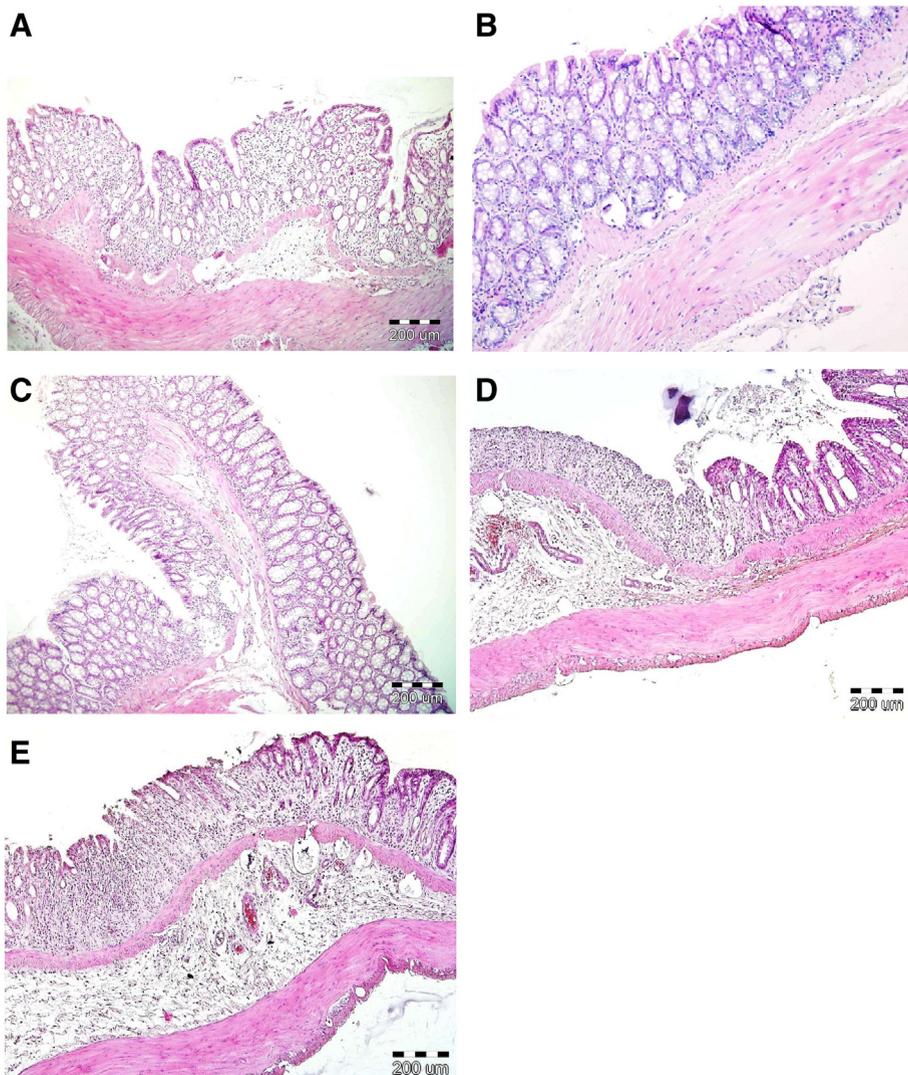
Fig. 3. Histological findings (H&E, magnification ×100). (A) Distal colon after five days of administration of 10% DSS in the drinking water. (B) Distal colon of the intact group, without the induction of colitis. (C) Distal colon after five days of administration of 10% DSS in the drinking water and treatment with diplacone (**1**) at a dose of 25 mg/kg, which was started two days before the induction of colitis. (D) Distal colon after five days of administration of 10% DSS in the drinking water and treatment with mimulone (**2**) at a dose of 25 mg/kg, which was started two days before the induction of colitis. (E) Distal colon after five days of administration of 10% DSS in the drinking water and treatment with sulfasalazine at a dose of 25 mg/kg, which was started two days before the induction of colitis.

were compared. Conversely, the application of SAS demonstrated the lowest therapeutic effect. Unfortunately, none of the test compounds (**1**, **2**, SAS) were able to fully protect the colon from histological damage and marked shortening. However, diplacone (**1**) has shown some cytoprotective effect in a study on alloxan-induced diabetes in mice [8].

The ability of **1** and **2** to ameliorate colitis can be associated with their antioxidant activity. Reactive oxygen species (ROS) are responsible for tissue damage in DSS-induced colitis as well as in IBD. These are produced largely by neutrophils and macrophages that infiltrate the mucosa [20]. Generally, many flavonoids are able to scavenge ROS and to interact with antioxidant enzymes [3]. SOD2 and CAT are enzymes involved in the antioxidant defensive mechanisms of cells. SOD catalyzes the dismutation of superoxide to hydrogen peroxide and molecular oxygen; CAT then converts the hydrogen peroxide to water and molecular oxygen. The levels and activities of these enzymes are related to the concentrations of their substrates [21]. Diplacone (**1**) and mimulone (**2**) have shown antiradical activity in previous studies [6,8]. The most potent activity is associated with the following structural conditions: 5,7-dihydroxy substitution on ring A, 4-oxo substitution on ring C, and 3′,4′-dihydroxy substitution on ring B (catechol moiety) of the flavonoid skeleton. Compound **1** acts as an efficient scavenger of superoxide and hydrogen peroxide [6]. The lower levels of SOD2 and CAT observed could be caused by a lower level of ROS, which are scavenged by **1**. Compound **2** showed several times lower antiradical activity, probably because of the presence of only one hydroxyl group (*para*-hydroxy) on ring B [8]. In harmony with the mentioned studies, diplacone (**1**) exhibited the greater lowering of the levels of SOD2 and CAT.
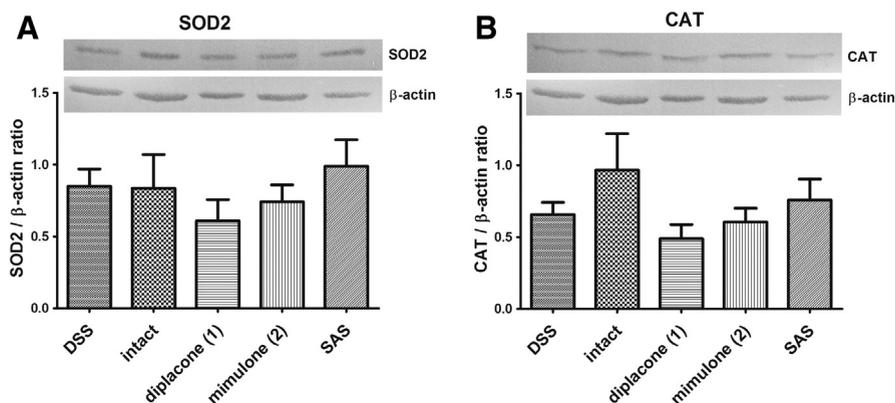
Fig. 4. Effect of the test compounds on the levels of SOD2 and CAT. Colitis was induced by administration of 10% DSS in the drinking water for five days. Administration of diplacone (**1**), mimulone (**2**), or sulfasalazine (SAS) at a dose of 25 mg/kg or the vehicle (the DSS group and the intact group) was started two days before the induction of colitis. Colitis was not induced in the intact group. (A) SOD2/β-actin ratio. (B) CAT/β-actin ratio. Values were obtained from Western blot analysis as described in Materials and methods. Representative blots are shown.

The inflammatory response is mediated by prostaglandins produced in large amounts by COX-2. Diplacone (**1**) has previously shown the ability to reduce the expression of COX-2 in the lipopolysaccharide (LPS)-stimulated murine macrophage cell line [6]. In our experiment, both diplacone (**1**) and mimulone (**2**) were able to reduce the increased levels of COX-2 in colonic tissue after the administration of DSS *in vivo*. The mechanism by which compounds **1** and **2** affect the levels of COX-2 is the subject of further testing.

Matrix metalloproteinases, particularly MMP2 and MMP9 (gelatinases 2 and 9), play an important role in the pathogenesis of IBD. These proteins are extracellular-matrix-degrading endopeptidases that affect the remodeling and destruction of tissue, the migration of immune cells, and the ulceration of the mucosa [22]. Increased levels and activities of both of these enzymes were found in the inflamed tissues of patients with CD and UC [23], as well as in an animal model of colitis [24]. Heimasaat *et al.* have demonstrated that the preventive use of a gelatinases blocker ameliorated acute DSS-induced colitis [25]. In addition, MMP2-deficient mice were protected from developing DSS-induced colitis [26]. Our results confirm the greater activity of MMP2 (smaller pro-MMP2/MMP2 ratio) in the untreated DSS group compared to the intact group. Prophylactic and curative administration of the test compounds (**1**, **2**, and SAS) showed an increase in the ratio of the pro-form to the
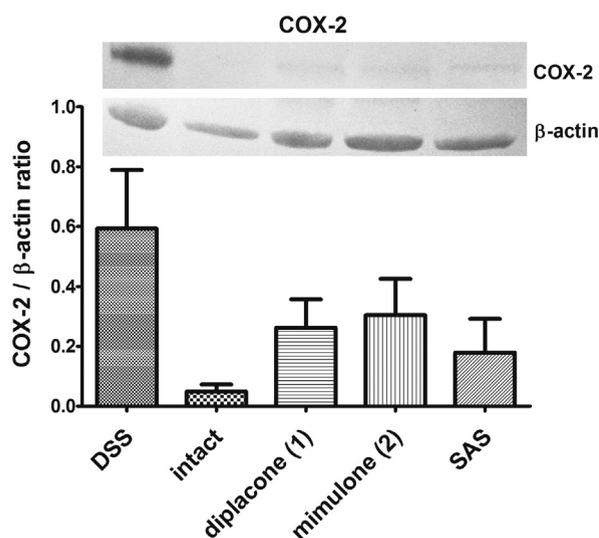
Fig. 5. Effect of the test compounds on the levels of COX-2. Colitis was induced by administration of 10% DSS in the drinking water for five days. Administration of diplacone (**1**), mimulone (**2**), or sulfasalazine (SAS) at a dose of 25 mg/kg or the vehicle (the DSS group and the intact group) was started two days before the induction of colitis. Colitis was not induced in the intact group. The graph indicates the COX-2/β-actin ratio. Values were obtained from Western blot analysis as described in Materials and methods. A representative blot is shown.
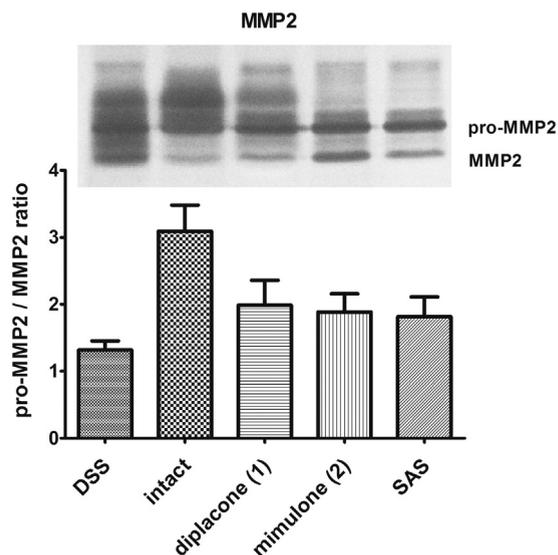
Fig. 6. Effect of the test compounds on the activity of MMP2. Colitis was induced by administration of 10% DSS in the drinking water for five days. Administration of diplacone (**1**), mimulone (**2**), or sulfasalazine (SAS) at a dose of 25 mg/kg or the vehicle (the DSS group and the intact group) was started two days before the induction of colitis. Colitis was not induced in the intact group. The graph indicates the pro-MMP2/MMP2 ratio. The activity of (pro-)MMP2 was detected by zymography as described in Materials and methods. A representative zymogram is shown.

active form of MMP2. ROS are also responsible for converting MMPs to the active form [27]. The antiradical activity of the test compounds may be one of the possible mechanisms that reduce the activation of MMP2.

The large number of natural compounds was evaluated on the DSS model of colitis [28], of the flavonoids namely hesperidin [29], chrysin [19], luteolin [30], icariin [16] and EGCG [31]. All of mentioned compounds apart from luteolin were also able to lower the activity of disease as compounds **1** and **2**. In addition, they protected colon against histological damage.

In conclusion, diplacone (**1**) and mimulone (**2**) exhibited greater effects than sulfasalazine following prophylactic/therapeutic administration. That the lowest disease activity index was achieved by **1** is probably associated with the greater antioxidant activity given by the presence of the catechol moiety. This statement is supported by previous *in vitro* and *in vivo* studies [6,8]. The ability of compounds **1** and **2** to increase the ratio of pro-MMP2/MMP2 activity and reduce the levels of COX-2 correlates with the values of DAI.

Conflict of interest

The authors have no conflicts to disclose.

Acknowledgment

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2015.01.012.

References

[1] Biasi F, Astegiano M, Maina M, Leonarduzzi G, Poli G. Polyphenol supplementation as a complementary medicinal approach to treating inflammatory bowel disease. Curr Med Chem 2011;18:4851–65.
[2] Kim H, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci 2004;96:229–45.
[3] Leonarduzzi G, Sottero B, Poli G. Targeting tissue oxidative damage by means of cell signaling modulators: the antioxidant concept revisited. Pharmacol Ther 2010;128:336–74.
[4] Costa G, Francisco V, Lopes MC, Cruz MT, Batista MT. Intracellular signaling pathways modulated by phenolic compounds: application for new anti-inflammatory drugs discovery. Curr Med Chem 2012;19:2876–900.
[5] Hošek J, Závalová V, Šmejkal K, Bartoš M. Effect of diplacone on LPS-induced inflammatory gene expression in macrophages. Folia Biol 2010;56:124–30.
[6] Hošek J, Toniolo A, Neuwirth O, Bolego C. Prenylated and geranylated flavonoids increase production of reactive oxygen species in mouse macrophages but inhibit the inflammatory response. J Nat Prod 2013;76:1586–91.
[7] Šmejkal K, Grycová L, Marek R, Lemiere F, Jankovská D, Forejtníková H, et al. C-Geranyl compounds from *Paulownia tomentosa* fruits. J Nat Prod 2007;70:1244–8.
[8] Zima A, Hošek J, Treml J, Muselík J, Suchý P, Pražanová G, et al. Antiradical and cytoprotective activities of several C-geranyl-substituted flavanones from *Paulownia tomentosa* fruit. Molecules 2010;15:6035–49.
[9] Navrátilová A, Schneiderová K, Veselá D, Hanáková Z, Fontana A, Dall'Acqua S, et al. Minor C-geranylated flavanones from *Paulownia tomentosa* fruits with MRSA antibacterial activity. Phytochemistry 2013;89:104–13.
[10] Tsune I, Ikejima K, Hirose M, Yoshikawa M, Enomoto N, Takei Y, et al. Dietary glycine prevents chemical-induced experimental colitis in the rat. Gastroenterology 2003;125:778–85.
[11] Araki Y, Andoh A, Fujiyama Y, Bamba T. Development of dextran sulphate sodium-induced experimental colitis is suppressed in genetically mast cell-deficient Ws/Ws rats. Clin Exp Immunol 2000;119:264–9.
[12] Štarha P, Hošek J, Vančo J, Dvořák Z, Suchý Jr P, Popa I, et al. Pharmacological and molecular effects of platinum(II) complexes involving 7-azaindole derivatives. PLoS One 2014;9:e90341.
[13] Šmejkal K, Svačinová J, Šlapetová T, Schneiderová K, Dall'acqua S, Innocenti G, et al. Cytotoxic activities of several geranyl-substituted flavanones. J Nat Prod 2010;73:568–72.
[14] Šmejkal K. Cytotoxic potential of C-prenylated flavonoids. Phytochem Rev 2014;13:245–75.
[15] Botta B, Vitali A, Menendez P, Misiti D, Delle Monache G. Prenylated flavonoids: pharmacology and biotechnology. Curr Med Chem 2005;12:717–39.
[16] Tao F, Qian C, Guo W, Luo Q, Xu Q, Sun Y. Inhibition of Th1/Th17 responses via suppression of STAT1 and STAT3 activation contributes to the amelioration of murine experimental colitis by a natural flavonoid glucoside icariin. Biochem Pharmacol 2013;85:798–807.
[17] Dou W, Zhang J, Sun A, Zhang E, Ding L, Mukherjee S, et al. Protective effect of naringenin against experimental colitis via suppression of Toll-like receptor 4/NF-κB signalling. Br J Nutr 2013;110:599–608.
[18] Al-Rejaie S, Abouhashish HM, Al-Enazi MM, Al-Assaf AH, Parmar MY, Ahmed MM. Protective effect of naringenin on acetic acid-induced ulcerative colitis in rats. World J Gastroenterol 2013;19:5633–44.
[19] Shin K, Kwon HS, Kim YH, Shin HK, Kim JK. Chrysin, a natural flavone, improves murine inflammatory bowel diseases. Biochem Biophys Res Commun 2009;381:502–7.
[20] Kitajima S, Takuma S, Morimoto M. Changes in colonic mucosal permeability in mouse colitis induced with dextran sulfate sodium. Exp Anim 1999;48:137–43.
[21] Crespo I, García-Mediavilla MV, Almar M, González P, Tuñón MJ, Sánchez-Campos S, et al. Differential effects of dietary flavonoids on reactive oxygen and nitrogen species generation and changes in antioxidant enzyme expression induced by proinflammatory cytokines in Chang Liver cells. Food Chem Toxicol 2008;46:1555–69.
[22] Medina C, Radomksi MW. Role of matrix metalloproteinases in intestinal inflammation. J Pharmacol Exp Ther 2006;318:933–8.
[23] Baugh M, Perry MJ, Hollander AP, Davies DR, Cross SS, Lobo AJ, et al. Matrix metalloproteinase levels are elevated in inflammatory bowel disease. Gastroenterology 1999;117:814–22.
[24] Oliveira L, Cunha AL, Duarte AC, Castanon MC, Chebli JM, Aguiar JA. Positive correlation between disease activity index and matrix metalloproteinases activity in a rat model of colitis. Arq Gastroenterol 2014;51:107–12.
[25] Heimesaat M, Dunay IR, Fuchs D, Trautmann D, Fischer A, Kühl AA, et al. Selective gelatinase blockage ameliorates acute DSS colitis. Eur J Microbiol Immunol 2011;1:228–36.
[26] Heimesaat M, Dunay IR, Fuchs D, Trautmann D, Fischer A, Kühl AA, et al. The distinct roles of MMP-2 and MMP-9 in acute DSS colitis. Eur J Microbiol Immunol 2011;1:302–10.
[27] Belkhiri A, Richards C, Whaley M, McQueen SA, Orr FW. Increased expression of activated matrix metalloproteinase-2 by human endothelial cells after sublethal H_2O_2 exposure. Lab Invest 1997;77:533–9.
[28] Vochyánová Z, Sikorová K, Šmejkal K, Hošek J. Plants in therapy of inflammatory bowel disease. Gastroenterol Hepatol 2014;68:248–54.
[29] Xu L, Yang ZL, Li P, Zhou YQ. Modulating effect of Hesperidin on experimental murine colitis induced by dextran sulfate sodium. Phytomedicine 2009;16:989–95.
[30] Karrasch T, Kim JS, Jang BI, Jobin C. The flavonoid luteolin worsens chemical-induced colitis in NF-kappaB(EGFP) transgenic mice through blockade of NF-kappaB-dependent protective molecules. PLoS One 2007;2:e596.
[31] Brückner M, Westphal S, Domschke W, Kucharzik T, Lügering A. Green tea polyphenol epigallocatechin-3-gallate shows therapeutic antioxidative effects in a murine model of colitis. J Crohns Colitis 2012;6:226–35.

Research Article

Flavonoid 4′-*O*-Methylkuwanon E from *Morus alba* Induces the Differentiation of THP-1 Human Leukemia Cells

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Aims. In this work we studied cytodifferentiation effects of newly characterized prenyl flavonoid 4′-*O*-methylkuwanon E (4ME) isolated from white mulberry (*Morus alba* L.). *Main Methods*. Cell growth and viability were measured by dye exclusion assay; cell cycle and surface antigen CD11b were monitored by flow cytometry. For the cytodifferentiation of cells the NBT reduction assay was employed. Regulatory proteins were assessed by western blotting. *Key Findings*. 4ME induced dose-dependent growth inhibition of THP-1 cells, which was not accompanied by toxic effect. Inhibition of cells proliferation caused by 4ME was associated with the accumulation in G1 phase and with downregulation of hyperphosphorylated pRb. Treatment with 4ME led to significant induction of NBT-reducing activity of PMA stimulated THP-1 cells and upregulation expression of differentiation-associated surface antigen CD11b. Our results suggest that monocytic differentiation induced by 4ME is connected with up-regulation of p38 kinase activity. *Significance*. Our study provides the first evidence that 4ME induces the differentiation of THP-1 human monocytic leukemia cells and thus is a potential cytodifferentiating anticancer agent.

1. Introduction

Specific types of neoplastic diseases, such as acute promyelocytic leukemia (APL), can be treated with a cytodifferentiating approach [1]. The therapy based on an induction of cytodifferentiating programme of malignant cells has shown good efficacy and low level of toxicity, when compared to a cytotoxic agent based treatment. Retinoids, the natural and synthetic derivatives of vitamin A, are known to play a crucial role in cellular and tissue differentiation [2]. All-*trans*-retinoic acid (ATRA) is used in the treatment/chemoprevention of hematologic and other malignancies [3]. However, the clinical use of ATRA is often limited by

resistance and toxicity (particularly retinoic acid syndrome) [4]. In general terms, there are two possible strategies for differentiation therapies: (a) the development of ATRA-based pharmacologic combinations that are more powerful and easily tolerated than the individual components [5] or (b) the identification of a novel agent capable of inducing the cytodifferentiation programme in cancer cells [2].

Previously we studied toxicity and biological effects of prenylated and geranylated flavonoids from plants of Moraceae and Paulowniaceae families with cytostatic activity in normal and cancer cell lines [6]. We showed that molecular mechanisms of the antiproliferative effects of geranylated flavanone tomentodiplacone B on human monocytic
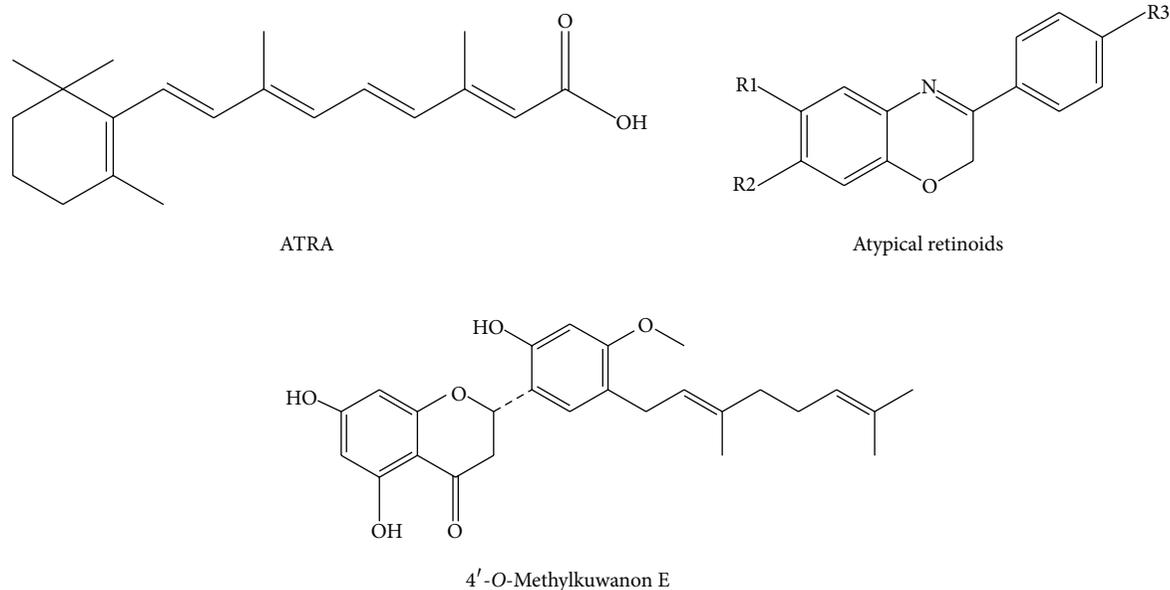
FIGURE 1: Chemical structures of all-*trans*-retinoic acid (ATRA), atypical retinoids, and 4′-*O*-methylkuwanon E (4ME).

leukemia cells are mediated through the direct inhibition of CDK2 activity followed by reduced pRb phosphorylation [7]. Flavonoid, cudraflavone B, isolated from *Morus alba* L. (Moraceae) (MA) exerted strong anti-inflammatory properties [8] together with the inhibition of G1/S transition, accompanied by the decreased proliferation and by apoptosis of four cancer cell lines [9]. However, in one of MA isolated flavonoids, 4′-*O*-methylkuwanon E (4ME, the novel compound detected and described in our laboratory), which was tested for its antiproliferative potential, its inhibitory activity on cell cycle could not be explained by significant proapoptotic effect [9]. Thus, we focused our attention on other mechanisms possibly underlying antiproliferative effect of 4ME (Figure 1) on THP-1 leukemia cells. Three different regions can be highlighted in the structure of ATRA: the hydrophobic trimethylhexene ring, the unsaturated linking chain, and the relatively hydrophilic moiety of the carboxylic acid. The basic structure of the atypical retinoids generated through modifications of ATRA using structure-based chemical design strategies is also shown [10]. From structure comparison clear similarities with the chemical formula of flavonoid 4ME can be found. Geranylated (prenylated) flavonoids are compounds usually showing two different structural regions: a lipophilic side chain and relatively hydrophilic ring B, connected to side chain *via* heterocyclic moiety. This general arrangement shows similarities with retinoids and gives to this type of flavonoids interesting properties affecting biological activity [11].

As a molecular structure of 4ME has a certain similarity with atypical retinoids [10] we hypothesized the ability of 4ME to induce differentiation programme in leukemia cells. Therefore, the present study has attempted to investigate the cytodifferentiation effect of prenylated flavonoid 4ME isolated from *M. alba* L. on THP-1 cells.

2. Materials and Methods

2.1. Test Compounds and Reagents. 4ME was isolated and supplied by the Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. The identification of substance was carried out using HRMS, ^{1}H and ^{13}C NMR analysis; and the purity exceeded 95% according to the HPLC analysis [6]. The compound is poorly soluble in water; therefore, a fresh 10 mM stock solution in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was prepared every time 1 day prior to experiments and stored at −20°C. This solution was further diluted in the culture media to the desired final concentrations. RPMI 1640 culture media, phosphate buffered saline (PBS), and antibiotics (penicillin and streptomycin) were purchased from Lonza (Verviers, Belgium). Foetal bovine serum (FBS) was purchased from PAA Laboratories (Pasching, Austria). Rabbit polyclonal antibodies against p38 MAPK [pT180/Y182] (9215S) and p38 MAPK (9212) were purchased from Cell Signaling Technologies (Beverly, MA, USA). Mouse monoclonal antibodies against pRb (554136) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit polyclonal antibody against phospho-Rb [Ser 780] (9307) was purchased from Cell Signaling Technologies (Beverly, MA, USA). PE-conjugated CD11b antibody was obtained from Beckman Coulter (Brea, CA, USA). ATRA and all other reagents were from Sigma-Aldrich.

2.2. Cell Culture. The human monocytic leukemia THP-1 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK; methods of characterization: DNA fingerprinting (multilocus probes) and isoenzyme analysis). Cells were cultured in RPMI 1640 medium supplemented with antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin), 10% FBS, and 2 mM L-glutamine. Cultures were

kept in an incubator at 37°C in a water-saturated 5% CO_2 atmosphere in air. Cells were passaged at approximately 1-week intervals. Cells were free from mycoplasma infection (Hoechst 33258 staining method).

2.3. In Vitro Analysis of Cell Growth and Viability. THP-1 cells were seeded (2×10^5 cells/mL) and incubated for 96 h at 37°C with 5% CO_2 with 4ME dissolved in DMSO (Sigma-Aldrich) in concentrations ranging from 5 to 20 μM in RPMI 1640 medium. The maximum concentration of DMSO in the assays never exceeded 0.1%. At the indicated time points (24 h, 72 h, and 96 h) cell population in each well was removed by gentle scraping with a cell scraper and harvested for further analysis. Numbers and viabilities of the cells were determined by counting with a hemocytometer as we previously described [7]. All data were evaluated using GraphPad Prism 5.00 software (GraphPad Software, San Diego, CA, USA, http://www.graphpad.com/).

2.4. Cell Cycle Analysis. THP-1 cells were incubated with increasing concentrations of 4ME for 24 h, washed in PBS (pH 7.4), and fixed for 30 min in an ice-cold 70% ethanol. Fixed cells were washed three times in PBS (pH 7.4) and incubated with RNaseA (0.02 mg/mL) (Boehringer, Ingelheim, Germany) for 30 min at 37°C. Nuclei were stained with propidium iodide (40 μg/mL) and analysed by flow cytometry using Cell Lab Quanta SC (Beckman Coulter, Brea, CA, USA). Cell cycle distribution was analysed using FlowJo software (http://www.flowjo.com/).

2.5. In Vitro Analysis of Cytodifferentiation. Functional assays of differentiation were based on the ability of phorbol myristate acetate-stimulated human monocytic leukemia cells to reduce nitroblue tetrazolium (NBT). Samples of 5×10^5 cells were incubated in 0.5 mL of RPMI 1640 with penicillin, streptomycin, glutamine, and 10% heat-inactivated FBS containing 0.25 mg/mL of NBT (Roche Applied Science, Mannheim, Germany) and 500 ng/mL phorbol-12-myristate-13-acetate (PMA). Cells were incubated at 37°C for 25 min after which the samples were centrifuged (3 000 g for 5 minutes) and 1% Triton X-100 (Sigma-Aldrich) was added in the amount of 1 mL/5×10^5 cells. Samples were sonicated (Sonicator S-3000, Misonix Inc., Farmingdale, USA) and intensity of dark-blue formazan was assessed by spectrophotometry analysis at OD 540 nm. For determination of the number of adherent cells THP-1 cells (2×10^5 cells/mL) were placed into 6-well tissue plate and cultured for 72 and 96 h. Nonadherent cells were removed by washing twice with PBS and then adherent cells were collected by gentle scraping with a cell scraper and by vigorous pipetting. The number of cells was counted by a hemocytometer. Morphological changes of THP-1 cells were detected using an inverted microscope (Axiovert 40 CFL, Zeiss, Germany).

2.6. Western Blotting. Cells were washed three times with PBS (pH 7.4) and lysed in 100 mM Tris-HCl (pH 6.8) containing 20% glycerol and 1% SDS. Protein concentrations were determined using the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Lysates were supplemented with bromophenol blue (0.01%) and β-mercaptoethanol (1%). Equal amounts of total protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE), electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA), immunodetected using the appropriate primary and secondary antibodies, and visualised with ECL Plus reagent (Amersham, Aylesbury, UK) according to the manufacturer's instructions.

2.7. Analysis of Surface Differentiation Marker CD11b by Flow Cytometry. THP-1 cells were plated at 3×10^5 cells/mL, 2 mL/well (6×10^5 cells/2 mL) in 6-well plate and incubated with ATRA (1 μM), 4ME (5, 10, and 20 μM, resp.), or RPMI 1640 only for 72 or 96 h in duplicate for each sample. A number of 5×10^5 cells from each well were collected at the indicated time points (72 h, 96 h), centrifuged (1.300 rpm for 3 min), washed twice with PBS, and incubated with PE-conjugated CD11b antibody (20 μL/5×10^5 cells, Beckman Coulter) for 30 min at room temperature in the dark. After incubation the cells were washed with PBS, suspended in PBS, and analysed by flow cytometry using Cell Lab Quanta SC flow cytometer and Kaluza Analysis Software (Beckman Coulter).

2.8. Statistical Analysis. Statistical significance was tested using the one-way ANOVA with Dunnett's and Tukey post test for comparisons between the mean values, and differences between two conditions were retained for $P < 0.05$. Statistical significance was determined at levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3. Results

3.1. Growth Inhibitory Effects and Morphological Changes Induced by 4ME to THP-1 Cells. To investigate how 4ME influences cell proliferation we examined its growth-inhibiting effects on THP-1 cells at three time points (after 24, 72, and 96 h, resp.). Simultaneously, we evaluated viability of cells upon 4ME treatment. Antiproliferative effect of 4ME on THP-1 cells was found to be time- and concentration-dependent, the most significantly observed ($P < 0.001$) at concentrations of 10 and 20 μM (Figure 2(a)). After 72 h, 4ME treatment of THP-1 cells leads to significant growth inhibition of THP-1 cells when concentrations of 10 μM ($P < 0.05$) and 20 μM ($P < 0.001$) were used. The lowest concentration of 4ME (5 μM) used was effective ($P < 0.05$) after 96 h of the treatment. No toxic effect of 4ME has been detected throughout the evaluation time as viability was not significantly changed, except the highest 4ME concentration at 96 h ($P < 0.05$) with 85% viability, which cannot be considered as toxic (Figure 2(b)). 4ME also induced the morphologic changes of THP-1 cells into macrophage-like cells with different cell shape and ability to attach the surface of plastic culture dishes (Figure 2(c)). The number of adherent cells after 72 and 96 h of cultivation increased in the presence of 4ME in a dose-dependent manner (Figure 2(d)). This

(a)

(b)

(1)

(2)

(c)

4ME concentration (μM)	Number of adherent cells ($\times 10^4$/mL)	
	72 h	96 h
0	0.1 ± 0.16	0.3 ± 0.08
5	0.2 ± 0.02	0.4 ± 0.21
10	1.7 ± 0.90	2.6 ± 0.91
20	10.0 ± 1.56	13.0 ± 2.33

(d)
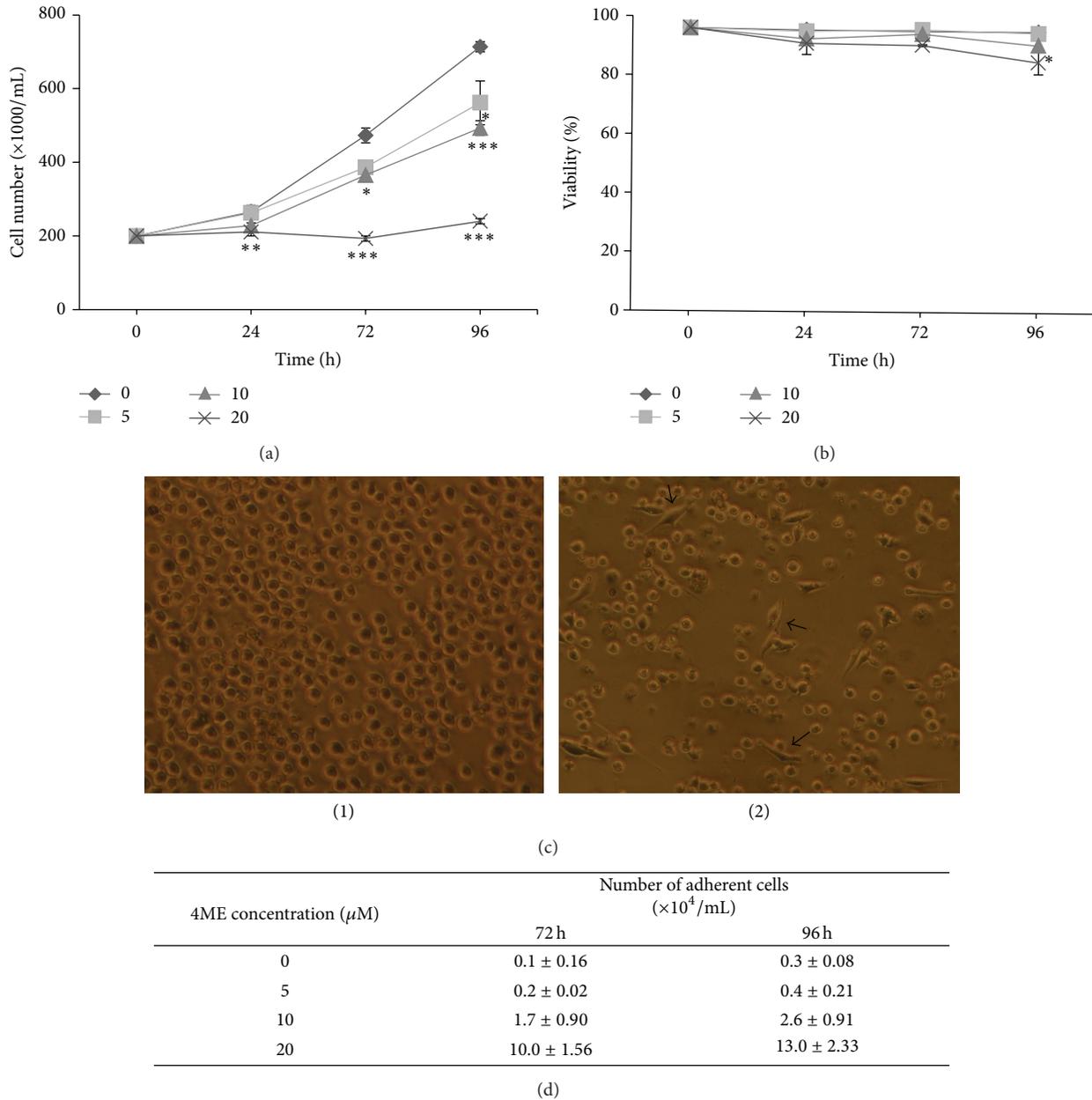
FIGURE 2: Growth inhibition and influence on viability and morphological changes of THP-1 cells by 4ME. Cells were seeded (2×10^5 cells/mL) and cultured with 0 (◆), 5 (■), 10 (▲), and 20 (×) μM 4ME for 96 h. Aliquots of cells were used for the determination of total number of cells (a) and cell viability (b). The results shown are expressed as the mean ± SD of two independent experiments, with each condition tested in triplicate. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from control. (c) Morphological changes of THP-1 monocytic leukemia cells after 72 h treatment with 4ME. (1) Vehicle treated THP-1 cells and (2) 4ME (20 μM) treated THP-1 cells. In comparison with monocytes, differentiated macrophages tend to adhere to the bottoms of the cultivation plates, as indicated with arrows (50x magnification). (d) THP-1 cells (2×10^5 cells/mL) were cultured for 72 and 96 h. The number of adherent cells was determined as described in Materials and Methods. Each value represents the mean ± SD.

effect was significant from 10 μM of the drug at both time points.

3.2. Effects of 4ME on Distribution of Cells in Cell Cycle Phases.
Antiproliferative effect of anticancer drugs is usually accompanied by significant changes of the cell cycle. To determine which changes in the cell cycle occur after 4ME treatment

of THP-1 cells we performed cell cycle analysis based on DNA content using flow cytometry. As shown in Figure 3 4ME caused the accumulation of human leukemia cells in G1/G0 phase dose-dependently after 24 h treatment. While the percentage of S phase cells decreased, the percentage of cells in G2/M phase remained unchanged upon 4ME treatment.
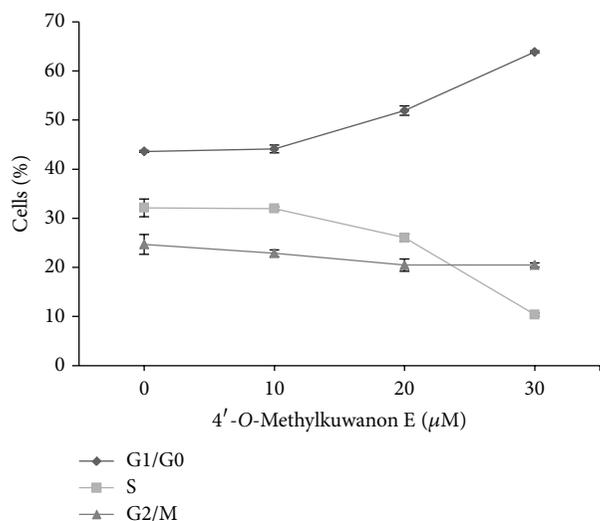
FIGURE 3: Treatment with 4ME causes accumulation of human monocytic leukemia THP-1 cells in G1/G0 phase. Cell cycle distribution at 24 h upon treatment of THP-1 cells with 4ME as determined by flow cytometry. Values shown are the mean ± SEM of the percentages of cells in individual phases of the cell cycle from two independent experiments.
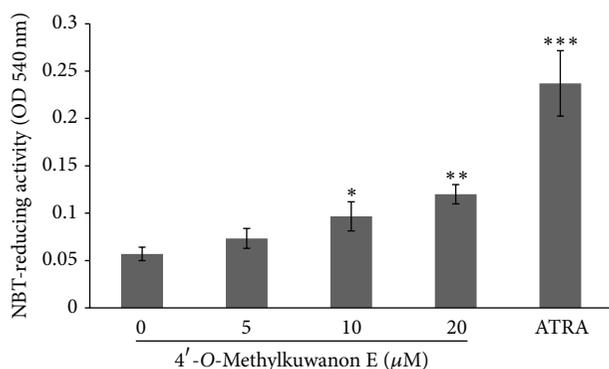
FIGURE 4: 4ME causes increased NBT reduction in PMA stimulated THP-1 cells. Cells were cultured with indicated concentrations of 4ME or 1 μM ATRA, added as a positive control, for 72 h. Aliquots of cells were used for the determination of NBT-reducing activity. The results shown are expressed as the mean ± SD of three independent experiments, with each condition tested in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, significantly different from control.

3.3. Effect of 4ME on NBT-Reducing Activity. Reduction of NBT is considered to be a typical marker of myelomonocytic differentiation in leukemia cells [12]. To investigate whether 4ME triggers the cytodifferentiating programme in monocytic leukemia cells, we have employed NBT reduction assay. Results showed that 4ME significantly induced NBT reduction in monocytes after 72 h in a dose-dependent manner (Figure 4). In concentration of 20 μM 4ME caused more than twofold higher ($P < 0.01$) induction of NBT reduction in comparison with the vehicle-treated cells. The NBT-reducing activity of THP-1 cells was also induced by 1 μM ATRA, added as a positive control.

3.4. Expression of Differentiation-Associated Cell Cycle Regulators in 4ME-Treated Cells. Based on the findings that 4ME causes growth inhibition, accumulation of cells in G1/G0 phase, and induction of NBT reduction in THP-1 cells we determined the expression and phosphorylation status of key cell cycle proteins involved in monocytes differentiation. The retinoblastoma protein (pRb) is currently known to have a prominent role in control of cellular proliferation and differentiation. Rb dephosphorylation leading to inhibition of G1/S transition is a prerequisite for initiating the process of cytodifferentiation. The proportion of hyperphosphorylated (phosphoserine 780) pRb was markedly reduced dose-dependently in THP-1 cells exposed for 72 h to 4ME treatment (Figure 5(a)). Since it has been known that the p38 signalling pathway plays a significant role in the differentiation process [13], THP-1 cells were cultivated with various concentrations of 4ME for 72 h and subjected to immunoblot analysis with anti-phospho-p38. As shown in Figure 5(b), the phosphorylation of p38 was upregulated by 4ME treatment at all concentrations used, suggesting that this kinase could be involved in 4ME-induced cytodifferentiation.

3.5. Effect of 4ME on Expression of Differentiation-Associated Surface Antigen CD11b. Monocytic differentiation is associated with increased expression of CD11b/CD18 [14]. Flow cytometric determination of THP-1 cells surface levels of the CD11b has been used as an index of 4ME-induced activation of these cells to macrophages. Monocytes were treated for 96 h with increasing 4ME concentrations or with 1 μM ATRA. The expression of CD11b, a cell surface marker of macrophage-like differentiation, was increased time- and dose-dependently by 4ME after both 72 and 96 h (Figure 6). The strongest effect was observed in ATRA-treated cells; still, the amount of cell-associated fluorescence (MFI) after 4ME treatment was more than fivefold higher when compared to control.

4. Discussion

Differentiation therapy is conceptually an elegant approach to the eradication of neoplastic cells from the human body because cytotoxicity is avoided, whereas normal mature cells are unaffected by the differentiation agents [15]. The most extensively studied differentiation agents in cancer medicine include ATRA, 9-*cis*-retinoic acid, and 13-*cis*-retinoic acid. ATRA is one of the most biologically active retinoids, and several clinical studies have established that ATRA can induce differentiation of leukemia cells and remission [16]. However, the occurring resistance and toxicity of ATRA therapy underline the importance of searching for new compounds capable to switch the differentiation programme in leukemia cells.

In this work we studied cytodifferentiation effects of newly characterized prenyl flavonoid 4ME isolated from white mulberry (*Morus alba* L.). Our previous results [9], some structural similarities with atypical retinoids, and unpublished observations turned our attention to mechanisms different from direct toxic effect, stress-related or apoptotic signaling pathways, which might be involved in

(a)

(b)

(c)
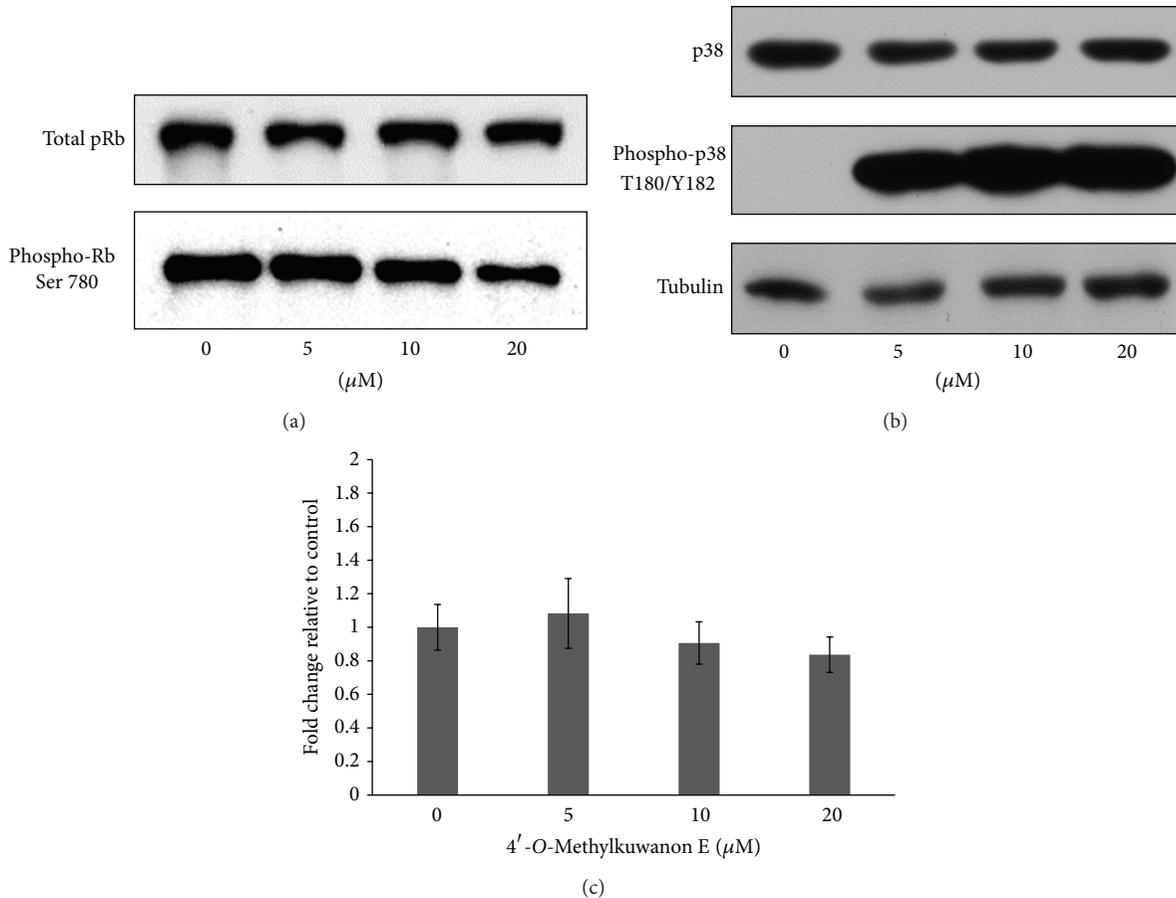
FIGURE 5: Expression of differentiation-associated cell cycle regulators after 72 h of 4ME treatment. (a) pRb phosphorylation on Ser 780 was reduced after 72 h of 4ME treatment, as determined by western blot analysis. (b) Following 72 h of challenge, 4ME increases p38 phosphorylation in all concentrations used. Data representative of two other experiments are shown. Panel (c) shows quantification of p38 expression at protein level in THP-1 cells after 72 h of 4ME treatment as determined by western blot analysis. Values shown are mean ± SD of two independent experiments performed in duplicate.

the action of this compound. We found that 4ME was able to induce dose-dependent growth inhibition of THP-1 cells, which was not accompanied by toxic effect (Figures 2(a) and 2(b)). Moreover, with 4ME added, the THP-1 cells morphologically resembled macrophages (Figure 2(c)) and increased the number of adherent cells (Figure 2(d)). These findings together with accumulation in G1 phase found by flow cytometry (Figure 3) prompt us to gain more detailed insight into the mechanism of 4ME action. The crucial process in terminal differentiation is to delay proliferation in G1/G0 phase of the cell cycle and thus establish somatic cell cycle programme. pRb is the key player involved in G1 phase regulation and its phosphorylation, which is required for G1/S transition was significantly inhibited by 4ME treatment (Figure 5(a)). We assume that this allowed THP-1 monocytes to escape from the cell cycle machinery and subsequently to initiate cytodifferentiation. Indeed, three-day treatment with 4ME led to significant induction of NBT-reducing activity following priming of the cells with PMA, accompanied by upregulated expression of differentiation-associated surface

antigen CD11b (Figures 4 and 6). Although these effects are lower than effects of ATRA still cytodifferentiation induced by 4ME could be considered as clearly evident. Since p38 pathway has been indicated to be involved in the differentiation of several human cells [13], we have focused on p38. Its activation can lead to biological outcomes such as proliferation, cell survival, and differentiation, depending on the context and the cell type [17]. As it is shown in Figure 5(c) after 72 h treatment with 4ME the expression of p38 protein was not enhanced, while its phosphorylated form was significantly increased (Figure 5(b)). These results suggest that monocytic differentiation induced by 4ME is associated with upregulation of p38 kinase activity. Still, the connection between differentiation and this signaling route remains to be elucidated.

In conclusion, this is the first study to demonstrate cytodifferentiating activity of prenylated flavanone 4ME in human monocytic leukemia cells. This promising effect was induced at nontoxic concentrations. However, our findings using THP-1 cell line do not completely reflect the situation in
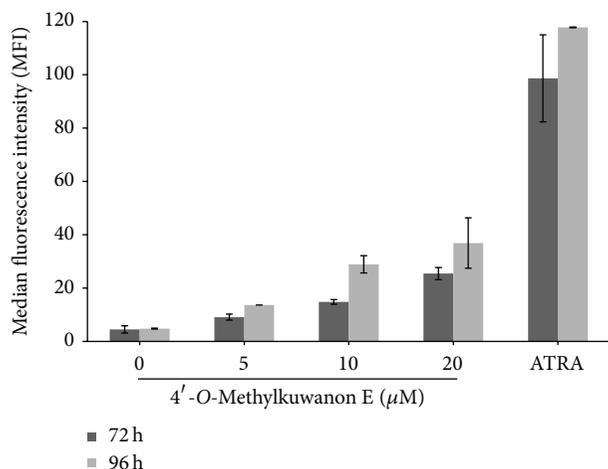
FIGURE 6: Increased expression of differentiation-associated surface antigen CD11b in 4ME-treated cells. THP-1 cells were cultured with indicated concentrations of 4ME or 1 μM ATRA, added as a positive control for 72 and 96 h. The level of expression of the indicated phenotypic marker was determined by flow cytometry using fluorescein-labelled monoclonal antibody against CD11b. The amount of cell-associated fluorescence in median fluorescence intensity (MFI) is illustrated in the graph. The results shown are expressed as the mean ± SD of three independent experiments, with each condition tested in duplicate.

patients with acute leukemia. Further experiments using mice models inoculated with L1210 mice leukemia cells or freshly isolated primary human leukemic cells are necessary to verify cytodifferentiation effect of 4ME.

5. Conclusion

The present study has shown that 4ME induced dose-dependent growth inhibition of THP-1 cells, which was not accompanied by toxic effect. Inhibition of cells proliferation caused by 4ME was associated with the accumulation in G1 phase and with downregulation of hyperphosphorylated pRb. Treatment with 4ME led to significant induction of NBT-reducing activity of PMA stimulated THP-1 cells and upregulated expression of differentiation-associated surface antigen CD11b. Our results suggest that monocytic differentiation induced by 4ME is connected with upregulation of p38 kinase activity.

Abbreviations

4ME: 4'-O-Methylkuwanon E
APL: Acute promyelocytic leukemia
ATRA: All-trans-retinoic acid
DMSO: Dimethylsulphoxide
FBS: Foetal bovine serum
NBT: Nitroblue tetrazolium
PBS: Phosphate buffered saline
PMA: Phorbol-12-myristate-13-acetate
pRb: Retinoblastoma protein
THP-1: Human monocyte leukemia cell line.

Conflict of Interests

All authors have no potential conflict of interests to disclose regarding this study.

References

[1] E. Garattini and M. Terao, "Cytodifferentiation: a novel approach to cancer treatment and prevention," *Current Opinion in Pharmacology*, vol. 1, no. 4, pp. 358–363, 2001.

[2] S. Zanardi, D. Serrano, A. Argusti, M. Barile, M. Puntoni, and A. Decensi, "Clinical trials with retinoids for breast cancer chemoprevention," *Endocrine-Related Cancer*, vol. 13, no. 1, pp. 51–68, 2006.

[3] E. Garattini, M. Gianni, and M. Terao, "Retinoids as differentiating agents in oncology: a network of interactions with intracellular pathways as the basis for rational therapeutic combinations," *Current Pharmaceutical Design*, vol. 13, no. 13, pp. 1375–1400, 2007.

[4] S. Waxman, "Differentiation therapy in acute myelogenous leukemia (non-APL)," *Leukemia*, vol. 14, no. 3, pp. 491–496, 2000.

[5] C. Pisano, P. Kollar, M. Gianní et al., "Bis-indols: a novel class of molecules enhancing the cytodifferentiating properties of retinoids in myeloid leukemia cells," *Blood*, vol. 100, no. 10, pp. 3719–3730, 2002.

[6] K. Šmejkal, J. Svačinová, T. Šlapetová et al., "Cytotoxic activities of several geranyl-substituted flavanones," *Journal of Natural Products*, vol. 73, no. 4, pp. 568–572, 2010.

[7] P. Kollár, T. Bárta, V. Závalová, K. Šmejkal, and A. Hampl, "Geranylated flavanone tomentodiplacone B inhibits proliferation of human monocytic leukaemia (THP-1) cells," *British Journal of Pharmacology*, vol. 162, no. 7, pp. 1534–1541, 2011.

[8] J. Hošek, M. Bartos, S. Chudík et al., "Natural compound cudraflavone B shows promising anti-inflammatory properties in vitro," *Journal of Natural Products*, vol. 74, no. 4, pp. 614–619, 2011.

[9] P. Kollar, T. Bárta, J. Hošek et al., "Prenylated flavonoids from *Morus alba* L. Cause Inhibition of G1/S transition in THP-1 human leukemia cells and prevent the lipopolysaccharide-induced inflammatory response," *Evidence-Based Complementary and Alternative Medicine*, vol. 2013, Article ID 350519, 13 pages, 2013.

[10] J. Anguiano, T. P. Garner, M. Mahalingam, B. C. Das, E. Gavathiotis, and A. M. Cuervo, "Chemical modulation of chaperone-mediated autophagy by retinoic acid derivatives," *Nature Chemical Biology*, vol. 9, no. 6, pp. 374–382, 2013.

[11] K. Šmejkal, "Cytotoxic potential of *C*-prenylated flavonoids," *Phytochemistry Reviews*, vol. 13, no. 1, pp. 245–275, 2014.

[12] Y. Ishii, S. Sakai, and Y. Honma, "Pyridinyl imidazole inhibitor SB203580 activates p44/42 mitogen-activated protein kinase and induces the differentiation of human myeloid leukemia cells," *Leukemia Research*, vol. 25, no. 9, pp. 813–820, 2001.

[13] T. K. P. Doan, K. S. Park, H. K. Kim, D. S. Park, J. H. Kim, and T. R. Yoon, "Inhibition of JNK and ERK pathways by SP600125-and U0126-enhanced osteogenic differentiation of bone marrow stromal cells," *Tissue Engineering and Regenerative Medicine*, vol. 9, no. 6, pp. 283–294, 2012.

[14] A. G. Rosmarin, S. C. Weil, G. L. Rosner, J. D. Griffin, M. A. Arnaout, and D. G. Tenen, "Differential expression of CD11b/CD18 (Mo1) and myeloperoxidase genes during myeloid differentiation," *Blood*, vol. 73, no. 1, pp. 131–136, 1989.

[15] Y. Ji and G. P. Studzinski, "Retinoblastoma protein and CCAAT/enhancer-binding protein beta are required for 1,25-dihydroxyvitamin D-3-induced monocytic differentiation of HL60 cells," *Cancer Research*, vol. 64, no. 1, pp. 370–377, 2004.

[16] L. Liu, J. B. Berletch, J. G. Green, M. S. Pate, L. G. Andrews, and T. O. Tollefsbol, "Telomerase inhibition by retinoids precedes cytodifferentiation of leukemia cells and may contribute to terminal differentiation," *Molecular Cancer Therapeutics*, vol. 3, no. 8, pp. 1003–1009, 2004.

[17] S. Uddin, J. Ah-Kang, J. Ulaszek, D. Mahmud, and A. Wickrema, "Differentiation stage-specific activation of p38 mitogen-activated protein kinase isoforms in primary human erythroid cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 1, pp. 147–152, 2004.
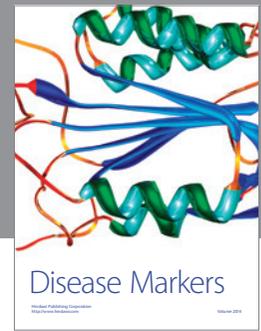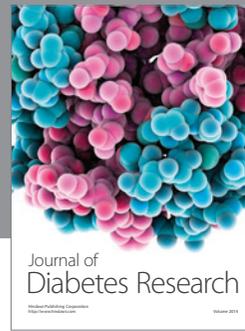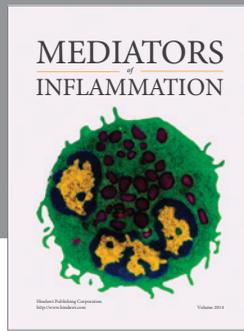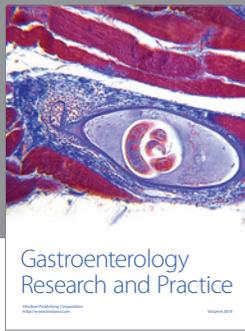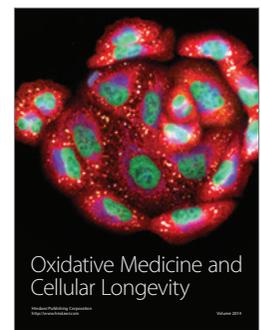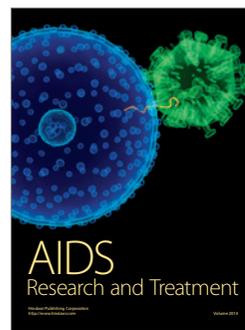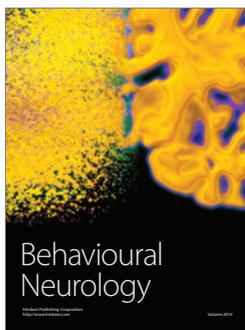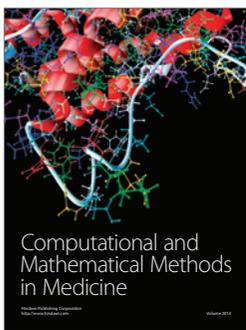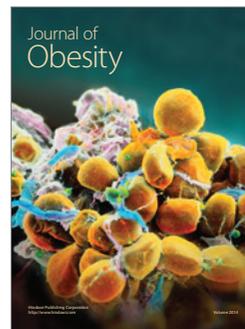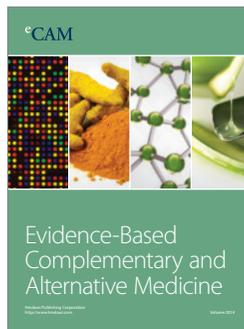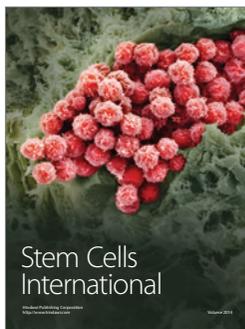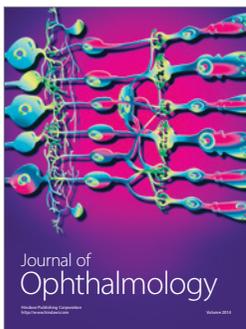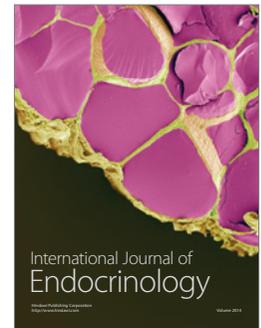
C-Geranylated Flavanones from *Paulownia tomentosa* Fruits as Potential Anti-inflammatory Compounds Acting via Inhibition of TNF-α Production

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S Supporting Information

ABSTRACT: Eleven new *C*-geranylated flavonoids, tomentodiplacones L, M, and N (**1**, **2**, **10**), tomentodiplacol B (**3**), 3′,4′-*O*-dimethyl-5′-hydroxydiplacone (**4**), mimulones F, G, and H (**5**, **6**, **7**), paulowniones A (**8**) and B (**9**), tomentone (**11**), and 3′,4′,5′-trimethoxyflavanone (**12**), together with 11 known flavonoids (**13**−**23**), were isolated from fruits of *Paulownia tomentosa*. The structures of the compounds isolated were determined by spectroscopic data interpretation. The ability of compounds **1**−**23**, together with the nonprenylated flavanones eriodictyol (**24**) and naringenin (**25**), to reduce the production of the pro-inflammatory cytokine TNF-α in THP-1 cells after bacterial lipopolysaccharide stimulation was evaluated using an in vitro screening test. The preliminary structure−activity relationships of these derivatives were also studied, and the correlation of their TNF-α inhibitory activity with their lipophilicity was investigated. The mechanism of action of compounds with significant antiphlogistic potential (**4**, **7**, **10**, **14**, **22**) was investigated. These compounds reduced both the secretion of TNF-α and the level of its corresponding mRNA. Compounds **4**, **7**, **10**, **14**, and **22** inhibited the nuclear translocation of NF-κB, which controls the expression of TNF-α, by blocking the degradation of IκB.
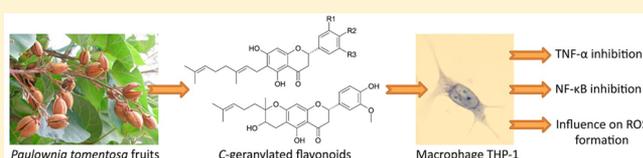
Paulownia tomentosa (Thunb.) Siebold et Zucc. ex Steud. (Paulowniaceae) is a medium-sized tree, native to mainland China and distributed all over the world. It has been used in Chinese folk medicine for the treatment of several diseases, such as upper respiratory tract infections and other bacterial infections.[1] This species is rich mainly in phenolics, such as flavonoids, phenylpropanoid glycosides, phenolic acids, and lignans.[2] Iridoids, sesquiterpene lactone, furanoquinone, and acylglycerols have also been isolated.[2] In particular, *C*-geranyl flavonoids obtained from *P. tomentosa* fruits have become the focus of phytochemical research because of their wide spectrum of biological activities. So far, 38 geranylated and two prenylated flavonoids have been isolated from *P. tomentosa*.[3−13] Twenty-six of these *C*-geranyl flavonoids possess a side-chain modified by oxidation, reduction, or cyclization. The significant bioactivities of these geranylated flavonoids have been investigated mainly using antibacterial, cytotoxic, antiphlogistic, or antioxidant assays.[2,4−10,13−15]

Inflammation is a complex response by the body to infection or injury. Flavonoids show pleiotropic effects and can modulate several key parts of the inflammatory process. They have antioxidant and/or pro-oxidant properties, interact directly with pro-inflammatory proteins, and affect signal pathways and inhibit the expression of inflammation-related genes.[16,17]

Besides inhibiting the activity of enzymes linked with inflammation (PLA₂, COX, LOX, iNOS, etc.) and their antioxidant and pro-oxidant actions, flavonoids also show an ability to interact with different intracellular signal-transducing pathways and thereby regulate the expression of inflammation-related genes. One of the most prominent among various known inflammatory pathways belongs to the transcription factor nuclear factor (NF)-κB. Published studies have validated the capability of flavonoids to reduce the activity of NF-κB by inhibiting events upstream, e.g., by reducing the phosphorylation of IKK (IκB kinase), leading to less degradation of IκB (an inhibitor of κB) or by attenuating the DNA-binding capability of NF-κB. Another important pro-inflammatory transcription factor is the activator protein (AP)-1, regulated by upstream mitogen-activated protein kinases (MAPKs). MAPKs not only influence AP-1 but also modulate the activity of NF-κB. Flavonoids are able to inhibit the phosphorylation of MAPKs and thereby lower the transcription of the target genes. Flavonoids are able to positively regulate the activity of several other signaling pathways. One of these is the signal pathway leading to the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2). Together with its negative regulator Keap-1 (Kelch-like ECH-associated protein 1), this protein is redox sensitive; therefore flavonoids possessing a redox potential are able to activate Nrf2, which leads to the transcription of genes coding antioxidant enzymes that help to resolve the inflammatory response. Transcription factors from the PPAR (peroxisome proliferator-activated receptor) family

Chart 1

	R1	R2	R3	R4
2	H	OH	OMe	
4	OH	OMe	OMe	
5	H	OH	H	
6	H	OH	H	
7	H	OH	H	
13	H	OMe	OMe	
14	H	OH	OMe	
15	H	OH	OMe	
16	H	OH	OMe	
17	H	OH	OMe	
18	H	OH	OMe	
19	H	OH	H	
21	OMe	OH	OMe	
22	H	OH	OH	

	R1	R2	R3	R4
3	OH	H	OH	OMe
11	H	OH	H	OH
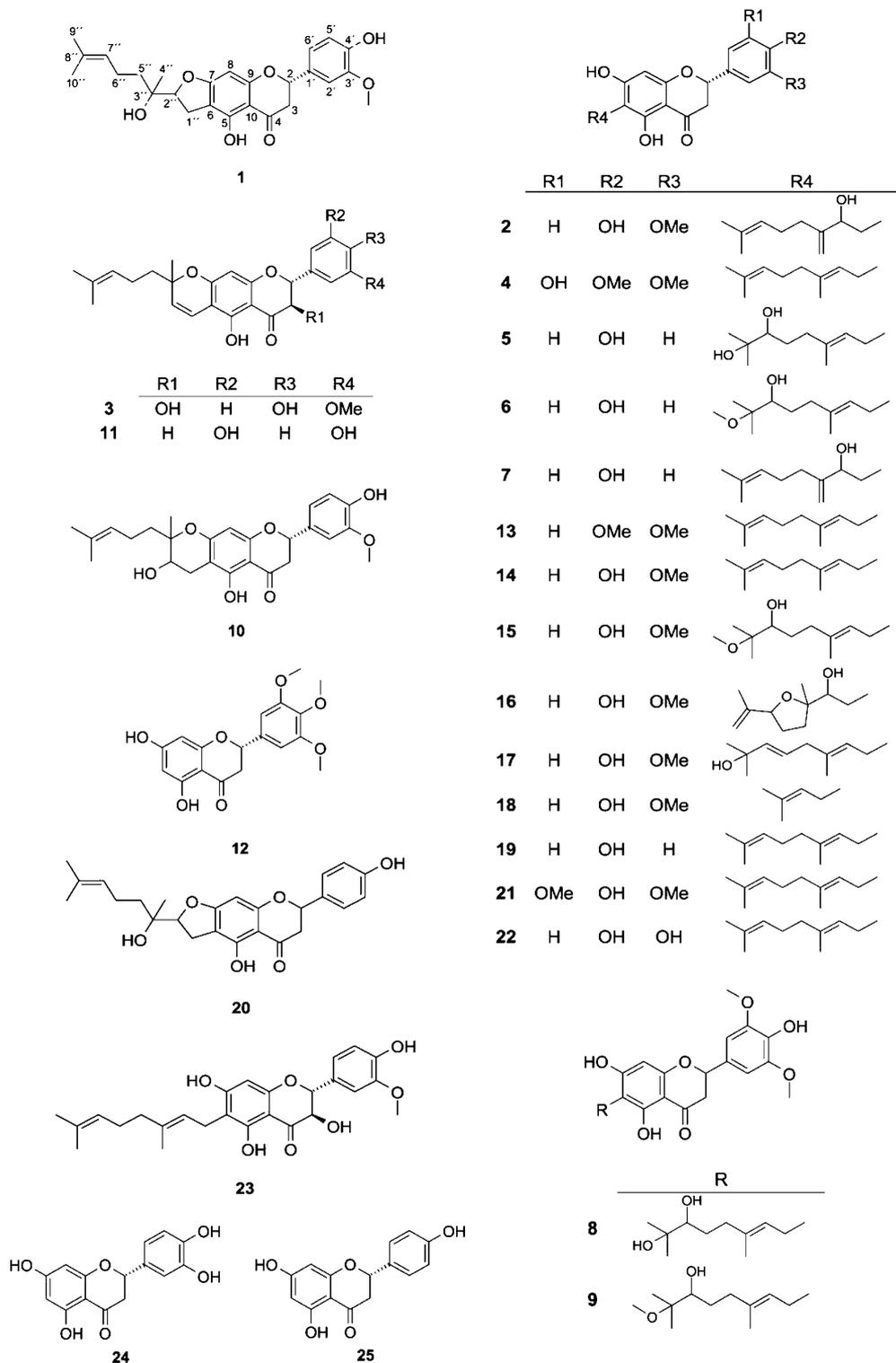
are also activated by several flavonoids. The activation of this pathway attenuates the activity of NF-κB.[17−19] As can be seen, the transcription of inflammation-related genes is regulated by multiple transcription factors, and the exact mechanism of action of flavonoids therefore remains unclear. These phytochemicals disturb the intracellular signaling network at several different sites and thereby contribute to the overall anti-inflammatory effect. The results of tests of the effects of

flavonoids in vivo have supported those previously observed in vitro. Various animal models have confirmed the anti-inflammatory properties of flavonoids. The modulation of pro-inflammatory gene expression has been determined to be one of the major mechanisms of action of flavonoids in vivo.[16]

As briefly summarized above, flavonoids can have abilities useful in combating inflammatory conditions. In particular, lipophilic prenylated or geranylated flavonoid derivatives can

display a wide spectrum of activities and may serve as potential lead compounds for the development of anti-inflammatory therapeutic agents. Therefore, this investigation was focused on the isolation of geranylated derivatives of flavonoids from *P. tomentosa* and the elucidation of their anti-inflammatory potential.

■ RESULTS AND DISCUSSION

Compound Structure Elucidation. Chromatographic separation of a methanolic partition of the ethanol extract of *P. tomentosa* fruits in several steps led to the isolation of 23 flavonoid derivatives. Compounds **1−11** were determined to be new geranylated derivatives. Also isolated was 3′,4′,5′-trimethoxyflavanone (**12**), a new nonprenylated derivative with an atypical substitution of ring B, obtained from a natural source for the first time. Moreover, 11 previously described prenylated flavonoids were identified by comparing their spectroscopic data with literature values, namely, 6-geranyl-5,7-dihydroxy-3′,4′-dimethoxyflavanone (**13**), 3′-O-methyldiplacone (**14**), tomentodiplacone G (**15**), tomentodiplacone H (**16**), tomentodiplacone B (**17**), 6-prenyl-3′-O-methyleriodyctiol (**18**), mimulone (**19**), bonannione B (**20**), 3′-O-methyl-5′-methoxydiplacone (**21**), diplacone (**22**), and 3′-O-methyldiplacol (**23**).[5,9,11,20,21]

The basic structural characteristics of the new isolated compounds were determined by analysis of their UV and IR spectra. Compounds **1**, **2**, **4−10**, and **12** exhibited similar UV spectra typical for a flavanone skeleton, with maxima at ∼205 and ∼295 nm and shoulder maxima at ∼230 and ∼335 nm. The UV spectra of compounds **3** and **11** showed maximal values at ∼204, ∼230, ∼275, ∼292 (sh), and ∼360 nm. By comparison to the common characteristics of flavanones, these shifts were assigned as being caused by the presence of a double bond conjugated with the ring A of a flavanone.[9] The infrared spectra obtained for the isolated compounds showed the broad absorption bands at ν_{max} 3500−3200 cm^{-1} typical for OH stretching vibrations, bands at ν_{max} 3000−2850 cm^{-1} showing the presence of methyl and methylene groups (intensive for prenylated/geranylated compounds), an intensive band at ν_{max} 1650−1580 cm^{-1}, indicating the presence of a carbonyl group, and a series of bands at 1600−1450 cm^{-1} typical for aromatic compounds.[9] The similarity of the acquired spectra to previously obtained analytical results supported the presence of further geranylated flavonoid derivatives.[9]

The NMR spectra obtained for the isolated compounds showed several well-observed similarities that were identified as signals typical for a flavonoid skeleton with a carbon chain moiety. Comparison of the NMR spectroscopic data obtained with those previously reported for *P. tomentosa* C-geranyl flavonoids allowed the assignment of a singlet at about δ_H 5.95 to H-8, because its HMBC correlations to C-6, C-7, C-9, and C-10 of ring A were observed. The connection of a geranyl chain to the flavonoid skeleton at C-6 was deduced on the basis of our previous work (the interactions of the OH-5 proton were not observed due to the use of *d*-methanol as the solvent).[4,5,9,11,12] In most cases, the ring C of the flavonoid was arranged as a flavanone (with the exception of compound **3**), with three doublets of doublets with approximate values at δ_{H-2} 5.3 (*J* = 3.0, 13.0 Hz), $\delta_{H-3\alpha}$ 2.7 (*J* = 3.0, 17.0 Hz), and $\delta_{H-3\beta}$ 3.1 (*J* = 13.0, 17.0 Hz). On the basis of their coupling constants, the geometrical isomerism of those hydrogens with *J* = 3.0 Hz was assigned as *cis* and of those with *J* = 13.0 Hz as *trans*.[4] Further analysis of the NMR data revealed that the phenolic

ring B of the flavonoid skeleton was either 4′-substituted, 3′,4′-disubstituted, or 3′,4′,5′-trisubstituted with hydroxy or methoxy groups. In compounds **5−7**, the 4′-hydroxyphenyl ring gave two doublets $\delta_{H-2′,6′}$ and $\delta_{H-3′,5′}$ (*J* = 8.6 Hz), each integrating for two protons. Compounds **1−3** and **10** possess a 3′-methoxy-4′-hydroxyphenyl arrangement of ring B, with two doublets $\delta_{H-2′}$ and $\delta_{H-5′}$ (*J* = 2.0 and 8.0 Hz, respectively) coupling with one doublet of doublets $\delta_{H-6′}$ (*J* = 2.0, 8.0 Hz). The position of the methoxy group was assigned on the basis of HMBC and NOESY correlations. A strong cross-peak of the methoxy protons with H-2′ in the NOESY spectrum was in general more diagnostic than the HMBC correlations, because of the similar carbon chemical shifts of C-3′ and C-4′. The ^{1}H NMR spectra of **8** and **9** showed only one singlet at $\delta_{H-2′,6′}$ 6.80 (integrating for two hydrogens), indicating a symmetric 3′,5′-substitution of ring B, which was subsequently assigned as a 3′,5′-dimethoxy-4′-hydroxyphenyl unit using both the HMBC and NOESY correlations of the methoxy protons. The HMBC experiments also confirmed the attachment of ring B to the flavonoid backbone at C-2 in all cases. Further evaluation of the NMR spectroscopic data provided information about the presence of geranyl side-chains in the compounds analyzed (with the exception of compound **12**). These consisted of two isoprenoid units displaying three CH$_3$ singlets, three CH$_2$ and two CH groups, and two quaternary carbons of the double bonds, and their coupling constant values ranged from 7.0 to 7.5 Hz.

Compound **1** was isolated as a brownish, amorphous substance. Its molecular formula was determined by HRESIMS to be C$_{26}$H$_{30}$O$_7$ on the basis of the presence of a [M − H]$^-$ ion at *m/z* 453.1916 (calcd for C$_{26}$H$_{29}$O$_7^-$, 453.1913). The structure of **1** was determined by evaluation of the NMR data using the ^{1}H NMR spectrum together with HSQC, HMBC, COSY, and NOESY experiments. Carbon signals at δ_C 72.9 and 90.7 suggested a modification of the geranyl chain and the presence of an oxygen-containing substituent. The combination of HMBC, COSY, and NOESY experiments allowed the inference to be made that C-2″ is linked to the oxygen present at C-7 and forms a 1″,2″-dihydrofuran ring and that C-3″ is substituted with an OH and a CH$_3$ group. The rest of the carbon side-chain (C-5″ to C-10″) remained unchanged when compared to the NMR signals of the geranyl chains present in related compounds.[5] The chemical shifts of the carbons and protons of this modified geranyl chain were also consistent with the NMR assignments of a similar compound, bonannione B, previously isolated from *Bonannia graeca*.[20] On the basis of previous work[9,11] and due to the character of the substitution of ring B, compound **1** was named tomentodiplacone L and assigned with the structure shown.

Compound **2** was obtained as a yellowish-brown, amorphous substance. HRESIMS analysis revealed the presence of a [M − H]$^-$ ion at *m/z* 453.1921 (calcd for C$_{26}$H$_{29}$O$_7^-$, 453.1913), and thus the molecular formula was determined as C$_{26}$H$_{30}$O$_7$. The NMR signals supported the presence of a 3′-methoxy-4′-hydroxyflavanone skeleton substituted with a 10-carbon chain. Three signals exhibited chemical shift values unusual for an unmodified geranylated derivative. The observation of a CH$_2$ unit with chemical shifts at δ_C 107.5 and δ_H 4.78 and 4.95, together with the quaternary carbon δ_C 151.5, suggested the presence of an sp^2 methylene group (C-4″) linked to C-3″. Diagnostic HMBC correlations of a triplet at δ_H 4.38 connected with an oxygenated carbon (δ_C 74.2) with C-4″ and C-3″ showed this proton to be H-2″. These data confirmed that the

geranyl chain was also modified by C-2″oxidation. Compound **2** (tomentodiplacone M) was assigned as shown.

Compound **3** was isolated as a yellow powder, with molecular formula determined by HRESIMS as $C_{26}H_{28}O_7$ on the basis of the presence of a $[M - H]^-$ ion at m/z 451.1741 (calcd for $C_{26}H_{27}O_7^-$, 451.1757). The unusual UV spectrum indicated the presence of a double bond attached to the chromophoric system of the flavonoid skeleton, and NMR experiments confirmed this hypothesis. Two doublets at δ_H 6.67 and 5.55 (J = 10.2 Hz) supported the presence of a double bond, and these signals correlated with carbons of ring A. Thus, they were assigned to C-1″ and C-2″, respectively. The signal of a quaternary carbon at δ_C 80.7 suggested a linkage to oxygen, and the formation of a pyran ring with OH-7 was deduced using HMBC correlations of other carbons from the geranyl chain. Unlike the other compounds obtained, the three proton signals of the flavanone nucleus AMX pattern were missing and two doublets characteristic for the flavanone-3-ol ring C were found instead.[4] This compound was named tomentodiplacol B and was assigned as shown.

Compound **4** was obtained as a yellowish, amorphous substance. Its molecular formula was determined by HRESIMS to be $C_{27}H_{32}O_7$ on the basis of the presence of a $[M - H]^-$ ion at m/z 467.2073 (calcd for $C_{27}H_{31}O_7^-$, 467.2070). The NMR data obtained closely resembled those of flavanones having an unmodified geranyl chain; therefore the elucidation procedure was focused only on differences in the proton signals of ring B. In the ^{1}H NMR spectrum, two distinguishable m-coupled (J = 1.8 Hz) doublets were observed at δ_H 6.66 and 6.64 (integrating for one proton each). On the basis of HMBC, COSY, and NOESY correlations of these protons and two methoxy groups, the structure of ring B was assigned as 3′,4′-dimethoxy-5′-hydroxyphenyl. Compound **4** was assigned as 3′,4′-O-dimethyl-5′-hydroxydiplacone.

Compound **5** was isolated as a yellow, amorphous substance. Its molecular formula was determined by HRESIMS to be $C_{25}H_{30}O_7$ on the basis of the presence of a $[M - H]^-$ ion at m/z 441.1935 (calcd for $C_{25}H_{29}O_7^-$, 441.1913). The signals of oxygen-bearing carbons (δ_C 72.3 and 77.5) were again present, but the observed HMBC correlations indicated that oxidation took place in a different part of the chain than in compounds **1**−**3**. The relatively lower chemical shifts of the two terminal methyl groups implied electronegative substitution at C-8″. The HMBC and COSY spectra were used to assign the two oxidized carbons as C-7″ and C-8″. With regard to the substitution pattern of ring B, analysis of the ^{1}H NMR spectrum revealed two proton signals typical for 4′-hydroxyphenyl substituents. Compound **5** (mimulone F) was thus assigned as shown.

Compound **6** was obtained as a brownish, amorphous substance. On the basis of HRESIMS analysis and the presence of a $[M - H]^-$ ion at m/z 455.2066 (calcd for $C_{26}H_{31}O_7^-$, 455.2070), the molecular formula was determined as $C_{26}H_{32}O_7$. The NMR data were mostly consistent with those of compound **5**, with the only difference being the presence of a methoxy group signal at δ_H 3.17, which could be located at C-8″ on the basis of the HMBC correlation observed. This methoxy substitution lowered the electron density, moving the chemical shift of C-8″ downfield. Compound **6**, named mimulone G, was assigned structurally as shown.

Compound **7** was isolated as a brownish, amorphous substance. Its molecular formula was determined by HRESIMS to be $C_{25}H_{28}O_6$ on the basis of the presence of a $[M - H]^-$ ion

at m/z 423.1778 (calcd for $C_{25}H_{27}O_6^-$, 423.1808). The NMR spectra closely resembled those obtained for compound **2**, the signals of the modified geranyl chain and flavanone skeleton were consistent, and the only difference was in the substitution of ring B. This compound (mimulone H) showed NMR signals typical for a 4′-hydroxyphenyl arrangement and was proposed structurally as shown.

Compound **8** was obtained as a yellow, amorphous substance. HRESIMS analysis revealed the presence of a $[M - H]^-$ ion at m/z 501.2157 (calcd for $C_{27}H_{33}O_9^-$, 501.2125), and thus the molecular formula was determined as $C_{27}H_{34}O_9$. The aromatic protons of the flavonoid ring B were represented by the presence of a singlet at δ_H 6.79 (2H), indicating a 3′,4′,5′-trisubstituted ring. Two equal methoxy groups at δ_H 3.88 (s, 6H) were assigned to carbons C-3′ and C-5′, respectively, on the basis of the NOESY cross-peak with H-2′ and H-6′, and the hydroxy group was assigned to C-4′. The upfield chemical shift of C-4′ corresponded to a higher electron density on this carbon caused by the two neighboring carbons bearing methoxy groups. NMR signals observed for the other part of the molecule were consistent with those of compound **5**. Compound **8** (paulownione A) was assigned, therefore, as shown.

Compound **9** was isolated as an orange, amorphous substance. On the basis of HRESIMS analysis and the presence of a $[M - H]^-$ ion at m/z 515.2277 (calcd for $C_{26}H_{35}O_9^-$, 515.2281), the molecular formula was determined to be $C_{28}H_{36}O_9$. As was the case with the difference between compounds **5** and **6**, this compound differed from compound **8** only by the methoxylation of C-8″. The other NMR signals were all equivalent. Compound **9** (paulownione B) was assigned structurally as shown.

Compound **10** was obtained as a brownish, amorphous substance. Its molecular formula was determined by HRESIMS to be $C_{26}H_{30}O_7$ on the basis of the presence of a $[M - H]^-$ ion at m/z 453.1965 (calcd for $C_{26}H_{29}O_7^-$, 453.1913). The NMR data included signals for a flavanone nucleus with a 3′-methoxy-4′-hydroxyphenyl ring B. Further analysis of the NMR data set allowed oxidized carbons to be inferred in the geranyl chain moiety. These carbons were assigned as C-2″ and C-3″, respectively. On the basis of HMBC correlations, a hydroxy group was assigned to C-2″ and a connection of C-3″ to OH-7 was deduced. Thus, the geranyl chain was cyclized to form a 2H-pyran ring. A search of the literature showed that the NMR signals for compound **10** were similar to those of bonanniol C, which possesses the same modification of the geranyl side-chain.[20] Compound **10** (tomentodiplacone N) was proposed structurally as shown.

Compound **11** was obtained as a yellow, amorphous substance. HRESIMS analysis revealed the presence of a $[M - H]^-$ ion at m/z 421.1688 (calcd for $C_{25}H_{25}O_6^-$, 421.1651), and thus the molecular formula was determined to be $C_{25}H_{26}O_6$. On the basis of the UV and NMR spectra, it was deduced that the geranyl chain is cyclized with OH-7 at C-3″, with a double bond formed between C-1″ and C-2″, leading to a structure similar to that of compound **3**. On the other hand, the signals of the protons of the flavonoid nucleus protons corresponded to a flavanone pattern, and those of the aromatic protons of ring B comprised two broad singlets integrating for one and two protons, respectively. These signals of ring B, unusual for flavonoids obtained from *P. tomentosa*, had previously been detected only in schizolaenone C, which displays a 3′,5′-dihydroxyphenyl ring B.[7] Although the
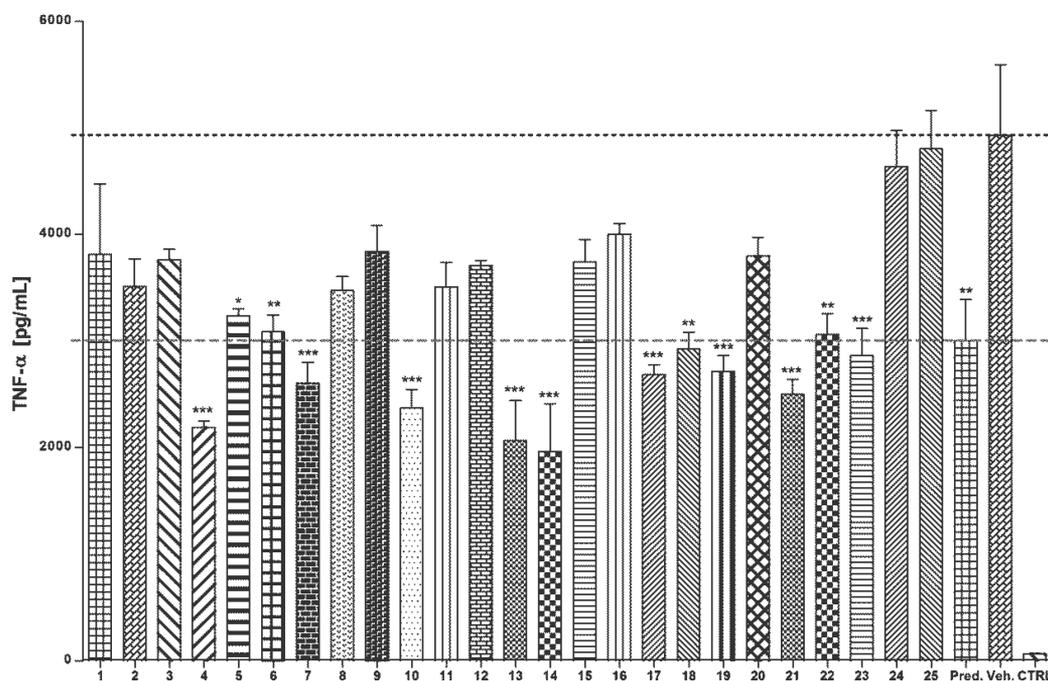
Figure 1. Effect of the 23 flavonoids on the secretion of TNF-α. THP-1 macrophages were pretreated with compounds **1–23** isolated from *P. tomentosa*, standards **24** and **25**, and prednisone (Pred.) at a concentration of 2 μM or with DMSO only (Veh. and CTRL) for 1 h. Subsequently, LPS (1 μg/mL) was added [except for the control cells (CTRL)] to trigger the secretion of pro-inflammatory cytokine TNF-α. After 24 h, the amount of TNF-α was evaluated by ELISA. The results are expressed as the mean $\pm$ SE for three independent experiments. The dotted black line indicates the amount of TNF-α secreted by the vehicle cells (untreated cells), and the dashed gray line indicates the amount of TNF-α secreted by the cells pretreated with prednisone. * indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.05$, ** indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.01$, and *** indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.001$.

symmetry of the aromatic ring suggested the assignment of the singlets at δ_H 6.81 to H-2′ and H-6′ and δ_H 6.93 to H-4′, the clear HMBC correlations of both signals to C-2 and the NOESY of both signals with H-2 and H-3β were convincing enough to assign δ_H 6.93 to H-2′ (or H-6′, respectively). Compound **11** (tomentone) was assigned as shown.

Compound **12** was isolated as a pale yellow powder. Its molecular formula was determined by HRESIMS to be $C_{18}H_{18}O_7$ on the basis of the presence of the $[M - H]^-$ ion at m/z 345.0935 (calcd for $C_{18}H_{17}O_7^-$, 345.0974). A lower molecular weight and an IR spectrum with reduced alkyl and alkylene bands compared to the other substances isolated suggested that no prenyl chain was present in this case. Two methoxy singlets (9H together) and two aromatic non-correlating singlets (2H each) were observed. The protons at δ_H 6.69 were assigned to ring B, and a 3′,4′,5′-trimethoxy-phenyl arrangement was determined on the basis of the HMBC correlations. The protons at δ_H 6.04 were assigned to H-6 and H-8 of the flavonoid ring A. Using CDCl$_3$ as the solvent, the OH-5 proton at δ_H 12.05 could be observed, as well as its HMBC correlations to carbons of ring A. Therefore, the structure of compound **12** was assigned as 3′,4′,5′-trimethoxy-flavanone.

The absolute configurations of compounds **1–12** were determined using analysis of their CD spectra. All of the compounds tested contain a chiral center at C-2, but not all of them displayed a CD curve. A positive Cotton effect for the n → π* electronic transition (320–340 nm) and a negative Cotton effect for the π → π* electronic transition (270–290 nm) led to the assignment of a 2*S*-configuration for compounds **1**, **2**, **4–7**, and **10–12**.[22] Compound **3** was determined to be a

2*R*,3*R*-flavanone-3-ol because of the *trans* relative configuration of its C-2 and C-3 substituents and also due to a positive n → π* Cotton effect at a high wavelength (350–370 nm).[22] Compounds **8** and **9** did not exhibit any Cotton effects, and therefore they were assigned as racemic mixtures of 2*S* and 2*R* enantiomers. The modifications of the geranyl chain resulted in the formation of other stereogenic centers in the compounds isolated (with the exception of compounds **4** and **12**). A quantum mechanical method was used to calculate the NMR parameters,[20] but the two calculated sets showed only small differences that were not sufficient to assign a relative configuration.

Cytotoxicity. The viability of THP-1 cells was measured after treatment with increasing concentrations of the flavonoids tested (**1–25**) with the aim of finding a nontoxic concentration for the following in vitro experiments. Compounds could be assumed to be potentially cytotoxic only when shown to exhibit IC$_{50}$ values of <10 μM. This criterion was fulfilled by compounds **1**, **2**, **4**, **10**, **13**, **14**, **19**, **21**, and **23**; the remaining compounds tested showed presumptive IC$_{50}$ values of >10 μM, and thus they can be deemed to be noncytotoxic (see Table S1 in the Supporting Information). Compound **7**, with an IC$_{50}$ value of 11.4 $\pm$ 0.2 μM, lies close to the border between being cytotoxic and noncytotoxic. All of the compounds tested showed values of IC$_{50}$ > 5 μM. The absence of an aliphatic side-chain (prenyl or geranyl) in the structure of a test compound significantly decreased the cytotoxicity, as has been described previously.[7,23] The simple, non-geranylated flavonoids **12**, **24**, and **25** were inactive in comparison to flavonoids with such side-chains (see Table S1). The cytotoxic effect of prenylated or geranylated flavonoids is possibly related to their lipophilicity

and to their greater ability to penetrate the membranes of cells.[23] The presence and position of hydroxy groups on the side-chain also influenced the cytotoxicity. The presence of a β-carbon (proximal) OH group on a geranyl chain did not affect the cytotoxicity of a compound, but an OH group on the distal end of the side-chain caused a loss of cytotoxic effect for such compounds. This effect can be seen for example in compound **2**, with a hydroxy group on the β-carbon of the geranyl chain (IC$_{50}$ 6.5 ± 0.1 μM), compound **14**, with an unmodified geranyl chain (IC$_{50}$ 7.1 ± 0.1 μM), and compound **17**, with a hydroxy group on the ω-carbon of the geranyl chain (IC$_{50}$ >20 μM). The substituents on ring B of the flavonoid structure seemed to have minor effects on the cytotoxic potential of the flavonoids tested. On the basis of the results obtained from cytotoxicity testing, a concentration of 2 μM of tested flavonoids **1**−**25** was selected as a noncytotoxic concentration for further evaluation of anti-inflammatory potential.

Anti-inflammatory Potential. The anti-inflammatory activity of flavonoids has long been well known. The production of a typical pro-inflammatory cytokine TNF-α in lipopolysaccharide (LPS)-stimulated macrophages was used to determine the ability of compounds **1**−**23** isolated from *P. tomentosa*, along with eriodictyol (**24**) and naringenin (**25**), to attenuate the production of this cytokine (Figure 1). As can be seen, several compounds affected the production of TNF-α in a statistically significant manner ($p < 0.001$). Thus, compounds **3**, **7**, **10**, **13**, **14**, **17**, **21**, and **23** affected the secretion of TNF-α as much as or more than the prednisone that was used as a positive control for the assay. This result confirmed the well-known fact that flavonoids possess anti-inflammatory potential.[24] Most of the compounds tested have a lipophilic character due to the presence of a prenyl or geranyl side-chain, so logarithmic *n*-octanol/water partition coefficient log (K_{ow}) values were determined and correlated with the TNF-α test results. This model showed only a relatively poor correlation with $R^2 = 0.42$ (Figure 2), which suggests that there is no simple relationship between the lipophilicity of the compounds and their ability to decrease the TNF-α secretion and that the pharmacological activity of these compounds is not dependent
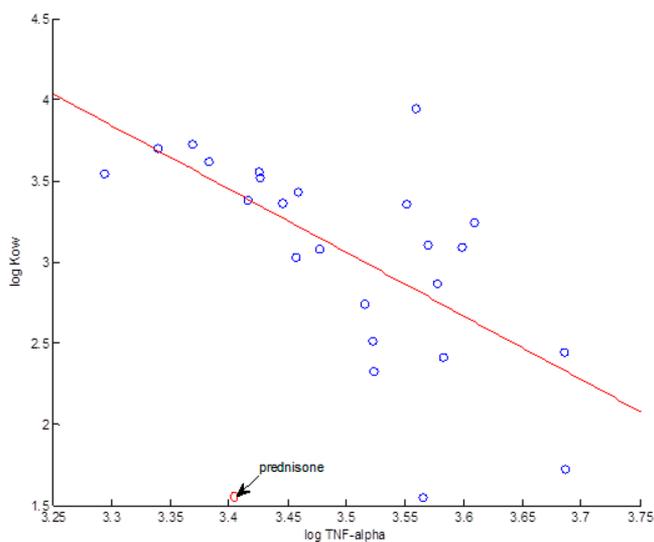
only on their lipophilic character. A 3D QSAR model was not evaluated due to the considerable flexibility of prenyl and geranyl groups, which take rather different conformations in hydrophilic and lipophilic environments, making it difficult to calculate correct values.

On the basis of the initial screening of their cytotoxicity and anti-inflammatory potential, compounds **4**, **7**, **10**, **14**, and **22** were selected for more detailed analysis of their activities. In order to determine whether the expression of TNF-α was modified by a post-transcriptional or pretranscriptional mechanism, the level of TNF-α mRNA was measured. A previous experiment with diplacone (**22**) showed its ability to diminish the gene expression of TNF-α.[14] The results obtained herein confirmed this result, as all of the tested compounds attenuated the expression of TNF-α mRNA (Figure 3), which
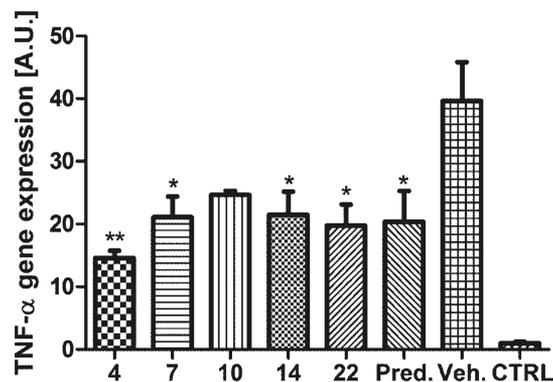
Figure 3. Effect of selected flavanones on the gene expression of TNF-α. THP-1 macrophages were pretreated with compounds **4**, **7**, **10**, **14**, **22**, and prednisone (Pred.) at a concentration of 2 μM or with DMSO only (Veh. and CTRL) for 1 h. Subsequently, LPS (1 μg/mL) was added [except for the control cells (CTRL)] to trigger the transcription of the TNF-α gene. After 4 h, the level of TNF-α mRNA was evaluated by RT-qPCR. The amount of TNF-α mRNA was normalized to β-actin mRNA. The results are expressed as the mean ± SE for three independent experiments. A.U. = arbitrary unit. * indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.05$, and ** indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.01$.

was clearly in accordance with the results of the secretion of the TNF-α cytokine. These data indicate that the regulation of the expression of TNF-α was upstream of the transcription. The expression of TNF-α is often shown to be controlled by the nuclear factor κB (NF-κB), and the effect of compounds **4**, **7**, **10**, **14**, and **22** on the activity of this transcription factor was therefore further investigated. Flavonoids are known to be able to modulate the activity of NF-κB.[24−26] NF-κB is kept inactive in the cytoplasm by its inhibitor IκB. When the signal pathway is activated, the IκB is degraded and NF-κB is translocated into the nucleus.[26] The pretreatment of cells with the test flavonoids **4**, **7**, **10**, **14**, and **22** led to attenuation of the IκB degradation after LPS stimulation (see Figure S60 in the Supporting Information) and subsequently to restriction of the uncovering of NF-κB transactivation domain detectable by used antibody (Figure 4). These results confirmed previous observations that describe the inhibition of NF-κB as one mode of anti-inflammatory action of flavonoids.[17]

Influence of the Flavonoids Tested on the Formation of ROS. Reactive oxygen species (ROS) play a significant role in inflammation. An elevated level of ROS can damage the

Figure 2. Dependence of the inhibition of the secretion of TNF-α on the lipophilicity of compounds **1**−**12** and **14**−**25**. The results are expressed as the dependence of log TNF-α on log K_{ow}.
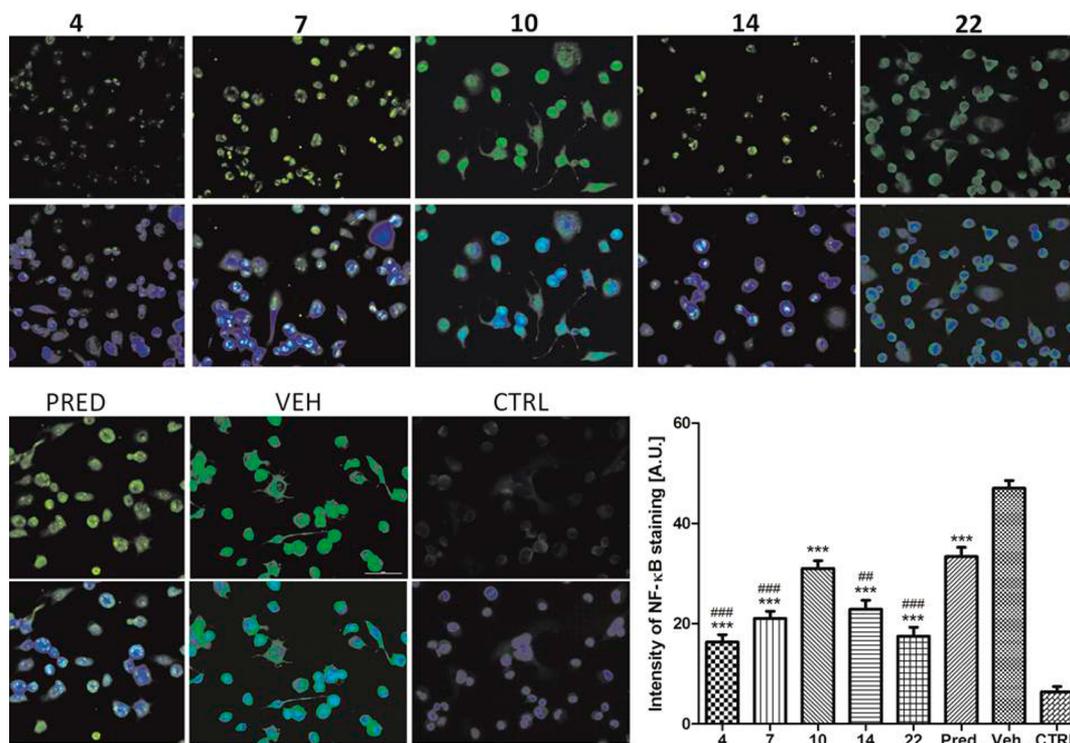
Figure 4. Effect of selected flavanones on the nuclear translocation of NF-κB. THP-1 macrophages were pretreated with compounds **4, 7, 10, 14, 22,** and prednisone (Pred.) at a concentration of 2 μM or with DMSO only (Veh. and CTRL) for 1 h. Subsequently, LPS (1 μg/mL) was added [except for the control cells (CTRL)] to trigger the NF-κB activation. After 4 h, the amount of NF-κB was detected by immunohistochemical analysis (green color for the p65 subunit and blue for the DAPI staining of nuclei). The first row of pictures represents p65 staining, and the second one a combination of p65 and DAPI staining. The results in the graph show the mean $\pm$ SE for three independent experiments for the p65 staining intensity. A.U. = arbitrary unit. *** indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.001$; ## indicates a significant difference in comparison with the prednisone-treated cells, $p < 0.01$; and ### indicates a significant difference in comparison with the prednisone-treated cells, $p < 0.001$.
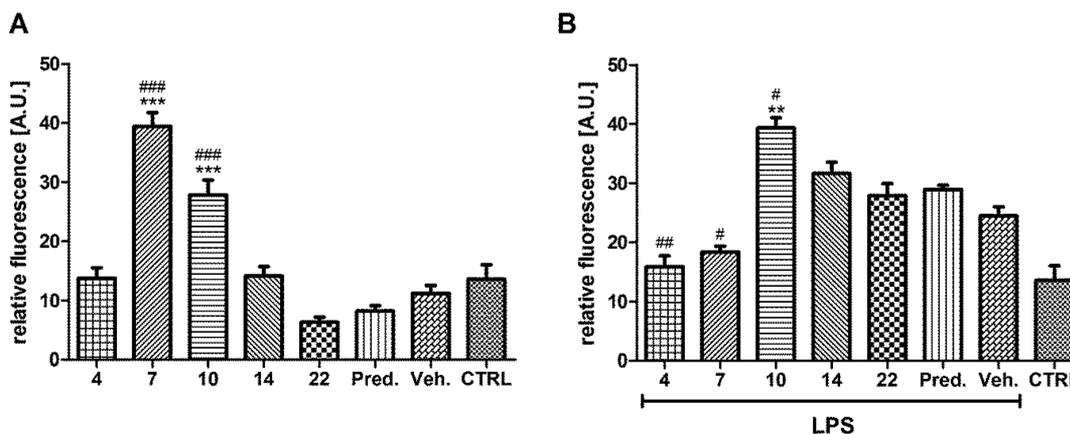
Figure 5. Effect of selected flavanones on the generation of ROS. THP-1 macrophages were pretreated with compounds **4, 7, 10, 14, 22,** and prednisone (Pred.) at a concentration of 2 μM or with DMSO only (Veh. and CTRL) for 1 h. The level of ROS was measured after 24 h (A). Alternatively, LPS (1 μg/mL) was added [except for the control cells (CTRL)] to trigger the ROS formation, and measurement was performed 24 h after the addition of LPS (B). The results are expressed as the mean $\pm$ SE for three independent experiments. A.U. = arbitrary unit. ** indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.01$; *** indicates a significant difference in comparison with the vehicle-treated cells, $p < 0.001$; # indicates a significant difference in comparison with the prednisone-treated cells, $p < 0.05$; ## indicates a significant difference in comparison with the prednisone-treated cells, $p < 0.01$; and ### indicates a significant difference in comparison with the prednisone-treated cells, $p < 0.001$.

surrounding biomolecules and contribute to the inflammatory response, partially even through the activation of NF-κB.[27] Flavonoids possess anti- and pro-oxidant activities, and they can affect the inflammatory processes by modulating redox-sensitive intracellular pathways.[28] Compounds **4, 7, 10, 14,** and **22** were

tested to evaluate the formation of ROS after stimulation of the THP-1 cells with bacterial LPS (Figure 5). Mimulone H (**7**) and tomentodiplacone N (**10**) significantly increased the level of ROS in the THP-1 cells without LPS stimulation. On the other hand, only compounds **10, 14,** and **22** augmented the

Table 1. NMR Spectroscopic Data (400 MHz, MeOD) for Tomentodiplacones L (1) and M (2) and Tomentodiplacol B (3)

	tomentodiplacone L (1)			tomentodiplacone M (2)			tomentodiplacol B (3)		
position	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC
2	79.4, CH	5.36, dd (3.0, 13.0)		78.7, CH	5.34, dd (2.7, 13.0)	1′	83.7, CH	5.02, d (11.6)	3, 2′, 6′
3	42.5, CH$_2$	2.71, dd (3.0, 17.1)	2, 4	42.5, CH$_2$	2.75, dd (2.7, 17.0)	2, 4, 1′	72.2, CH	4.63, d (11.6)	2, 4, 1′
		3.16, dd (13.0, 17.1)			3.14, dd (13.0, 17.0)				
4	196.7, C			196.5, C			197.2, C		
5	165.4, C			161.4, C			162.3, C		
6	105.6, C			105.7, C			102.1, C		
7	168.4, C			165.2, C			162.4, C		
8	89.8, CH	5.99, s	6, 7, 9, 10	94.5, CH	5.97, s	6, 7, 9, 10	95.2, CH	5.91, s	6, 7, 9, 10
9	163.4, C			161.5, C			160.8, C		
10	102.5, C			101.4, C			100.6, C		
1′	130.2, C			130.2, C			127.9, C		
2′	109.8, CH	7.09, br s	2, 4′, 6′	109.8, CH	7.09, d (1.5)	2, 4′, 6′	111.1, CH	7.13, d (1.9)	2, 4′, 6′
3′	147.3, C			147.8, C			147.6, C		
4′	146.7, C			146.8, C			146.9, C		
5′	114.7, CH	6.83, d (7.8)	1′, 3′	114.6, CH	6.83, d (8.0)	1′, 3′	114.5, CH	6.87, d (8.1)	1′, 3′
6′	119.0, CH	6.93, br d (7.8)	2, 2′, 4′	118.9, CH	6.93, dd (1.5, 8.0)	2, 2′, 4′	120.8, CH	6.99, dd (1.9, 8.1)	2, 2′, 4′
1″	25.2, CH$_2$	3.06, d (8.4)	6, 7, 2″, 3″	28.4, CH$_2$	2.78, m	5, 6, 7, 2″, 3″	115.0, CH	6.67, d (10.2)	5, 6, 7, 3″
					2.93, m				
2″	90.7, CH	4.77, t (8.4)	4″	74.2, CH	4.38, t (6.4)	6, 1″, 3″, 4″	124.8, CH	5.55, d (10.2)	6, 3″
3″	72.9, C			151.5, C			80.7, C		
4″	20.4, CH$_3$	1.23, s	2″, 3″, 5″	107.5, CH$_2$	4.78, s	2″, 5″	26.2, CH$_3$	1.411, s	2″, 3″, 5″
					4.95, s			1.414, s	
5″	37.5, CH$_2$	1.54, t (8.4)	2″, 3″, 6″, 7″	30.9, CH$_2$	2.18, m	3″, 4″, 6″, 7″	41.2, CH$_2$	1.72, m	6″
6″	21.4, CH$_2$	2.13, m	7″	26.3, CH$_2$	2.19, m	5″, 8″	22.1, CH$_2$	2.10, m	5″, 7″, 8″
7″	124.1, CH	5.15, t (7.0)	6″, 9″, 10″	124.2, CH	5.18, br s	6″	123.4, CH	5.12, m	
8″	130.7, C			130.7, C			131.0, C		
9″	24.3, CH$_3$	1.70, s	7″, 8″, 10″	24.5, CH$_3$	1.70, s	7″, 8″, 10″	24.2, CH$_3$	1.67, s	7″, 8″, 10″
10″	16.2, CH$_3$	1.65, s	7″, 8″, 9″	16.2, CH$_3$	1.65, s	7″, 8″, 9″	16.1, CH$_3$	1.58, s	7″, 8″, 9″
MeO-3′	54.9, CH$_3$	3.89, s	3′	54.9, CH$_3$	3.89, s	3′	54.9, CH$_3$	3.91, s	3′

formation of ROS in the presence of LPS. Interestingly, **4** and **7** insignificantly decreased the level of ROS after LPS stimulation. The ability of **10** to hold a greater level of ROS independently on the presence of inflammatory stimuli (bacterial LPS) could explain, at least partially, its lower activity in inhibiting the NF-κB pathway as compared with the other flavonoids tested. The discrepancy in the behavior of **7** in comparison with **10** might be explained by different structural moieties responsible for anti- and pro-oxidant properties. Mimulone H (**7**) probably acts as a pro-oxidant, but after inflammatory response has been stimulated, it could block the formation of ROS acting as an antioxidant with any pro-oxidant effect eliminated.

Conclusions. This work has demonstrated that *P. tomentosa* is a rich source of various *C*-geranylated flavonoids, which because of their lipophilicity, are able to affect biological targets and become interesting leads for the development of new drugs. The isolation of 12 new flavonoids and 11 previously known compounds from *P. tomentosa* fruits is presented herein. These compounds were tested for their anti-inflammatory potential on the macrophage-like cell line THP-1, and the mechanism of action was determined for the five compounds with the most significant and promising results. The compounds tested showed a significant antiphlogistic potential, caused, at least partly, by interaction with the NF-κB pathway. However, the redox potentials of these flavonoids can also influence inflammation. Nevertheless, the interaction between ROS and the inflammatory signaling pathways, e.g., the NF-κB pathway, in the presence of flavonoids with redox and antiphlogistic potential needs additional investigations, especially in in vivo models.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured with a JASCO P-2000 digital polarimeter (Jasco, Easton, MD, USA). UV spectra were measured with a UV/Vis Lambda 25 spectrometer (PerkinElmer, Waltham, MA, USA). Circular dichroism (CD) spectra were obtained using a JASCO J-810 polarimeter (Jasco). IR spectra (ATR) were measured with a Nicolet Impact 400D FT-IR instrument (Thermo Scientific, Waltham, MA, USA). NMR (1D and 2D) spectra were obtained on a Bruker Avance III 400 spectrometer (Bruker, Billerica, MA, USA) with TMS as the internal standard. ESIMS were recorded on a Varian 500 MS ion trap spectrometer (Varian, Palo Alto, CA, USA) in the negative mode. HRMS were measured using an API-TOF Mariner spectrometer (Applied Biosystems, Thermo Scientific) in the negative mode. Analytical HPLC measurements were obtained on an Agilent 1100 chromatographic system with a 1100 series diode array detector (Agilent Technologies, Santa Clara, CA, USA). Preparative HPLC was performed using a YL 9100 HPLC System (Young Lin, The Republic of Korea) with a FOXY R2 fraction collector (Teledyne Isco, Lincoln, NE, USA).

Column chromatography was performed using silica gel with a particle size of 40−63 μm (Merck, Billerica, MA, USA). For analytical purposes, silica gel 60 F$_{254}$, 20 × 20 cm, 200 μm TLC plates (Merck) and an Ascentis Express RP-Amide, 10 cm × 2.1 mm, particle size 2.7 μm, analytical HPLC column (Sigma-Aldrich, St. Louis, MO, USA) were used. Preparative separations were carried out with preparative

Table 2. NMR Spectroscopic Data (400 MHz, MeOD) for 3′,4′-O-Dimethyl-5′-hydroxydiplacone (4) and Mimulones F (5) and G (6)

	3′,4′-O-dimethyl-5′-hydroxydiplacone (4)			mimulone F (5)			mimulone G (6)		
position	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC
2	78.8, CH	5.32, dd (3.0, 12.8)	1′	78.9, CH	5.31, dd (2.9, 13.1)	1′	78.7, CH	5.30, br d (ov)	2′, 6′
3	42.9, CH₂	2.73, dd (3.0, 17.2)	2, 4, 1′	42.7, CH₂	2.70, dd (2.9, 17.0)	2, 4, 1′	42.7, CH₂	2.69, dd (3.0, 17.1)	2, 4, 1′
		3.07, dd (12.8, 17.2)			3.07, dd (13.1, 17.0)			3.06, dd (13.0, 17.1)	
4	196.7, C			195.7, C			196.1, C		
5	160.8, C			160.9, C			160.8, C		
6	108.8, C			108.2, C			108.1, C		
7	164.9, C			164.7, C			165.0, C		
8	93.9, CH	5.99, s	4, 6, 7, 9, 10	94.3, CH	5.95, s	4, 6, 7, 9, 10	94.1, CH	5.93, s	4, 6, 7, 9, 10
9	160.9, C			161.2, C			161.1, C		
10	101.7, C			101.4, C			101.5, C		
1′	135.2, C			130.0, C			129.7, C		
2′	101.5, CH	6.66, d (1.8)	2, 3′, 4′, 6′	127.5, CH	7.32, d (8.5)	2, 4′, 6′	127.5, CH	7.32, d (8.6)	2, 4′, 6′
3′	152.6, C			114.8, CH	6.83, d (8.5)	1′, 4′, 5′	114.8, CH	6.83, d (8.6)	1′, 4′, 5′
4′	136.4, C			157.4, C			157.1, C		
5′	150.5, C			114.8, CH	6.83, d (8.5)	1′, 3′, 5′	114.8, CH	6.83, d (8.6)	1′, 3′, 5′
6′	106.8, CH	6.64, d (1.8)	2, 2′, 4′, 5′	127.5, CH	7.32, d (8.5)	2, 2′, 4′	127.5, CH	7.32, d (8.6)	2, 2′, 4′
1″	20.2, CH₂	3.23, d (7.3)	5, 6, 7, 2″, 3″	20.2, CH₂	3.25, d (7.1)	5, 6, 7, 2″, 3″	20.3, CH₂	3.25, d (7.1)	5, 6, 7, 2″, 3″
2″	122.2, CH	5.21, t (7.3)	6, 1″, 4″, 5″	122.5, CH	5.27, t (7.1)	1″, 4″, 5″	122.9, CH	5.28, t (7.1)	1″, 4″, 5″
3″	133.9, C			133.9, C			133.5, C		
4″	14.7, CH₃	1.77, s	2″, 3″, 5″	14.7, CH₃	1.80, s	2″, 3″, 5″	14.3, CH₃	1.78, s	2″, 3″, 5″
5″	39.4, CH₂	1.96, t (7.2)	2″, 3″, 4″, 6″	36.4, CH₂	2.01, m	2″, 3″, 4″, 6″, 7″	36.1, CH₂	2.03, m	2″, 3″, 4″, 6″, 7″
					2.24, m			2.22, m	
6″	26.2, CH₂	2.07, q (7.2)	3″, 5″, 7″, 8″	29.2, CH₂	1.34, m	3″, 5″, 7″	28.9, CH₂	1.33, m	3″, 5″, 7″
					1.73, m			1.66, m	
7″	124.3, CH	5.08, t (7.2)	9″, 10″	77.5, CH	3.24, ov	5″, 6″, 8″, 9″, 10″	74.7, CH	3.40, br d (10.1)	5″, 6″, 8″, 9″, 10″
8″	130.8, C			72.3, C			77.0, C		
9″	25.4, CH₃	1.63, s	7″, 8″, 10″	24.0, CH₃	1.15, s	7″, 8″, 10″	19.8, CH₃	1.13, s	7″, 8″, 10″
10″	16.4, CH₃	1.58, s	7″, 8″, 9″	23.4, CH₃	1.13, s	7″, 8″, 9″	19.2, CH₃	1.08, s	7″, 8″, 9″
MeO-3′	54.8, CH₃	3.86, s	3′						
MeO-4′	59.5, CH₃	3.80, s	4′						
MeO-8″							47.9, CH₃	3.17, s	8″
								3.18, s	

Eriodictyol (24) and naringenin (25) were used as non-prenylated flavanones for anti-inflammatory potential testing and were obtained from Sigma-Aldrich, with declared purity exceeding 95%.

Plant Material. *Paulownia tomentosa* fruits were collected during October 2010 (a mixture of immature and mature fruits) and November 2010 (mature fruits) on the grounds of the University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic. Botanical identification was confirmed by Dr. Petr Babula (UVPS Brno). A voucher specimen is deposited in the herbarium of the Department of Natural Drugs, UVPS Brno (no. PT102010).

Extraction and Isolation. A total of 12.9 kg of the fruits was extracted in 96% ethanol (5 × 24 h), and the extract was dried using a Rotavapor. Then, 401 g of this dried extract was suspended in 10% ethanol (4 L) and subsequently extracted with CHCl₃ (3 × 4 L). The organic fraction was dissolved in 90% methanol (1 L) and treated with hexane (3 × 1 L) to yield an MeOH portion (115.8 g after removal of the solvents in vacuo). The MeOH extract was applied to a silica gel column and eluted with benzene−acetone, 95:5 (27 L) and 9:1 (11 L). Fractions of 130 mL were collected and combined on the basis of TLC and HPLC analysis to give 19 final fractions, PT4-1 to PT4-19.

Fraction PT4-5 (120 mg) yielded compound 13 (54 mg) after semipreparative RP-HPLC (with a gradient of redistilled MeCN and 0.2% HCOOH). Fraction PT4-8 (8232 mg) was subjected to column chromatography using CHCl₃−MeOH−benzene, 8.5:0.5:1 (1.5 L), to give six subfractions, PT4-8-A to PT4-8-F. Subfraction PT4-8-E (120 mg) was purified using preparative TLC (benzene−EtOAc, 1:4), and compound 17 (75 mg) was obtained. Subfraction PT4-8-C (4945 mg) was separated on a silica gel column and eluted with benzene−EtOAc (3:2, 1.5 L) to provide seven components. PT4-8-C-1 yielded 850 mg of compound 14. The 2847 mg of fraction PT4-8-C-2 was subjected to semipreparative RP-HPLC (with a gradient of MeOH and 0.2% HCOOH). Fraction PT4-8-C-2/2 (27 mg) was then separated using preparative TLC (benzene−EtOAc, 2:3) to obtain compound 15 (15 mg) and compound 16 (5 mg). Fraction PT4-10 was subjected to column chromatography using CHCl₃−MeOH−benzene, 8.5:0.5:1 (2 L), to give eight subfractions, PT4-10-A to PT4-10-H. Subfraction PT4-10-C was separated using semipreparative RP-HPLC (with a gradient of redistilled MeCN and 0.2% HCOOH) to purify compounds 1 (51 mg), 2 (71 mg), and 4 (46 mg); compounds 18 (18 mg) and 3 (3 mg) were then obtained by purification using preparative TLC (benzene−EtOAc, 3:2). Purification of subfraction PT4-10-D using RP-HPLC (with a gradient of redistilled MeCN and 0.2% HCOOH) yielded compound 19 (707 mg). Subfraction PT4-10-E was separated analogously using RP-HPLC to give compound 7 (8

Table 3. NMR Spectroscopic Data (400 MHz, MeOD) for Mimulone H (7) and Paulowniones A (8) and B (9)

	mimulone H (7)			paulownione A (8)			paulownione B (9)		
position	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC	δ_C, type	δ_H (J in Hz)	HMBC
2	78.9, CH	5.33, dd (2.5, 12.9)	2′, 6′	79.0, CH	5.31, dd (3.1, ov)	4, 1′, 2′	79.1, CH	5.31, dd (ov, 13.0)	1′, 2′, 6′
3	42.6, CH₂	2.71, dd (2.5, 17.1)	2, 4	42.8, CH₂	2.67, dd (3.1, 17.0)	4	42.1, CH₂	2.67, dd (2.9, 17.0)	4
		3.12, dd (12.9, 17.1)			3.10, dd (13.0, 17.0)			3.12, dd (13.0, 17.0)	
4	196.5, C			195.1, C			196.9, C		
5	161.5, C			161.1, C			161.2, C		
6	105.6, C			108.7, C			109.0, C		
7	166.0, C			167.9, C			168.6, C		
8	94.6, CH	5.95, s	6, 7, 9, 10	94.9, CH	5.92, s	6, 7, 9, 10	94.9, CH	5.92, s	6, 7, 9, 10
9	161.3, C			160.9, C			161.4, C		
10	101.2, C			100.8, C			100.2, C		
1′	129.6, C			129.6, C			130.3, C		
2′	127.5, CH	7.33, d (8.4)	2, 4′, 6′	103.5, CH	6.79, s	2, 1′, 3′, 4′, 5′, 6′	103.5, CH	6.80, s	2, 1′, 3′, 4′, 5′, 6′
3′	114.8, CH	6.83, d (8.4)	1′, 4′, 5′	147.7, C			148.0, C		
4′	157.5, C			135.1, C			135.2, C		
5′	114.8, CH	6.83, d (8.4)	1′, 3′, 5′	147.7, C			148.0, C		
6′	127.5, CH	7.33, d (8.4)	2, 2′, 4′	103.5, CH	6.79, s	2, 1′, 2′, 3′, 4′, 5′	103.5, CH	6.80, s	2, 1′, 2′, 3′, 4′, 5′
1″	28.3, CH₂	2.78, m	5, 6, 7, 2″	20.3, CH₂	3.24, br d (ov)	5, 6, 7, 2″, 3″	20.3, CH₂	3.25, d (7.1)	5, 6, 7, 2″, 3″
		2.94, m							
2″	74.4, CH	4.37, m	6, 1″, 4″, 5″	123.2, CH	5.29, t (ov)	4″, 5″	123.4, CH	5.29, t (7.1)	5″
3″	151.3, C			133.7, C			133.6, C		
4″	107.9, CH₂	4.77, s	2″, 5″	14.6, CH₃	1.80, s	2″, 3″, 5″	14.7, CH₃	1.80, s	2″, 3″, 5″
		4.96, s							
5″	31.1, CH₂	2.18, m	4″, 6″, 7″	36.2, CH₂	2.03, m	2″, 3″, 6″, 7″	35.8, CH₂	2.03, m	3″, 4″, 6″
					2.24, m			2.22, m	
6″	26.1, CH₂	2.18, m	3″, 5″, 8″	29.1, CH₂	1.33, m		29.1, CH₂	1.34, m	7″
					1.73, m			1.66, m	
7″	124.1, CH	5.18, br s		77.5, CH	3.25, br s	5″, 8″	74.6, CH	3.41, br s	8″
8″	130.8, C			72.2, C			77.1, C		
9″	24.4, CH₃	1.70, s	7″, 8″, 10″	24.1, CH₃	1.16, s	7″, 8″, 10″	19.8, CH₃	1.13, s	7″, 8″, 10″
10″	16.2, CH₃	1.65, s	7″, 8″, 9″	23.4, CH₃	1.13, s	7″, 8″, 9″	19.1, CH₃	1.09, s	7″, 8″, 9″
MeO-3′				55.3, CH₃	3.88, s	3′	55.4, CH₃	3.88, s	3′
MeO-5′				55.3, CH₃	3.88, s	5′	55.4, CH₃	3.88, s	5′
MeO-8″							47.8, CH₃	3.176, s	8″
								3.182, s	

mg), and a subsequent preparative TLC separation yielded compounds **5** (20 mg), **6** (17 mg), and **20** (3 mg). Finally, fraction PT4-14 was separated using RP-HPLC (with a gradient of redistilled MeCN and 0.2% HCOOH) and yielded compounds **10** (20 mg), **11** (71 mg), and **21** (242 mg), as well as compounds **12** (4 mg), **8** (3 mg), and **9** (3 mg), which were purified using preparative TLC (benzene−EtOAc, 3:2). The isolation procedure used to obtain compounds **22** and **23** has been described in previous work.[5] The purity of the isolated compounds was evaluated using HPLC-DAD analysis and exceeded 95% in all cases. (Note: To eliminate hazardous effects on health, all separation procedures using benzene were performed in a fumehood using proper protective aid.)

Tomentodiplacone L (1): brownish, amorphous substance; $[\alpha]^{22}_D$ −15.9 (c 1.0, CH₃OH); UV (CH₃OH) λ_{max} (log ε) 206 (4.89), 295 (4.59), 336 (sh) (3.79); CD (CH₃OH) Θ_{332} +7670, Θ_{293} −33 913, Θ_{222} +22 987; IR (ATR) ν_{max} 3428, 3271, 2967, 2919, 1653, 1608, 1515, 1454 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; ESIMS (negative) m/z 453 [M − H]⁻; HRESIMS (negative) m/z 453.1916 [M − H]⁻, calcd for C₂₆H₂₉O₇⁻, 453.1913.

Tomentodiplacone M (2): brown, amorphous substance; $[\alpha]^{22}_D$ −7.6 (c 1.0, CH₃OH); UV (CH₃OH) λ_{max} (log ε) 205 (4.84), 232 (sh) (4.55), 292 (4.42), 338 (sh) (3.71); CD (CH₃OH) Θ_{332} +9299, Θ_{293} −42 528, Θ_{223} +32 948; IR (ATR) ν_{max} 3368, 3229, 2908, 2852,

1635, 1593, 1515, 1454 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; ESIMS (negative) m/z 453 [M − H]⁻; HRESIMS (negative) m/z 453.1921 [M − H]⁻, calcd for C₂₆H₂₉O₇⁻, 453.1913.

Tomentodiplacol B (3): yellow, amorphous substance; $[\alpha]^{22}_D$ +9.6 (c 0.3, CH₃OH); UV (CH₃OH) λ_{max} (log ε) 204 (4.83), 230 (4.51), 237 (sh) (4.48), 277 (4.62), 291 (sh) (4.45), 365 (4.09); CD (CH₃OH) Θ_{370} +3646, Θ_{296} −14 162, Θ_{265} +9192; IR (ATR) ν_{max} 3360, 2967, 2919, 2851, 1629, 1514, 1454 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; ESIMS (negative) m/z 451 [M − H]⁻; HRESIMS (negative) m/z 451.1741 [M − H]⁻, calcd for C₂₆H₂₇O₇⁻, 451.1757.

3′,4′-O-Dimethyl-5′-hydroxydiplacone (4): brown, amorphous substance; $[\alpha]^{22}_D$ −21.4 (c 1.0, CH₃OH); UV (CH₃OH) λ_{max} (log ε) 209 (4.85), 232 (sh) (4.58), 294 (4.42), 336 (sh) (3.71); CD (CH₃OH) Θ_{331} +13 273, Θ_{292} −65 239; IR (ATR) ν_{max} 3336, 2913, 2848, 1630, 1589, 1509, 1446 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; ESIMS (negative) m/z 467 [M − H]⁻; HRESIMS (negative) m/z 467.2073 [M − H]⁻, calcd for C₂₇H₃₁O₇⁻, 467.2070.

Mimulone F (5): yellow, amorphous substance; $[\alpha]^{22}_D$ −2.1 (c 0.85, CH₃OH); UV (CH₃OH) λ_{max} (log ε) 204 (4.58), 227 (4.50), 294 (4.31), 334 (sh) (3.60); CD (CH₃OH) Θ_{331} +8762, Θ_{291} −44 776; IR (ATR) ν_{max} 3127, 2973, 1624, 1584, 1516, 1487, 1442 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; ESIMS (negative) m/z 441

Table 4. NMR Spectroscopic Data (400 MHz, MeOD for 10 and 11, CDCl₃ for 12) for Tomentodiplacone N (10), Tomentone (11), and 3′,4′,5′-Trimethoxyflavanone (12)

position	tomentodiplacone (10) δ_C, type	δ_H (J in Hz)	HMBC	tomentone (11) δ_C, type	δ_H (J in Hz)	HMBC	3′,4′,5′-trimethoxyflavanone (12) δ_C, type	δ_H (J in Hz)	HMBC
2	79.1, CH	5.33, br d (12.1)		79.2, CH	5.32, dd (3.0, 13.0)	3, 1′	78.9, CH	5.36, br s	4, 1′, 2′, 6′
3	42.1, CH₂	2.72, br d (16.7)	2, 4	42.6, CH₂	2.74, dd (3.0, 17.0)	2, 4, 1′	42.8, CH₂	2.85, dd (2.8, 17.0)	4
		3.15, br d (16.7)			3.12, dd (13.0, 17.0)			3.09, dd (13.0, 17.0)	
4	196.9, C			196.8, C			194.8, C		
5	160.2, C			157.8, C			164.5, C		
6	100.4, C			102.1, C			95.9, CH	6.04, s	7, 10
7	162.1, C			162.3, C			164.3, C		
8	95.4, CH	5.93, s	6, 7, 9, 10	95.1, CH	5.90, s	6, 7, 9, 10	95.9, CH	6.04, s	7, 10
9	161.1, C			160.3, C			n.d.		
10	102.2, C			100.4, C			103.0, C		
1′	130.5, C			130.1, C			133.7, C		
2′	109.8, CH	7.08, br s	2, 4′, 6′	113.2, CH	6.93, br s	2, 1′, 3′, 4′	103.2, CH	6.69, s	2, 1′, 3′, 4′, 6′
3′	147.9, C			145.5, C			153.6, C		
4′	146.9, C			117.8, CH	6.81, br s	2′, 5′	138.4, C		
5′	114.5, CH	6.83, d (7.7)	1′, 3′	145.1, C			153.6, C		
6′	119.2, CH	6.92, br d (7.7)	2, 2′, 4′	114.8, CH	6.81, br s	2, 1′, 2′, 5′	103.2, CH	6.69, s	2, 1′, 2′, 4′, 5′
1″	24.0, CH₂	2.54, m	5, 6, 7, 2″, 3″	115.2, CH	6.64, d (10.2)	5, 6, 7, 3″			
		2.86, m							
2″	66.3, CH	3.85 (ov)	6, 4″	124.7, CH	5.53, d (10.2)	6, 3″, 5″			
3″	80.3, C			80.6, C					
4″	17.7, CH₃	1.27, s	2″, 3″, 5″	26.0, CH₃	1.40, s	2″, 3″, 5″			
		1.28, s			1.41, s				
5″	37.5, CH₂	1.67, t (ov)	3″, 6″	41.2, CH₂	1.70, m	6″, 7″			
6″	21.1, CH₂	2.16, m	5″, 7″, 8″	22.1, CH₂	2.09, q (7.5)	5″, 7″, 8″			
7″	123.8, CH	5.13, t (6.7)	6″, 9″, 10″	123.5, CH	5.11, t (7.5)	9″, 10″			
8″	131.0, C			131.2, C					
9″	24.3, CH₃	1.69, s	7″, 8″, 10″	24.2, CH₃	1.66, s	7″, 8″, 10″			
10″	16.2, CH₃	1.62, s	7″, 8″, 9″	16.1, CH₃	1.58, s	7″, 8″, 9″			
MeO-3′	54.8, CH₃	3.88, s	3′				56.1, CH₃	3.92, s	3′
MeO-4′							60.8, CH₃	3.89, s	4′
MeO-5′							56.1, CH₃	3.92, s	5′

[M − H]⁻; HRESIMS (negative) m/z 441.1935 [M − H]⁻, calcd for C₂₅H₂₉O₇⁻, 441.1913.

Mimulone G (6): yellow, amorphous substance; [α]²²_D −3.1 (c 0.85, CH₃OH); UV (CH₃OH) λ_max (log ε) 203 (4.51), 227 (4.42), 295 (4.24), 337 (sh) (3.53); CD (CH₃OH) Θ₃₃₂ +6813, Θ₂₉₁ −33 636; IR (ATR) ν_max 3243, 2927, 1632, 1599, 1515, 1446 cm⁻¹; ¹H and ¹³C NMR data, see Table 2; ESIMS (negative) m/z 455 [M − H]⁻; HRESIMS (negative) m/z 455.2066 [M − H]⁻, calcd for C₂₆H₃₁O₇⁻, 455.2070.

Mimulone H (7): brownish, amorphous substance; [α]²²_D −0.1 (c 0.8, CH₃OH); UV (CH₃OH) λ_max (log ε) 204 (4.47), 226 (sh) (4.35), 294 (4.14), 334 (sh) (3.52); CD (CH₃OH) Θ₃₃₅ +6898, Θ₂₉₁ −34 360; IR (ATR) ν_max 3242, 2961, 2920, 1635, 1599, 1516, 1450 cm⁻¹; ¹H and ¹³C NMR data, see Table 3; ESIMS (negative) m/z 423 [M − H]⁻; HRESIMS (negative) m/z 423.1778 [M − H]⁻, calcd for C₂₅H₂₇O₆⁻, 423.1808.

Paulownione A (8): yellowish, amorphous substance; [α]²²_D +4.8 (c 0.3, CH₃OH); UV (CH₃OH) λ_max (log ε) 208 (4.63), 233 (sh) (4.29), 293 (4.14), 336 (sh) (3.55); IR (ATR) ν_max 3385, 2931, 1634, 1520, 1456 cm⁻¹; ¹H and ¹³C NMR data, see Table 3; ESIMS (negative) m/z 501 [M − H]⁻; HRESIMS (negative) m/z 501.2157 [M − H]⁻, calcd for C₂₇H₃₃O₉⁻, 501.2125.

Paulownione B (9): yellowish, amorphous substance; [α]²²_D −4.0 (c 0.3, CH₃OH); UV (CH₃OH) λ_max (log ε) 207 (4.35), 234 (sh) (3.97), 294 (3.83), 335 (sh) (3.29); IR (ATR) ν_max 3352, 2924, 2850,

1632, 1605, 1519, 1452 cm⁻¹; ¹H and ¹³C NMR data, see Table 3; ESIMS (negative) m/z 515 [M − H]⁻; HRESIMS (negative) m/z 515.2277 [M − H]⁻, calcd for C₂₈H₃₅O₉⁻, 515.2281.

Tomentodiplacone N (10): brown, amorphous substance; [α]²²_D −6.7 (c 0.6, CH₃OH); UV (CH₃OH) λ_max (log ε) 205 (4.84), 232 (sh) (4.56), 294 (4.48), 328 (sh) (3.75); CD (CH₃OH) Θ₃₃₄ +14 516, Θ₂₉₅ −60 729, Θ₂₂₄ +49 652; IR (ATR) ν_max 3371, 2967, 2915, 2846, 1632, 1605, 1580, 1515, 1444 cm⁻¹; ¹H and ¹³C NMR data, see Table 4; ESIMS (negative) m/z 453 [M − H]⁻; HRESIMS (negative) m/z 453.1965 [M − H]⁻, calcd for C₂₆H₂₉O₇⁻, 453.1913.

Tomentone (11): brown, amorphous substance; [α]²²_D +12.6 (c 0.5, CH₃OH); UV (CH₃OH) λ_max (log ε) 204 (4.56), 230 (sh) (4.17), 265 (sh) (4.30), 274 (4.38), 295 (sh) (4.07), 310 (sh) (3.90), 356 (4.33); CD (CH₃OH) Θ₃₃₃ +771, Θ₂₉₄ −5320; IR (ATR) ν_max 3323, 2965, 2924, 1594, 1520, 1446 cm⁻¹; ¹H and ¹³C NMR data, see Table 4; ESIMS (negative) m/z 421 [M − H]⁻; HRESIMS (negative) m/z 421.1688 [M − H]⁻, calcd for C₂₅H₂₅O₆⁻, 421.1651.

3′,4′,5′-Trimethoxyflavanone (12): yellowish, amorphous substance; [α]²²_D −52.8 (c 0.3, CH₃OH); UV (CH₃OH) λ_max (log ε) 207 (4.59), 229 (sh) (4.22), 289 (4.08), 329 (sh) (3.65); CD (CH₃OH) Θ₃₂₈ +6936, Θ₂₈₈ −33 751; IR (ATR) ν_max 3356, 2921, 2847, 1617, 1588, 1507, 1454 cm⁻¹; ¹H and ¹³C NMR data, see Table 4; ESIMS (negative) m/z 345 [M − H]⁻; HRESIMS (negative) m/z 345.0935 [M − H]⁻, calcd for C₁₈H₁₇O₇⁻, 345.0974.

Cell Culture and Differentiation to Macrophages. The THP-1 human monocytic leukemia cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 medium (PAA) supplemented with antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Sigma-Aldrich), 10% FBS (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich). Cultures were kept in an incubator at 37 °C in a water-saturated atmosphere of air containing 5% CO_2. Cells were passaged at approximately three-day intervals. Stabilized cells (3rd–15th passage) were split into microtitration plates to obtain a concentration of 500 000 cells/mL, differentiation to macrophages was induced by phorbol myristate acetate (PMA) dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 50 ng/mL, and the cells were incubated for 24 h. In comparison with monocytes, differentiated macrophages tend to adhere to the bottom of the cultivation plates. Unattached cells were washed out with PBS (PAA) and incubated with fresh complete RPMI medium, i.e., containing antibiotics and FBS but without PMA, for the next 24 h. The medium was then aspirated, and the cells were washed with PBS and cultivated for another 24 h in serum-free RPMI 1640 medium. These prepared macrophages were used for the detection of the inflammatory-like response.

Cytotoxicity Testing. THP-1 cells (floating monocytes, 500 000 cells/mL) were incubated in 100 μL of a serum-free RPMI 1640 medium and seeded into 96-well plates in triplicate at 37 °C. Measurements were taken 24 h after treatment with increasing concentrations of the test compounds dissolved in DMSO. Viability was measured by the cell proliferation reagent WST-1 (Roche, Basel, Switzerland) according to the manufacturer's manual. The amount of formazan created (which correlates to the number of metabolically active cells in the culture) was calculated as a percentage of the control cells, which were treated only with DMSO and were assigned as 100%. The cytotoxicity of tomentodiplacone B (**17**) and diplacone (**22**) has been measured previously.[4,29]

Test Compound Treatment and Evaluation of Anti-inflammatory Potential. Differentiated macrophages (500 000 cells/mL; 100 μL in the 96-well plate, 1 mL in the 24-well plate, 3 mL in the six-well plate) were pretreated for 1 h with 2 μM solutions of the test compounds or prednisone dissolved in DMSO (the final DMSO concentration was 0.1%) and with 0.1% DMSO solution alone (the vehicle); the used concentrations of the test compounds did not have cytotoxic effects (cell viability > 94%). The inflammatory-like response was triggered by adding 1.0 μg/mL lipopolysaccharide isolated from *Escherichia coli* 0111:B4 (Sigma-Aldrich) and dissolved in water to the pretreated macrophages; the control cells were left without LPS treatment. Each experiment was run in triplicate.

Isolation of RNA and Evaluation of Gene Expression. THP-1 macrophages were pretreated with compounds **4**, **7**, **10**, **14**, **22**, or prednisone at a concentration of 2 μM or only with DMSO for 1 h. LPS was then added to trigger an inflammatory-like response. Four hours later, the medium was aspirated and the total RNA was isolated directly from the cells in cultivation plates using a RealTime Ready cell lysis kit (Roche), according to the manufacturer's instructions. The gene expression of TNF-α and β-actin was quantified by two-step reverse-transcription quantitative (real-time) PCR (RT-qPCR). The reverse transcription step was performed with a Transcriptor Universal cDNA Master (Roche) using cell lysate as the template. The reaction consisted of three steps: (1) primer annealing at 29 °C for 10 min, (2) reverse transcription at 55 °C for 10 min, and (3) transcriptase inactivation at 85 °C for 5 min. A Fast Start Universal Probe Master (Roche) and Gene Expression assays (Applied Biosystems) were used for qPCR. These assays contain specific primers and TaqMan probes that bind to an exon−exon junction to avoid DNA contamination. The parameters for the qPCR work were adjusted according to the manufacturer's recommendations: 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The results were normalized to the amount of ROX reference dye, and the change in gene expression was determined by the $2^{-\Delta\Delta CT}$ method.[30] Transcription of the control cells was set as 1.0, and other experimental groups were multiples of this value.

Evaluation of Cytokine Secretion by ELISA. Macrophages, which had been pretreated with the test compounds for 1 h, were incubated with LPS for the next 24 h. After this period, the medium was collected and the concentration of TNF-α was measured using an Instant ELISA kit (eBioscience, San Diego, CA, USA), according to the manufacturer's manual.

Determination of the Degradation of IκB by Western Blot. Macrophage-like THP-1 cells were pretreated with the test compounds and stimulated by LPS as described above. Thirty minutes after the addition of LPS, the medium was aspirated and the cells were washed with cold PBS. Subsequently, the cells were collected using lysis buffer [50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 270 mM sucrose, and 0.1% (v/v) Triton X-100] and a scraper. The lysis of cells was facilitated by a short (∼1 min) incubation in an ultrasonic water bath. The protein concentration was measured using a Bradford method protein assay kit (Amresco, Solon, OH, USA) according to the manufacturer's instructions. For the separation of protein, 18 μg of the proteins was loaded onto a 12% polyacrylamide gel. Then, they were transferred electrophoretically to PVDF (polyvinylidene fluoride) membranes that were subsequently blocked using 5% BSA dissolved in TBST buffer [150 mM NaCl, 10 mM Tris base, and 0.1% (v/v) Tween-20]. The membranes were incubated with the primary mouse anti-IκB-α antibody (Cell Signaling, Danvers, MA, USA) at a concentration of 1:500 or with the primary mouse anti-β-actin (Abcam, Cambridge, UK) at a concentration of 1:5000 at 4 °C for 16 h. After washing, the secondary anti-mouse IgG antibody (Sigma-Aldrich), diluted 1:2000, was applied to the membranes, and they were incubated for 1 h at the laboratory temperature (∼22 °C). The amount of the bound secondary antibody was determined colorimetrically using an Opti-4CN kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instruction.

Detection of Activation of the NF-κB. Prepared macrophage-like THP-1 cells were prepared at a concentration of 500 000 cells/mL in serum-free RPMI 1640 medium and seeded onto chambered Permanox slides (Thermo Fisher Scientific) in volumes of 300 μL. Cells were pretreated for 1 h with the test compounds **4**, **7**, **10**, **14**, and **22** and prednisone at a concentration of 2 μM. After the preincubation of the test samples, 1 μg/mL LPS was added to activate the NF-κB pathway, and the cells were cultivated for the next 4 h. The transcription factor NF-κB (p65) was visualized immunohistochemically using rabbit polyclonal anti-NF-κB p65 antibody (Abcam, ab7970) as the primary antibody and anti-rabbit IgG−FITC conjugate (Sigma-Aldrich) as the secondary antibody, as described previously.[31] Used primary anti-p65 antibody recognizes the C-terminal end of p65, which also serves as a binding site for IκB-α. Hence, this antibody is able to bind to p65 only after IκB-α degradation. An Axioskop 40 equipped with an appropriate set of filters (Carl Zeiss, Oberkochen, Germany) was used for the microscopic observations. Photographs were taken using a digital microscope camera (ProgRess MF, Jenoptik, Jena, Germany). The NIS-element program (Czech Republic) was used to process the images.

Detection of the Generation of ROS. Macrophage-like cells were prepared as described for the translocation of the NF-κB nucleus. To detect the potential ability of the test flavonoids **4**, **7**, **10**, **14**, and **22** and prednisone to inhibit the production of ROS in the presence of LPS, the cells were pretreated for 1 h with the test compounds at a concentration of 2 μM. After preincubation with the test samples, LPS (1 μg/mL) was added to stimulate the generation of ROS, and the cells were cultivated for the next 24 h. To measure the ability of the test compounds to induce the generation of reactive oxygen species, the experiment was repeated with cells cultivated without LPS. The amount of ROS was detected by using CellROX Deep Red Reagent (Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Briefly, the cells were incubated with the reagent (5 mM) for 30 min at 37 °C, washed three times with PBS (0.05 M, pH 7.4), and observed under a fluorescence microscope (Axioskop 40, Carl Zeiss) equipped with an appropriate set of filters. Photographs were taken using a digital microscope camera (ProgRess

MF, Jenoptik). The NIS-element program (Czech Republic) was used to process the images.

Statistical Evaluation. The cytotoxicity data were expressed as the percentage of viability, with 100% representing the treatment with vehicle (DMSO). The IC_{50} values were calculated from viability curves. The results are presented as arithmetic mean ± standard error of the mean (SE). The statistically significant differences between individual groups during anti-inflammatory activity testing were assessed by the one-way ANOVA test, followed by Tukey's post hoc test for multiple comparisons. GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA) was used for the analysis.

Test Method for log K_{ow} Determination. *n*-Octanol (Sigma-Aldrich) saturated with water and water saturated with *n*-octanol were used. Each of the compounds was weighed (with an accuracy of 0.000 01 g) into a 25 mL volumetric flask, and 15 mL of the organic phase was added. The concentration of each compound was controlled at 0.008 mol/L. Ten milliliters of the organic phase solution was put into a 250 mL conical glass flask, and 40 mL of the water phase was added. The system was shaken at 25 °C for 1 h and then centrifuged. After allowing 24 h for the mixture to stabilize, the final concentration was measured using an Agilent 1100 Series HPLC with a DAD detector.[32] Measurements were made at 40 °C on an Ascentis Express RP-amide column (10 cm × 2.1 mm, particle size 2.7 μm, Sigma-Aldrich). A combination of gradient and isocratic elution was used: at time zero 10% MeCN and 90% 0.2% HCOOH, at the 36th minute 100% MeCN, and at the 40th minute 100% MeCN. A flow rate of 0.3 mL/min was applied, and the injection volume was adjusted to 20 μL for the aqueous phase and 0.5 μL for the organic phase (the peak area PAo was calculated to be 40-fold then). K_{ow} values were calculated as the peak area in the organic phase divided by the peak area in the water phase, and the logarithm was then calculated as follows:

$$\log(K_{ow}) = \log\left(\frac{PAo}{PAw}\right)$$

Prednisone was excluded from the data set because it has a different structure and thus a different mechanism of action. A linear regression model was tested.

$$\log(TNF - alpha) = -3.91 \log(k') + 16.75$$

ASSOCIATED CONTENT

ⓈSupporting Information

Additional figures illustrating the NMR spectra (Figures S1–S60) and IC_{50} values (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
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REFERENCES

(1) Duke, J. A.; Ayensu, E. S. *Medicinal Plants of China*; Reference Publications, Inc.: Algonac, MI, 1985; p 582.

(2) Schneiderová, K.; Šmejkal, K. *Phytochem. Rev.* **2014**, DOI: 10.1007/s11101-014-9376-y.

(3) Wollenweber, E.; Wehde, R.; Christ, M.; Dörr, M. *Nat. Prod. Commun.* **2008**, 3, 1285−1287.

(4) Šmejkal, K.; Grycová, L.; Marek, R.; Lemiére, F.; Jankovská, D.; Forejtníková, H.; Vančo, J.; Suchý, V. *J. Nat. Prod.* **2007**, 70, 1244−1248.

(5) Šmejkal, K.; Chudík, S.; Klouček, P.; Marek, R.; Cvačka, J.; Urbanová, M.; Julínek, O.; Kokoška, L.; Šlapetová, T.; Holubová, P.; Zima, A.; Dvorská, M. *J. Nat. Prod.* **2008**, 71, 706−709.

(6) Asai, T.; Hara, N.; Kobayashi, S.; Kohshima, S.; Fujimoto, Y. *Phytochemistry* **2008**, 69, 1234−1241.

(7) Šmejkal, K.; Svačinová, J.; Šlapetová, T.; Schneiderová, K.; Dall'Acqua, S.; Innocenti, G.; Závalová, V.; Kollár, P.; Chudík, S.; Marek, R.; Julínek, O.; Urbanová, M.; Kartal, M.; Csöllei, M.; Doležal, K. *J. Nat. Prod.* **2010**, 73, 568−572.

(8) Šmejkal, K.; Holubová, P.; Zima, A.; Muselík, J.; Dvorská, M. *Molecules* **2007**, 12, 1210−1219.

(9) Navrátilová, A.; Schneiderová, K.; Veselá, D.; Hanáková, Z.; Fontana, A.; Dall'Acqua, S.; Cvačka, J.; Innocenti, G.; Novotná, J.; Urbanová, M.; Pelletier, J.; Čížek, A.; Žemličková, H.; Šmejkal, K. *Phytochemistry* **2013**, 89, 104−113.

(10) Kim, S.-K.; Cho, S.-B.; Moon, H.-I. *Phytother. Res.* **2010**, 24, 1898−1900.

(11) Schneiderová, K. *Identification of Secondary Metabolites from Paulownia tomentosa Fruits*. Ph.D. Thesis, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic, 2013; p 216.

(12) Schneiderová, K.; Šlapetová, T.; Hrabal, R.; Dvořáková, H.; Procházková, P.; Novotná, J.; Urbanová, M.; Cvačka, J.; Šmejkal, K. *Nat. Prod. Res.* **2013**, 27, 613−618.

(13) Cho, J. K.; Curtis-Long, M. J.; Lee, K. H.; Kim, D. W.; Ryu, H. W.; Yuk, H. J.; Park, K. H. *Bioorg. Med. Chem.* **2013**, 21, 3051−3057.

(14) Hošek, J.; Závalová, V.; Šmejkal, K.; Bartoš, M. *Folia Biol.* **2010**, 56, 124−130.

(15) Zima, A.; Hošek, J.; Treml, J.; Muselík, J.; Suchý, P.; Pražanová, G.; Lopes, A.; Žemlička, M. *Molecules* **2010**, 15, 6035−6049.

(16) Procházková, D.; Boušová, I.; Wilhelmová, N. *Fitoterapia* **2011**, 82, 513−523.

(17) Kim, P. K.; Son, K. H.; Chang, H. W.; Kang, S. S. *J. Pharmacol. Sci.* **2004**, 96, 229−245.

(18) García-Lafuente, A.; Guillamón, E.; Villares, A.; Rostagno, M. A.; Martínez, J. A. *Inflamm. Res.* **2009**, 58, 537−552.

(19) Costa, G.; Francisco, V.; Lopes, M. C.; Cruz, M. T.; Batista, M. T. *Curr. Med. Chem.* **2012**, 19, 2876−2900.

(20) Rosselli, S.; Bruno, M.; Maggio, A.; Bellone, G.; Formisano, C.; Mattia, C. A.; di Micco, S.; Bifulco, G. *Eur. J. Org. Chem.* **2007**, 15, 2504−2510.

(21) Wollenweber, E.; Roitman, J. N. *Nat. Prod. Commun.* **2007**, 2, 385−389.

(22) Slade, D.; Ferreira, D.; Marais, J. P. J. *Phytochemistry* **2005**, 66, 2177−2215.

(23) Šmejkal, K. *Phytochem. Rev.* **2014**, 13, 245−275.

(24) González, R.; Ballester, I.; López-Posadas, R.; Suárez, M. D.; Zarzuelo, A.; Martínez-Augustin, O.; Sánchez de Medina, F. *Crit. Rev. Food Sci. Nutr.* **2011**, 51, 331−362.

(25) Hošek, J.; Bartoš, M.; Chudík, S.; Dall'Acqua, S.; Innocenti, G.; Kartal, M.; Kokoška, L.; Kollár, P.; Kutil, Z.; Landa, P.; Marek, R.; Závalová, V.; Žemlicka, M.; Šmejkal, K. *J. Nat. Prod.* **2011**, 74, 614−619.

(26) Perkins, N. D.; Gilmore, T. D. *Cell Death Differ.* **2006**, 13, 759−772.

(27) Gloire, G.; Legrand-Poels, S.; Piette, J. *Biochem. Pharmacol.* **2006**, 72, 1493−1505.

(28) Hošek, J.; Toniolo, A.; Neuwirth, O.; Bolego, C. *J. Nat. Prod.* **2013**, 76, 1586−1591.

(29) Kollár, P.; Bárta, T.; Závalová, V.; Šmejkal, K.; Hampl, A. *Br. J. Pharmacol.* **2011**, 162, 1534−1541.

(30) Livak, K. J.; Schmittgen, T. D. *Methods* **2001**, 25, 402−408.

(31) Zelová, H.; Hanáková, Z.; Čermáková, Z.; Šmejkal, K.; Dall'Acqua, S.; Babula, P.; Cvačka, J.; Hošek, J. *J. Nat. Prod.* **2014**, 77, 1297−1303.

(32) Shi, J.-Q.; Cheng, J.; Wang, F.-Y.; Flamm, A.; Wang, Z.-Y.; Yang, X.; Gao, S.-X. *Ecotoxicol. Environ. Saf.* **2012**, *78*, 134−141.

Turkish *Scorzonera* Species Extracts Attenuate Cytokine Secretion via Inhibition of NF-κB Activation, Showing Anti-Inflammatory Effect *in Vitro*

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Abstract: *Scorzonera* species are used in different folk medicines to combat many diseases, including the illnesses connected with inflammation. Previous experiments showed anti-inflammatory activity of *Scorzonera* extracts *in vivo*. *S. latifolia*, *S. cana* var. *jacquiniana*, *S. tomentosa*, *S. mollis* ssp. *szowitsii*, *S. eriophora*, *S. incisa*, *S. cinerea*, and *S. parviflora* extracts were, therefore, evaluated for their inhibitory activities of TNF-α and IL-1β production, and NF-κB nuclear translocation in THP-1 macrophages. The HPLC analysis was carried out to elucidate and to compare the composition of these extracts. Major compounds of the tested extracts have been isolated using different chromatographic techniques and further tested for their inhibitory activities on TNF-α and IL-1β production. Several extracts showed promising anti-inflammatory activity in these *in vitro* tests. Results of HPLC analysis revealed chlorogenic acid as a compound present in all tested extracts. Hyperoside, quercetin-3-*O*-β-D-glucoside and rutin were also present in varying amount in some *Scorzonera* species analyzed. Furthermore, eight phenolics which were identified as quercetin-3-*O*-β-D-glucoside (**1**), hyperoside (**2**), hydrangenol-8-*O*-glucoside (**3**), swertisin (**4**), 7-methylisoorientin (**5**), 4,5-*O*-dicaffeoyl-quinic acid (**6**), 3,5-di-*O*-caffeoyl-quinic acid (**7**), and chlorogenic acid (**8**) have been isolated as major phenolic compounds of the tested extracts and, together with eight terpenoids (**9–16**) previously obtained from different *Scorzonera* species, have been tested for the inhibition of TNF-α production, unfortunately with no activity comparable with standard.

Keywords: anti-inflammatory activity; IL-1β; NF-κB; *Scorzonera*; phenolic; TNF-α; triterpen

1. Introduction

Inflammation is a coordinated and complex biological reaction involving various pro-inflammatory and anti-inflammatory cellular proteins, enzymes, and cytokines [1–5]. Among the pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin 1-β (IL-1β) have a wide range of biological activities on numerous cell types and are reported to be involved in the pathogenesis of various inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel disease, osteoarthritis, psoriasis, endotoxemia, and/or toxic shock syndrome, different types of cancer, and degenerative diseases of the central nervous system [3,6,7]. In response to infection or injury, these cytokines are released by tissue macrophages, and serve to recruit circulating neutrophils to the site of inflammation. They may also directly modulate neutrophil functions across the vascular endothelium [6]. TNF-α, which is reported as a key cytokine in the inflammation, has a wide range of functions for maintaining the normal cellular physiology such as triggering of apoptosis, influence on secretion of cytokines e.g., IL-1, IL-6 and IL-10 as well as activation of T cells and other inflammatory cells [3,8]. TNF-α also causes further activation of the transcription factors NF-κB [9] which regulates about 200 immune, growth, and inflammation-related genes [4]. It is well established that inappropriate and prolonged activation of NF-κB has been linked to several diseases while normal activation of NF-κB is required for cell survival and immunity. Due to the pathophysiological importance of an enhanced production of inflammatory mediators through NF-κB activation, selective inhibitors of NF-κB activation may have broad application as novel therapeutics, for example, anti-inflammatory and anticancer agents [10,11].

Plants used in folk medicine serve as one of the main sources of drug discovery and development [1]. In Turkey, numerous plant species are known for their therapeutic properties and have been used in traditional Turkish folk medicine to treat a wide range of diseases. Plants from the genus *Scorzonera*, belonging to Asteraceae family, are used as food as well as medicinal plants not only in Turkey, but also in some other European countries [12–14]. Different species of *Scorzonera* have been used in European traditional medicine against pulmonary diseases, colds, for the treatment of wounds as well as for their stomachic, diuretic, galactagogue, antipyretic, and appetizing effects [12,13,15]; in Mongolian traditional medicine for the treatment of diarrhea, lung edema, parasitic diseases, and fever caused by bacterial, and viral infections [13]; in Libyan folk medicine for the treatment of hepatic pains [16]; and in Chinese, as well as in Tibetan folk medicine against breast inflammation and abscess due to their antipyretic and anti-inflammatory activities [17]. Additionally, in Turkish folk medicine different species of this genus have been reported to be used in treatment of rheumatism, pain, wound healing, as well as arteriosclerosis, kidney diseases, hypertension, and diabetes [18,19].

The extracts from some *Scorzonera* species showed hepatoprotective activity *in vivo* in CCl$_4$ induced liver damage in rats and also anti-ulcerative effect (acetic acid-induced gastric ulcer in rats), showing decrease of inflammatory markers during microscopic evaluation of stomach tissue [20]. In order to verify traditional usage of *Scorzonera* species, anti-nocieptive, anti-inflammatory, and wound healing activities have been evaluated and promising results have been obtained in our previous research [21–24]. The aim of the current study was to evaluate the ability of *Scorzonera* extracts to inhibit TNF-α and IL-1β production and NF-κB nuclear translocation in LPS-stimulated THP-1 macrophages, which may be responsible for the observed anti-inflammatory activity. Eight different *Scorzonera* species: *S. cana* (C.A. Meyer) Hoffm. var. *jacquiniana* (W. Koch) Chamb., *S. cinerea* Boiss., *S. eriophora* DC., *S. incisa* DC., *S. mollis* Bieb. ssp. *szowitzii* (DC.) Chamb., *S. latifolia* (Fisch. and Mey.) DC., *S. parviflora* Jocq., and *S. tomentosa* L., which displayed potent anti-inflammatory activity *in vivo* test models in our previous researches were selected for activity tests [22–24]. HPLC analysis of the tested *Scorzonera* extracts was also performed with aim to elucidate their composition. HPLC analytical method previously published by [22] was optimized and composition of the extracts was tested qualitatively to select main compounds for activity testing. According to this and previous work, eight phenolics: quercetin-3-*O*-β-D-glucoside (**1**), hyperoside (**2**), hydrangenol-8-*O*-glucoside (**3**), swertisin (**4**), 7-methylisoorientin (**5**), 4,5-*O*-dicaffeoyl-quinic acid (**6**), 3,5-*O*-dicaffeoyl-quinic acid (**7**), and chlorogenic acid (**8**), which were isolated as the major phenolic compounds in current study,

and terpenoids **9-16**, which have been isolated previously [taraxasterol acetate (**9**), lupeol (**10**), lupeol acetate (**11**), β-sitosterol (**12**), 3-β-hydroxy-fern-8-en-7-one-acetate (**13**), urs-12-en-11-one-3-acetyl (**14**), 3-β-hydroxy-fern-7-en-6-one-acetate (**15**), and olean-12-en-11-one-3-acetyl (**16**)] were also tested for their inhibitory effects on the TNF-α and IL-1β production.

2. Results and Discussion

This study was focused on determination of TNF-α and IL-1β production as well as NF-κB nuclear translocation inhibitory activities of *S. latifolia* (Fisch. & Mey.) DC., *S. cana* (C.A. Meyer) Hoffm. var. *jacquiniana* (W. Koch) Chamb., *S. tomentosa* L., *S. mollis* Bieb. ssp. *szowitzii* (DC.) Chamb., *S. eriophora* DC., *S. incisa* DC., *S. cinerea* Boiss., *S. parviflora* Jocq. aerial part water/methanolic extracts to reveal, if these mechanisms are playing an important role in the anti-inflammatory activity of tested extracts, which was previously observed.

LPS-activated macrophages were used for testing of the activity of the extracts. As shown in Figure 1, pretreatment of LPS activated THP-1 cells with *Scorzonera* extracts led to inhibition of TNF-α production. When compared with the vehicle-treated group, activities of all extracts, except of Ex. 2, Ex. 6 and Ex. 8 were found to be significant. Among the tested extracts, *S. tomentosa* aerial part extract has been established as the most active. Additionally, *S. latifolia* aerial part extract displayed notable activity.

Figure 1. TNF-α production inhibitory activity of *Scorzonera* species. The cells were pretreated with extracts 1–8 (Ex. 1–8) (25 μg/mL), and prednisone (1 μM), or the vehicle (Veh., DMSO) only. After 1 h of the incubation, the inflammatory response was induced by LPS [except of the control cells (CTRL)]. The secretion of TNF-α was measured 24 h after the LPS addition. The results are expressed as mean ± SE for three independent experiments. * Significant difference in comparison to vehicle only treated cells ($p < 0.05$), ** significant difference in comparison to vehicle only treated cells ($p < 0.01$), *** significant difference in comparison to vehicle only treated cells ($p < 0.001$).

The ability of the *Scorzonera* extracts to decrease IL-1β production after inflammatory stimulation was also evaluated. Inhibitory activity was observed for all *Scorzonera* extracts tested and results similar to TNF-α production inhibitory test were obtained (Figure 2). When compared with the vehicle-treated group, activities of several extracts, except of Ex. 2, Ex. 5, Ex. 6, and Ex. 8 were found to be significant in testing of IL-1β secretion inhibition.

Both cytokines, TNF-α and IL-1β, are under transcription control of NF-κB, which is activated by LPS. As visible from the Figure 3, the effect of *Scorzonera* extracts on NF-κB nuclear activation was evaluated. The anti-p65 antibody used for the assay recognizes the binding site of IκB-α on the p65 protein. The antibody binds to p65 only if the inhibitor IκB-α is degraded and NF-κB could enter to nucleus. In partial accordance with results of TNF-α and IL-1β inhibitory activity testing, Ex. 3, 4, 5,

and 7 were assigned to be most potent in inhibition of NF-κB nuclear activation. The results show that *S. tomentosa* aerial part extract has been the most active one.

Figure 2. IL-1β production inhibitory activities of *Scorzonera* species. The cells were pretreated with extracts 1–8 (Ex. 1–8) (25 μg/mL), and prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of the incubation, the inflammatory response was induced by LPS [except of the control cells (CTRL)]. The secretion of IL-1β was measured 24 h after the LPS addition. The results are expressed as mean $\pm$ SE for three independent experiments. * Significant difference in comparison to vehicle only treated cells ($p < 0.05$), ** significant difference in comparison to vehicle only treated cells ($p < 0.01$), *** significant difference in comparison to vehicle only treated cells ($p < 0.001$).

Figure 3. Graphical comparison of inhibitory activity of *Scorzonera* species on NF-κB activation. The cells were pre-treated with extracts 1–8 (Ex. 1–8) (25 μg/mL), and prednisone (1 μM), or the vehicle (DMSO) only. After 1 h of the incubation, the inflammatory response was induced by LPS [except of the control cells (CTRL)]. The NF-κB activation was measured 3 h after the LPS addition. The results are expressed as mean $\pm$ SE for three independent experiments (**A**) and quintessential pictures obtained by fluorescent camera (**B**). *** Significant difference in comparison to vehicle only treated cells ($p < 0.001$).

Later, the analysis of phenolic and terpenoid compounds isolated from *Scorzonera* species was carried out with aim to establish the compound responsible for the effect.

The chromatograms of the extract with greatest NF-κB nuclear translocation inhibitory activity Ex. 1 (*S. latifolia*) and Ex. 3 were (*S. tomentosa*) selected as representative for analysis of Ex. 1–8, and as visible from the Figure 4, compounds **1–8** [quercetin-3-*O*-β-D-glucoside (**1**), hyperoside (**2**), hydrangenol-8-*O*-glucoside (**3**), swertisin (**4**), 7-methylisoorientin (**5**), 3,5-*O*-dicaffeoyl-quinic acid (**6**), 4,5-*O*-dicaffeoyl-quinic, acid (**7**) and chlorogenic acid (**8**)] are the main UV-detectable substances observable, which have been later isolated as the major compounds from *S. latifolia* aerial part. According to our knowledge, this is the first phytochemical study related *S. latifolia* aerial part and isolation of swertisin (**4**) as well as 7-methylisoorientin (**5**) from *Scorzonera* species. Further, isolated phenolic compounds **1–8** and later also terpenoids **9–16** were tested in TNF-α and IL-1β production inhibition assay, compared to effect of prednisone. Unfortunately, no statistically significant activity of these compounds in concentration of 10 μM (lacking cytotoxic effect; Supplementary Materials: Figure S1) has been observed in comparison with prednisone, which was used as the standard.
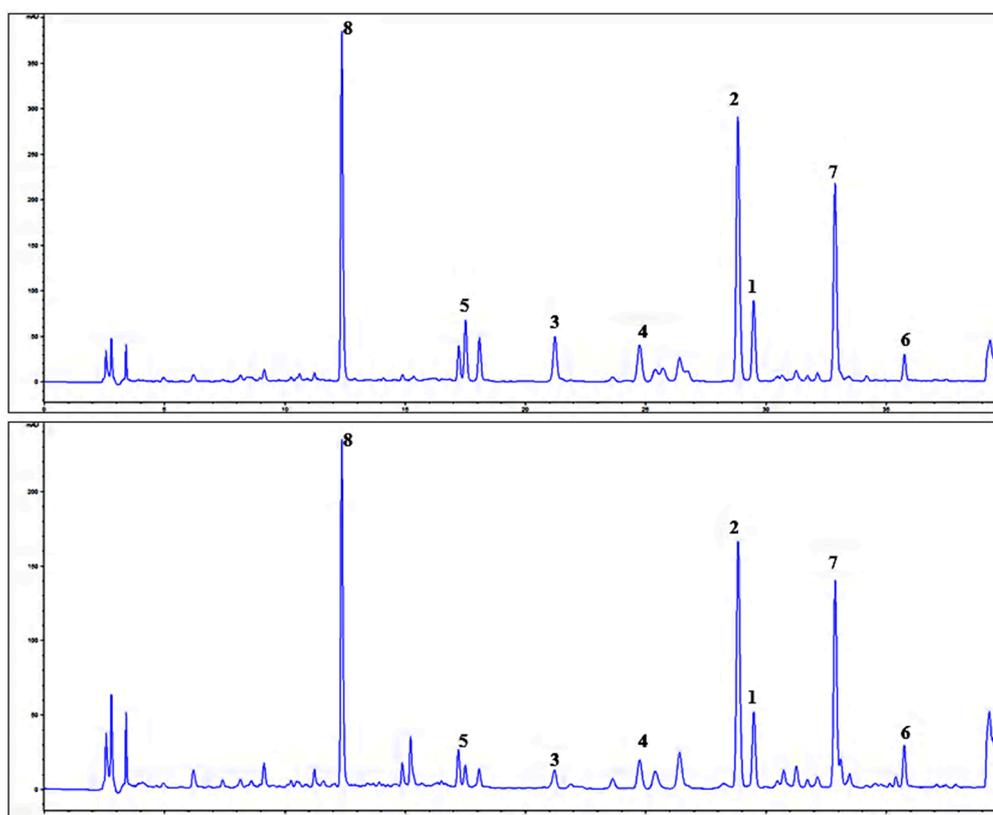
Figure 4. Chromatograms of *S. latifolia* (Ex. 1) and *S. tomentosa* (Ex. 3) aerial part extract; λ 254 nm; quercetin-3-*O*-β-D-glucoside (**1**), hyperoside (**2**), hydrangenol-8-*O*-glucoside (**3**), swertisin (**4**), 7-methylisoorientin (**5**), 3,5-*O*-dicaffeoyl-quinic acid (**6**), 4,5-*O*-dicaffeoyl-quinic acid (**7**), and chlorogenic acid (**8**).

Scorzonera species were in focus of our previous research touching testing of analgesic, anti-inflammatory, and wound healing activities. Aerial part extracts of *Scorzonera* showed significant inhibitory effects in carrageenan and PGE$_2$ induced hind paw edema model *in vivo* [21–23]. The concentrations of extracts showing the activity was 100 mg/kg of body weight of mice, and the effect was comparable with indomethacin used as the standard at the some concentration. Carrageenan-induced hind paw edema acute inflammation test model is biphasic. First phase starts with the release of histamine, serotonin, and kinins, while the second phase is related to the release

of prostaglandins like substances [25]. All of the tested *Scorzonera* extracts were found be inactive in serotonin-induced hind paw edema model [23]. Furthermore, mouse ear edema induced with TPA was also employed for the anti-inflammatory evaluation of *Scorzonera* species and the results have revealed that some *Scorzonera* displayed potent activity while some are completely inactive [23]. Mouse ear edema induced with topically-applied TPA is also an excellent acute inflammation animal model, closely related with the infiltration of neutrophil and macrophages, the induction of pro-inflammatory cytokines including TNF-α and IL-1β, and the generation of ROS including superoxide anion [26]. The anti-inflammatory activity was assayed also by acetic acid-induced capillary permeability test [24] and *S. latifolia*, *S. tomentosa*, *S. mollis* ssp. *szowitsii* showed potent effect. In the present study, extracts of *Scorzonera* species which were found previously to be active in acetic acid-induced capillary permeability test (Ex. 1 and 3) showed notable inhibitory activities on TNF-α and IL-1β production in LPS stimulated THP-1 cell lines. As visible, for example, from a comparison of the concentration used in this experiment with concentrations in previously published studies [27,28], the effect is promising enough to perform further experiments. Moreover, no sign of toxicity of extracts in our *in vitro* assay, and also in previous *in vivo* tests supports the idea of further testing.

Aerial part extracts of the *Scorzonera* species were also investigated for their effects on NF-κB nuclear translocation. The transcription factor NF-κB plays a critical role in inducible expression of genes involved in diverse biological processes, including development, immune and inflammatory responses, cell growth, cell death (apoptosis), and stress responses. NF-κB is found as an inactive form in the cytoplasm in complex bound to its inhibitory subunit IκB. Numerous stimuli activate NF-κB, mostly through IκB kinase-dependent (IKK-dependent) phosphorylation and subsequent degradation of IκB proteins. The released NF-κB dimers translocate to the nucleus, bind to DNA and activate gene transcription. NF-κB regulates a wide variety of important target genes. Among the numerous target genes of NF-κB are those encoding inflammatory and chemotactic cytokines such as interleukin-1 (IL-1), IL-2, IL-6, IL-8, and TNF, cell adhesion molecules, major histocompatibility complex class molecules, cytokine receptors, and pro-inflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2. The activation of NF-κB has been implicated in cancers and in many human chronic inflammatory diseases, such as asthma, arthritis, and inflammatory bowel disease. Therefore, the NF-κB signaling pathway is clearly established as one of most important targets for discovering drugs for the treatment of a wide variety of inflammatory diseases, autoimmune diseases as well as cancer [29,30]. Current study results show that extracts of the *Scorzonera* species (Ex. 1 and 3) inhibited significantly NF-κB activation and consequently TNF-α and IL-1β production at concentration of 25 μg/mL. This concentration was selected according to concentration used in previous assays used for analysis of *Scorzonera* anti-inflammatory potential *in vivo* [23], and it is overall comparable with concentrations of different extract selected as active in literature [31]. Therefore, this assay brings further support of the possible uses of *Scorzonera* in traditional medicines as an anti-inflammatory agent.

HPLC analysis was performed in order to identify the main content compounds of tested extracts (Ex. 1–8). The HPLC method described by Küpeli Akkol *et al.* was improved and used [22]. The results of our analyses confirmed the presence of compounds observed previously (hyperoside (**2**) and chlorogenic acid (**8**) [22–24]), and further chromatographic separation and later HPLC analysis also confirmed the presence of other phenols (**1**, **3**–**7**). According to the results, all *Scorzonera* extracts analyzed contain chlorogenic acid (**8**) as one of the main compound [22]. Furthermore, all extracts are found to be rich in flavonoid content such as hyperoside (**2**), rutin, as well as quercetin-3-*O*-β-D-glucoside (**1**). As visible, with exception of **8**, all phenolics identified in *Scorzonera* aerial part extracts are glycosides, in majority flavonoids. The presence of all these compounds (**1**–**8**) in the most active extracts (Ex. 1 and Ex. 3) and previous assays showing relatively high content of phenolics [22–24], together with our qualitative HPLC analysis and literature reports about the activity of content compounds lead us to idea of testing the isolated content compounds. Antioxidant, anti-inflammatory, and anti-nociceptive activities of chlorogenic acid (**8**) have previously

been reported [2,32,33]. It was isolated as anti-inflammatory compound from *Sambucus ebulus* L. [2] and as anti-inflammatory and analgesic compound from *Cheilanthes farinose* (Forsk.) Kaulf. [33]. An analgesic and anti-inflammatory activity of this compound was also confirmed by Dos Santos *et al.* [32] and chlorogenic acid (**8**) was found to inhibit TNF-α expression in active in reducing the arachidonic acid metabolites, nitric oxide, and pro-inflammatory cytokine production in a dose-dependent manner and under some conditions effect observed was almost comparable to ibuprofen [5]. Thus, it has been suggested that chlorogenic acid (**8**) probably could be one of the compound responsible for the anti-nociceptive and anti-inflammatory activities of *Scorzonera* species. Additionally, flavonoid glycosides and aglycones are mentioned many times as anti-inflammatory principles of food or medicinal plants, with different mode of activity, including the effect on gene expression and production of pro-inflammatory cytokines [33].

Some studies have shown that some flavonoids are modulators of pro-inflammatory cytokine production. Luteolin, apigenin, quercetin, naringenin, genistein were reported to have inhibitory effects on TNF-α production [34,35]. The properties of the flavonoids, such as quercetin and myricetin, may be mediated through down-regulation of the NF-κB pathway [36,37]. The greatest effect on the NF-κB pathway was observed after the treatment of cells with *S. tomentosa* extract (Ex. 3), which contain the highest amounts of chlorogenic acid (**8**) and hyperoside (**2**) among investigated extracts. From the above mentioned, we suggested that chlorogenic acid (**8**), derivatives of dicaffeoyl-quinic acid (**6** and **7**), and flavonoids, especially quercetin-3-*O*-β-D-glucoside (**1**) and hyperoside (**2**) could be responsible for the anti-inflammatory activity of tested extracts. Therefore, further experiments were carried out with phenolic compounds previously obtained from *Scorzonera* species (**1–8**), to assay the inhibition of TNF-α and IL-1β production in THP-1 cells, and later also with triterpenes **9–16**, because taraxasterol acetate (**9**) isolated from *S. latifolia* showed previously analgesic activity *in vivo* in writhing and tail-flick tests [21] and also other triterpenoid substances like lupeol derivatives (**10** and **11**), β-sitosterol (**12**) and other triterpenes (**13–16**) could potentially demonstrate anti-phlogistic effect [38,39]. Unfortunately, only weak activity of compounds tested was observed, and no statistically significant activity has been observed in comparison with standard used (prednisone, Supplementary Materials, Figure S1). However, the activity was assayed at concentration of 10 μM only, literature touching chlorogenic acid (**8**) showed majority of experiments published the usage and effect in substantially greater concentrations (milimolar) [32,33,40], therefore the concentration used by us could be counted as under-dosed, and the effect was not observed. The same can be said for some flavonoids, represented for example by hyperoside (**2**), which previously showed anti-inflammatory effect mediated by decreasing the production of TNF-α and IL-1β [41], but well observable at concentrations greater than 10 μM. Similarly, the activity of flavonoids reported previously [34,35] was observed at concentrations of 50 μM. Touching the triterpenes, our results confirmed the study of Srivastava *et al.* [42] showing low effect of **9** only on LPS-induced neuroinflammation in C6 rat glial cells (low inhibition of TNF-α, IFN-γ, and IL-6 release). However, this compound inhibited the superoxide radical anion generation and elastase release in assay using human neutrophils [43]. This is a good example how the method and concentration used can affect the interpretation of results, even when some anti-inflammatory effects of compounds, which we used for assay, were carried out by different techniques *in vivo*, what makes the comparison with literature more difficult. For us, the important is the comparison of compounds tested (**1–16**) with prednisone, which is commonly used as a reference compound, and which showed better effect. Other possibility explaining the discrepancy of results obtained for extracts and compounds tested is the presence of yet undetected substances in extracts, or a synergic activity could be involved. Therefore, further studies on determination of active substances responsible for the TNF-α and IL-1β production inhibition should be carried out.

As conclusion, the anti-inflammatory potential of some *Scorzonera* extracts has been confirmed using *in vitro* TNF-α and IL-1β production inhibition assay on LPS-stimulated THP-1 macrophages, which were supported by inhibition of NF-κB activation. Several compounds isolated from these

extracts were further tested for TNF-α and IL-1β production but none of the compounds presented activity. More efforts should be spent on the determination of active substances.

3. Experimental Section

3.1. Plant Material

Scorzonera species were collected in different parts of Anatolia. The taxonomic identification of the plant was confirmed by H. Duman, a plant taxonomist of the Department of Biological Sciences, Faculty of Art and Sciences, Gazi University. Voucher specimens are kept in the herbarium of Ankara University, Faculty of Pharmacy (Table 1).

Table 1. Locality of the plant material harvest and extract coding.

Plant Species	Locality	Date	Identification/Voucher Specimen No.	Code Name
S. latifolia (Fisch. & Mey.) DC.	Kars, Arpaçay	2005	H. Duman/23830	Ex. 1
S. cana (C.A. Meyer) Hoffm. var. *jacquiniana* (W. Koch) Chamb.	Ankara, Çamlıdere	2008	H. Duman/23834	Ex. 2
S. tomentosa L.	Yozgat, Akdağmadeni	2005	H. Duman/23841	Ex. 3
S. mollis Bieb. ssp. *szowitzii* (DC.) Chamb.	Ankara, Kızılcahamam	2006	M. Koyuncu/23844	Ex. 4
S. eriophora DC.	Ankara,Çubuk	2007	H. Duman/23832	Ex. 5
S. incisa DC.	Konya, Ermenek	2005	H. Duman/23833	Ex. 6
S. cinerea Boiss.	Sivas, Çetinkaya	2005	H. Duman/23829	Ex. 7
S. parviflora Jocq.	Ankara, Gölbaşı	2008	H. Duman/25894	Ex. 8

3.2. Extraction of Plant Materials for Activity

Dried and powdered aerial parts of the plants (10 g) were separately extracted with 20% aqueous methanol (100 mL) at room temperature for 24 h in three days using continual stirring. Each extract was filtered and concentrated to dryness under reduced pressure and low temperature (40–50 °C) to yield crude extracts (Ex. 1–8).

3.3. Isolation and Identification of Compounds

Compounds **1**–**8** were isolated from *S. latifolia* ethyl acetate extract (Supplementary Materials, Figure S2). Aerial part extract of the *S. latifolia* was selected due to its anti-inflammatory activity potential and sufficient quantity. Dried and powdered aerial parts of the plant (1.5 kg) were macerated in methanol (2.5 L × 5) at room temperature for 24 h. The extract was filtered and MeOH removed under reduced pressure and 40–50 °C using a rotary evaporator to get crude extract (231 g). This methanol extract was subjected to liquid-liquid fractionation. *n*-Hexane, chloroform and ethyl acetate were used. Including water part, four fractions of *S. latifolia* extract were obtained. The ethyl acetate part (21.5 g) was used for further separation by column chromatograpy. Elution was performed on silica gel (40–63 μm, Merck) column with EtOAc:MeOH:Water (100:13.5:10, *v/v/v*) solvent system to obtain 92 subfraction (120 mL). **1** was purified from subfraction 18–19 by preparative TLC on silica gel plates (Merck 5744) using EtOAc:MeOH:Water (70:13.5:10, *v/v/v*) as mobile phase. Yellow precipitate was occurred in subfraction 21, later identified as **2**. Compounds **3** and **4** were obtained from subfraction 23–24 and 30–31 in crystalline form. Subfraction 41 gave **5** as white amorphous precipitate. Substances **6**–**8** were obtained on reverse phase TLC plates (Merck 5559) with MeOH: Water (1:1, *v/v*) solvent system as eluent from subfraction 60–64 and 86–92 respectively. Their identification was carried out using HRMS and ^{1}H- and ^{13}C-NMR and comparison of data obtained with that in literature [44–46]. The exact masses were measured using LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an electrospray ion source. The mobile

phase consisted of methanol/water (4:1), flow rate of 100 μL/min, and the samples diluted with the mobile phase were injected using a 2-μL loop. The mass spectra were internally calibrated using protonated phthalic anhydride or deprotonated stearic acid as a lock mass. The ^{1}H- and ^{13}C-NMR spectra were measured on a Bruker AVANCE-600 spectrometer (^{1}H at 600.13 MHz, ^{13}C at 150.9 MHz) using cryo-probe (5 mm CPTCI ^{1}H-^{13}C/^{15}N/D Z-GRD) in DMSO-d_6 at 298 K. Structural assignment of proton and carbon signals was achieved using 2D-H,H-COSY, 2D-H,H-ROESY, 2D-H,C-HSQC, and 2D-H,C-HMBC spectra.

3.4. HPLC Analysis

HPLC analyses were carried out using Agilent LC 1100 chromatograph (Agilent Technologies, Darmstadt, Germany). The diode array detector (DAD) was set at wavelength of 254 nm and peak areas were integrated automatically using Agilent ChemStation Software. Separation was carried out using a Supelco Ascentis$^®$ (Bellefonte, Pennsylvania, PA, USA) Express RP-Amide (150 mm × 4.6 mm; 2.7 μm) column. The mobile phase was composed of acetonitrile (A) and 0.2% HCOOH (B) using gradient elution: initial A:B (8:92, *v/v*), in 10th min A:B (18:82), in 20th min. A:B (20:80, *v/v*), in 30th min 30:70 (*v/v*). This was followed isocratic flow of A:B (30:70, *v/v*) to 45th min. The flow rate was 0.5 mL/min, column temperature was maintained at 41 °C. The sample injection volume was 10 μL. The identification of compounds in extracts was carried out using the comparison of retention time and UV spectrum obtained from the analysis of single compounds previously isolated or obtained from commercial sources (**1**–**8**, MeOH solutions, Figure 5). Furthermore, a combined injection of single compound with extract was used to confirm the compound presence.
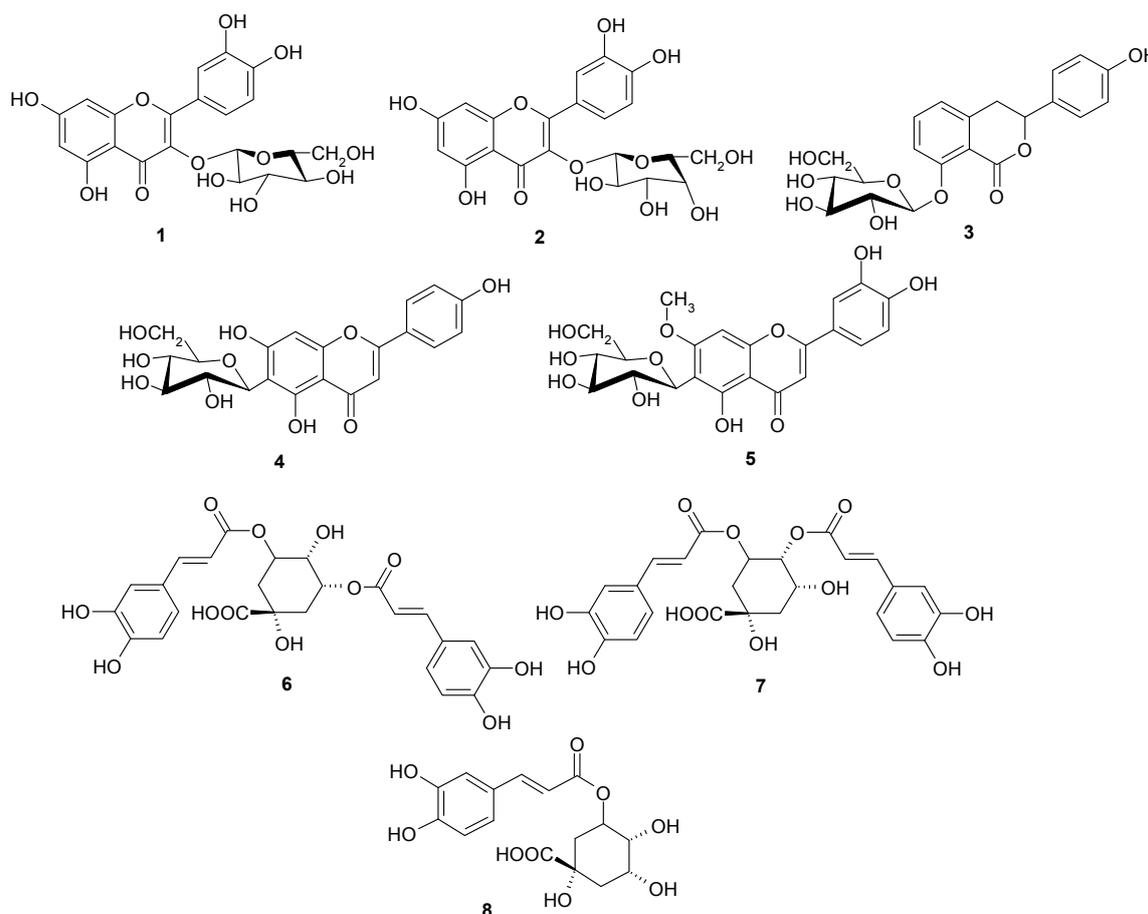
Figure 5. Phenolic constituents of *Scorzonera* species found in *Scorzonera* extracts and tested for TNF-α production inhibition.

3.5. Anti-Inflammatory Assay

3.5.1. Compounds for *In Vitro* Anti-Inflammatory Assays

Phenolic compounds **1**–**8** [quercetin-3-*O*-β-D-glucoside (**1**), hyperoside (**2**), hydrangenol-8-*O*-glucoside (**3**), swertisin (**4**), 7-methylisoorientin (**5**), 3,5-*O*-dicaffeoyl-quinic acid (**6**), 4,5-*O*-dicaffeoyl-quinic acid (**7**), chlorogenic acid (**8**)] (Figure 5) and terpenoids **9**–**16** [taraxasterol acetate (**9**), lupeol (**10**), lupeol acetate (**11**), β-sitosterol (**12**), 3-β-hydroxy-fern-8-en-7-one-acetate (**13**), urs-12-en-11-one-3-acetyl (**14**), 3-β-hydroxy-fern-7-en-6-one-acetate (**15**), olean-12-en-11-one-3-acetyl (**16**)] (Figure 6) used for assays were isolated from *Scorzonera* species according to the procedures showed in [47–49].
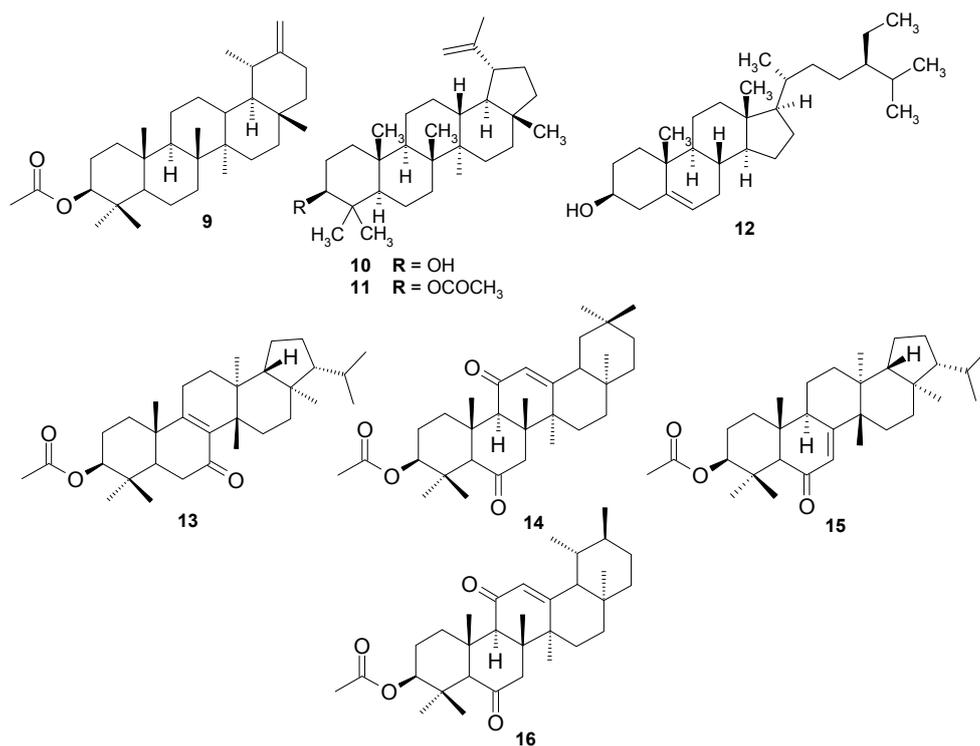
Figure 6. Terpenoid constituents of *Scorzonera* species found in *Scorzonera* extracts and tested for TNF-α production inhibition.

3.5.2. TNF-α and IL-1β Inhibitory Activity Measurement

The RPMI 1640 medium and the penicillin–streptomycin mixture were purchased from Lonza (Belgium). Foetal bovine serum (FBS), phorbol myristate acetate (PMA), prednisone, and the lipopolysaccharide (LPS) obtained from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich (Germany). Instant ELISA TNF-α Kit (eBioscience, Vienna, Austria) was used to evaluate the production of TNF-α and IL-1β. The human monocytic leukemia cell line THP-1 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK).

The cells were cultivated at 37 °C in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL of penicillin, and 100 µg/mL of streptomycin in a humidified atmosphere containing 5% of CO_2. The growth medium was changed twice a week, when cells had reached a concentration of 5×10^5–7×10^5 in mL. The viability of cells was greater than 94% throughout the experiment. Stabilized cells were split into 24-well plates to get a concentration of 100,000 cells/mL, and the differentiation into macrophages was induced by phorbol myristate acetate (PMA). To promote differentiation of monocytes to macrophages, PMA was added to the final concentration 50 ng/mL and cells were stimulated for 24 h. In comparison with monocytes, differentiated macrophages tend

to adhere on the bottom of cultivation plates. Next 24 h cells were incubated with fresh complete medium without PMA, then medium was aspirated, cells were washed with PBS and cultivated another 24 h in the serum-free RPMI 1640 medium. Prepared macrophages were consequently used for following experiments.

Differentiated macrophages were pretreated for 1 h with *Scorzonera* water methanolic extracts or compounds **1–16** dissolved in DMSO [25 μg/mL for extracts and 10 μM for compounds, respectively; this concentration lacks cytotoxic effect (data not shown)]. For comparison with conventional drugs, 1 μM prednisone dissolved in DMSO was used. Vehicle-treated cells contained the vehicle (DMSO) only. The concentration of DMSO was 0.1% in each well. The inflammatory response was triggered by adding LPS dissolved in water (1 μg/mL) to drug-pretreated macrophages, control cells remained without LPS stimulation, and the cells were incubated for another 24 h. After this time period, the medium was harvested and the concentration of TNF-α and IL-β was measured by using an Instant ELISA kits.

3.5.3. Measurement of Inhibition of Activation of NF-κB

Differentiated macrophages were treated by tested extracts and LPS as was described above. Three hours after LPS adding, the cultivation medium was removed and cells were three times carefully washed by PBS (pH = 7.4) at room temperature (2 min per each washing). After this, cells were fixed (ice-cold acetone, 3 min) and washed two times by cold PBS. Incubation of cells in PBS containing 1% BSA (*w/v*) for 30 min and in the primary antibody (rabbit polyclonal Anti-NF-κB p65 antibody (product number ab7970), Abcam, UK) overnight at 4 °C followed. Used primary anti-p65 antibody recognizes C-terminal end of p65, which also serves as binding site for IκB-α. Hence, this antibody is able to bind to p65 only after IκB-α degradation. After incubation, cells were washed three times by PBS (5 min each wash) and incubated in secondary antibody (anti-rabbit IgG—FITC conjugate, PBS containing 1% BSA, *w/v*) for 1 h at room temperature. Finally, mixture was decanted, cells were washed by PBS (three times, 5 min, in dark), incubated with DAPI (0.5 μg/mL, PBS) for 1 min and washed by PBS. Cells were observed under a fluorescence microscope (Axioskop 40, Carl Zeiss, Germany) equipped by FITC and DAPI filters (Carl Zeiss). Photographs were taken using digital microscope camera (ProgRess MF, Jenoptik, Germany). NIS-element program (Czech Republic) was used for image processing—converting into color scale concentration images—and analysis—evaluation of intensity of emission. All chemicals used in this part of experiment were purchased from Sigma-Aldrich unless otherwise specified.

3.6. Statistical Analysis

All results were presented as the mean $\pm$ standard error of the mean (S.E.M.) or as a percentage. Analysis of variance (ANOVA) was used for the statistical analysis of the data. The Tukey HSD test (Tukey´s honestly significant difference test) was used to determine the significance. Results with $p < 0.05$ were considered to be statistically significant.

4. Conclusions

The anti-inflammatory potential of some *Scorzonera* extracts has been proved in *in vitro* TNF-α and IL-1β production inhibition assay on THP-1 macrophages, and supported by analysis of NF-κB nuclear translocation inhibition, but none of the compounds tested proved the activity in present study. More effort should be spend on the determination of active substances responsible for activity.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Atanasov, A.G.; Waltenberger, B.; Pferschy-Wenzig, E.M.; Linder, T.; Wawrosch, C.; Uhrin, P.; Temml, V.; Wang, L.; Schwaiger, S.; Heiss, E.H.; *et al.* Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* **2015**. [CrossRef] [PubMed]
2. Yesilada, E. Evaluation of the anti-inflammatory activity of the Turkish medicinal plant *Sambucus ebulus*. *Chem. Nat. Prod.* **1997**, *33*, 539–540.
3. Bandgar, B.P.; Patil, S.A.; Totre, J.V.; Korbad, B.L.; Gacche, R.N.; Hote, B.S.; Jalde, S.S.; Chavan, H.V. Synthesis and biological evaluation of nitrogen-containing benzophenone analogues as TNF-a and IL-6 inhibitors with antioxidant activity. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 2292–2296. [CrossRef] [PubMed]
4. Leiro, J.M.; Varela, M.; Piazzon, M.C.; Arranz, J.A.; Noya, M.; Lamas, J. The anti-inflammatory activity of the polyphenol resveratrol may be partially related to inhibition of tumour necrosis factor-α (TNF-α) pre-mRNA splicing. *Mol. Immunol.* **2010**, *47*, 1114–1120. [CrossRef] [PubMed]
5. Chauhan, P.S.; Satti, N.K.; Sharma, V.K.; Dutt, P.; Suri, K.A.; Bani, S. Amelioration of inflammatory responses by chlorogenic acid via suppression of pro-inflammatory mediators. *J. Appl. Pharm. Sci.* **2011**, *1*, 67–75.
6. Benbarek, H.; Deby-Dupont, G.; Deby, C.; Serteyn, D. Direct stimulation of the oxidative activity of isolated equine neutrophils by TNF-α and IL-1β. *Vet. Immunol. Immunopathol.* **2008**, *121*, 101–106. [CrossRef] [PubMed]
7. Rao, P.P.N.; Kabir, S.N.; Mohamed, T. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs): Progress in Small Molecule Drug Development. *Pharmaceuticals* **2010**, *3*, 1530–1549. [CrossRef]
8. Zelova, H.; Hosek, J. TNF-α signalling and inflammation: Interactions between old acquaintances. *Inflamm. Res.* **2013**, *62*, 641–651. [CrossRef] [PubMed]
9. Cheng, J.; Chen, M.; Wallace, D.; Tith, S.; Arrhenius, T.; Kashiwagi, H.; Ono, Y.; Ishikawa, A.; Sato, H.; Kozono, T.; *et al.* Discovery and structure-activity relationship of coumarin derivatives as TNF-α inhibitors. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2411–2415. [PubMed]
10. Folmer, F.; Jaspars, M.; Solano, G.; Cristofanon, S.; Henry, E.; Tabudravu, J.; Black, K.; Green, D.H.; Küpper, F.C.; Aalbersberg, W.; *et al.* The inhibition of TNF-α-induced NF-κB activation by marine natural products. *Biochem. Pharmacol.* **2009**, *78*, 592–606. [CrossRef] [PubMed]
11. Nam, N.H.; Jae, Y.Y. NF-κB Inhibitory Activities of the Methanol Extracts and some Constituents therein of some Vietnamese Medicinal Plants. *Sci. Pharm.* **2009**, *77*, 389–399. [CrossRef]
12. Zidorn, C.; Ellmerer, E.P.; Sturm, S.; Stuppner, H. Tyrolobibenzyls E and F from *Scorzonera humilis* and distribution of caffeic acid derivatives, lignans and tyrolobibenzyls in European taxa of the subtribe Scorzonerinae (Lactuceae, Asteraceae). *Phytochemistry* **2003**, *63*, 61–67. [CrossRef]
13. Tsevegsuren, N.; Edrada, R.A.; Lin, W.; Ebel, R.; Torre, C.; Ortlepp, S.; Wray, V.; Proksch, P. Biologically Active Natural Products from Mongolian Medicinal Plants *Scorzonera divaricata* and *Scorzonera pseudodivaricata*. *J. Nat. Prod.* **2007**, *70*, 962–967. [CrossRef] [PubMed]
14. Wang, Y.; Edrada-Ebel, R.A.; Tsevegsuren, N.; Sendker, J.; Braun, M.; Wray, V.; Lin, W.; Proksch, P. Dihydrostilbene derivatives from the Mongolian medicinal plant *Scorzonera radiata*. *J. Nat. Prod.* **2009**, *72*, 671–675. [CrossRef] [PubMed]
15. Zidorn, C.; Ellmerer-Müller, E.P.; Stuppner, H. Sesquiterpenoids from *Scorzonera hispanica* L. *Pharmazie* **2000**, *55*, 550–551. [PubMed]
16. Auzi, A.R.; Hawisa, N.T.; Sherif, F.M.; Sarker, S.D. Neuropharmacological properties of *Launaea resedifolia*. *Rev. Bras. Farmacogn.* **2007**, *17*, 160–165. [CrossRef]
17. Zhu, Y.; Wu, Q.; Hu, P.; Wu, W. Biguaiascorzolides A and B: Two novel dimeric guaianolides with a rare skeleton, from *Scorzonera austriaca*. *Food Chem.* **2009**, *114*, 1316–1320. [CrossRef]
18. Sezik, E.; Yeşilada, E.; Tabata, M.; Honda, G.; Takaishi, Y.; Fujita, T.; Tanaka, T.; Takeda, Y. Traditional medicine in Turkey VIII. Folk medicine in East Anatolia; Erzurum, Erzincan, Ağrı, Kars, Iğdır Provinces. *Econ. Bot.* **1997**, *51*, 195–211. [CrossRef]
19. Baytop, T. *Türkiye'de Bitkiler ile Tedavi (Theraphy with Medicinal Plants in Turkey)*; Nobel Publishers: Istanbul, Turkey, 1999.

20. Donia, A.M. Phytochemical and pharmacological studies on *Scorzonera alexandrina* Bioss. *J. Saudi Chem. Soc.* **2013**. in press. [CrossRef]

21. Bahadir, Ö.; Saltan Çitoğlu, G.; Šmejkal, K.; Dall'Acqua, S.; Özbek, H.; Cvačka, J.; Žemlička, M. Analgesic Compounds from *Scorzonera latifolia* (Fisch. and Mey.) DC. *J. Ethnopharmacol.* **2010**, *131*, 83–87. [CrossRef] [PubMed]

22. Küpeli Akkol, E.; Acıkara Bahadır, Ö.; Süntar, İ.; Çitoğlu Saltan, G.; Keleş, H.; Ergene, B. Enhancement of wound healing by topical application of *Scorzonera* species: Determination of the constituents by HPLC with new validated reverse phase method. *J. Ethnopharmacol.* **2011**, *137*, 1018–1027. [CrossRef] [PubMed]

23. Küpeli Akkol, E.; Acıkara Bahadır, Ö.; Süntar, İ.; Ergene, B.; Çitoğlu Saltan, G. Ethnopharmacological evaluation of some *Scorzonera* species: *In vivo* anti-inflammatory and antinociceptive effects. *J. Ethnopharmacol.* **2012**, *140*, 261–270. [CrossRef] [PubMed]

24. Süntar, İ.; Acıkara Bahadır, Ö.; Çitoğlu Saltan, G.; Keleş, H.; Ergene, B.; Küpeli Akkol, E. *In vivo* and *In vitro* Evaluation of the Therapeutic Potential of Some *Scorzonera* Species as Wound healing Agent. *Curr. Pharm. Des.* **2011**, *137*, 1018–1027.

25. Amdekar, S.; Roy, P.; Singh, V.; Kumar, A.; Singh, R.; Sharma, P. Anti-Inflammatory Activity of Lactobacillus on Carrageenan-Induced Paw Edema in Male Wistar Rats. *Int. J. Inflamm.* **2012**, *2012*. [CrossRef] [PubMed]

26. Kim, K.R.; Jeong, C.K.; Park, K.K.; Choi, J.H.; Park, J.H.Y.; Lim, S.S.; Chung, W.Y. Anti-Inflammatory Effects of Licorice and Roasted Licorice Extracts on TPA-Induced Acute Inflammation and Collagen-Induced Arthritis in Mice. *J. Biomed. Biotechnol.* **2010**, *2010*. [CrossRef] [PubMed]

27. Yeşilada, E.; Üstün, O.; Sezik, E.; Takaishi, Y.; Ono, Y.; Honda, G. Inhibitory effects of Turkish folk remedies on inflammatory cytokines: Interleukin-1α, interleukin-1β and tumor necrosis factor α. *J. Ethnopharmacol.* **1997**, *58*, 59–73. [CrossRef]

28. Schwaiger, S.; Zeller, I.; Pölzelbauer, P.; Frotschnig, S.; Laufer, G.; Messner, B.; Pieri, V.; Stuppner, H.; Bernhard, D. Identification and pharmacological characterization of the anti-inflammatory principal of the leaves of dwarf elder (*Sambucus ebulus* L.). *J. Ethnopharmacol.* **2011**, *133*, 704–709. [CrossRef] [PubMed]

29. Kaileh, M.; Berghe, W.V.; Boone, E.; Essawi, T.; Haegeman, G. Screening of indigenous Palestinian medicinal plants for potential anti-inflammatory and cytotoxic activity. *J. Ethnopharmacol.* **2007**, *113*, 510–516. [CrossRef] [PubMed]

30. Siriwatanametanon, N.; Fiebich, B.L.; Efferth, T.; Prieto, J.M.; Heinrich, M. Traditionally used Thai medicinal plants: *In vitro* anti-inflammatory, anticancer and antioxidant activities. *J. Ethnopharmacol.* **2010**, *130*, 196–207. [CrossRef] [PubMed]

31. Borchers, A.T.; Keen, C.L.; Stern, J.S.; Gershwin, M.E. Inflammation and Native American medicine: The role of botanicals. *Am J Clin Nutr* **2000**, *72*, 339–347. [PubMed]

32. Dos Santos, M.D.; Almeida, M.C.; Lopes, N.P.; Souza, G.E.P. Evaluation of the anti-inflammatory, analgesic and antipyretic activities of natural polyphenol chlorogenic acid. *Biol. Pharm. Bull.* **2006**, *29*, 2236–2240. [CrossRef] [PubMed]

33. Yonathan, M.; Asres, K.; Assefa, A.; Bucar, F. *In vivo* anti-inflammatory and antinociceptive activities of *Cheilanthes farinose*. *J. Ethnopharmacol.* **2006**, *108*, 462–470. [CrossRef] [PubMed]

34. Hošek, J.; Šmejkal, K. Flavonoids as Anti-inflammatory Agents. In *Encyclopedia of Inflammatory Diseases*; Parnham, M., Ed.; Springer: New York, NY, USA; ISBN: 978-3-0348-0620-6. (Online).

35. Comalada, M.; Ballester, I.; Bailon, E.; Sierra, S.; Xaus, J.; Galvez, J.; Sanchez de Medina, F.; Zarzuelo, A. Inhibition of pro-inflammatory markers in primary bone marrow-derived mouse macrophages by naturally occurring flavonoids: Analysis of the structure-activity relationship. *Biochem. Pharmacol.* **2006**, *72*, 1010–1021. [CrossRef] [PubMed]

36. Paul, A.T.; Gohil, V.M.; Bhutani, K.K. Modulating TNF-α signaling with natural products. *Drug Discov. Today* **2006**, *11*, 725–732. [CrossRef] [PubMed]

37. Yamamoto, Y.; Gaynor, R.B. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. *J. Clin. Investig.* **2001**, *107*, 135–142. [CrossRef] [PubMed]

38. Jeong, G.-S.; Bae, J.-S. Anti-Inflammatory Effects of Triterpenoids; Naturally Occurring and Synthetic Agents. *Mini-Rev. Org. Chem.* **2014**, *11*, 316–329. [CrossRef]

39. Sultana, N.; Saify, Z.S. Naturally occurring and synthetic agents as potential anti-inflammatory and immunomodulants. *Anti-Inflamm. Anti-Allergy Agents Med. Chem.* **2012**, *11*, 3–19. [CrossRef]

40.   Shen, W.; Qi, R.; Zhang, J.; Wang, Z.; Wang, H.; Hu, C.; Zhao, Y.; Bie, M.; Wang, Y.; Fu, Y.; Chen, M.; Lu, D. Chlorogenic acid inhibits LPS-induced microglial activation and improves survival of dopaminergic neurons. *Brain Res. Bull.* **2012**, *88*, 487–494. [CrossRef] [PubMed]

41.   Ku, S.K.; Zhou, W.; Lee, W.; Han, M.S.; Na, M.; Bae, J.S. Anti-inflammatory effects of hyperoside in human endothelial cells and in mice. *Inflammation* **2015**, *38*, 784–799. [CrossRef] [PubMed]

42.   Srivastava, P.; Mohanti, S.; Bawankule, D.U.; Khan, F.; Shanker, K. Effect of *Pluchea lanceolata* bioactives in LPS-induced neuroinflammation in C6 rat glial cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **2014**, *387*, 119–127. [CrossRef] [PubMed]

43.   Chen, J.-J.; Tsai, Y.-C.; Hwang, T.-L.; Wang, T.-C. Thymol, Benzofuranoid, and Phenylpropanoid Derivatives: Anti-inflammatory Constituents from *Eupatorium cannabinum*. *J. Nat. Prod.* **2011**, *74*, 1021–1027.

44.   Hashimoto, T.; Tori, M.; Asakawa, Y. Three dihydroisocoumarine glucosides from *Hydrangea macrophylla* subsp. Serrate. *Phytochemistry* **1987**, *26*, 3323–3330. [CrossRef]

45.   Frank, J.H.; Powder-George, Y.M.; Ramsewak, R.S.; Reynolds, W.F. Variable-Temperature ^{1}H-NMR Studies on Two *C*-Glycosylflavones. *Molecules* **2012**, *17*, 7914–7926. [CrossRef] [PubMed]

46.   Pukalskas, A.; Venskutonis, P.R.; Dijkgraaf, I.; van Beek, T.A. Isolation, identification and activity of natural antioxidants from costmary (*Chrysanthemum balsamita*) cultivated in Lithuania. *Food Chem.* **2010**, *122*, 804–811. [CrossRef]

47.   Acikara Bahadir, Ö.; Saltan Citoglu, G.; Dall'Acqua, S.; Özbek, H.; Cvačka, J.; Žemlička, M.; Šmejkal, K. Bioassay-guided isolation of the antinociceptive compounds motiol and β-sitosterol from *Scorzonera latifolia* root extract. *Pharmazie* **2014**, *69*, 711–714.

48.   Paraschos, S.; Magiatis, P.; Kalpoutzakis, E.; Harvala, C.; Skaltsounis, A.-L. Three New Dihydroisocoumarins from the Greek Endemic Species *Scorzonera cretica*. *J. Nat. Prod.* **2001**, *64*, 1585–1587. [CrossRef] [PubMed]

49.   Acikara Bahadir, Ö.; Saltan Citoglu, G.; Dall'Acqua, S.; Šmejkal, K.; Cvačka, J.; Žemlička, M. A new triterpene from *Scorzonera latifolia* (Fisch. and Mey.) DC. *Nat. Prod. Res.* **2012**, *26*, 1892–1897. [CrossRef] [PubMed]

Sample Availability: Samples of the compounds **1**–**16** are available from the authors.

Article

The Chemical Composition of *Achillea wilhelmsii* C. Koch and Its Desirable Effects on Hyperglycemia, Inflammatory Mediators and Hypercholesterolemia as Risk Factors for Cardiometabolic Disease

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Abstract: This study was done to identify the content compounds of *Achillea wilhelmsii* (*A. wilhelmsii*) and to evaluate its hypoglycemic and anti-hypercholesterolemic activity and effect on inflammatory mediators. The extracts and fractions of *A. wilhelmsii* were thoroughly analyzed using high performance liquid chromatography (HPLC), and the total content of phenols and flavonoids was determined. The hypoglycemic activity was evaluated *in vivo* using alloxan-induced diabetic mice. The effect upon inflammatory mediators was evaluated *in vitro* using the human monocytic leukemia cell line (THP-1). The anti-hypercholesterolemic activity was evaluated *in vitro* using the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase assay kit. The water extract (WE)-treated group showed the highest reduction in the fasting blood glucose levels (FBGL). The chloroform fraction (CF) and ethyl acetate fraction (EAF) both showed a significant ability to reduce the secretion of tumor necrosis factor alpha (TNF-α). The EAF, however, also attenuated the levels of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9). The CF showed the most significant 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) inhibition activity. The five main compounds in the CF were isolated and identified. Out of the five compounds in the CF, 1β,10β-epoxydesacetoxymatricarin (CP1) and leucodin (CP2) showed the highest anti-hypercholesterolemic potential. A molecular docking study provided corresponding results.

1. Introduction

Achillea is an herb that belongs to the family Asteraceae. This genus includes more than 100 species worldwide [1]. *Achillea* species occur in the high mountains of the Mediterranean [2] and are native to Europe and Western Asia [1]. Due to their medicinally-useful properties, several *Achillea* species have been used since ancient times as traditional remedies for abdominal pain, cough, inflammation, jaundice, fever, diarrhea and wound healing [3,4]. Various species of *Achillea* have been analyzed, and more than 100 compounds have been identified [1]. *Achillea* extracts showed anti-oxidant, anti-inflammatory, analgesic, anti-pyretic, anti-spasmodic, anti-ulcerogenic, anti-bacterial, cytotoxic, immunosuppressive and hypoglycemic properties [2–11]. *Achillea wilhelmsii* (*A. wilhelmsii*) C. Koch has previously demonstrated anti-hyperlipidemic, anti-hypertensive and anti-mycobacterial properties [12,13].

Diabetes mellitus (DM) is a metabolic disorder that is primarily characterized by hyperglycemia. This disease had been known and treated with traditional herbal therapies long before the chemical agents we know today became available [14].

Hypercholesterolemia, a form of hyperlipidemia, is nowadays, together with other non-communicable diseases, such as myocardial infarction, stroke, obesity and diabetes, among the leading causes of death worldwide in all income groups equally [15]. It is the combination of genetic and environmental risk factors that is responsible for the high levels of cholesterol in blood [16]. Nowadays, one of the most effective ways of lowering plasma cholesterol levels is controlling *de novo* synthesis of endogenous cholesterol by the inhibition of HMGR. The inhibition of this enzyme represents the rate-limiting step of the mevalonate pathway for the synthesis of sterol isoprenoids, such as cholesterol, and non-sterol isoprenoids, such as dolichol, heme-A, isopentenyl tRNA and ubiquinone [17,18].

A leading cause of premature death is cardiovascular disease, arising from increased rates of cardiometabolic risk factors, such as obesity, hypertension, hyperglycemia and dyslipidemia [19]. Inflammation is a key component of several cardiometabolic diseases, including obesity, type II diabetes and atherosclerotic cardiovascular disease [20]. Cardiometabolic risk factors, including elevated lipids, glucose and hypertension, are expected to continue to rise, especially as the population ages [21]. Adipose tissue in abdominal obesity is considered as an endocrinal organ orchestrating key pathophysiological pathways in inflammation and lipid metabolism. Adipose tissue synthesizes and secretes various adipocytokines that create a pro-inflammatory environment [22–24].

This study was done to evaluate the hypoglycemic and anti-hypercholesterolemic activities of *A. wilhelmsii* and to explore its influence on selected inflammatory mediators. To the best of our knowledge, this is the first time this plant has been studied thoroughly for its desirable effects on the major risk factors for cardiometabolic disease.

2. Results

2.1. Phytochemical Analyses

A. wilhelmsii extracts and fractions, the water extract (WE), the ethyl acetate fraction (EAF), the hydro-alcoholic extract (HAE) and the chloroform fraction (CF), were examined using high performance liquid chromatography (HPLC) with the aim to analyze their content of phenolic compounds. The compounds present were identified by their UV spectra and mass spectrometric ions through library search and comparison with the literature. It is important to note that this identification is tentative. The profile of the different phenolic compounds in the WE is shown in Figure 1. *C*-glycosides of luteolin and apigenin are the most abundant components. Caffeic acid is also present. The EAF chromatogram is shown in Figure 2. *C*-glycosides, ferulic acid and two sesquiterpenoids, 1β,10β-epoxydesacetoxymatricarin (CP1) and leucodin (CP2), are present. The same two sesquiterpenoid compounds are also present in the HAE and CF. Three methoxylated flavonoid aglycones are present in the HAE and CF: 2-(3,4-dimethoxyphenyl)-5-hydroxy-6,7-dimethoxychromen-4-one (CP3),

2-(3,4-dimethoxyphenyl)-5,6,7-dimethoxychromen-4-one (CP4) and salvigenin (CP5). Figures 3 and 4 show the chromatograms of the HAE and CF, respectively. Figure 5 shows the structures of the compounds identified tentatively in *A. wilhelmsii*. Figure 6 shows the structures of the compounds isolated and identified from the CF of *A. wilhelmsii*.
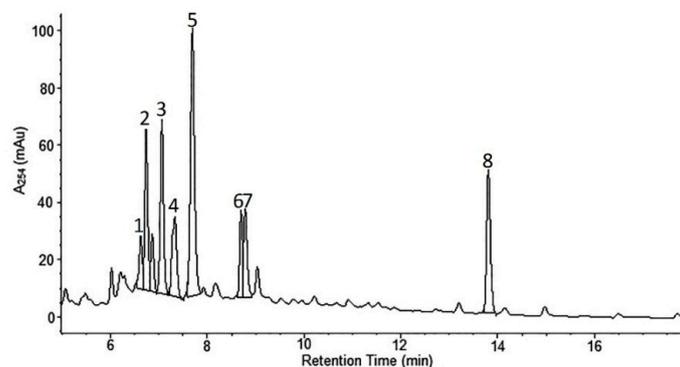
Figure 1. The selected segment of HPLC-DAD chromatogram of the water extract (WE) recorded at 254 nm. Peak assignments (tentative identification): 1. isoschaftoside; 2. schaftoside; 3. vicenin-2; 4. vicenin-3; 5. caffeic acid; 6. isoorientin; 7. isovitexin; 8. leucodin.
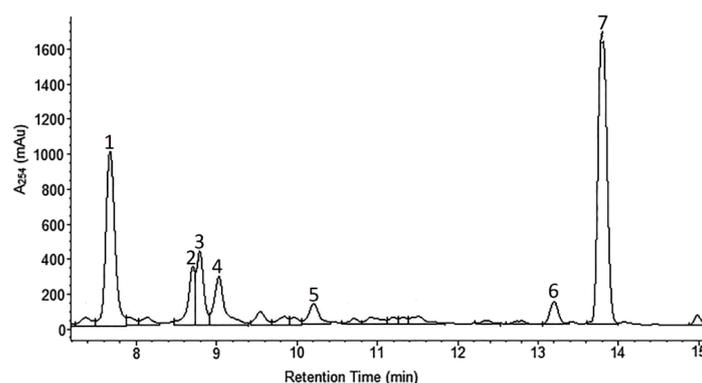
Figure 2. The selected segment of HPLC-DAD chromatogram of the ethyl acetate fraction (EAF) recorded at 254 nm. Peak assignments (tentative identification): 1. caffeic acid; 2. isoorientin; 3. isovitexin; 4. swertisin; 5. ferulic acid; 6. 1β,10β-epoxydesacetoxymatricarin; 7. leucodin.
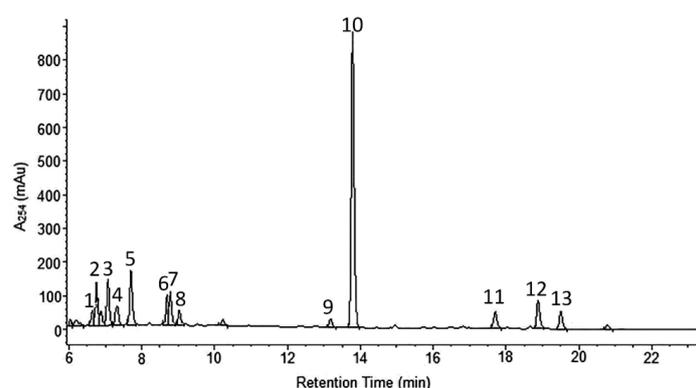
Figure 3. The selected segment of HPLC-DAD chromatogram of the hydro-alcoholic extract (HAE) recorded at 254 nm. Peak assignments (tentative identification): 1. isoschaftoside; 2. schaftoside; 3. vicenin-2; 4. vicenin-3; 5. caffeic acid; 6. isoorientin; 7. isovitexin; 8. swertisin; 9. 1β,10β-epoxydesacetoxymatricarin; 10. leucodin; 11. 5-demethylsinensetin; 12. 2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one; 13. salvigenin.
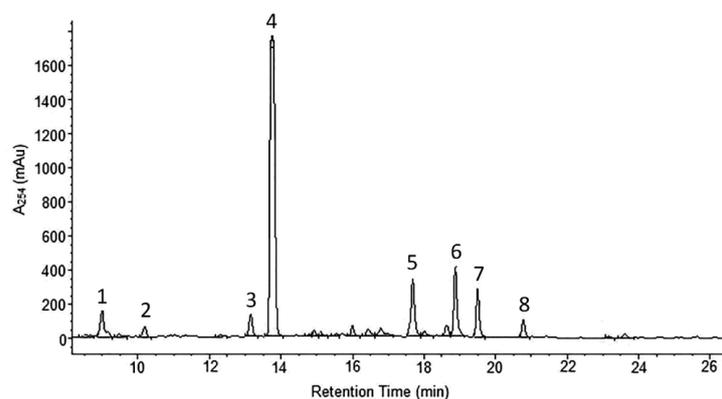
Figure 4. The selected segment of HPLC-DAD chromatogram of the chloroform fraction (CF) recorded at 254 nm. Peak assignments (tentative identification): 1. unknown; 2. ferulic acid; 3. 1β,10β-epoxydesacetoxymatricarin (CP1); 4. leucodin (CP2); 5. 5-demethylsinensetin (CP3); 6. 2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one (CP4); 7. salvigenin (CP5); 8. unknown.

isoschaftoside

schaftoside

vicenin-2

vicenin-3

isoorientin

isovitexin

swertisin

caffeic acid

ferulic acid
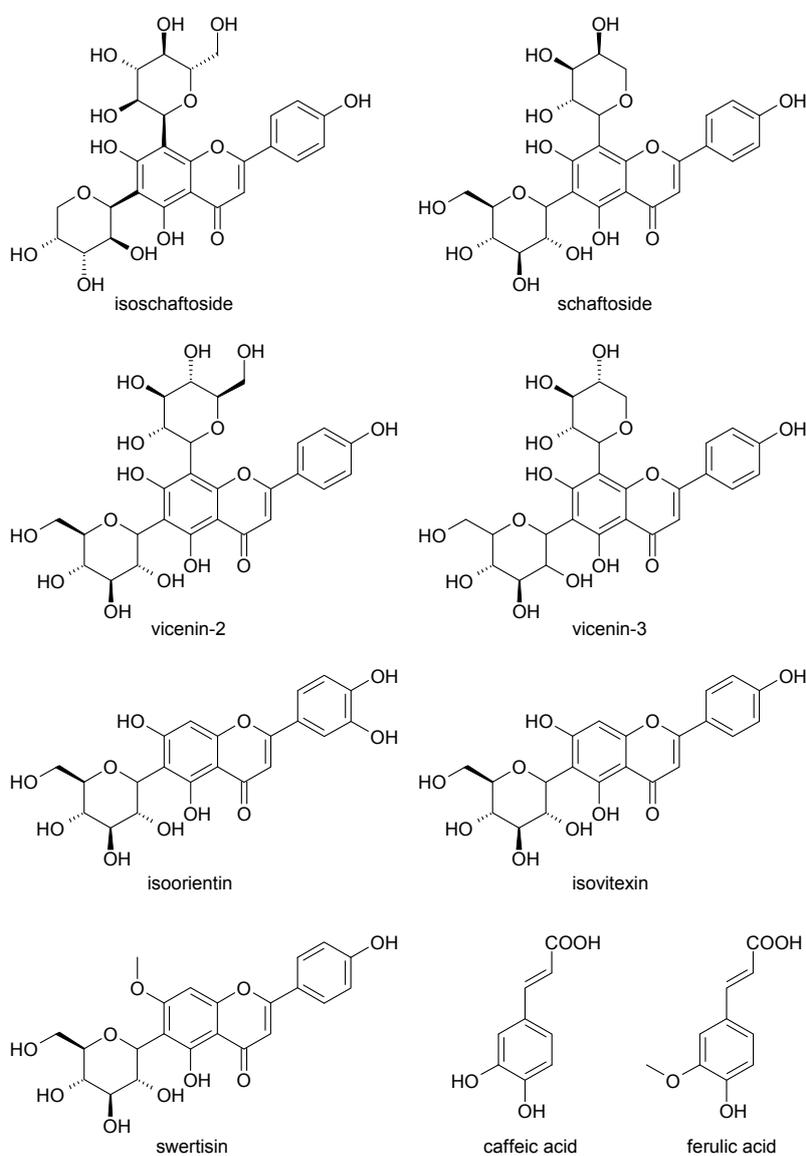
Figure 5. The structures of the compounds identified tentatively in *A. wilhelmsii*.

1ß,10ß-epoxydesateoxymatricarin (CP1)

Leucodin (CP2)

5-demethylsinensetin (CP3)

2-(3,4-dimethoxy-phenyl)-3-hydroxy-
-5,6,7-trimethoxy-chromen-4-one (CP4)

Salvigenin (CP5)
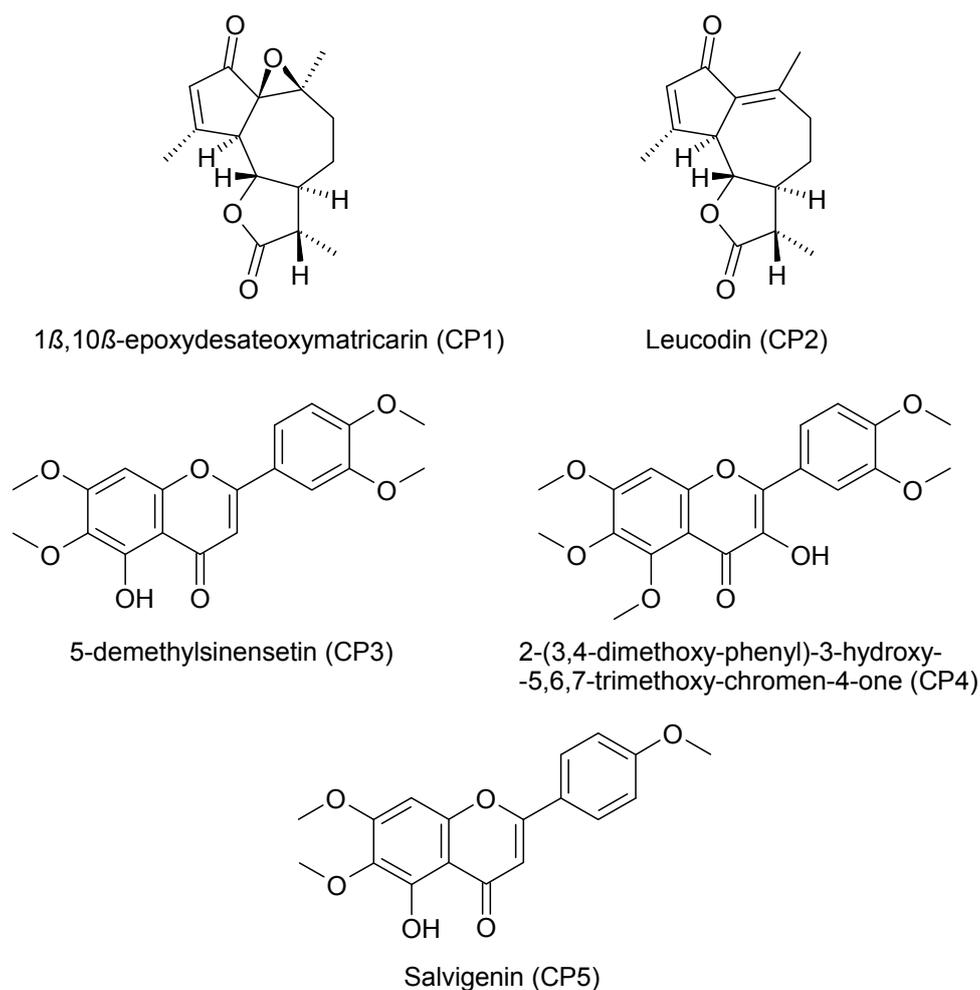
Figure 6. The structures of the compounds isolated and identified from the CF of *A. wilhelmsii.*

The total content of polyphenols was determined by a modified Folin-Ciocalteu colorimetric method, and the results were expressed as gallic acid equivalent (GAE) per gram of dry extract weight. Flavone and flavonol contents were analyzed using a colorimetric method, and the results were expressed as quercetin equivalent (QE) per gram of dry extract weight [25]. The findings are summarized in Table 1. EAF showed the highest content of phenols among the tested samples, while CF showed the highest content of flavonoids. The WE showed the least content of phenols and flavonoids.

Table 1. Total content of polyphenols, flavones and flavonols in the studied extracts and fractions. Each value represents the mean $\pm$ SD of three independent measurements.

Sample	Total Content of Phenolic Compounds (GAE mg/g)	Total Content of Flavones and Flavonols (QE mg/g)
WE	33.11 $\pm$ 1.12	3.64 $\pm$ 0.21
EAF	104.75 $\pm$ 6.12	11.51 $\pm$ 0.85
HAE	55.77 $\pm$ 2.94	11.28 $\pm$ 1.05
CF	46.40 $\pm$ 2.22	13.27 $\pm$ 0.45

2.2. The Hypoglycemic Activity

The hypoglycemic activity was tested *in vivo.* Plant extracts and fractions were administered for 20 consecutive days. Quercetin was used as a standard for comparison. The results of the hypoglycemic activity assay are summarized in Table 2. On the first day of the experiment, the differences in

the fasting blood glucose levels (FBGL) of the diabetic groups were statistically insignificant when compared to each other. On the fourth day, the FBGL of both EAF- and quercetin-treated groups started to show a significant difference when compared to the diabetic group. On the last day of the experiment, the WE-, EAF- and HAE-treated groups showed significant differences when compared to the diabetic groups. However, the differences of the FBGL of the WE-treated group were statistically insignificant when compared to the healthy group.

Table 2. The *in vivo* hypoglycemic activity assay results for the tested groups. Each value is the mean ± SD for six mice in each group. The empty cells in the table are because the mice in the CF-treated group did not survive past Day 15.

Day	Healthy Group	Diabetic Group	EAF-Treated Group	HAE-Treated Group	CF-Treated Group	WE-Treated Group	Quercetin-Treated Group
	FBGL (mmol/L)	FBGL (mmol/L)	FBGL (mmol/L)	FBGL (mmol/L)	FBGL (mmol/L)	FBGL (mmol/L)	FBGL (mmol/L)
0	6.00 ± 0.84 [a]	30.80 ± 2.65 [b]	30.11 ± 2.84	30.27 ± 1.88	31.23 ± 1.19	30.71 ± 2.83	29.63 ± 2.67
4	5.67 ± 1.11 [a]	30.69 ± 1.97 [b]	24.71 ± 2.99 [a]	29.58 ± 1.94	33.45 ± 1.55	28.13 ± 2.93	27.58 ± 2.09 [a]
8	5.75 ± 1.05 [a]	29.46 ± 2.74 [b]	20.07 ± 1.95 [a]	26.27 ± 2.17 [a]	32.00 ± 2.25	15.18 ± 2.89 [a]	21.02 ± 1.63 [a]
12	6.35 ± 0.99 [a]	29.13 ± 1.98 [b]	18.04 ± 2.79 [a]	21.22 ± 1.45 [a]	34.35 ± 1.06	12.73 ± 2.16 [a]	18.70 ± 1.26 [a]
16	6.12 ± 0.90 [a]	30.46 ± 2.41 [b]	13.19 ± 2 .22 [a]	16.77 ± 1.46 [a]	-	8.10 ± 2.48 [a]	17.55 ± 1.61 [a]
20	5.85 ± 0.97 [a]	31.09 ± 2.06 [b]	12.40 ± 2.11 [a]	14.08 ± 1.34 [a]	-	4.39 ± 1.54 [a]	14.02 ± 2.06 [a]

[a] The number is statistically significant when compared to the diabetic group (the comparison is between the readings taken on the same day) ($p < 0.05$). [b] The number is statistically significant when compared to the healthy group (the comparison is between the readings taken on the same day) ($p < 0.05$).

The histology study revealed signs of acute enteritis in the jejunum of the CF-treated group that were not present in the other groups. The control diabetic group exhibited severe liver tissue injuries and lipid accumulations, shrinkage and severe damage of Langerhans islets with fat deposits and tubular edema and glomerular hemorrhage in the juxtamedullary region of the kidney. All of these morphological changes can be attributed to the administration of alloxan [26,27]. Both the WE- and quercetin-treated groups presented a higher number of functional Langerhans islets when compared to the other treated groups. Improvements in the liver and kidney tissue were also observed in the WE- and quercetin-treated groups.

2.3. The Effect on Inflammatory Mediators

The effect on inflammatory mediators of *A. wilhelmsii* was evaluated *in vitro* using the model of LPS-stimulated macrophage-like cells THP-1. The production of pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α), and the activity of inflammation-related proteinases, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) were used as markers of the inflammatory response in these cells.

All extracts tested significantly attenuated TNF-α secretion (Figure 7). EAF and CF reduced the level of this cytokine almost to the basal level (control cells). Three *A. wilhelmsii* extract fractions, EAF, HAE and CF, were significantly better than 1 μM prednisone, whereas the WE was not as effective as this commercially available drug. The next evaluated marker of inflammation was proteinase MMP-2 (Figure 8A). The EAF, HAE and CF all had the ability to reduce the amount of this enzyme. CF had an even greater effect than prednisone. For the biological activity of MMP-2, it is necessary to truncate the inactive pro-MMP-2 form to an active MMP-2 form. Thus, the ratio between pro-MMP-2 and MMP-2 is more important than the total amount of MMP-2 itself. From this point of view, only the EAF significantly changed the pro-MMP-2/MMP-2 ratio towards pro-MMP-2 (Figure 8C). A typical pro-inflammatory marker is an elevated level of MMP-9. Both the EAF and CF were able to significantly attenuate the level of this enzyme (Figure 8B).
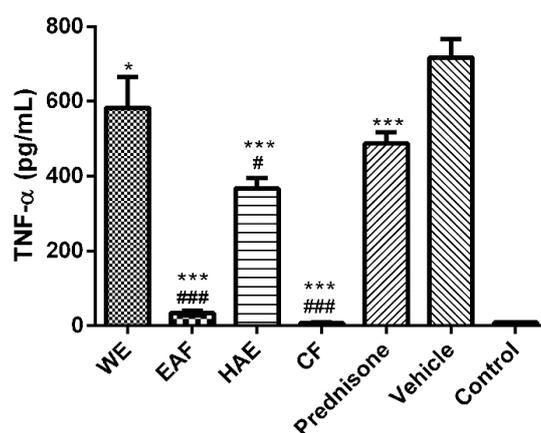
Figure 7. Effects of the tested *A. wilhelmsii* extracts and fractions and the reference drug prednisone, on TNF-α secretion. Cells were pre-treated with the given extracts and fractions (25 μg/mL), prednisone (1 μM) or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by the addition of lipopolysaccharide (LPS) (except for the control cells). Results are expressed as means ± SD for three independent experiments. * Significant difference in comparison to vehicle-treated cells ($p < 0.05$); *** significant difference in comparison to vehicle-treated cells ($p < 0.001$); # significant difference in comparison to prednisone-treated cells ($p < 0.05$); ### significant difference in comparison to prednisone-treated cells ($p < 0.001$).
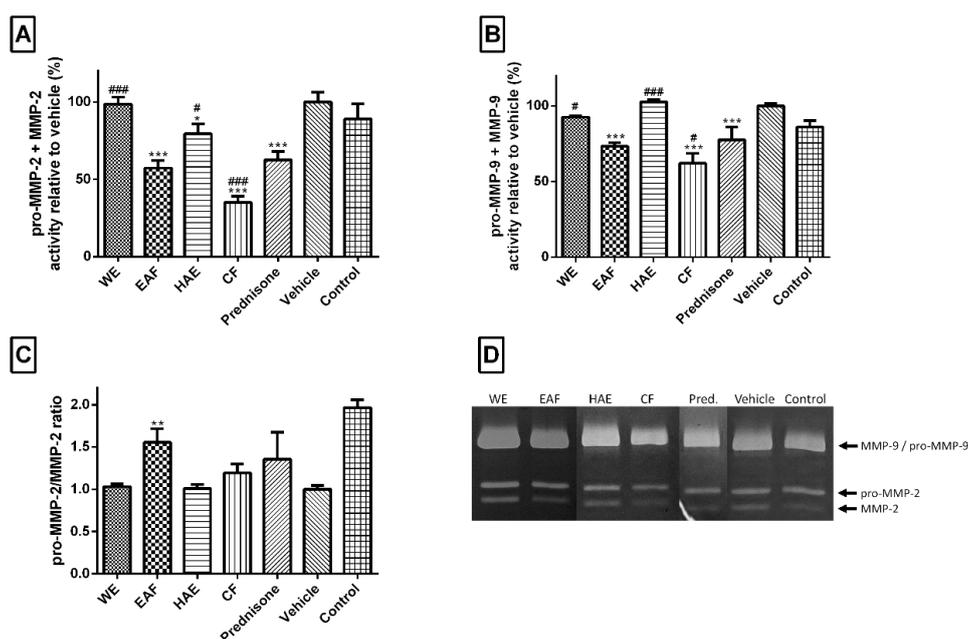
Figure 8. Effects of tested *A. wilhelmsii* extracts and fractions and the reference drug prednisone on LPS-induced matrix metalloproteinases (MMP) activity. Cells were pre-treated with given extracts (25 μg/mL), prednisone (1 μM; Pred.) or the vehicle (DMSO) only. After 1 h of incubation, the inflammatory response was induced by the addition of LPS (except for the control cells). The activities of MMP-2 (**A**) and MMP-9 (**B**) were detected by zymography. The intensity of the digested bands was analyzed by densitometry analysis; (**C**) The pro-MMP-2/MMP-2 ratio. The results are expressed as means ± SD for three independent experiments. * Significant difference in comparison to vehicle-treated cells ($p < 0.05$); ** significant difference in comparison to vehicle-treated cells ($p < 0.01$); *** significant difference in comparison to the vehicle-treated cells ($p < 0.001$); # significant difference in comparison to prednisone-treated cells ($p < 0.05$); ### significant difference in comparison to prednisone-treated cells ($p < 0.001$); (**D**) The gel representative results of one of the three independent experiments.

2.4. The Anti-Hypercholesterolemic Activity

2.4.1. The 3-Hydroxy-3-methyl-glutaryl-CoA Reductase Inhibition Assay

The results of the HMGR inhibition assays of the extract and fractions are summarized in Table 3.

Table 3. HMGR percentage inhibition values for the extracts and fractions of *A. wilhelmsii,* along with the control (pravastatin). Each value represents the mean $\pm$ SD of three independent measurements.

Sample	HMGR Inhibition (%)
WE	36.11 ± 4.21
EAF	64.70 ± 2.50
HAE	70.15 ± 3.84
CF	89.21 ± 7.24
Pravastatin	98.02 ± 3.34

The CF showed the highest HMGR inhibition potential, so the five main compounds present in the CF (1β,10β-epoxydesacetoxymatricarin (CP1), leucodin (CP2), 5-demethylsinensetin (CP3), 2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one (CP4) and salvigenin (CP5)) were isolated, identified (structures and spectral data are in the Supplementary Materials) and tested separately. The dose-dependent inhibition of HMGR for the compounds is shown in Figure 9. Both 1β,10β-epoxydesacetoxymatricarin (CP1) and leucodin (CP2) showed powerful inhibition of HMGR, whereas 5-demethylsinensetin (CP3), 2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one (CP4) and salvigenin (CP5) did not reach 50% inhibition, even at higher concentrations (80 μM). The IC$_{50}$ values for 1β,10β-epoxydesacetoxymatricarin (CP1) and leucodin (CP2) were 6.37 and 3.88 μM, respectively. For the standardization of the method, preliminary screening of pravastatin inhibition activity was performed. The IC$_{50}$ value for pravastatin was 72.12 nM.
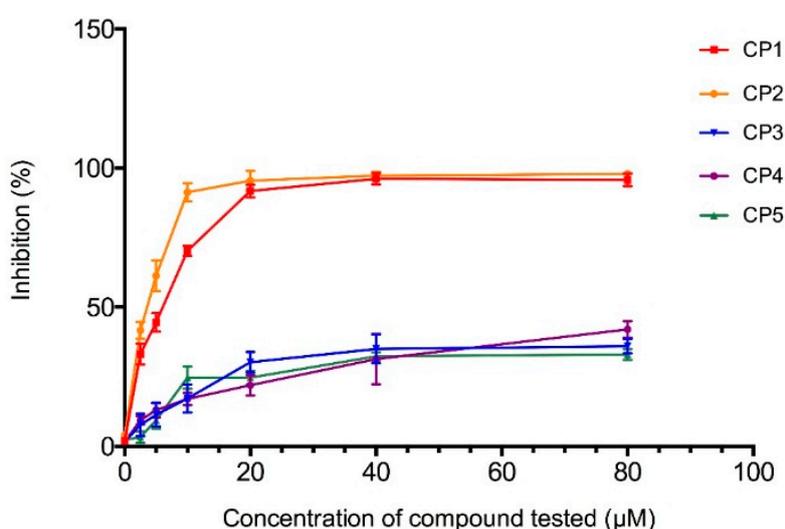
Figure 9. Inhibition (%) of each one of the five compounds (1β,10β-epoxydesacetoxymatricarin (CP1), leucodin (CP2), 5-demethylsinensetin (CP3), 2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one (CP4) and salvigenin (CP5)) plotted against the tested concentrations (0, 2.5, 5, 10, 20, 40, 80 μM).

2.4.2. Computational Docking

The five compounds isolated from the CF were subjected to molecular docking study. For comparison purposes, statins were also docked into the active pocket to calculate the binding

affinities to be compared with the active compounds found in CF. Statins had binding affinities that ranged from −7.0 to −9.6 Kcal/mol. Compounds 1β,10β-epoxydesacetoxymatricarin (CP1) and leucodin (CP2) (Figures 10 and 11) showed the highest affinities and were perfectly docked into the active pocket site. Atorvastatin showed the highest affinity at −9.6 kcal/mol (Figure 12), while pravastatin had a binding affinity of −7.0 Kcal/mol (Figure 13). Similar findings were observed with the tested compounds with a range from −7.4 to −8.0 Kcal/mol (well within the range of the statins) (Table 4.). The slightly lower affinity in comparison with atorvastatin can be explained by the fact that 1β,10β-epoxydesacetoxymatricarin (CP1) and leucodin (CP2) are smaller molecules than atorvastatin with a smaller molecular surface and less specific shape for the binding site. It has to be noted that the other compounds, 5-demethylsinensetin (CP3), 2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one (CP4) and salvigenin (CP5), also showed good binding affinity, but all the hits found by the docking program were not in the active pocket site, which corresponds with the fact that these compounds could not reach 50% inhibition at the tested concentrations.
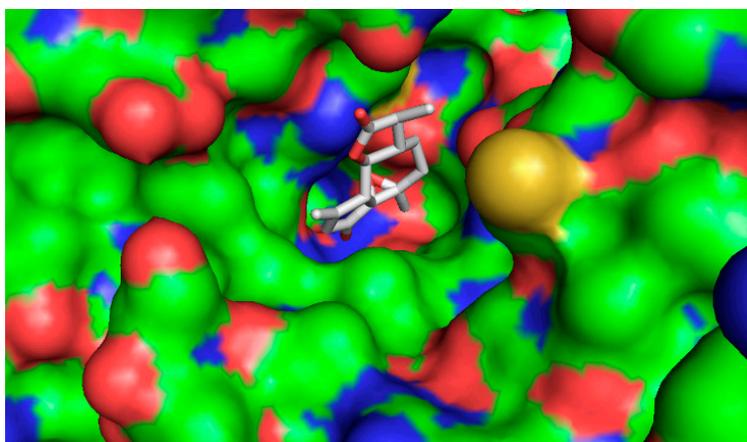
Figure 10. Bound conformer of ligands interacting with the substrate binding sites of HMGR for 1β,10β-epoxydesacetoxymatricarin (CP1).
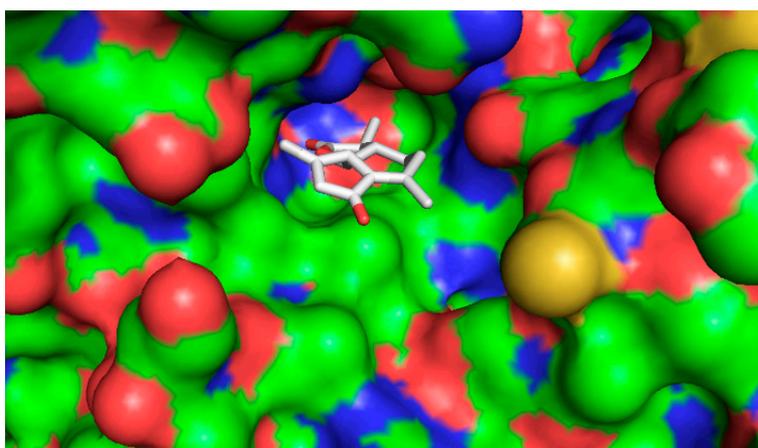
Figure 11. Bound conformer of ligands interacting with the substrate binding sites of HMGR for Leucodin (CP2).
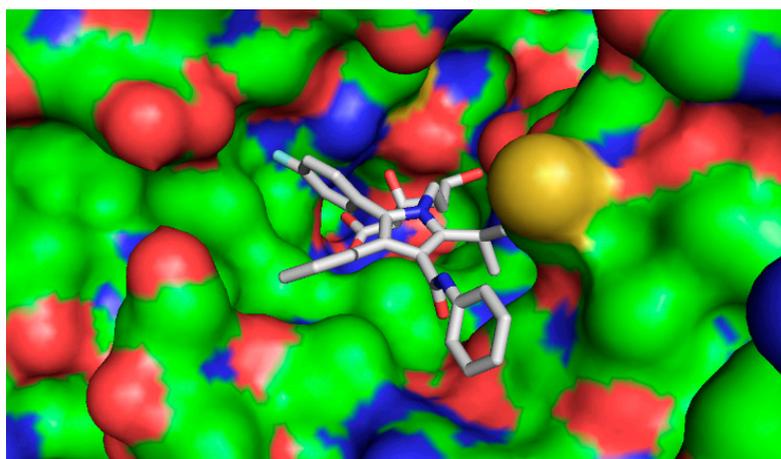
Figure 12. Bound conformer of ligands interacting with the substrate binding sites of HMGR (atorvastatin).
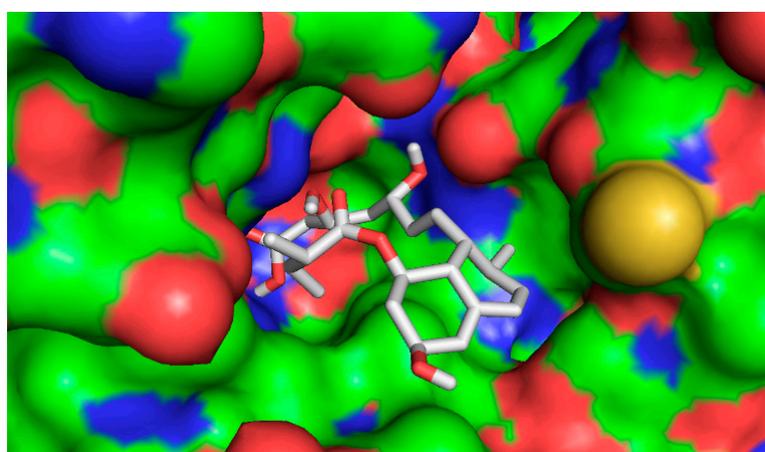
Figure 13. Bound conformer of ligands interacting with the substrate binding sites of HMGR (pravastatin).

Table 4. The binding affinity of different ligands (the 5 compounds and 2 selected statins).

Ligand	Binding Affinity (Kcal/mol)
1β,10β-epoxydesacetoxymatricarin (CP1)	−7.6
Leucodin (CP2)	−8.0
5-demethylsinensetin (CP3)	−7.5
2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one (CP4)	−7.4
Salvigenin (CP5)	−7.4
Atorvastatin	−9.6
Pravastatin	−7.0

3. Discussion

C-glycosides, especially luteolin and apigenin *C*-glycosides, were previously reported to occur in the genus *Achillea* [11,28]. Vicenin-2, schaftoside, isoschaftoside and isovitexin have all been reported from *Achillea setacea* [29]. Leucodin and deacetylmatricarin have been reported from *Achillea millefolium* [30]. Salvigenin has been reported before from *Achillea tenuifolia* [31]. Derivatives of caffeic acid and ferulic acid are also known to occur in the genus *Achillea* [32]. However, this is the first time the content compounds of *A. wilhelmsii* have been analyzed in detail. Our investigation led to the identification of two sesquiterpenoids and three methoxylated flavonoids that have not yet been

reported in *A. wilhelmsii*. The determination of the total content of phenols and flavonoids enabled us to quantify the amount of the phenolic compounds present in *A. wilhelmsii*.

Quercetin is one of the most abundant flavonol-type flavonoids found in fruit and vegetables, and it is known to be a strong antioxidant. Several investigators reported the hypoglycemic effect of quercetin along with other important biological effects [14,33]. This was the reason why we used quercetin as a standard for comparison in the hypoglycemic activity assay. The results obtained showed that the WE normalized the FBGL back to normal and had an even greater hypoglycemic effect than quercetin. The WE was found to be rich in apigenin *C*-glycosides. One apigenin *C*-glycoside (apigenin-6-*C*-β-fructopyranoside) had been reported to possess anti-diabetic properties [34]. The histology study showed that the WE and quercetin both had a desirable effect on the number of functional Langerhans islets in the pancreas, which could partially explain their hypoglycemic effects. The enteritis seen in the CF-treated group could be due to the high concentration of the fraction administered to the mice. The enteritis in this group was associated with a noticeable weight loss that could ultimately explain why the mice in this group did not survive the whole 20 days of the experiment. The WE showed a very promising hypoglycemic effect that justifies the use of this plant as a natural hypoglycemic by the traditional healers in the Middle East.

Plants from the genus *Achillea* are used in the Middle East region not only for their medical benefits, but are also casually consumed as a hot beverage. Because the anti-phlogistic potential of *A. wilhelmsii*, especially the lipophilic fractions, was proven in this work, it is possible to say that casual consumption of this plant as a part of the diet could attenuate the inflammatory state, such as that induced by *Helicobacter pylori* or ethanol-induced gastric ulcers [9,35], and improve the outcome of many inflammation-related disorders. The EAF had the most potent effect among the tested samples. It reduced the TNF-α secretion, attenuated the level of MMP-2 and MMP-9 and was the only fraction to inhibit the digestion of the pro-MMP-2 proenzyme to a mature, biologically-active form.

Persistent high serum levels of cholesterol are a cause of cardiovascular diseases and possible death and contribute to the formation of atherosclerotic plaques in arteries throughout the body. Atherosclerosis is one of the leading causes of deaths out of all = non-communicable diseases, such as cancer, hypertension and diabetes [15]. The atherosclerotic process, together with the development of metabolic syndrome, affects a large number of the adult population worldwide. Pravastatin is a representative of the statin class of drugs that in their active hydrolysed form are specific inhibitors of HMGR. Both 1β,10β-epoxydesacetoxymatricarin (CP1) and leucodin (CP2) showed an inhibition activity comparable to that of pravastatin.

4. Materials and Methods

4.1. Plant Material

A. wilhelmsii air-dried aerial parts were obtained from Syria. The plant was collected from an area close to the Anti-Lebanon Mountains (July 2010). The plant was identified and authenticated by Necmi Aksoy, Department of Forest Botany, Faculty of Forestry, Duzce University, Turkey. The voucher specimen (DUOF No: 1602) was deposited at the herbarium of The Faculty of Forestry (DUOF), Department of Forest Botany, Faculty of Forestry, Duzce University, Turkey. The identification of this plant was confirmed by Jiří Danihelka, Department of Botany and Zoology, Faculty of Science, Masaryk University, Czech Republic.

4.2. Extraction

4.2.1. Preparation of *A. wilhelmsii* Crude Extracts

Crushed plant material (500 g) was defatted with n-hexane, dried and then extracted three times with distilled water. The filtrate was combined, freeze-dried and refrigerated (WE). The yield of this was 51.84 g. The plant material was dried again and extracted with a hydro-alcoholic mixture

(75% ethanol in water) three times. The filtrate was combined, concentrated under reduced pressure, freeze-dried and refrigerated. The yield of this was 24.33 g.

4.2.2. Fractionation by Solvent-Solvent Extraction

Half of the water extract (WE) was utilized for immiscible liquid-liquid extraction using ethyl acetate. The ethyl acetate fraction (EAF) was collected and dried to give a yield of 2.22 g. Half of the hydro-alcoholic extract (HAE) was also separated into two parts by immiscible liquid-liquid extraction using chloroform. The chloroform fraction (CF) was collected and dried to give a yield of 8.49 g.

4.3. Phytochemical Analyses

4.3.1. HPLC-DAD-MS Analysis

The analysis of *A. wilhelmsii* extracts and fractions was performed by reversed-phase LC equipped with DAD and negative ion ESI with MS/MS. LC was performed with an Agilent (Santa Clara, CA, USA) 1100 Series LC system. The data were processed using Agilent Rev.B.04.01 (481) ChemStation. MS detection was performed using an Agilent 1100 LC-MSD Trap system VL series. The gas flow rate of N_2 was 10 L/min; the capillary voltage was 3.5 kV; the nebulization was pressure 80 psi; and the gas temperature was 300 °C. Spectra were recorded in negative ion mode between *m/z* 150 and 1500. The data were processed using Agilent LC/MSD Trap Software 5.3. A SUPELCOSIL ABZ+PLUS, (3 μm, 15 cm × 4.6 mm) column, thermostated at 40 °C, was used. The solvents were (A) 0.2% formic acid in water and (B) MeCN. The elution gradient was 10% to 100% B in A over 36 min using a flow rate of 1 mL/min. The absorbance was recorded at 215, 230, 254, 280 and 350 nm.

4.3.2. Determination of Phenolic Compounds Content

The total content of polyphenols was determined by a modified Folin-Ciocalteu colorimetric method [25]. A sample at a concentration of 5 mg/mL was dissolved either in water or DMSO depending on its solubility. The results were expressed as gallic acid equivalent (GAE) per gram of dry extract weight. All measurements were done in triplicate.

4.3.3. Determination of Flavone and Flavonol Content

Flavone and flavonol contents were analyzed using a colorimetric method. The method was described in detail in the literature [25]. A sample at a concentration of 5 mg/mL was dissolved either in water or DMSO depending on its solubility. The results were expressed as quercetin equivalent (QE) per gram of dry extract weight. All measurements were done in triplicate.

4.4. The Hypoglycemic Activity

4.4.1. Animals

Adult male mice CD1 (standard laboratory mouse) weighing 20 to 25 g purchased from Masaryk University (Brno, Czech Republic, 30048/2007-10001, number: CZ-62760157) were used. The mice were housed in an air-conditioned animal laboratory under standard conditions; that is, a temperature of 22 °C, a relative humidity of 50% and a 12-h light/dark cycle. The animals were allowed to acclimatize for 5 days and were fed with a pellet diet and tap water *ad libitum*.

4.4.2. Induction of Experimental Diabetes

Alloxan monohydrate (Sigma-Aldrich, Munich, Germany) was freshly dissolved in sterile normal saline and injected intravenously (120 mg/kg body weight) in the tail vein of the test subjects. Two days after the administration of alloxan, fasting blood glucose levels (FBGL) were measured using One Touch Ultra Easy glucometer (Johnson & Johnson, division LifeScan, New Brunswick, NJ, USA), and diabetic mice with levels higher than 20 mmol/L were selected and distributed into 7 groups

randomly (6 mice in each group). All aspects of animal care complied with the ethical guidelines and technical requirements and were proven to be consistent with the Animal Scientific Procedures Act 86/609/EC. The state of health of all animals was regularly examined several times a day during both the period of the animal's acclimation and the whole course of the experiment, by the working team whose members are holders of the Certificate on Professional Competence issued by the Central Commission for the Animal Protection pursuant to § 17 of the Act on Protection of Animals against Cruelty (No. 246/1992 Collection) of the Czech National Council.

4.4.3. Experimental Design

A total of 42 mice (6 mice in each group) were used. The plant extracts and fractions were prepared by dissolving the samples in normal saline prior to use. Kolliphor EL (Sigma-Aldrich) was used to solubilize the HAE, EAF and CF in percentages of 3%, 2% and 5%, respectively. The quercetin suspension was prepared by suspending quercetin in saline using 3% Kolliphor EL and used as a standard for comparison. These prepared solutions were administered by gavage (i.g.) to the groups for 20 consecutive days according to the following:

Group 1: Healthy mice;
Group 2: Diabetic control;
Group 3: Diabetic mice + 200 mg/kg WE;
Group 4: Diabetic mice + 200 mg/kg EAF;
Group 5: Diabetic mice + 200 mg/kg HAE;
Group 6: Diabetic mice + 200 mg/kg CF;
Group 7: Diabetic mice + 50 mg/kg quercetin suspension.

4.4.4. Histology Study

The mice were sacrificed by cervical dislocation. Immediately after, samples of the pancreas, liver, kidney and gut were collected and fixed in 10% buffered formaldehyde (PH 7.2–7.4). Samples were further dehydrated by an ascending ethanol line (30%, 50%, 70%, 80%, 95%, 100%), lightened by xylene and fixed in paraffin. Paraffin blocks were cut using a microtome (Leica SM 2000, Prague, Czech Republic) into sections of 3.5 μm and stained by hematoxylin-eosine pigment.

4.5. The Effect upon Inflammatory Mediators

4.5.1. Materials

The RPMI 1640 medium and the penicillin-streptomycin mixture were purchased from Lonza (Brussel, Belgium). Fetal bovine serum, phorbol myristate acetate, prednisone and the LPS obtained from *Escherichia coli* 0111:B4 were purchased from Sigma-Aldrich. Instant ELISA Kits (eBioscience, Vienna, Austria) were used to evaluate the production of TNF-α.

4.5.2. Cell Maintenance and Macrophage Preparation

The human monocytic leukemia cell line THP-1 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were cultivated at 37 °C in an RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere containing 5% CO_2. Monocytes' differentiation to macrophages was induced by phorbol myristate acetate [36].

4.5.3. Drug Treatment, Induction of Inflammation and Evaluation of TNF-α Secretion

Differentiated macrophages were pretreated for 1 hour with *A. wilhelmsii* extracts and fractions dissolved in either water or DMSO to obtain a final concentration of 25 μg/mL (this concentration lacked the cytotoxic effect). For comparison, 1 μM prednisone dissolved in DMSO was used as a

standard. Vehicle-treated cells contained a vehicle (DMSO) only, and control cells were without the lipopolysaccharide treatment. The concentration of DMSO was 0.1% in each well. The inflammatory response was triggered by adding the lipopolysaccharide dissolved in water (1 µg/mL) to the drug-pretreated macrophages, and the cells were incubated another 24 h. After this time period, the medium was collected, and the concentration of TNF-α was measured. The lowest detection limit was 7.8 pg/mL of TNF-α.

4.5.4. Zymography

Conditioned media obtained by the same way as for TNF-α evaluation were used for measurement of matrix-metalloproteinases (MMP) activity by zymography [37]. Briefly, 20 µL of collected medium were loaded onto polyacrylamide gel impregnated with 0.1% gelatin. After electrophoresis, the SDS from the gels was washed out by 2.5% (*v/v*) Triton X100, and the gels were incubated for 30 min at room temperature (23 °C) and overnight (16 to 20 h) at 37 °C in the developing buffer (50 mM Tris pH 8.8, 5 mM $CaCl_2$, 3 mM NaN_3 and 0.5% Triton ×100). Gels were stained by Coomassie blue. The intensity of digested regions was calculated by AlphaEasy FC 4.0.0 software (Alpha Innotech, San Leandro, CA, USA) for densitometric analysis.

4.6. Statistical Analysis

Statistical analysis was performed by one-way analysis of variance using GraphPad Prism (Version 5, GraphPad, La Jolla, CA, USA). The significant differences were assessed with Tukey's honestly significant difference test ($p < 0.05$). For the effect upon inflammatory mediators assay, statistical significance was determined at levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

4.7. The Anti-Hypercholesterolemic Activity

4.7.1. *In Vitro* HMGR Inhibition Assay

The commercially available HMGR assay kit from Sigma-Aldrich, Catalog Number CS1090, was used to evaluate the HMGR inhibition according to the manufacturer's instructions. The concentration of the purified human enzyme stock solution (Sigma) was 0.50 to 0.70 mg protein/mL. Reference statin drug pravastatin (from Sigma) was used as the positive control. To characterize HMGR inhibition under defined assay conditions, reactions containing 4 µL of NADPH and 12 µL of HMG-CoA substrate in a final volume of 0.2 mL of 100 mM potassium phosphate buffer, pH 7.4, were initiated (Time 0) by the addition of 2 µL of the catalytic domain of human recombinant HMGA incubated in BioTek Synrgy HT (Winooski, VT, USA) at 37 °C in the presence or absence (control) aliquots of the tested samples dissolved in water or DMSO. The blank experiment did not contain HMG-CoA reductase nor any of the studied substances. The rates of NADPH consumed were monitored every 20 s for up to 10 min by scanning spectrophotometrically.

A 2.5-mg/mL concentration of each extract or fraction was used for the measurement. Two microliters were the amount of the sample used in the well. All measurements were done in triplicate. The percentage of inhibition was calculated as follows:

$$\% \text{ Inhibition} = \frac{\Delta A340 \text{control} - \Delta A340 \text{sample}}{\Delta A340 \text{control}} \times 100\%$$

4.7.2. The Isolation, Identification and HMGR Inhibition Activity Testing of the Main Compounds in the CF

The main 5 compounds (Peaks 3, 4, 5, 6 and 7) in the CF were separated using reversed-phase preparative HPLC on an YL 9100 HPLC System (Young Lin, Korea) with a Foxy R2 Fraction Collector (Teledyne Isco, Lincoln, NE, USA). The column was SUPELCOSIL RP-amide, 250 cm × 10 mm, particle size 5 µm. Gradient elution employed 0.2% HCOOH and MeCN, in the gradient: initial composition 30% MeCN, final composition 100% MeCN in the 30th min; flow rate 5 mL/min. Fractions were

acquired according to the detector response at $\lambda = 254$ nm. The purity of all compounds exceeded 95%, as checked via analytical HPLC.

The identity of the compounds was validated using nuclear magnetic resonance NMR (^{1}H-NMR) (Billerica, MA, USA). The carbon chemical shifts were obtained by NMR (HSQC and HMBC) spectra that were obtained on a Bruker Avance 400 spectrometer (Billerica, MA, USA) with TMS as an internal standard. HRMS were measured using an ESI-TOF spectrometer (Mariner Biosystems, Applied Biosystems, Waltham, MA, USA) using ESI in the positive mode of ionization.

The 5 isolated compounds (1β,10β-epoxydesacetoxymatricarin (CP1), leucodin (CP2), 5-demethylsinensetin (CP3), 2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one (CP4) and salvigenin (CP5)) were evaluated for their HMGR inhibition activity using the same protocol. The compounds were tested at 0, 2.5, 5, 10, 20, 40 and 80 μM. All measurements were done in triplicate. All results were expressed as the mean $\pm$ SD of the three repetitions, and IC$_{50}$ values, *i.e.*, the half maximal inhibition concentration, were calculated using GraphPad Prism software.

4.7.3. Computational Docking

Ligand Preparation

All ligands were modelled using Marvin 15.4.20.0, 2015, ChemAxon, and the conformer with the lowest potential energy was used as an input to PyRX 0.8. Ligands were then prepared using PyRX with default settings.

Protein Preparation

PyRX was also used for the protein preparation with default settings. The crystal structure of human HMG-CoA reductase inhibited by atorvastatin (PDB ID: 1HWK) was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) [38]. Extraneous atoms, alternate amino acid residue conformations, ligands, ions and solvent molecules were removed. The protein was then prepared using the PyRX program with default settings.

Molecular Docking

PyRX was used in conjunction with AutoDock Vina [39]. As a binding site, we used the position of atorvastatin determined by the crystallographic experiment. Search exhaustiveness for AutoDock Vina was set to 30, and the edge of the cube defining the searching space was set to 30 Angstroms. The first 10 best solutions according to the binding affinity were stored. For the graphical evaluation of the results, PyMOL was used, and the best solution, ranked by binding affinity, was chosen.

The compounds screened were in accordance with Lipinski's rule of five, a compound having not more than 5 hydrogen bond donors (OH and NH groups), not more than 10 hydrogen bond acceptors (notably N and O), molecular weight under 500 g/mol, partition coefficient log P of less than 5 and rotatable bonds of less than 10.

Author Contributions: Elian Khazneh, Karel Šmejkal and Pavel Suchý conceived of and designed the experiments. Elian Khazneh, Petra Hřibová, Jan Hošek, Zuzana Hanaková, Gabriela Pražanová, Jan Muselík and Jiří Václavík performed the experiments. Elian Khazneh, Karel Šmejkal, Jaroslav Legáth and Michal Miłek analyzed the data. Karel Šmejkal, Pavel Suchý, Peter Kollár and Jaroslav Legáth provided the reagents, materials and analysis tools. Elian Khazneh wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

A. wilhelmsii	*Achillea wilhelmsii*
CF	chloroform fraction
CP1	1β,10β-epoxydesacetoxymatricarin
CP2	Leucodin
CP3	5-demethylsinensetin
CP4	2-(3,4-dimethoxy-phenyl)-3-hydroxy-5,6,7-trimethoxy-chromen-4-one
CP5	Salvigenin
DUOF	Herbarium of the Faculty of Forestry
EAF	ethyl acetate fraction
FBGL	fasting blood glucose levels
GAE	gallic acid equivalent
HAE	hydro-alcoholic extract
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
MMP	matrix-metalloproteinases
QE	quercetin equivalent
THP-1	human monocytic cell line derived from an acute monocytic leukemia patient
TNF-α	tumor necrosis factor α
WE	water extract

References

1. Dokhani, S.H.; Cottrell, T.; Khajeddin, J.; Mazza, G. Analysis of aroma and phenolic components of selected *Achillea* species. *Plant Foods Hum. Nutr.* **2005**, *60*, 55–62. [CrossRef] [PubMed]
2. Trifunovic, S.; Vajs, V.; Juranic, Z.; Zizak, Z.; Tesevic, V.; Macura, S.; Milosavljevic, S. Cytotoxic constituents of *Achillea clavennae* from Montenegro. *Phytochemistry* **2006**, *67*, 887–893. [CrossRef] [PubMed]
3. Rezaeipoor, R.; Saeidnia, M.; Kamalinejad, M. Immunosuppressive activity of *Achillea talagonica* on humoral immune responses in experimental animals. *J. Ethnopharmacol.* **2005**, *14*, 221–227. [CrossRef]
4. Konyalioglu, S.; Karamenderes, C. The protective effects of *Achillea L.* species native in Turkey against H₂O₂-induced oxidative damage in human erythrocytes and leucocytes. *J. Ethnopharmacol.* **2005**, *14*, 221–227. [CrossRef] [PubMed]
5. Kotan, R.; Cakir, A.; Dadasoglu, F.; Aydin, T.; Cakmakci, R.; Ozar, H.; Kordali, S.; Mete, E.; Dikbas, N. Antibacterial activities of essential oils and extracts of *Turkish achillea, Satureja* and *Thymus* species against plant pathogenic bacteria. *J. Sci. Food Agric.* **2010**, *15*, 145–160. [CrossRef] [PubMed]
6. Stojanovic, G.; Radulovic, N.; Hashimoto, T.; Palic, R. *In vitro* antimicrobial activity of extracts of four *Achillea* species: The composition of *Achillea clavennae* L. (*Asteraceae*) extract. *J. Ethnopharmacol.* **2005**, *101*, 185–190. [CrossRef] [PubMed]
7. Yazdanparast, R.; Ardestani, A.; Jamshidi, S.H. Experimental diabetes treated with *Achillea santolina*: Effect on pancreatic oxidative parameters. *J. Ethnopharmacol.* **2007**, *112*, 13–18. [CrossRef] [PubMed]
8. Karamenderes, C.; Apaydin, S. Antispasmodic effect of *Achillea nobilis L.* subsp. Sipylea (O. Schwarz) Bassler on the rat isolated duodenum. *J. Ethnopharmacol.* **2003**, *84*, 175–179. [CrossRef]
9. Potrich, F.B.; Allemand, A.; da Silva, L.M.; dos Santos, A.C.; Baggio, C.H.; Freitas, C.S.; Mendes, D.A.G.B.; Andre, E.; Werner, M.F.P.; Marques, M.C.A. Antiulcerogenic activity of hydroalcoholic extract of *Achillea millefolium* L.: Involvement of the antioxidant system. *J. Ethnopharmacol.* **2010**, *130*, 85–92. [CrossRef] [PubMed]
10. Cavalcanti, A.M.; Baggio, C.H.; Freitas, C.S.; Rieck, L.; de Sousa, R.S.; Da Silva-Santos, J.E.; Mesia-Vela, S.; Marques, M.C.A. Safety and antiulcer efficacy studies of *Achillea millefolium* L. after chronic treatment in Wistar rats. *J. Ethnopharmacol.* **2006**, *107*, 277–284. [CrossRef] [PubMed]

11. Tuberoso, C.I.G.; Montoro, P.; Piacente, S.; Corona, G.; Deiana, M.; Dessi, M.A.; Pizza, C.; Cabras, P. Flavonoid characterization and antioxidant activity of hydroalcoholic extracts from *Achillea ligustica* All. *J. Pharm. Biomed. Anal.* **2009**, *50*, 440–448. [CrossRef] [PubMed]

12. Kaliora, A.C.; Dedoussis, G.V.Z. Natural antioxidant compounds in risk factors for CVD. *Pharmacol. Res.* **2007**, *56*, 99–109. [CrossRef] [PubMed]

13. Tosun, F.; Kizilay, C.A.; Sener, B.; Vural, M.; Palittapongarnpim, P. Antimycobacterial screening of some *Turkish* plants. *J. Ethnopharmacol.* **2004**, *95*, 273–275. [CrossRef] [PubMed]

14. Shetty, A.K.; Rashmi, R.; Rajan, M.G.R.; Sambaiah, K.; Salimath, P.V. Antidiabetic influence of quercetin in Streptozotocin-induced diabetic rats. *Nutr. Res.* **2004**, *24*, 373–381. [CrossRef]

15. Jamkhande, P.G.; Chandak, P.G.; Dhawale, S.C.; Barde, S.R.; Tidke, P.S.; Sakhare, R.S. Therapeutic approaches to drug targets in atherosclerosis. *Saudi Pharm. J.* **2014**, *22*, 179–190. [CrossRef] [PubMed]

16. Bhatnagar, D.; Soran, H.; Durrington, P.N. Hypercholesterolaemia and its management. *BMJ* **2008**, *337*. [CrossRef] [PubMed]

17. Buhaescu, I.; Izzedine, H. Mevalonate pathway: A review of clinical and therapeutical implications. *Clin. Biochem.* **2007**, *40*, 575–584. [CrossRef] [PubMed]

18. Manzoni, M.; Rollini, M. Biosynthesis and biotechnological production of statins by filamentous fungi and application of these cholesterol-lowering drugs. *Appl. Microbiol. Biotechnol.* **2002**, *58*, 555–564. [PubMed]

19. Ferguson, J.F.; Ryan, M.F.; Gibney, E.R.; Brennan, L.; Roche, H.M.; Reilly, M.P. Dietary isoflavone intake is associated with evoked responses to inflammatory cardiometabolic stimuli and improved glucose homeostasis in healthy volunteers. *Nutr. Metab. Cardiovasc. Dis.* **2014**, *24*, 996–1003. [CrossRef] [PubMed]

20. Braun, L.M.; Rodríguez, D.A.; Evenson, K.R.; Hirsch, J.A.; Moore, K.A.; Roux, A.V.D. Walkability and cardiometabolic risk factors: Cross-sectional and longitudinal associations from the Multi-Ethnic Study of Atherosclerosis. *Health Place* **2016**, *39*, 9–17. [CrossRef] [PubMed]

21. Carney, R.; Cotter, J.; Bradshaw, T.; Firth, J.; Yung, A.R. Cardiometabolic risk factors in young people at ultra-high risk for psychosis: A systematic review and meta-analysis. *Schizophr. Res.* **2016**, *170*, 290–300. [CrossRef] [PubMed]

22. Bastien, M.; Poirier, P.; Lemieux, I.; Despres, J.P. Overview of epidemiology and contribution of obesity to cardiovascular disease. *Prog. Cardiovasc. Dis.* **2014**, *56*, 369–381. [CrossRef] [PubMed]

23. Mathieu, P.; Poirier, P.; Pibarot, P.; Lemieux, I.; Després, J.P. Visceral obesity: The link among inflammation, hypertension, and cardiovascular disease. *Hypertension.* **2009**, *53*, 577–584. [CrossRef] [PubMed]

24. Moy, F.M.; Loh, D.A. Cardiometabolic risks profile of normal weight obese and multi-ethnic women in a developing country. *Maturitas* **2015**, *81*, 389–393. [CrossRef] [PubMed]

25. Bernatoniene, J.; Petkeviciute, Z.; Kalveniene, Z.; Masteikova, R.; Draksiene, G.; Muselik, J.; Bernatoniene, R.; Lazauskas, R.; Savickas, A. The investigation of phenolic compounds and technological properties of *Leonurus*, *Crataegus* and Ginkgo extracts. *J. Med. Plants Res.* **2010**, *4*, 925–931. [CrossRef]

26. Soto, C.; Mena, R.; Luna, J.; Cerbon, M.; Larrieta, E.; Vital, P.; Uriae, E.; Sancheza, M.; Recobaa, R.; Barrona, H.; *et al.* Silymarin induces recovery of pancreatic function after alloxan damage in rats. *Life Sci.* **2004**, *75*, 2167–2180. [CrossRef] [PubMed]

27. Ahlem, S.; Khaled, H.; Wafa, M.; Sofiane, B.; Mohamed, D.; Jean-Claude, M.; Abdelfattah, E.F. Oral administration of *Eucalyptus globulus* extract reduces the alloxan-induced oxidative stress in rats. *Chem. Biol. Interact.* **2009**, *181*, 71–76. [CrossRef] [PubMed]

28. Valant-Vetschera, K.M. Flavonoid glycoside accumulation trends of *Achillea nobilis* L. and related species. *Biochem. Syst. Ecol.* **1987**, *15*, 45–52. [CrossRef]

29. Marchart, E.; Kopp, B. Capillary electrophoretic separation and quantification of flavone-*O*- and *C*-glycosides in *Achillea setacea* W. et K. *J. Chromatogr. B* **2003**, *792*, 363–368. [CrossRef]

30. Glasl, S.; Mucaji, P.; Werner, I.; Presser, A.; Jurenitsch, J. Sesquiterpenes and flavonoid aglycones from a Hungarian taxon of the *Achillea millefolium* group. *Z. Naturforsch. C* **2002**, *57*, 976–982. [CrossRef] [PubMed]

31. Moradkhani, S.; Ayatollahi, A.M.; Ghanadian, M.; Moin, M.R.; Razavizadeh, M.; Shahlaei, M. Phytochemical analysis and metal-chelation activity of *Achillea tenuifolia* Lam. *Iran. J. Pharm. Res.* **2012**, *11*, 177–183. [PubMed]

32. Saeidnia, S.; Gohari, A.R.; Mokhber-Dezfuli, N.; Kiuchi, F. A review on phytochemistry and medicinal properties of the genus Achillea. *Daru* **2011**, *19*, 173–186. [PubMed]

33. Vessal, M.; Hemmati, M.; Vasei, M. Antidiabetic effects of quercetin in streptozocin-induced diabetic rats. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **2003**, *135*, 357–364. [CrossRef]

34. Cazarolli, L.H.; Kappel, V.D.; Pereira, D.F.; Moresco, H.H.; Brighente, I.M.; Pizzolatti, M.G.; Silva, F.R.M.B. Anti-hyperglycemic action of apigenin-6-*C*-β-fucopyranoside from *Averrhoa carambola*. *Fitoterapia* **2012**, *83*, 1176–1183. [CrossRef] [PubMed]

35. Zaidi, S.F.; Muhammad, J.S.; Shahryar, S.; Usmanghani, K.; Gilani, A.H.; Jafri, W.; Sugiyama, T. Anti-inflammatory and cytoprotective effects of selected Pakistani medicinal plants in *Helicobacter pylori*-infected gastric epithelial cells. *J. Ethnopharmacol.* **2012**, *141*, 403–410. [CrossRef] [PubMed]

36. Hosek, J.; Zavalova, V.; Smejkal, K.; Bartos, M. Effect of diplacone on LPS-induced inflammatory gene expression in macrophages. *Folia Biol. Praha* **2010**, *56*, 124–130. [PubMed]

37. Talhouk, R.S.; Chin, J.R.; Unemori, E.N.; Werb, Z.E.N.A.; Bissell, M.J. Proteinases of the mammary gland: Developmental regulation *in vivo* and vectorial secretion in culture. *Development* **1991**, *112*, 439–449. [PubMed]

38. Bernstein, F.C.; Koetzle, T.F.; Williams, G.J.; Meyer, E.F.; Brice, M.D.; Rodgers, J.R.; Kennard, O.; Shimanouchi, T.; Tasumi, M. The Protein Data Bank: A computer-based archival file for macromolecular structures. *Arch. Biochem. Biophys.* **1978**, *185*, 584–591. [CrossRef]

39. Trott, O.; Olson, A.J. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *J. Comput. Chem.* **2010**, *31*, 455–461. [CrossRef] [PubMed]

Sample Availability: Samples of the compounds are not available from the authors.

F

Flavonoids as Anti-inflammatory Agents

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Synonyms

Bioflavonoids; Flavonoids; Phenylchromones; Vitamin P

Definition

The flavonoids are a group of plant secondary metabolites biosynthetically derived from shikimic acid and polyketide pathways. They are plant pigments containing benzopyrone substituted with a phenyl ring at position 2 or 3 and possess a wide spectrum of biological activities, including antioxidant and anti-inflammatory activities.

Chemical Structures and Properties

The technical term "flavonoid" arises from the basic skeleton of these plant pigments that are derived from benzo-γ-pyrone, structure well known as chromone, modified at position 2 or 3 by the addition of a phenyl ring at position 2 yielding a flavonoid and at position 3 an isoflavonoid (Fig. 1). The biosynthesis of flavonoids is based on the crossing of shikimic acid and acylphloroglucinol metabolic pathways. The three-ring skeleton may be substituted with hydroxyl groups. Substitution of the A ring typically occurs at positions 5 and 7 (a *meta* arrangement characteristic for acylphloroglucinols). On the B ring positions 4′ or 3′ and 4′ (typical for shikimates) are preferred (Havsteen 2002; Fig. 1). There can be exceptions, for example, in Moraceae flavonoids, where the B ring is substituted with *meta* (2′, 4′) hydroxyl groups (Bruneton 1999; Havsteen 2002).

The hydroxyl groups of flavonoids are commonly substituted with one or more sugar units. The sugar can also be connected via a C-C bond. Glycosides of the flavonoids are relatively hydrophilic. The aglycones can be lipophilic, and their lipophilicity can be further enhanced by methylating the hydroxyl groups to form methoxyl groups or by prenylation or geranylation at different positions on the skeleton. The prenyl or geranyl moiety may also be modified in different ways (oxidation, reduction, cyclization, etc.) (Smejkal 2014).
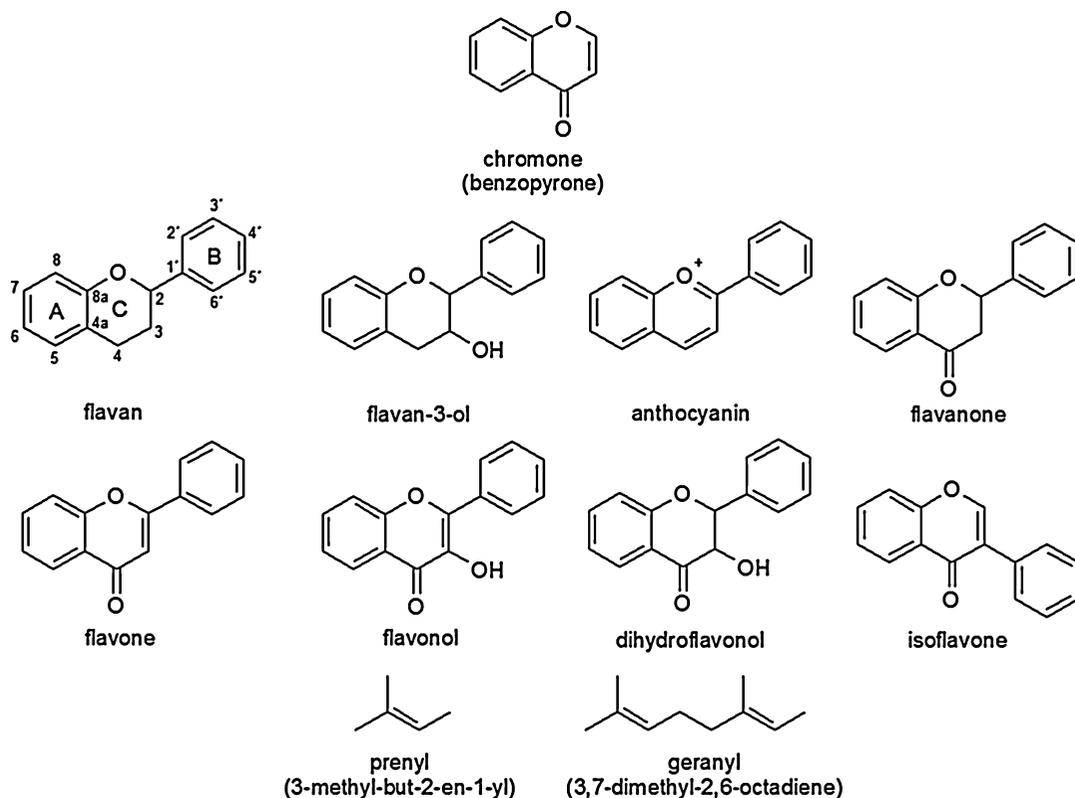
Flavonoids as Anti-inflammatory Agents, Fig. 1 The overview of basic flavonoid skeletons and their substituents increasing lipophilicity

As a general rule, flavonoid glycosides are soluble in water and alcohols but sometimes sparingly (rutin, hesperidin). Aglycones are usually well soluble in nonpolar solvents, and when they have at least one hydroxyl group free, they can be dissolved in alkaline solutions to form salts (e.g., sodium, potassium salts, etc.). Flavonoids are usually stable compounds, relatively resistant to heat, oxygen, and moderate degrees of acidity, but preparation of fruits and vegetables for consumption can lead to some loss in flavonoid content. Some technological procedures used in laboratory can lead to partial decomposition and formation of artifacts. Especially compounds with prenyl-like modification could undergo oxidative procedures. Also some food technology processing, such as peeling, skinning, and leaf selection may remove and reduce the total flavonoid content (Bruneton 1999; Havsteen 2002).

Different plants contain predominantly different types of flavonoids. Well known are so-called dietary flavonoids present in common foods, but there are many other flavonoid species present in medicinal plants.

Flavanones can be found in chick peas and cumin, and the major sources of flavanones are citrus fruits and juices. Cumin and peppermint are rich in flavanones like hesperidin. Narirutin and naringenin glycosides were obtained also from hawthorn berry and rowanberry. The flavanone liquiritigenin is found in liquorice roots. Glycosides, like neohesperidose-substituted flavanones (good example is naringin from grapefruit), are usually bitter; rutinose-substituted flavanones (hesperidin in oranges) are usually tasteless. For examples of common flavanones, see Fig. 2 (Peterson and Dwyer 1998; Del Rio et al. 2013).
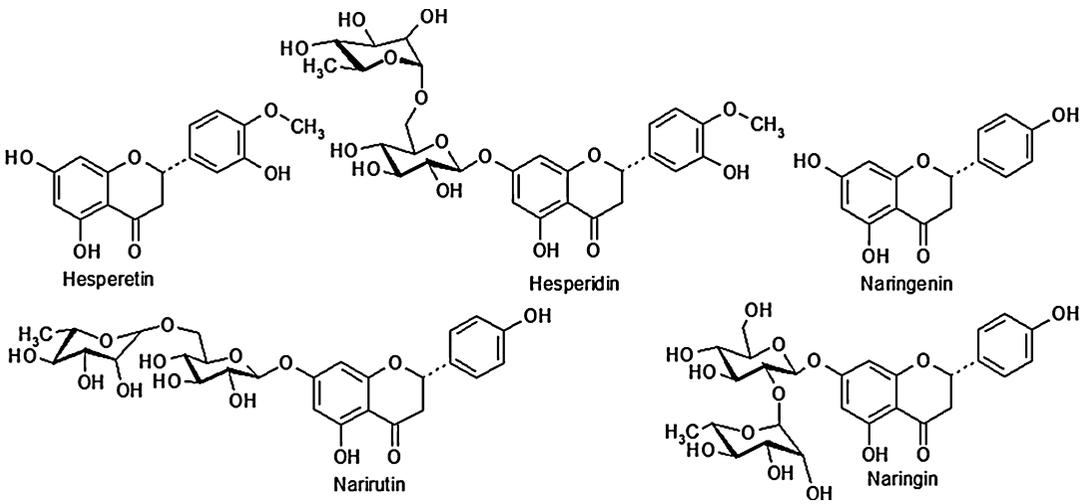
Flavonoids as Anti-inflammatory Agents, Fig. 2 Structures of some common flavanones
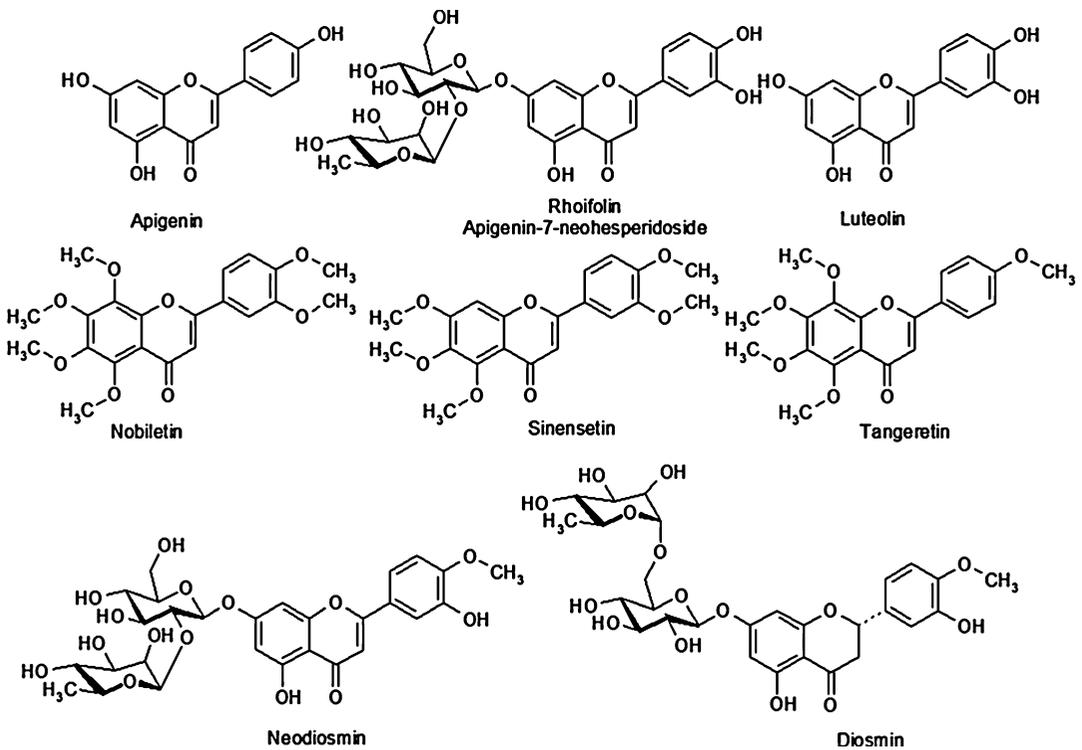
Flavonoids as Anti-inflammatory Agents, Fig. 3 Structures of some common flavones

Flavones are not common in fruit, but they are often found in grains and herbs. The most common flavone aglycones are apigenin and luteolin (Fig. 3). Apigenin and its glycosides are commonly found in cereal grains, some herbs, and some vegetables. Luteolin is found primarily in cereals and herbs. Glycosides of luteolin and apigenin are commonly present in vegetables and
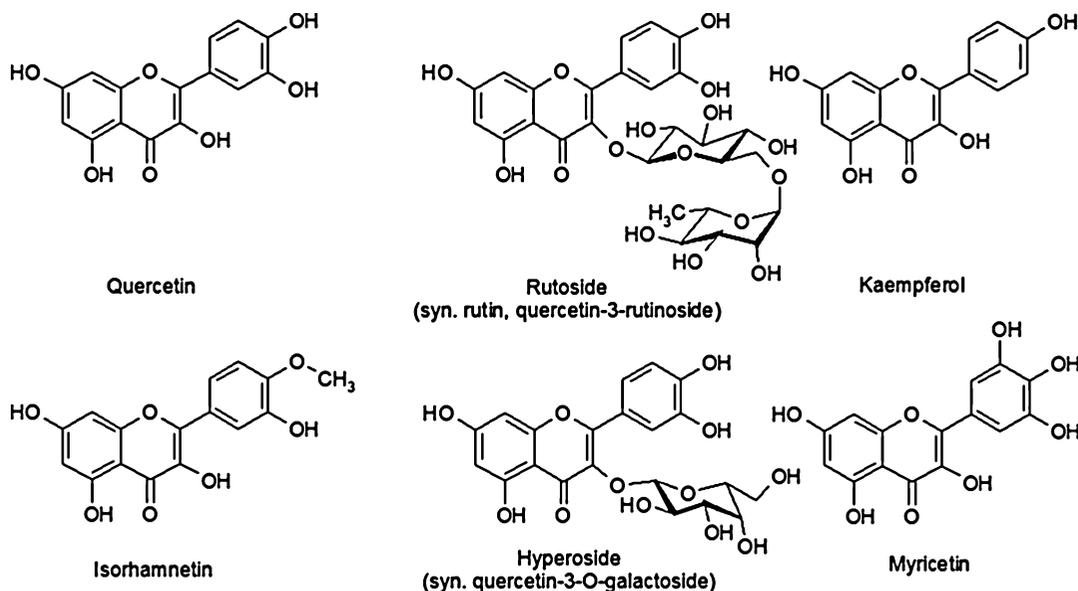
Flavonoids as Anti-inflammatory Agents, Fig. 4 Structures of some common flavonols

the leaves of vegetables. Some medicinal and aromatic herbs, such as rosemary and thyme, contain flavones. If they occur in high concentrations or are complexed with metal ions, flavones can contribute to color of plant tissue, and they also contribute to their taste. Nobiletin, sinensetin, and tangeretin are nonglycosidic, highly methoxylated, bitter citrus flavones that contribute to taste. On the other hand, the glycosylated flavones neodiosmin and rhoifolin reduce the bitterness of plant bitter substances (Peterson and Dwyer 1998; Del Rio et al. 2013).

The best known flavonols are quercetin and kaempferol. Quercetin is ubiquitous in fruits and vegetables (Fig. 4). Quercetin glycosides predominate in vegetables or in the leaves of various vegetables. The most common quercetin glycoside is rutoside (also known rutin). Kaempferol is most common among fruits and leafy vegetables; it can be also found in some berries, herbs, legumes, and root vegetables. Isorhamnetin occurs in onions and pears. Myricetin is found most often in berries, maize, and tea. In fruits, flavonols and their glycosides are found predominantly in the skin (Peterson and Dwyer 1998; Del Rio et al. 2013).

The isoflavonoids are a separate group of flavonoids, well known for their estrogenic properties. The group can be divided into isoflavanones, isoflavones, and isoflavonols. The best-known isoflavonoids are daidzein (the aglycone of the glycoside daidzin) and genistein (the aglycone of the glycoside genistin). Other known isoflavonoid aglycones found in food are biochanin A and formononetin (Fig. 5). The isoflavonoids are found predominantly in legumes. Soybeans are the major source of daidzein and genistein, but these are also found in black beans, green peas, and clover sprouts. Other isoflavonoids, such as biochanin A and formononetin, have been found in green beans, chick peas, lima beans, split peas, alfalfa and clover sprouts, and sunflower seeds (Peterson and Dwyer 1998; Del Rio et al. 2013).

Flavans, especially flavan-3-ols, can be found under different names: catechins, leucoanthocyanins, proanthocyanins, and tannins. They occur mainly as monoflavans, biflavans, and triflavans. Flavans are rarely glycosylated, but they may be esterified with gallic acid. Monoflavans are found in ripe fruits and fresh leaves. There is little information on the monoflavan content in vegetables. Tea contains
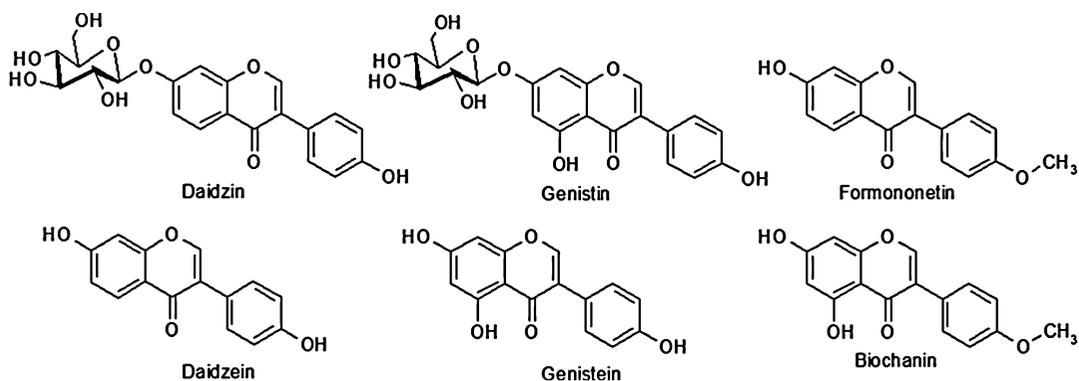
Flavonoids as Anti-inflammatory Agents, Fig. 5 Structures of some common isoflavones
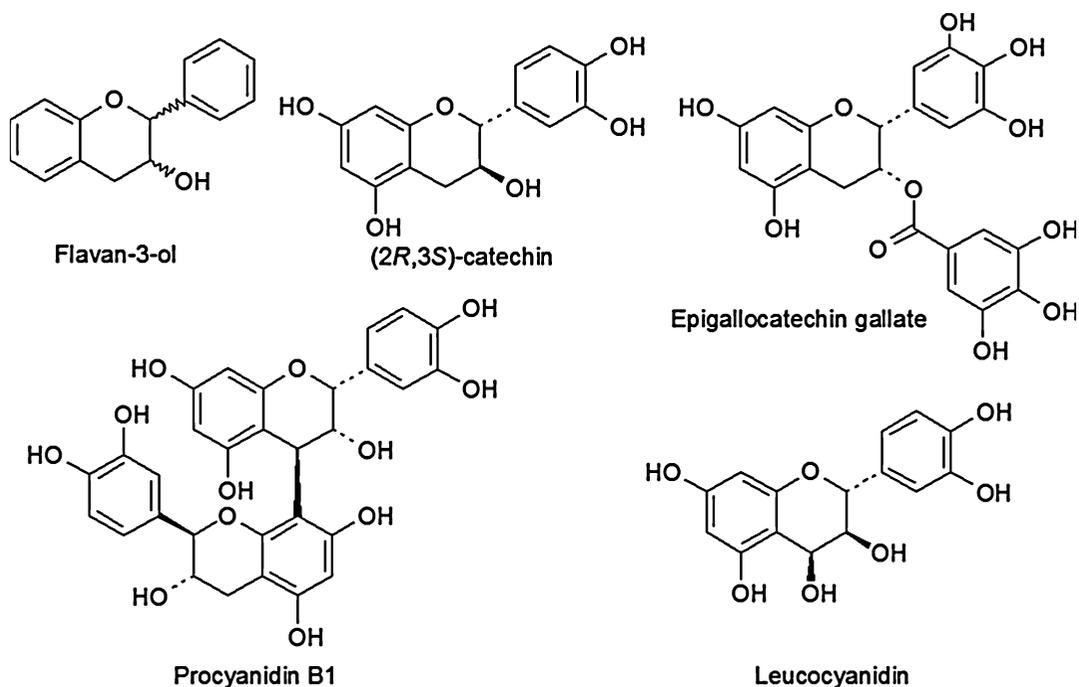
Flavonoids as Anti-inflammatory Agents, Fig. 6 Structures of some common flavans

large amounts of flavans. Biflavans and triflavans occur in fruits and cereals. They are found in apples, blackberries, black currants, cranberries, grapes, peaches, and strawberries. In cereals, they are found in sorghum and barley grains. Examples of some flavan derivatives are displayed in Fig. 6 (Peterson and Dwyer 1998; Del Rio et al. 2013).

Anthocyanins are responsible for the blue and red pigmentation of berries, cherries, and plums, as well as eggplant, red cabbage, and radishes. The color of anthocyanins is pH dependent. An anthocyanin is red at pH <3 (the stable form); it turns blue or purple when the pH is increased to 4–6. At higher pH values it becomes colorless and later decomposed. Anthocyanins often occur in complex mixtures. Grape extracts possess the glucosides, acetyl glucosides, and coumaryl glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin (Fig. 7). In
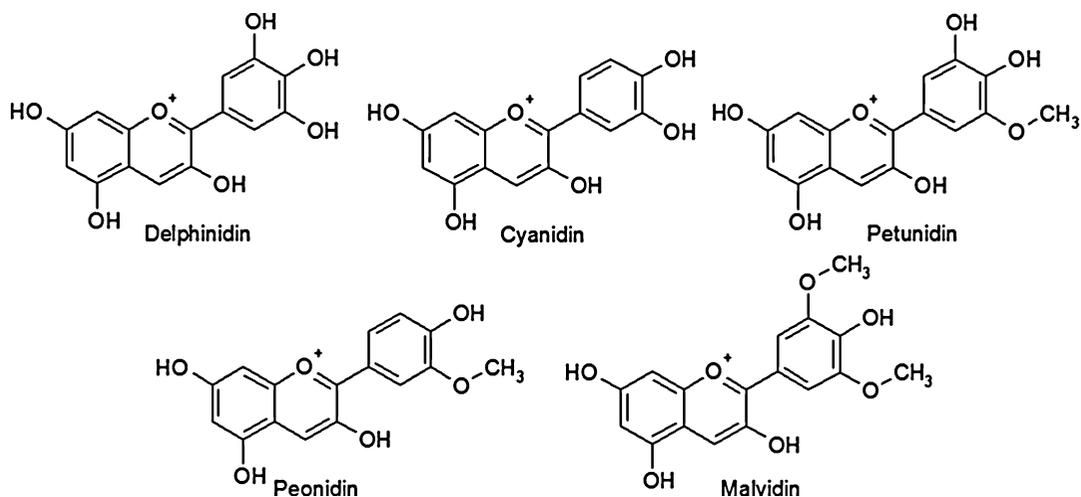
Flavonoids as Anti-inflammatory Agents, Fig. 7 Structures of some common anthocyanidins. Cl^- is the most common anion

flowers, anthocyanins may form complexes with flavones or metal ions such as iron or magnesium. The anthocyanin content of a fruit usually increases as the fruit ripens (Peterson and Dwyer 1998; Del Rio et al. 2013).

Derivatives with a modified basic flavonoid skeleton can occur in many plants used medicinally. Prenylated flavonoids occur in several plant families, for example, Paulowniaceae (*Paulownia* spp., formerly Scrophulariaceae), Leguminosae (*Sophora* spp.), Guttiferae (*Garcinia* spp.), Rutaceae (*Neoraputia* spp.), and Cannabaceae (*Cannabis* and *Humulus* spp.), but probably the largest number of prenylated flavonoids can be found in the Moraceae family (*Morus* and *Artocarpus* spp.). Prenylated flavonoids are a large group of compounds that combine a flavonoid skeleton with a lipophilic prenyl side chain (Fig. 8). In general, the majority of these flavonoids are *C*-prenylated or geranylated, but some *O*-prenylated compounds also occur. As mentioned above, the side chain can be modified, especially by oxidation, and this variation contributes many structural possibilities. Adding the lipophilicity of a prenyl side chain to the flavonoid skeleton gives the resultant compound great potential for biological activity (Yazaki et al. 2009; Smejkal 2014).

Pharmacological Activities

Experimental *in vitro* and *in vivo* studies have revealed many biological and pharmacological activities of flavonoids. Their antioxidant and anti-inflammatory properties are the most studied, but these phytochemicals also show antibacterial, antiviral, hypolipidemic, and even anticancer effects (Havsteen 2002). The process of identifying a particular structure-activity relationship is complicated, especially *in vivo*, because flavonoids can have pleiotropic effects that target many cellular proteins or mechanisms.

Antioxidant Activity

Oxidative stress is one of the main hallmarks of inflammation. Reactive oxygen species (ROS) are produced by immune cells, especially macrophages and neutrophils, to kill invading bacteria and viruses. However, excessive or prolonged oxidative stress can damage the surrounding molecules, e.g., the unsaturated lipids in plasma membranes, proteins, or DNA (Havsteen 2002). Antioxidant compounds could therefore be promising as anti-inflammatory agents. The antioxidant capabilities of flavonoids have been well described using many biochemical models, both *in vitro* (cell-based) and *in vivo*.
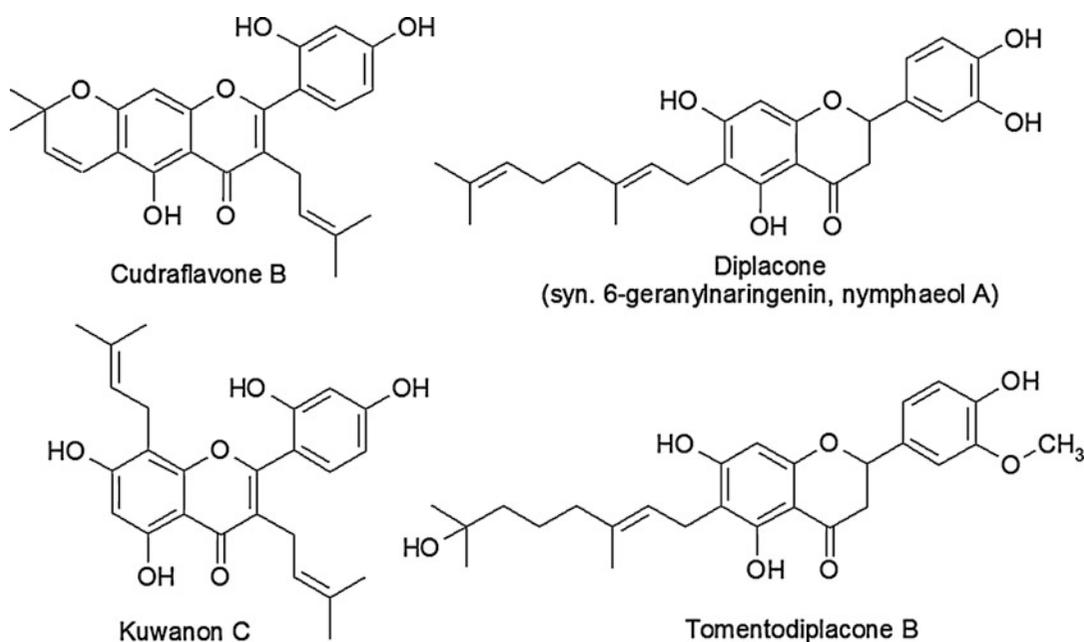
Flavonoids as Anti-inflammatory Agents, Fig. 8 Examples of some prenylated or geranylated flavonoids

Flavonoids as Anti-inflammatory Agents, Table 1 Mechanisms of antioxidant activity of flavonoids

	Responsible structural elements (shown in bold)	
Chemical or biological effect		References
ROS scavenging	3′,4′-hydroxyl groups	Pietta 2000
	2,3-double bond in conjugation with 4-carbonyl group	Procházková et al. 2011
	3,5,7-hydroxyl groups	
Peroxynitrite scavenging	3′,4′-hydroxyl groups	Procházková et al. 2011
	3-hydroxyl group	
Metal-chelating activity	3′,4′-hydroxyl groups	Pietta 2000
	4-carbonyl group in conjugation with 3-hydroxyl group	Procházková et al. 2011
	4-carbonyl group with 5-hydroxyl group	
PKC inhibition	Coplanar flavone structure	Procházková et al. 2011
	3′,4′-hydroxyl groups	
	7-hydroxyl group	

ROS = reactive oxygen species
PKC = protein kinase C

The main mechanisms of the antioxidant action of flavonoids are listed in Table 1 (Procházková et al. 2011). Flavonoids can scavenge ROS and reactive nitrogen species (RNS) directly, chelate metal ions, and inhibit oxidases. Moreover, they are involved in the

regeneration of α-tocoferol and ascorbic acid radicals.

The flavonoids scavenge ROS directly by donating the hydrogen atom of a hydroxyl group. This radical scavenging activity is connected with the oxidation-reduction potential of the flavonoids. Flavonoids interact with radicals, donating one hydrogen atom and forming a flavonoid phenoxyl radical (flavonoid-O$^{\bullet}$). This radical may then react with other radicals to form a stable quinoid structure. The number of hydroxyl groups and their positions on the flavonoid skeleton significantly affect the radical scavenging activity. Substituents on the B ring (especially catechol and pyrogallol moieties) are more important than those on rings A and C. The antiradical activity is diminished, when only one hydroxyl group is present on the B ring. The second structural element responsible for eliminating radicals is the 2,3-double bond conjugated with the 4-oxo group on ring C. On the other hand, glycosylation attenuates the scavenging activity. Flavonoids are also able to trap the peroxynitrite radical, which can occur as a product of the reaction of superoxide with NO. This activity is attributed to the presence of the 3′ and 4′-hydroxyl groups along with the 3-hydroxyl group. (Pietta 2000; Havsteen 2002; Procházková et al. 2011).

Another feature contributing to the antioxidant activity of flavonoids is their ability to chelate metal ions, especially Fe^{2+} and Cu^{2+}. Free metal ions can take part in Fenton's reaction and thus enhance the formation of ROS. Chelation of these ions neutralizes their ability to serve as redox catalysts. On the other hand, trace metal ions are essential cofactors in many biological systems, e.g., Fe^{2+} in hemoglobin or Cu^{2+} and Zn^{2+} in a superoxide dismutase (SOD), where the chelating ability of flavonoids would be detrimental. Metal ions can bind to the two hydroxyl groups of a catechol moiety of the B ring, the 3-hydroxyl and 4-oxo groups of ring C, or the 4-oxo and 5-hydroxyl groups on rings A and C (Pietta 2000; Procházková et al. 2011).

The antioxidant action of flavonoids is also attributable to their ability to inhibit enzymes that produce radicals, such as xanthine oxidase

(XO) or NADH oxidase (NOX). They also interact with protein kinase C (PKC), which is responsible for the activation of NOX, and thereby attenuate the functioning of NOX. It has been postulated that a coplanar flavone structure with free hydroxyl substituents at positions 3′, 4′, and 7 should diminish the activity of PKC (Pietta 2000; Procházková et al. 2011).

Fully evaluating the antioxidant activity of flavonoids *in vivo* is more complicated because of their relatively low bioavailability and destruction by metabolism. Flavonoid metabolites usually possess less antioxidative activity than the parent structure. It seems likely that flavonoids and flavonoid metabolites work in different ways, the flavonoids by scavenging up ROS and the flavonoid metabolites by preventing the formation of such radicals (Pietta 2000; Procházková et al. 2011).

Prooxidant Activity

In addition to their antioxidant activity, flavonoids can also act as prooxidants. Their ability to trigger oxidative stress could explain some of the toxic effects of flavonoids observed *in vivo*. On the other hand, a mild prooxidant effect could also induce the expression of antioxidant enzymes, such as SOD or catalase (CAT), and thereby contribute to the overall antioxidant cytoprotection (Procházková et al. 2011).

It has been postulated that a greater number of hydroxyl groups (especially on rings A and B) means a greater prooxidant capacity. The presence of the 2,3-double bond and the 4-carbonyl group may also promote the formation of ROS. The antioxidant or prooxidant effect is usually concentration dependent. At low concentrations, flavonoids act as antioxidants, but at higher concentrations they are prooxidants (Procházková et al. 2011).

Anti-inflammatory Activity

Inflammation is a multiple and complex response by the body to infection or injury. Flavonoids show pleiotropic effects and can modulate a broad spectrum of inflammatory regulatory nodes (Fig. 9). Their antiphlogistic action combines many particular effects. The antiphlogistic
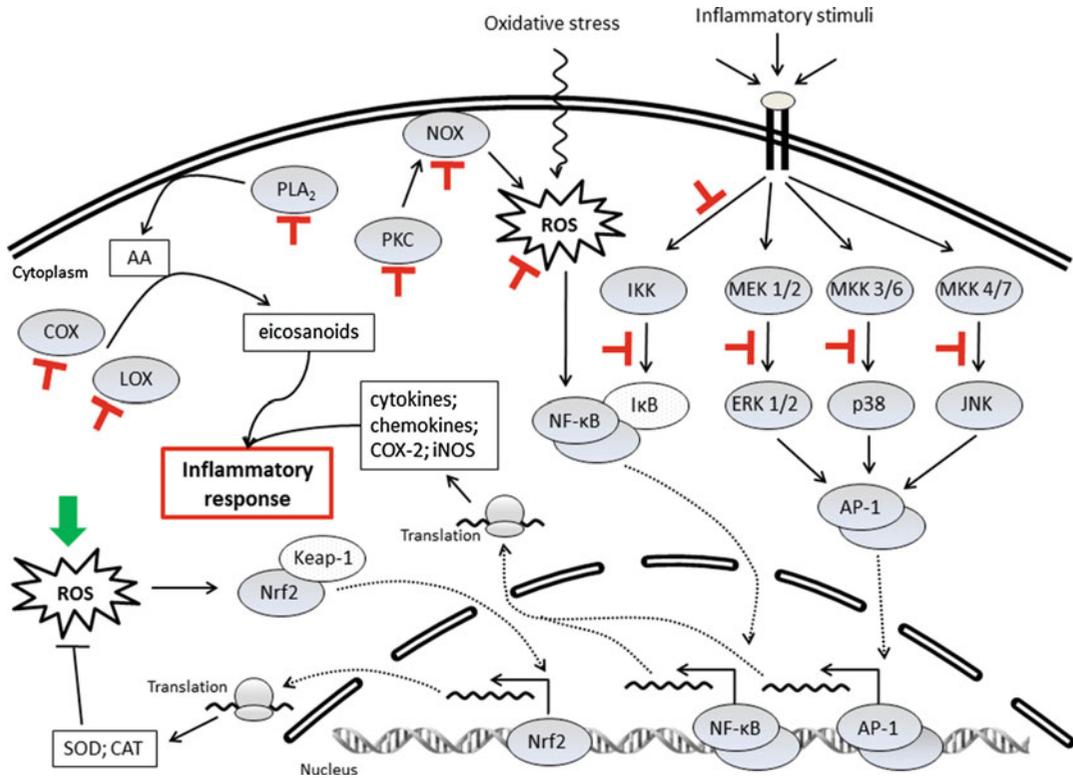
Flavonoids as Anti-inflammatory Agents,
Fig. 9 Mechanisms of anti-inflammatory action of flavo-
noids. *Full arrows* mean direct interaction, *dashed arrows*
indicate translocation. *Thick red* **T** marks show points, at
which flavonoids inhibit; the *thick green arrow* indicates
the prooxidant effect of flavonoids. *AA* arachidonic acid,
AP-1 activator protein 1, *CAT* catalase, *COX* cyclooxy-
genase, *ERK 1/2* extracellular signal-regulated kinase 1/2,
IκB inhibitor of κB, *IKK* IκB kinase, *JNK* c-Jun
N-terminal kinase, *Keap-1* Kelch-like ECH-associated
protein 1, *LOX* lipoxygenase, *MEK 1/2* MAPK/ERK
kinase 1/2, *MKK* mitogen-activated protein kinase,
NF-κB nuclear factor κB, *NOX* NADH oxidase, *Nrf2*
nuclear factor erythroid 2-related factor 2, *PKC* protein
kinase C, *PLA$_2$* phospholipase A$_2$, *ROS* reactive oxygen
species, *SOD* superoxide dismutase

action of flavonoids can be mediated by several
pathways (Table 2): *via* antioxidant and
prooxidant effects (see above), by interacting
directly with pro-inflammatory proteins, and by
interacting with signal pathways and inhibiting
the expression of inflammation-related genes.

Modulation of the activity of
pro-inflammatory enzymes is one of the most
important mechanisms of flavonoid action.
Pro-inflammatory enzymes, such as phospholi-
pase A$_2$ (PLA$_2$), cyclooxygenase (COX),
lipoxygenase (LOX), and NO synthase (NOS),
produce very potent inflammatory mediators,
and inhibiting them can contribute to the overall
antiphlogistic potential of flavonoids. Special
attention is focused on enzymes metabolizing

arachidonic acid (AA). PLA$_2$ stands at the begin-
ning of this AA process. Many studies have dem-
onstrated the ability of flavonoids to inhibit
different isoforms of this enzyme, with prefer-
ence given to PLA$_2$-IIA. The most potent are
flavonoids with a 2,3-double bond, and especially
biflavonoids. Following its enzymatic release,
AA is oxidized to different eicosanoids in a pro-
cess mediated by COX and LOX enzymes. Fla-
vonoids diminish the activity of COX, but they
inhibit COX-1 preferentially over COX-2. Only a
limited number of observations of anti-COX-2
effects of flavonoids have been reported, experi-
ments that have shown the positive influence of
the 2,3-double bond and the presence of a prenyl
group at C3 on this anti-inflammatory action. The

Flavonoids as Anti-inflammatory Agents, Table 2 Mechanisms of anti-inflammatory action of some flavonoids

Responsible structural elements (shown in bold)

Biological effect		References
PLA$_2$ inhibition	2,3-double bond	Kim et al. 2004
COX-1 inhibition	2,3-double bond	Kim et al. 2004
COX-2 inhibition	2,3-double bond 3-isoprenyl residue Galloyl moiety	Kim et al. 2004
5-LOX, 12-LOX inhibition	2,3-double bond 3-hydroxyl group	Kim et al. 2004
Inhibition of inflammation-related gene expression (modification of signal-transducing pathways)	5,7-hydroxyl groups 3′,4′-hydroxyl groups 2,3-double bond 4-carbonyl group	Kim et al. 2004 Procházková et al. 2011 Costa et al. 2012
In vivo anti-inflammatory activity	5,7-hydroxyl groups 3′,4′-hydroxyl or methoxyl groups 2,3-double bond	Kim et al. 2004 Costa et al. 2012

PLA2 = phospholipase A$_2$
COX = cyclooxygenase
LOX = lipoxygenase

presence of a galloyl moiety also favors the inhibition of COX-2 activity. Another way of using AA is via LOX enzymes. Whereas flavone derivatives have been described as demonstrating anti-COX activity, flavonol derivatives have shown anti-LOX effects. This confirms the importance of a hydroxyl group at C3. Furthermore, the 2,3-double bond has been found to be a very important moiety for the inhibition of LOX activity. The majority of anti-LOX experiments have been performed using 5-LOX and 12-LOX, and information about any effects on other isoforms of LOX is limited. Another enzyme that produces pro-inflammatory molecules is inducible NO synthase (iNOS), which produces NO in stimulated immune cells. Many studies have confirmed the ability of flavonoids to diminish the production of NO, but the effect was probably caused by reduced iNOS expression rather than direct inhibition (Kim et al. 2004; García-Lafuente et al. 2009).

Another well-established potential mechanism for the anti-inflammatory activity of flavonoids comes from their ability to interact with intracellular signal-transducing pathways and thereby regulate the expression of inflammation-related genes. The most prominent position among inflammatory pathways belongs to the transcription factors nuclear factor (NF)-κB. Many studies have confirmed the ability of flavonoids to diminish the activity of NF-κB by inhibiting events upstream, e.g., reducing the phosphorylation of IKK (IκB kinase) which leads to less degradation of IκB (inhibitor of

κB) or attenuating the DNA-binding capability of NF-κB. Another important pro-inflammatory transcription factor is activator protein (AP)-1, the activity of which is regulated by upstream mitogen-activated protein kinases (MAPKs). The three most frequently studied MAPKs are ERK 1/2 (extracellular signal-regulated kinase 1/2), p38, and JNK (c-Jun N-terminal kinase). These kinases not only influence AP-1 but also modulate the activity of NF-κB. Flavonoids are able to inhibit the phosphorylation of all of the abovementioned MAPKs and thereby lower the transcription of the target genes. Analysis of the structure-activity relationship shows the importance of the 2,3-double bond, the carbonyl group of C4, and the pattern hydroxylation for the ability of flavonoids to modulate signal pathways. Flavonoids are able to positively regulate the activity of several other signaling pathways. One of these is the signal pathway leading to the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2). Together with its negative regulator Keap-1 (Kelch-like ECH-associated protein 1), this protein is redox sensitive. Flavonoids possessing a redox potential are therefore able to trigger Nrf2, which leads, subsequently, to the transcription of genes coding antioxidant enzymes that help to resolve the inflammatory response. Transcription factors from the PPAR (peroxisome proliferator-activated receptor) family are also activated by several flavonoids. The activation of this pathway attenuates the activity of NF-κB (Kim et al. 2004; García-Lafuente et al. 2009; Costa et al. 2012). Because the transcription of inflammation-related genes is regulated by multiple transcription factors, the exact mechanism of flavonoid action remains unclear. It is safe to say that these phytochemicals disturb the intracellular signaling network at several different sites and thereby contribute to the overall anti-inflammatory effect.

In vivo tests have confirmed all of the effects of flavonoids previously observed in *in vitro* experiments. Various animal models using different ways to induce inflammation have shown the antiphlogistic ability of flavonoids. The modulation of pro-inflammatory gene expression has been determined to be one of the major mechanisms of flavonoid action *in vivo*. However, because flavonoids are very quickly metabolized in the body, the particular cell types or proteins that they target remain unclear. Nevertheless, it has been shown that a 2,3-double bond and increased levels of hydroxylation or methoxylation of the flavonoid skeleton enhance their action (Kim et al. 2004).

Metabolism and Pharmacokinetics

As mentioned above, flavonoids occur both as aglycones and glycosides. The majority of the so-called dietary flavonoids occur as glycosides, with the exception of the flavan-3-ols and many of the flavonoids present in medicinal plants (especially the lipophilic prenylated flavonoids). The bioavailability of flavonoids is generally low and can vary widely between different classes of flavonoid and between individual compounds within one class. For example, following ingestion, the relative urinary excretion of unchanged anthocyanins and daidzin has been found to be 0.3 % and 43 %, respectively. There are several structural factors that can affect the bioavailability of flavonoids. The molecular weight of a specific flavonoid strongly affects its absorption. Compared to the monomeric and dimeric proanthocyanidins, for example, the oligomeric proanthocyanidins, with much higher molecular weights, are practically not absorbed at all. Unabsorbed and unchanged proanthocyanidins can reach the colon and be metabolized by the microflora in the gut, resulting in microbial metabolites that can then be absorbed into the circulating blood. It has been suggested that the colonic breakdown products of oligomeric proanthocyanidins show bioactivity (Del Rio et al. 2013).

Following ingestion, flavonoids pass to the small intestine (Fig. 10). Some absorption occurs at this stage, usually connected with the
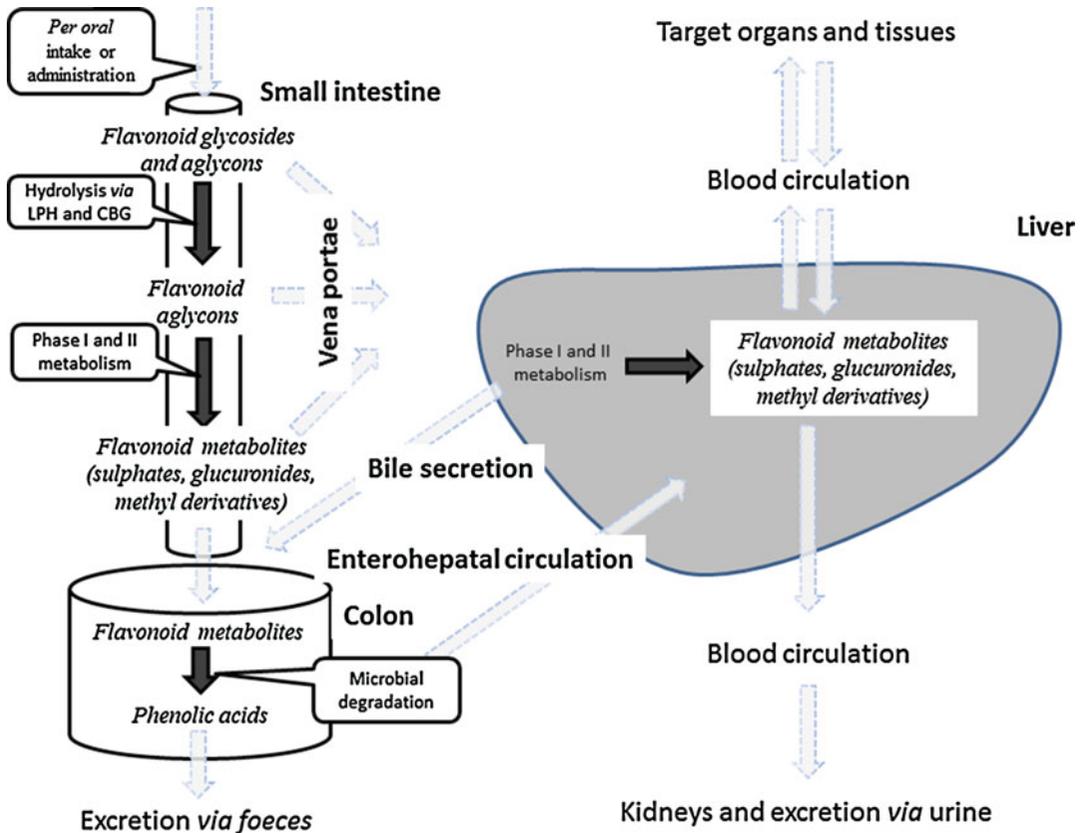
Flavonoids as Anti-inflammatory Agents, Fig. 10 Schematic image of flavonoid metabolism. *LPH* lactase-phlorizin hydrolase, *CBG* cytosolic β-glucosidase

hydrolysis of glycosides. The corresponding aglycone is released as a result of the activity of lactase-phlorizin hydrolase (LPH), present in the brush-border epithelial cells of the small intestine. LPH possesses broad substrate specificity for flavonoid-*O*-β-ᴅ-glucosides. The more lipophilic aglycones that are released then enter epithelial cells by passive diffusion facilitated by the proximity of the cell membrane. An alternative route of hydrolysis is mediated by cytosolic β-glucosidase (CBG), present in epithelial cells. But, CBG hydrolysis requires that polar glycosides be transported into cells, possibly by the active sodium-dependent glucose transporter (SGLT1). For this reason, the glycosylation of a flavonoid can strongly affect its absorption, which can be very fast or very slow, depending on the type of the sugar, the number of sugar

molecules attached, and the position of attachment on the flavonoid skeleton (Crozier et al. 2010). For example, the bioavailability of the quercetin glycosides obtained from an apple was only 30 % compared to that of similar glycosides obtained from an onion. The sugar moiety greatly influences the absorption; quercetin glucosides are absorbed 10 times as rapidly, and the plasma concentration peaks are 20 times as high as for quercetin rutinosides. It has been suggested that glucosides are absorbed in the small intestine, whereas quercetin rutinosides may be absorbed in the colon after deglycosylation. As mentioned previously, flavonoids are metabolized at the site of their absorption, and this metabolic conversion is known to be a major factor affecting the bioavailability of the flavonoid. Sulfation (sulfotransferases,
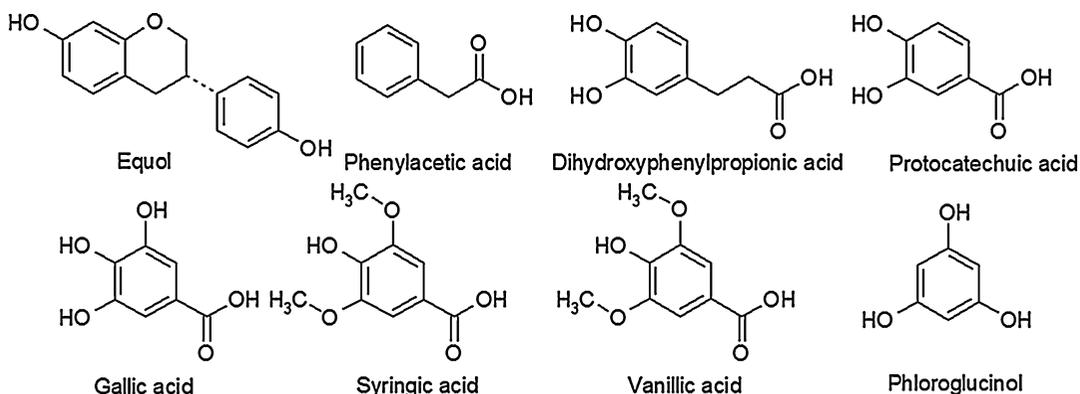
Flavonoids as Anti-inflammatory Agents, Fig. 11 Examples of some flavonoid metabolites produced by microflora in the gut

SULTs), methylation (*via* catechol-*O*-methyltransferases, COMTs), and glucuronidation (uridine-50-diphosphate glucuronosyltransferases, UGTs) occur in the enterocytes and liver, and generally, most of the flavonoids undergo these metabolic conversions. For example, quercetin aglycone and its glycosides are not usually found in the plasma, but the corresponding sulfate, methyl, and glucuronyl conjugates are. Catechin is found exclusively in the plasma as sulfate, methyl, and glucuronic acid conjugates.

Proportions of some flavonoid metabolites are returned to the lumen of the small intestine as efflux in a process mediated by members of the adenosine triphosphate (ATP)-binding cassette (ABC) family of transporters, including multidrug resistance protein (MRP) and P-glycoprotein (P-gp). On the other hand, flavonoids have been shown to inhibit the activity of these transporters (Crozier et al. 2010).

After metabolites are absorbed into the portal blood, they rapidly reach the liver, where they can again undergo several phase I and phase II metabolic conversions and, in some cases, enter the enterohepatic circulation by way of the bile and be returned to the small intestine. In humans, the metabolizing enzymes SULTs and UGTs are classified into several subfamilies that are expressed differently in the liver. The sulfation process is regioselective under the control of different isoforms of SULT. SULT1A1 exhibits

the greatest ability to catalyze the sulfation of the isoflavonoid hydroxyl groups at positions 7 and 4′. Flavones are preferentially sulfated at position 7, catalyzed by SULT1A1 and SULT1A3. For flavonols, SULT1A1 is the main catalyst, with some contributions from SULT1A3 and SULT1E1. Flavanones are preferentially metabolized by SULT1A2 (and also by SULTs 1A3, 1B1, and 1E1) to generate mostly 3′-*O*-sulfate; SULT1C4 leads to 7-*O*-sulfates. One description covers the glucuronidation of flavonoids – the flavones, flavonols, and flavanones – with the regioselectivity of UGT1A3, summarized as 3′-OH > 7-OH > 3-OH > 5-OH. Glucuronidation of isoflavonoids is carried out by UGT1A1 in the following order: 7-OH > 4′-OH > 5-OH. The UGTs 1A1, 1A8, and 1A9 preferentially glucuronize catechins at position 3′-OH (Thilakarathna and Rupasinghe 2013).

Substantial quantities of flavonoids and their metabolites can pass through to the colon. Gut microflora have the ability to decompose conjugates and release aglycones. The rings in these can undergo scission to produce smaller molecules, which, together with unchanged aglycones, can be reabsorbed, metabolized in phase II in the liver, and enter the enterohepatic circulation or (and this is predominant) be excreted in the urine. Gut microflora degrade flavonoids into simple phenolic acids that can be absorbed into the circulating blood. The key point of this process is the scission of the C ring and the loss of carbons

C5 to C8 as oxalacetate, which is eventually metabolized to carbon dioxide. The interaction of flavonoids with colonic microflora strongly influences their bioavailability and could consequently change their biological effects. Some examples of flavonoid metabolites produced by the microflora in the gut are shown in Fig. 11. Proanthocyanidins (flavan-3-ols) are metabolized to phenylacetic acid, mono- and dihydroxyphenylacetic acids, mono- and dihydroxyphenylpropionic acids, or hydroxybenzoic acid. Anthocyanins are converted mainly into protocatechuic acid, gallic acid, syringic acid, vanillic acid, and phloroglucinol. The metabolism of isoflavones is more complicated. It is strongly influenced by the composition of the microflora in the gut. For example, the production of equol, the main metabolite created following consumption of daidzein, is greatly influenced by differences in the microflora of the gut between different individuals, and not all adult persons are able to fully metabolize this substance. This could hamper the clinical effectiveness of isoflavones and affect their bioactivity, helping to explain why the activity of equol has been reported to be stronger than that of daidzein (de Pascual-Teresa et al. 2010; Crozier et al. 2010).

Some simple flavonoid metabolites are known to express biological activities and have potential health properties, even though the ingested flavonoids were not absorbed primarily in the small intestine. As concerns their anti-inflammatory activity, substances such as protocatechuic acid from anthocyanins, methyl-derivatives of flavan-3-ols, or metabolites of flavonols (e.g., quercetin) commonly possess antioxidant properties. Protocatechuic acid inhibits the adhesion of monocyte to tumour necrosis factor (TNF)-α-activated mouse aortic endothelial cells, the expression of intercellular adhesion molecule 1 (ICAM-1), and the binding activity of NF-κB. It also reduces the amount of vascular cell adhesion molecule 1 (VCAM-1). Some flavan-3-ol glucuronides (in physiologically appropriate concentrations) inhibit the release of arachidonic acid and its metabolites *in vitro*. Flavonol glucuronides and sulfates inhibit the expression of

ICAM-1 and E-selectin in human aortic endothelial cells. It must be mentioned, however, that contradictory reports about the bioactivity of flavonoid metabolites have been published, and many studies on their activity were carried out *in vitro* or otherwise not in humans. This topic deserves more effort.

Clinical Use

Flavonoids are usually used in traditional and modern medicine and in the cosmetic industry in the form of crude plant extracts. More than one hundred flavonoid-containing medicinal plants are used in Europe. Flavonoids are also part of a diet rich in vegetables, fruits, and some other products. It has been reported that the daily intake of flavonoids in the human diet is as much as 650 mg. However, several flavonoids have found practical uses only as pure substances or simple standardized mixtures of compounds. For example, rutoside (quercetin-3-*O*-rutinoside, rutin), its semisynthetic derivative troxerutin (hydroxyethylrutoside), diosmin, and hesperidin have found the clinical application. The main clinical use of the flavonoids is their so-called venoactivity (ability to reduce the permeability and fragility of capillaries). When tested in animal models, reduced symptoms of vitamin C deficiency led to their old designation as vitamin P. The flavonoids are primarily indicated as venotonic and vasoprotective agents that increase the tonus and resistance of the walls of blood vessels. They decrease swelling and have an anti-inflammatory effect. Preparations with flavonoids are used to combat chronic venous insufficiency of lower limbs in adult patients: feelings of heavy limbs, pain, night cramps, edema, and trophic and inflammatory changes. These compounds are also used to treat hemorrhoids. On the other hand, some authors have expressed doubts about the therapeutic effects of flavonoids (Bruneton 1999).

Despite the fact that various experiments *in vitro* and *in vivo* have shown the anti-inflammatory potential of flavonoids, studies on humans are still insufficient and sometimes

controversial, as reflected in reviews by Hamer and Steptoe (2006), de Pascual-Teresa et al. (2010), and Izzi et al. (2012). Most studies have not dealt with pure compounds but rather with mixtures or with flavonoid-rich material. In some cases they have assumed that a diet rich in raw vegetables, berries, fruits, or nuts should be rich in flavonoids. For example, a vegan diet has been found to reduce pain and stiffness of joints in fibromyalgic patients and in patients with rheumatoid arthritis. It has been observed that consuming red wine reduces the expression of major adhesion molecules on monocytes and T-lymphocytes. Significant reduction in the levels of ICAM-1 and VCAM-1 in the blood has been observed in volunteers after treatment with a polyphenol-rich food concentrate. Administration of polyphenols extracted from grapes significantly reduced the levels of TNF-α and interleukin (IL)-6 in the plasma. Ingestion of an anthocyanin-rich extract derived from blueberries significantly reduced the plasma levels of pro-inflammatory cytokines and chemokines regulated by NF-κB signaling in a group of volunteers. Different studies have suggested that a high consumption of vegetables, fruits, and legumes by healthy volunteers correlates inversely with inflammation markers in the blood. It has been reported that a supplement containing mixed tocopherols, flavonoids, and docosahexaenoate significantly reduced the serum levels of IL-6 and C-reactive protein (CRP) in untrained males after eccentric exercise, but another report showed that except for apples and tea, flavonoid-rich foods did not reduce the risk of type-2 diabetes and did not affect the serum levels of IL-6 or CRP. Long-term administration of cocoa powder reduced the expression of very late antigen 4 (VLA-4), CD40, and CD36 in monocytes, as well as the serum concentrations of the soluble adhesion molecules P-selectin and intercellular adhesion molecule, derived from the endothelium in patients with high risk of atherosclerosis (Hamer and Steptoe 2006; de Pascual-Teresa et al. 2010; Izzi et al. 2012).

Soya and isoflavonoids obtained from soya are a relatively commonly target of clinical studies that yield controversial results. The administration of soya isoflavones elevates the serum concentrations of IL-6 in women, although it has no effect on acute-phase proteins or other pro-inflammatory cytokines. Nevertheless, it has been proposed that the estrogenic effect of isoflavones be considered as a mechanism for potentiating immune surveillance and a possible explanation for the lower incidence of certain types of cancer in parts of the world where soya is eaten. Further reports showed no significant effect of soya isoflavones on the concentrations of interferon (IFN)-γ, IL-2, TNF-α, and CRP in the plasma, or of 8-isoprostane in the urine. A larger randomized study on the dietary intake of soya also reported that no significant differences could be observed in the levels of leptin, adiponectin, monocyte attractant protein 1 (MCP-1), macrophage inflammatory protein-1β (MIP-1β), IL-6, or CRP after supplementation. On the other hand, soya isoflavones lowered the level CRP in hemodialyzed patients (Hamer and Steptoe 2006; de Pascual-Teresa et al. 2010; Izzi et al. 2012).

Reduced levels of TNF-α and oxidative stress have been found after ingestion of black currants, and quercetin and EGCG have mediated reductions in the plasma levels of granulocyte colony-stimulating factor, CRP, IL-6, and IL-10. On the other hand, further study has shown that supplementation with quercetin does not affect the activity of natural killer cells, the granulocyte oxidative burst, or phagocytosis in women. Oral administration of a flavonoid-rich extract of purple passion fruit peel has been shown to suppress the clinical symptoms of asthma and osteoarthritis, a phenomenon which is consistent with the reported ability of apple polyphenols to reduce the clinical symptoms of allergic rhinitis and of muscadine grape seeds to significantly increase the resting brachial diameter in subjects with increased cardiovascular risk. Recent results from the Polyp Prevention Trial indicate that diminished concentrations of cytokines resulting from a high consumption of flavonols may prevent colorectal neoplasms (Hamer and Steptoe 2006; de Pascual-Teresa et al. 2010; Izzi et al. 2012).

Potential Adverse Effects

Flavonoid compounds, which are present in common diet, are usually consumed in relatively low quantities. Moreover, their occurrence is typified by the presence of relatively complex mixtures of flavonoid derivatives in low doses. Therefore, the probability of adverse effects is relatively low. On the other hand, if the plant material is rich in some type of flavonoid (e.g., the citrus peel in flavanones) or the flavonoids are used as pure substances in large doses, some adverse effects could occur. A special class of flavonoids is the isoflavonoids which show well-described estrogenic properties (Egert and Rimbach 2011; Skibola and Smith 2000).

Drug Interactions

For example, the interaction of grapefruit juices rich in flavanones (naringin, naringenin, hesperidin, hesperetin, quercetin, kaempferol, phloridzin, phloretin) and grapefruit-derived preparations are relatively well known, although recent reports show that besides flavanones, also so-called furanocoumarins (bergamottin, bergapten, bergaptol, $6',7'$-dihydroxybergamottin) are compounds responsible for grapefruit interaction with CYP, P-glycoprotein, and organic anion-transporting polypeptides (Dolton et al. 2012; Srinivas 2013). The pharmacokinetic interaction of grapefruit is connected, for example, with alteration of bioavailability of carbamazepine, fexofenadine, antimalarials (artemether, primaquine), anxiolytics/hypnotics (midazolam, triazolam, buspirone), antiarrhythmics (amiodarone, propafenone), cardiovascular agents (verapamil, dihydropyridines), cholesterol-lowering agents (statins), some cytostatics and immunosuppressants, and phosphodiesterase V inhibitors (sildenafil) (Seden et al. 2010). Some authors also discuss the ability of flavonoids to alter the trace element bioavailability and the

interaction with folate and vitamin C absorption (Egert and Rimbach 2011).

Cross-References

▶ Asthma
▶ Cancer and Inflammation
▶ Cytokines
▶ Eicosanoids
▶ Interleukin-10
▶ Interleukin-6
▶ MAP Kinase Pathways
▶ Nitric Oxide
▶ Non-steroidal Anti-inflammatory Drugs (NSAIDs)
▶ Nuclear Factor kB
▶ Osteoarthritis
▶ Phospholipase A2 Inhibitors
▶ Prostaglandins
▶ Rheumatoid Arthritis

References

Bruneton, J. (1999). *Pharmacognosy, phytochemistry, medicinal plants*. Lavoisier, Paris, France.

Costa, G., Francisco, V., Lopes, M. C., Cruz, M. T., & Batista, M. T. (2012). Intracellular signaling pathways modulated by phenolic compounds: Application for new anti-inflammatory drugs discovery. *Current Medicinal Chemistry, 19*, 2876–2900.

Crozier, A., Del Rio, D., & Clifford, M. N. (2010). Bioavailability of dietary flavonoids and phenolic compounds. *Molecular Aspects of Medicine, 31*, 446–467.

de Pascual-Teresa, S., Moreno, D. A., & García-Viguera, C. (2010). Flavanols and anthocyanins in cardiovascular health: A review of current evidence. *International Journal of Molecular Science, 11*, 1679–1703.

Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects again chronic diseases. *Antioxidants & Redox Signaling, 18*, 1818–1892.

Dolton, M. J., Roufogalis, B. D., & McLachlan, A. J. (2012). Fruit juices as perpetrators of drug interactions: The role of organic anion-transporting

polypeptides. *Clinical Pharmacology & Therapeutics, 92*, 622–630.

Egert, S., & Rimbach, G. (2011). Which sources of flavonoids: Complex diets or dietary supplements? *Advances in Nutrition, 2*. doi:10.3945/an.110.000026.

García-Lafuente, A., Guillamón, E., Villares, A., Rostagno, M. A., & Martínez, J. A. (2009). Flavonoids as anti-inflammatory agents: Implications in cancer and cardiovascular disease. *Inflammation Research, 58*, 537–552.

Hamer, M., & Steptoe, A. (2006). Influence of specific nutrients on progression of atherosclerosis, vascular function, haemostasis and inflammation in coronary heart disease patients: A systematic review. *British Journal of Nutrition, 95*, 849–859.

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology & Therapeutics, 96*, 67–202.

Izzi, V., Masuelli, l., Tresoldi, I., Sacchetti, P., Modesti, A., Galvano, F., & Bei, R. (2012). The effects of dietary flavonoids on the regulation of redox inflammatory networks. *Frontiers in Bioscience, 17*, 2396–2418.

Kim, P. K., Son, K. H., Chang, H. W., & Kang, S. S. (2004). Anti-inflammatory plant flavonoids and cellular action mechanisms. *Journal of Pharmacological Sciences, 96*, 229–245.

Peterson, J., & Dwyer, J. (1998). Flavonoids: Dietary occurrence and biochemical activity. *Nutrition Research, 18*, 1995–2018.

Pietta, P. G. (2000). Flavonoids as antioxidants. *Journal of Natural Products, 63*, 1035–1042.

Procházková, D., Boušová, I., & Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia, 82*, 513–523.

Seden, K., Dickinson, L., Khoo, S., & Back, D. (2010). Grapefruit-drug interactions. *Drugs, 70*, 2373–2407.

Skibola, C. F., & Smith, M. T. (2000). Potential health impacts of excessive flavonoid intake. *Free Radical Biology and Medicine, 29*, 375–383.

Smejkal, K. (2014). Cytotoxic potential of *C*-prenylated flavonoids. *Phytochemistry Reviews, 13*, 245–275.

Srinivas, N. R. (2013). Is pomegranate juice a potential perpetrator of clinical drug-drug interactions? Review of the in vitro, preclinical and clinical evidence. *European Journal of Drug Metabolism and Pharmacokinetics, 38*, 223–229.

Thilakarathna, S. H., & Rupasinghe, H. P. V. (2013). Flavonoids bioavailability and attempts for bioavailability enhancement. *Nutrients, 5*, 3367–3387.

Yazaki, K., Sasaki, K., & Tsurumaru, Y. (2009). Prenylation of aromatic compounds, a key diversification of plant secondary metabolites. *Phytochemistry, 70*, 1739–1745.

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Pro-oxidant Activity of Flavonoids and Their Possible Effects

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ABSTRACT

Flavonoids are secondary metabolites showing pleiotropic effects. They are present in many medicinal plants or are part of a diet. The anti-oxidant activity of flavonoids is often discussed and many beneficial effects are accounted to the anti-oxidant activity of flavonoid derivatives. However, the pro-oxidant activity of some flavonoid derivatives is known. The pro-oxidant effect is connected both with positives and negatives. The negatives of pro-oxidant activity are linked up mainly with mutagenic or cancenogenic potential of high doses of flavonoids,via possible changes and interaction with biomolecules including DNA; positive effects are connected with stimulation of anti-oxidant defense of organism, improvement in effectiveness of antibiotics or with role of ROS species in acute inflammatory process. However, many studies bring inconsistent results and controversies about anti- and pro-oxidant effect of flavonoids exist.

Key words: Antibacterial, Antioxidant, Cancer, Flavonoid, Inflammation, Pro-oxidant

INTRODUCTION

The word "flavonoid"is derived from the yellow color of the plant pigments containing in their structure benzo-ã-pyrone, structure well known as

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chromone, modified at position 2 or 3 by the addition of a phenyl ring at position 2 or 3 (Fig. 1). Their skeleton may be substituted with hydroxyl groups on the ring A typically at positions 5 and 7 (a *meta* arrangement characteristic for acylphloroglucinols), on the ring B at positions 4´ or 3´ and 4´ (typical for shikimates) (Havsteen 2002) (Fig. 1); with exceptions, for example flavonoids in Moraceae, where the ring B is substituted with *meta* (2´, 4´) hydroxyl groups (Bruneton, 1999; Havsteen, 2002). The *O*-glycosylation occurs commonly, the sugar unit can also be connected directly to carbon of flavonoid skeleton. Glycosides of the flavonoids are relatively hydrophilic but the aglycones are lipophilic moreover, their methylation, prenylation or geranylation can solubility in hydrophobic solvents further enhance. The prenyl or geranyl moiety may also be modified in different ways (oxidation, reduction, cyclization, etc.) (Smejkal, 2014). Therefore, flavonoid glycosides are soluble in water and alcohols sometimes sparingly (rutin, hesperidin) and aglycones are usually well soluble in non-polar solvents.
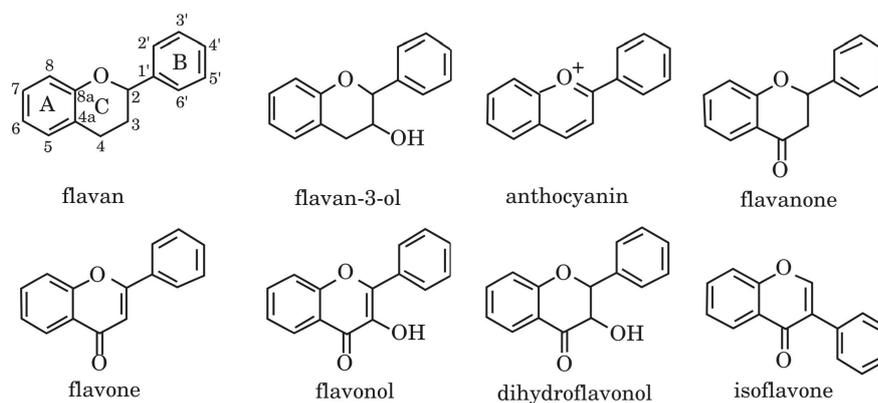
Fig. 1: Structures of flavonoid skeletons

Various plants contain predominantly different subclasses of flavonoids. dietary flavonoids present in common food aree well known but there are many other flavonoid species present in medicinal plants.

Flavonoids are known to possess various bioactivities. They have been proved *in vitro*, some of them *in vivo* or in clinical studies. Especially their anti-oxidant and anti-inflammatory properties are well known, but anti-bacterial, anti-viral, hypolipidemic and even anti-cancer effects were also described (Havsteen, 2002; Nijveldt *et al*., 2001). But, the identification of structure-activity relationships and especially elucidation of their *in vivo* activity is very difficult, because flavonoids have pleiotropic effects that target many cellular proteins or mechanisms. This chapter attempts to highlight the effect of flavonoids in oxidative processes *in vitro* and *in vivo*, with special attention laid on their possible pro-oxidative effects.

Anti-oxidant Activity of Flavonoids

The anti-oxidant activity of flavonoids is very well described. In case of anti-oxidant activity of flavonoids, the attention is concentrated especially on the presence of hydroxyl substitution on the flavonoid skeleton because the electron or hydrogen donor properties or metal chelating potential is based on the presence and position of such as oxygenated substituents. It is postulated that one of the anti-oxidant mechanisms of action of flavonoids is a donation of hydrogen to existing radical substance and further formation of stable compounds with character of radical (due to the delocalization of radical electron), which further do not promote the radical reaction and thus, the radical reaction is terminated. The stability of such a substances can be different according to the conditions during reaction. The presence of 2,3 double bond, 3-OH, 5-OH at flavonoid skeleton and also catechol or pyrogallol arrangement of the substitution at ring B are often mentioned as crucial of the anti-radical activity of flavonoids (Pietta, 2000; Prochazkova *et al.*, 2011).

Moreover, flavonoids have been found to inhibit the activity or expression of some enzymes connected with oxidation stress such as cyclooxygenase, lipoxygenase and NADPH oxidase. Therefore, they can diminish the levels of radicals independently on direct scavenging of formed radicals (Bodewes *et al.*, 2011; Amic *et al.*, 2007; Izzi *et al.*, 2012).

Methods for Evaluation of Anti-Oxidant or Pro-Oxidant Activity of Flavonoids

There is no method, which can be completely universally used to evaluate the "true" anti-oxidant or pro-oxidant capacity or power of single compound (for example flavonoid) or complex mixture (for example plant extract). The comparison of results of different tests is difficultbecause assays used differ; when the type of assay is the same, different concentration, media and conditions of measurement can affect the result. There are attempts to use some standardization, for example usage of gallic acid or quercetin as standard of test, or recalculation of observed effect into Trolox equivalents, but these approaches are not always used and are not suitable every time. The good anti-radical activity *in vitro* in biochemical model does not automatically mean the good antioxidant potential *in vivo*.

The need to use standardized and generally applicable protocols has resulted into attempt to describe recommended procedures: (i) the identification of main components and quantification of phenolic (anti-oxidant substances, possible flavonoids) in plant extract; (ii) the analysis and quantification of the radical scavenging activity, determination of reduction potential; (iii) the analysis of lipid oxidation in biological model; (iv) the studies *in vivo* using relevant markers of oxidative stress or oxidative damage.

To effectively elucidate anti-oxidant properties of a compound or extract, it is possible to determine in different ways the efficacy of prevention of the oxidation of relevant substrates (lipids, lipoproteins, DNA) against relevant free radical species such as the peroxyl, superoxide or hydroxyl radical (many others). The selection of assay must be relevant to the material tested and to expected result (Litescu *et al*., 2010). The mechanisms involved and the type of assessment of anti-oxidant capacity assays can be divided into two main categories. The first category is an "assessment of anti-oxidant efficacy in relation to free radical species" (Litescu *et al*., 2010). This category includes different reaction mechanism models such as reactions based on transfer of hydrogens, reactions based on transfer of single electron or reactions combining the hydrogen and electron transfer. The most frequently used methods include Oxygen Radical Absorbance Capacity (ORAC), Trolox Equivalent Anti-oxidant Capacity (TEAC), Ferric Reducing Anti-oxidant Power (FRAP) assays or scavenging effect of the compounds towards specific reactive species (ABTS (2,2´-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), peroxyl radical etc.) (Cao and Prior, 1998; Apak *et al*., 2013). The second category represents an "assessment of anti-oxidant efficacy using biological significant markers and significant substrates". This category involves the determination of anti-oxidant efficacy through the evaluation of the damaging effects produced by reactive species of oxygen (ROS) or reactive nitrogen species (RNS) on a biological substrate;the reacting substrate can be biomolecules like phospholipids, fatty acids, proteins, DNA or others, or cell compartments like phospholipid membranes, mitochondria etc. (Wood *et al*., 2006).

Methods used for the determination of the anti-oxidant activity can also be divided into cell-free models and methods using live biological systems (isolated cells, cell tissues or organs). The cell-free models are based on the measurement of a decrease of concentration of the starting substrate or increase of the final product, influenced by the production or reaction of reactive species or scavenging reactive species (DPPH, ABTS, hydrogen peroxide, hypochlorous acid etc.). The starting substrate can be the radical substance itself. Many of these assays are based on the scavenging effect of the compounds tested and are suitable to be used for an assessment of an anti-oxidant activity of compounds tested but usually not for the evidence of their pro-oxidative effect. Anti-oxidant activity is often determined as a proportional decrease of amount of reactive species or effect of reactive species on target molecule. It is determined as the ratio of activity of compound tested and negative control and then the activity can be expressed in relation to positive control of assay (for example catechin, gallic acid, Trolox). The percentage of the activity of sample usually cannot be higher than control, and the presence of reactive species in reaction mixture is often essential. The exception can be for example analysis of effects of potential anti-/pro-oxidants in model of Fenton reaction, where compounds

tested can stimulate formation of hydroxyl radical. If the amount of hydroxyl radical released is increased, compound tested can have the pro-oxidant effect. The precondition for the participation the substance (flavonoid) in Fenton reaction is its ability to reduce transition metal to a lower oxidation status. The reduced metal is then the most often reacting with hydrogen peroxide to form a hydroxyl radical or may react with molecular oxygen to form superoxide. It can be assumed that compounds with a higher redox potential will behave in presence of the transition metals like pro-oxidants (Gomes *et al*., 2008).

One of the few ways to determine the pro-oxidative activity of compound tested in cell-free models is an effect of the compoundon thestimulation ofthe enzymatic activity of pro-oxidant enzymes (NADPH-oxidase, NO-synthase, xanthine-oxidase, myeloperoxidase) and thus increase the corresponding ROS or RNS production, or the inhibition of activity of the anti-oxidant enzymes (catalase, superoxide-dismutase, glutathione-reductase) (Wassmann *et al*., 2004).

Biological systems for determination of anti-oxidant or pro-oxidant activity are more complex. Cellular systems are often used. Effects of compounds tested on the production of ROS/RNS or damage of cells are determined from comparison of results obtained on non-treated cells with treated and cells with an inductor of oxidative damage. Methods using biological systems are useful both for assessment of anti-oxidant and pro-oxidant activity, and their results are more suitable for prediction of results in *in vivo* assays.

The compounds tested can activate signaling pathways; they can induce or repress the expression of genetic information, or affect the activity of enzymes. The amount of pro-oxidant andanti-oxidant enyzymes in cells can be analyzed on the level of gene transcription (by mRNA analysis), on the level of the translation of mRNA to protein (by immunodetection) or on the level of activity of particular proteins (activity of enzymes based on the amount of formed products) (Wood *et al*., 2006; Niki, 2010).

Because the majority of natural ROS or RNS has a very short life-time, they are often detected indirectly using color and fluorescent probes or the detection is based on markers arising from the reaction of ROS and RNS with different biomolecules. In some case the markers of an oxidative damage in cellular systems can be idetected continually in the time by non-invasive spectroscopic methods (taurine chloramine, NADH/NAD$^+$) (Senthilmohan *et al*., 2006).

The lipid peroxidation can be detected *vby* determination of malondialdehyde levels or by the determination of thiobarbituric acid reactive substances (TBARS). The fragmentation of DNA or the determination of modified nucleosides concentration is used for the

evaluation of oxidative damage of DNA, when ROS or RNS react with nucleosides and cause their oxidation (for example the formation of 8-oxo-2´-deoxyguanosine).The myeloperoxidase activity can be detected by the analyzing of chlorinated products of the reaction. The chlorinated compounds are not common in the body (except of xenobiotics). They can be formed during the inflammation by a reaction of hypochlorous acid and biomolecules. Hypochlorous acid preferably reacts with the amino groups of the proteins or amino acids, but is able to react also with the aromatic ring of tyrosine to yield 3-chlorotyrosine or 3,6-dichlortyrosine (Senthilmohan *et a*l. 2006). It can be detected in the solution or in the body fluids. Pro-oxidant compounds may interfere with the protective and reparative mechanism of the cells (the oxidized glutathione vs. glutathione-reductase, 8-oxo-2´-deoxyguanosine vs. DNA reparation). The above mentioned methods can also be used for testing *in vivo*.

Pro-oxidant Activity of Flavonoids

It is clear that flavonoid anti-oxidant properties can positively affect the oxidative balance of organism. But, the word balance must be emphasized, because from some specific reasons, the promotion of local oxidative stress can be important for organism, and the increase of the localized amount of radical species is produced to combat pathological conditions. These conditions represent for example an aging of cells, the first phases of inflammation, a stimulation of apoptosis, or a fight of organism against the invading bacteria or viruses. (Narayana *et al*., 2001; Juranek *et al*., 2013). On the other hand, the excessive amounts of ROS or NOS can damage tissues and cause harmful effects. The flavonoids can act as pro-oxidants under specific conditionsand therefore, can promote the oxidation of other compounds.

Some structure activity relationship studies carried out by Cao *et al*. (1997) or Hanasaki *et al*. (1994) showed that flavonoid pro-oxidant action in Fenton reaction is dependent on the presence of multiple hydroxyl groups, possibly located on the ring B of flavonoid skeleton. The localization of hydroxyls is not limited on the ring B, for example baicalein with pyrogallol arrangement of ring A also shows increased production of hydrogen peroxide. Mono and dihydroxy flavonoid derivatives showed negligible activity (Cao *et al*. 1997; Hanasaki *et al*. 1994). This was later summarized by Heim *et al*. (2002). Structure activity relationships can also be deduced from the study of Miura *et al*. (1998) where flavonoids tested were found to generate H_2O_2 in an acetate buffer of pH 7.4. The ability of flavonoids to produce H_2O_2 decreased in the order of myricetin > baicalein > quercetin > (-)-epicatechin > (+)-catechin > fisetin = 7,8-dihydroxyflavone. Flavonoids with a pyrogallol and catechol structure and the pyrogallol-type flavonoids generated more H_2O_2 than the catecholtypes. Concentration, reaction time and the amount of oxygen dissolved in medium affected the reaction. The

H_2O_2 formation was inhibited by the addition of SOD therefore, these flavonoids generated H_2O_2 by donating a hydrogen, through a superoxide anion radical. Interestingly, flavonoids which generated more H_2O_2 were more powerful anti-oxidants in the NADPH-dependent lipid peroxidation of rat microsomes (Miura *et al.,* 1998).

The further assay using a promotion or inhibition of formation of superoxide anion radical showed that the flavones (2,3 double bond, 4-oxo group) possess higher activity in comparison with other flavonoids (Sun *et al.*, 2010). On the other hand, *in vitro* biochemical assays using OH•-dependent oxidation of deoxyribose showed that flavanones (no 2,3 double bond, naringenin and hesperetin) showed higher pro-oxidant activity than flavonols (2,3 double bond, quercetin and morin). But, unlike naringenin and hesperetin, quercetin and morin were able to induce the formation of hydrogen peroxide. Morin induced the formation of hydrogen peroxide at concentrations higher than 100 µM. All these four flavonoids increased concentration of superoxide, similarly to increase of lipoperoxidation (Yen *et al.*, 2003). The kaempferol-induced lipoperoxidation in isolated rat-liver nuclei was significantly stimulated by the addition of catalase and sodium azide in the presence of Fe(III) (Sahu and Gray, 1994), similarly to morin and naringenin (Sahu and Gray, 1997). When assayed in model using lymphocytes, only morin and naringenin significantly decreased the activity of glutathione reductase, but with glutathione transferase (GST) the situation was more complicated when quercetin at 200 µM decreased the GST activity, while naringenin at concentration of 25–50 µM enhanced the GST activity and in higher concentration the activity was inhibited, similarly to hesperetin. All four flavonoids tested showed decrease of membrane thiols in dose dependent manner (Yen *et al*. 2003). The concentration dependent pro-oxidant effect of quercetin, rutin, catechin, and epigallocatechin gallate was observed also in assay of Labuda *et al*. (2003), showing that the potential of flavonoids to cause a DNA degradation by pro-oxidant action in presence of atmospheric oxygen was in order quercetin>rutin>epigallocatechin gallate>catechin.

The results of structure activity relation studies are strongly dependent on the conditions used for assay. Especially the presence of a high concentration of transient metals in environment of cell or in reaction mixture *in vitro* leads to pro-oxidative behavior of flavonoids. They can reduce Fe(III) to Fe(II), resulting in further production of hydroxyl radical in Fenton reactions (Rapta *et al*., 1995). The reduction of Cu(II) to Cu(I) was also observed (Cao *et al*., 1997). This was confirmed *in vitro* using different chemical/biochemical reactions, or *in vitro* assays using cell-based models (Hadi *et al*., 2007).

Series of flavonoids was tested to confirm their pro-oxidative or anti-oxidative potential in lipoperoxidation (Sugihara *et al*., 1999). Their activity

was strongly dependent on the concentration and the structure of compounds tested and on the presence of metal ions in reaction mixture. Briefly, luteolin, apigenin, and chrysin (flavones) were anti-oxidant at low iron ion concentrations, but were pro-oxidative at high iron ion concentrations. Taxifolin (flavanone) showed both anti- and pro-oxidative activity, depending on iron ions concentration, but with other metal showed only anti-oxidant activity.

The importance of these effects is questionable. Under physiological conditions, the transient metals are usually compartmentalized or secured from interaction with possible pro-oxidants by bond with special carrier or storage compound; however, under pathological conditions they can be released and could interact with flavonoids (Pietta, 2000; Amiæ *et al*., 2007; Jomova and Valko, 2011).

The decision, if the flavonoid will act as anti-oxidant or pro-oxidant, seems to be concentration dependent. The relatively high concentrations of flavonoids are necessary to evoke the pro-oxidant effect, as tens to hundreds micromole concentrations were needed (Yen *et al*., 2003). The other proof of this concentration dependent effect is the activity observed when quercetin and myricetin were analyzed; they caused diminishing of iron-induced lipid peroxidation in rat liver microsomes in micromolar concentrations, while at 100 µM increased the hydroxyl radical formation (Laughton *et al* 1989). The human leucocytes treated with quercetin in concentration up to 50 µM showed reduced level of DNA damage induced by superoxide, while at higher concentration of quercetin this damage was promoted (Wilms *et al*., 2008).

It is possible that pro-oxidant activity of flavonoids is linked to their auto-oxidation in presence of high concentration of dissolved oxygen, which is transformed to superoxide and can dismutate into hydrogen peroxide. This auto-oxidation is pH dependent for example, in quercetin, it does not occur at physiological pH levels. But, quercetin auto-oxidation was increased in presence of iron and also after addition of superoxide dismutase. However, the autoxidation at these conditions was not observed for other flavonoids like kaempferol or rutoside. Series of structurally related flavonoids under physiological conditions showed the possibility of auto-oxidation and ROS production, and the contribution of electrochemical properties of flavonoids to their bioactivity was clear, as they showed possibility to inhibit mitochondrial succinate-CoQ reductase (complex II) (Hodnick *et al*., 1988). This inhibition was also showed previously by Hodnick *et al*. (1986), when fourteen flavonoids were systematically analyzed to elucidate their abilities to inhibit succinoxidase and generate ROS in beef heart mitochondria. Flavonoids with a catechol moiety on the ring B exhibited the following general order of potency: chalcone Ã flavone Ã flavonol Ã dihydroflavonol Ã anthocyanidin. Catechins showed no activity. 3,5,7-trihydroxyflavones

possessing adjacent pyrogallol and ring B catechol configurations were the most potent inhibitors of succinoxidase. Four of the fifteen flavonoids tested exhibited substrate-independent, KCN-insensitive respiration. Two flavonols with a pyrogallol configuration, myricetin and quercetagetin produced the greatest respiratory burst and were found to auto-oxidize. The mitochondrial respiratory burst induced by both flavonols and their auto-oxidation resulted in the generation of O_2^- and H_2O_2 (Hodnick *et al*., 1986). The effect of electrochemical properties of phenolics on lipid peroxidation was showed also in study of Simiæ *et al*. (2007) but flavonoids quercetin and rutin did not prove their pro-xidant activity (anti-oxidant effect was observed).

The reaction of products which are formed during the oxidative changes of flavonoids is also worthy to mention. The typical is formation of phenoxyl radicals, which are unstable and their lifespan can persist hundreds of miliseconds (Neta and Grodkowski 2005). They later further oxidize and different semiquinones and quinones are formed (Hodnick *et al*. 1989). These quinoid structures are still reactive, and must be cleared out by anti-oxidant defense of organism (Hernández *et al*., 2009; Awad *et al*., 2002; Torres *et al*., 2006; Kanakis *et al*., 2005). The examples of the pro-oxidant activity of phenoxyl radicals are well known. Phenoxyl radicals formed from flavonoids apigenin, naringenin and naringin react with NADH showing high O_2 consumption and formation of large amount of superoxide (Galati *et al*., 1999 and 2002; Chan *et al,*. 1999).

The effect of several flavonoids on respiratory burst was assayed on isolated mitochondria under different conditions. Auto-oxidation of flavonoids connected with formation of ROS was observed (Hodnick *et al*., 1989 and 1994). The hydroxyl group arrangement was important for the auto-oxidation effect, compounds with three hydroxyl groups at ring A of flavonoid skeleton showed higher rate of auto-oxidation than compounds with dihydroxyl moiety only. The effect was potentiated by addition of cyanide into assay buffer.

Flavonols with catechol group at the ring B (*e.g.,* quercetin and fisetin) are able to form *o*-semiquinones and they are able to oxidize NADH. It is the ratio of NADH to NAD^+ that affects the rate of superoxide production (Kussmaul and Hirst, 2006). Quercetin and fisetin decreased a mitochondrial NADH to NAD^+ ratio in isolated liver cells in a dose-dependent manner, the effect was accompanied by reduced ketogenesis, stimulation of citric acid cycle and uncoupling effect on oxidative phosphorylation. Moreover, 25 ìM concentrations of quercetin increasedoxygen uptake, while concentrations of 50-300 ìM decreased oxygen uptake and stimulated citric acid cycle. Quercetin therefore shifts the overall cellular conditions to a more oxidized state (possess pro-oxidant activity) (Buss *et al*., 2005; Constantin *et al*., 2011; Procházková *et al*., 2011).

The flavonoids can also be oxidized into phenoxyl radicals by different peroxidases. For example, myeloperoxidases are active in neutrophils and macrophages during inflammation, and here flavonoid phenoxyl radicals are described to increase lipoperoxidation. Phenoxyl radicals produced from apigenin, naringenin and naringin further oxidize NADH and trigger consumption of O_2 and formation of superoxide (Galati *et al*., 1999 and 2002; Chan *et al*., 1999). Galati *et al*. (2001) also described the reaction of flavonoids with glutathione, H_2O_2 and peroxidase, and the formation of products dependent on the redox potential of the flavonoid tested (Fig. 2). Catalytic amounts of apigenin and naringenin but not kaempferol (flavonoids that contain a phenol B ring) when oxidized by H_2O_2 and peroxidase co-oxidized GSH to GSSG *via* a thiyl radical, while quercetin, luteolin (flavonoids that contain a catechol B ring) or kaempferol depleted GSH stoichiometrically without forming a thiyl radical or GSSG. Quercetin, luteolin and kaempferol formed mono-GSH and bis-GSH conjugates, whereas apigenin and naringenin did not form GSH conjugates. Spectral studies of products show that GSH was oxidized by apigenin/naringenin phenoxyl radicals, whereas GSH conjugate formation involved the *o*-quinone metabolite of luteolin or the quinoid (quinone methide) product of quercetin/kaempferol. The presence of hydroxyl group at flavonoid ring B is not completely necessary for interaction with glutathione, because not only *p*-hydroxyl or *o*-dihydroxyl substituted flavonoids are easily oxidized to corresponding quinones. Galangin, a flavonol with no substitution of ring B and 3,5,7 trihydroxy-chromen-4-one moiety also oxidizes and reacts with thiol groups (Michels *et al*., 2004)

Some schemes showing the potential oxidation products of some flavonoid derivatives were proposed (Fig. 3). The formation of the products is strongly affected by the overall redox potential of these compounds, caused by structural parameters. The pyranone ring is probably the place of reaction, as the presence or character of R1 and presence/absence of 2,3 double bond are usually determinants of anti-oxidant activity (Havsteen, 2000; Andersen and Markham, 2005).

As visible, the descriptions of pro-oxidant activity of flavonoids are not numerous and are mainly reported from different biochemical or *in vitro* experiments. However, flavonoids are almost never used solely *in vivo* or in real life. The consumption of flavonoids is usually accompanied by consumption of other compounds including low molecular anti-oxidants like vitamin C, vitamin E, or possible glutathione (Andersen and Markham, 2005). Therefore, some experiments were carried out *in vitro* and showed, that catechol bearing moiety flavonoids oxidize glutathione or ascorbic acid. It is known that anti-oxidants "can work in cooperation", oxidized anti-oxidants can be recycled by "fresh" anti-oxidants in reduced state to make kind of network. It was described that *o*-quinones formed from flavonoids can be recycled by glutathione or ascorbate, which are getting oxidized.
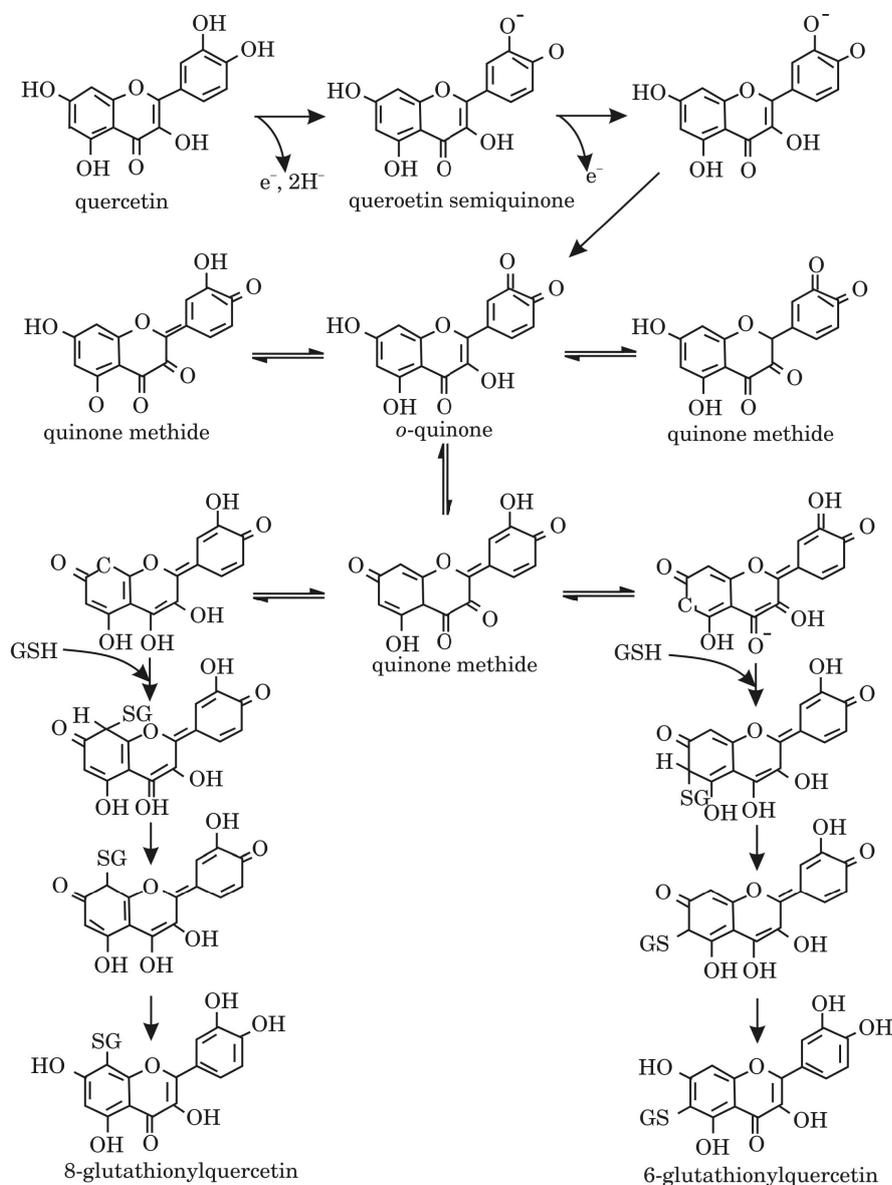
Fig. 2: Proposed scheme of quercetin conjugation with glutathione (Galati *et al.*, 2001)

This effect can potentiate the overall antioxidant activity of mixture of compounds. But, the consumption of such as recycling undergoing substances can lead to the depletion of antioxidants and overall decrease of anti-oxidant capacity of system, as it was shown for kaempferol, quercetin

and myricetin. These substances induced concentration dependent decrease of glutathione level and GST activity in model system of isolated rat liver cell nuclei, when myricetin (pyrogallol at B ring) showed strongest effect (Sahu and Gray, 1996).
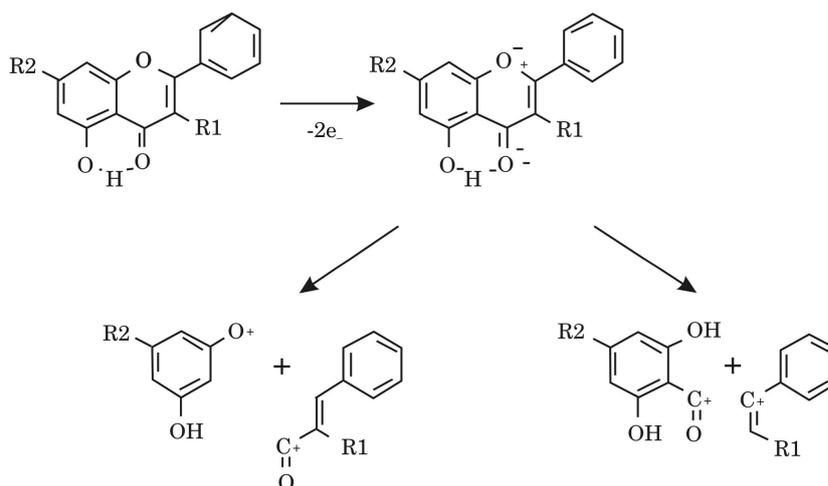
Fig. 3: Proposed scheme of oxidative decomposition of flavonoid

Flavonoids Can Damage DNA and Proteins *via* Produced ROS or RNS

The DNA damage is often observed during toxic activity of flavonoids. However, the mechanisms of the damage remain unclear, as both participation of pro-oxidant effect and absence of pro-oxidant processes are reported during DNA damage. Moreover, flavonoids can also stimulate DNA reparation processes and cause DNA protection (Spencer *et al*., 2003).

The direct damage represented by the induction of single strand DNA breakage was caused by flavonoids having pyrogallol arrangement of A or B ring in presence of NRS (Ohshima *et al*., 1998). Catechol group of quercetin can be oxidized by Cu(II) and when bound to DNA, the formation of ROS can cause the cleavage. The cleavage was not observed when luteolin and kaempferol were tested in the same manner. The excessive amount of quercetin further inhibited copper-dependent quercetin-induced DNA damage. The damage on DNA was site specific on thymine and cytosine, the formation of 8-oxo-2´-deoxyguanosine was observed (Yamashita *et al*., 1999; Amiæ *et al*., 2007). In contrary to observation of Yamashita *et al*. (1999), kaempferol induced concentration-dependent DNA damage when tested under aerobic conditions in isolated rat liver nuclei. This effect was accompanied by lipoperoxidation, and enhanced by presence of Fe(III) or

Cu(II) (Sahu and Gray, 1994). Similar results were observed when activity of morin and naringenin were tested. ROS scavengers catalase, superoxide dismutase and mannitol had no effect on the flavonoid-induced nuclear DNA damage in the presence of the metal ions; nuclear lipid peroxidation was partially inhibited only by mannitol. Hydroxyl radicals are the initiators of the lipoperoxidation, producing peroxidation products such as peroxyl radicals possibly causing the DNA strand breaks. But, also the hydroxyl radicals produced close to the DNA backbone could induce direct site-specific strand breaks insensitive to presence of ROS scavengers (Sahu and Gray, 1997). However, catechin and taxifolin did not show pro-oxidant behavior through the oxidation of 2´-8-deoxyguanosine, induced by a Fenton reaction catalyzed by copper (Teixeira *et al.*, 2005).

Quercetin, morin, naringenin and hesperidin showed the ability to induce DNA damage in lymphocytes, however in high concentration of 200 µM (Yen *et al.*, 2003). The human leucocytes treated with quercetin in concentration up to 50 µM showed reduced level of DNA damage induced by superoxide, while in higher concentration this damage was promoted (Wilms *et al.*, 2008). Pro-oxidant properties of high concentrations of quercetin (200 ìM) might be related to the formation of a quinoid-type oxidation product, which can react very efficiently with lysine residues in proteins to induce its oxidative degradation into the corresponding semialdehyde. Quercetin may also be able to form stable phenol-protein complexes (Utrera *et al.*, 2013).

According to various spectrophotometric studies the dominant binding mode of quercetin, kaempferol and luteolin with DNA *in vitro* is intercalation. Once the flavonoid structure is intercalated into DNA, they could manifest two distinct effects: (a) cytotoxic and DNA-damaging activity or (b) protective activity against oxidative stress (Mariniæ *et al.*, 2006; Rusak, 2010).

Rusak *et al.* concluded that kaempferol and luteolin cause DNA damage, which could be detected by alkaline comet assay. The toxicity was mainly attributed to their ability to cause oxidative damage to DNA. The pro-oxidative activity of flavonoids may be related to the ability of these compounds to undergo auto-oxidation catalyzed by transition metals to produce superoxide anions. An alternative mechanism for the pro-oxidant toxicity involves the numerous peroxidases that catalyze the oxidation of phenols. Among the flavonoids tested, the most effective inducer of DNA damage in lymphocytes was luteolin. Surprisingly, luteolin showed the best results as a protective agent against oxidative stress in hydrogen peroxide stressed lymphocytes (Rusak *et al.*, 2010). The finding that luteolin and kaempferol intercalate into DNA could be correlated to their ability to produce the single-strand breaks detectable by comet assay. This presumption is based on the fact that quercetin inhibits topoisomerases II

and IV. These enzymes cleave the superhelical form of the DNA chain to create additional turns or to unravel superhelical turns prior to reconnection of the DNA ends. This final step can be inhibited by quercetin most likely due to intercalation of quercetin into DNA. The outcomes are DNA single-strand breaks (Rusak *et al.*, 2010).

There are several attributes connected to the oxidative damage of flavonoids to DNA. Firstly, the oxidative damage is mostly dependent on concentration of corresponding flavonoid. The human leucocytes treated with quercetin in concentration up to 50 µM showed reduced level of DNA damage induced by superoxide, while in higher concentration reaching 100 µM this damage was promoted. Pro-oxidant effects of quercetin can be the consequence of conversion into *o*-semiquinone and quinone-methide isomers (Wilms *et al.*, 2008).

Similarly, procyanidin B_2 reduced the oxidative DNA damage at optimal concentration. The damage was measured *via* decrease of the 8-oxo-2´-deoxyguanosin formation in cultured cells treated with H_2O_2-generating system. On the other hand, higher concentration of procyanidin B_2 showed a pro-oxidant activity. Possible mechanism is that procyanidin B_2 was auto-oxidized into a semiquinone radical, and further to a corresponding *o*-quinone form (Sakano *et al.*, 2005). Johnson *et al.* presented resembling results with epigallocatechin gallate and quercetin. Relatively low concentrations (10 µM) of both flavonoids inhibited H_2O_2-induced DNA damage. But at higher concentration, *e.g.*, 100 µM, both epigallocatechin gallate and quercetin induced oxidative damage to DNA (Johnson *et al.*, 2000).

Other common feature of oxidative damage caused by flavonoids is presence of metal ions and their involvement in the reactions. For example, myricetin, baicalein and quercetin caused strand scission of DNA in presence of copper ions. The reaction probably involved hydrogen peroxide, because it was prevented by catalase (Yoshino *et al.*, 1999). Analogous activity in presence of copper ions was shown by flavonoids isolated from stem of *Rhus javanica* var. *roxburghiana* taxifolin, fisetin and fustin (Lin *et al.*, 2008). Flavonols with pyrogallol or catechol ring B have also been shown to auto-oxidize in the presence of transition metals to produce ROS, witch accelerate low-density lipoprotein oxidation. The green tea catechin, epigallocatechin gallate, was recently shown to induce H_2O_2 generation and cause subsequent oxidative damage to isolated cellular DNA in presence of transition metal ions (Galati *et al.* 2004). Propolis flavonoids (such as galangin, chrysin and pinocembrin) also showed the ability to induce H_2O_2-mediated DNA damage (Tsai *et al.* 2012).

Different mechanism of DNA damage independent on ROS was found when leukemia cells were treated with apigenin. The treatment resulted

in the induction of DNA damage mediated by p38 and protein kinase C-delta (PKCä), yet was independent of reactive oxygen species or caspase activity. The DNA damage was followed by activation of apoptosis program (Arango *et al*., 2012).

Antibacterial Defense, Activity against Pathogenic Bacteria and Pro-Oxidant Effect

It is now known, that effect of certain antibiotics is connected with oxidative stress. Some antibiotics show the increase of intracellular oxidants, disruption of iron and Fe-S cluster homeostasis, DNA damage and also up-regulation of many cellular defensive mechanisms. It is suggested that these antibiotics can promote the formation of radical species *via* Fenton reaction, it means that they are promoting the formation of hydroxyl radical using the hydrogen peroxide decomposition in presence of Fe(II). The hydroxyl radical is very toxic substance causing harm to membrane lipids, proteins and also DNA of invading pathogenic species. In case of unbalance and no control, it can also damage also the body´s own structures. The oxidation of guanosine to produce hydroxyl guanosine is probably the action responsible for majority of the antibacterial effect. Induction of some enzymes like catalase, catalyzing the decomposition of hydrogen peroxidecan also occur (Hassett and Imlay 2007).

Some strong anti-oxidants like glutathione or ascorbic acid (however, ascorbic acid can have pro-oxidative effect) show prevention of the death of bacteria caused by fluoroquinolones and aminoglycosides, but enhance the effect of â-lactam antibiotics against *E. coli*. The antibacterial effect can be either enhanced or inhibited by polyphenols in dependence on the bacterial strain used, on the method, and also on the antibiotic used for assaying.

Medicinal plant extracts, commonly used in traditional and modern phytotherapy, often show anti-oxidant activity possibly connected with presence of phenolic substances (including flavonoids) (Rice-Evans *et al*., 1997; Pietta 1998). On the other hand, plant extracts can also possess pro-oxidant effects (Tang and Halliwell, 2010). Antibiotics and medicinal herbs may be used simultaneously for the therapy of infectious diseases. However, influence of plant extracts on sensitivity of bacteria to antibiotics has not been well studied. Some data show that anti-oxidant activity of plant extracts correlates with protection of microbials against H_2O_2 (Oktyabrsky *et al*., 2009). Recent data show that medicinal plant extracts and phenols may protect bacterial cells against ciprofloxacin toxicity (Smirnova *et al*., 2012). In parallel, flavonoids are reported as antimicrobial compounds. Many papers dealing with the antibacterial activity of different flavonoids were publishedand later well reviewed (Cushnie and Lamb, 2005 and 2011). One of the proposed modes of antibacterial action of flavonoids is the

interaction with energetic metabolism of bacteria (caused by NADH-cytochrome *c* reductase inhibition) (Haraguchi *et al*., 1998); however, there are also other proved mechanisms (Dzoyem *et al*., 2013).

The group of Haraguchi *et al*. hypothesized that the licochalcones may be interfering with energy metabolismin a similar way to respiration-inhibiting antibiotics, because fluent transfer of energy is required for active uptake of many metabolitesand for further biosynthetic procedures (Haraguchi *et al*., 1998). The licochalcones were shown to strongly suppress oxygen consumption in *M. luteus* and S. aureus but not in *E. coli*. It correlated well with the observed antibacterial activity. The group of Haraguchi *et al*. (1998) further demonstrated that licochalcones A and C inhibited the activity of NADH-cytochrome *c* reductase, but not cytochromecoxidase or NADH-CoQ reductase. Therefore, the inhibition site of the seretrochalcones was probably between CoQ and cytochrome*c*in the bacterial respiratory electron transport chain. But, these results were later shown to be dubious because there is possibility that flavonoid treatment of bacterial population causes their aggregation and smaller surface of bacterial colony population decreases the consumption of oxygen (Cushnie *et al*., 2007).

Some flavonoids have been found to damage the cytoplasmatic membrane, possibly *via* generating the formation of hydrogen peroxide (Cushnie and Lamb, 2005; Arakawa *et al*., 2004). Epigallocatechin gallate (EGCG) efficiently generated hydrogen peroxide in rate dependent on solution pH; the generation rate increased with rising pH. Hydrogen peroxide is generated from catechin by one electron reduction of dissolved oxygen; therefore reactive oxygen species are generated. However, this could not be assigned as pro-oxidant effect due to the ongoing reduction of molecular oxygen. The generated H_2O_2 was responsible for the antibacterial effect, and moreover, it can further be metabolized *via* Fenton reaction to hydroxyl radical (Arakawa *et al*., 2004).

The study of Samoilova *et al*. (2014a) has tried to evaluate the potential of green and black tea extracts (rich in flavan-3-ol derivatives) and some other commonly available medicinal plants to chelate the iron, to stimulate/inhibit production of hydrogen peroxide (horse-radish peroxidase inhibition) and antiradical activity using DPPH assay. Tested extracts do not inhibited the growth of tested *E. coli* with the exception of *Arctostaphyllos uvae ursi* extract in the absence of hydrogen peroxide, but the pretreatment of growing bacteria culture with several extracts (showing high anti-oxidant activity) inhibited the toxic effect of H_2O_2.Green and black tea, *A. uvae ursi* and *Vaccinium vitis-idaea* extracts in aerobic conditions exhibited pro-oxidant effects, producing H_2O_2. The presence of higher amount of H_2O_2 induced expression of the katG gene encoding catalase HPI in *E. coli* cells to protect them (Samoilova *et al*., 2014a), as confirmed in other report showing that

pretreatment of *E. coli* with quercetin and tannic acid up-regulated anti-oxidant genes katG and sodA due to pro-oxidant and chelating activity and resulted in noteworthy protective effects against oxidative stress (Smirnova *et al.*, 2009). The ability of extracts to chelate iron was tested using *in vitro* chelating assay and *in vivo* in *E. coli*, in assay evaluating the iucC::lacZ fusion expression. The *iucC* gene is part of the aerobactin iron uptake operon and is negatively controlled by the global regulator Fur. Chelators trigger de-repression of theFur regulon and induce the *iucC* due to a decrease in intracellulariron. Some of the extracts tested showed chelating activity; however this did not correlate directly with raised expression of *tucC* gene, maybe because of the lowered permeation of catechins in extracts into bacterial cells (Samoilova *et al.*, 2014a; Smirnova *et al.*, 2009). Later, it has been shown that different extract delayed the *E. coli* lysis by ciprofloxacin, some of them protected them also against ampicillin. Black tea extracts promoted the lysis of *E. coli* caused by ampicillin. There was a correlation between the lysis delay and inhibition of *tucC* gene expression. Several extracts increased lysis caused by application of kanamycin. When the level of *katG* expression (marker of oxidative stress) was analyzed, both ciprofloxacin and kanamycin elevated its level and these antibiotics caused to *E. coli* oxidative stress. The extracts than lowered the *katG* expression when applied alone, later lowered it in combination with ciprofloxacin, and induced it when combined with kanamycin (Samoilova *et al.*, 2014a). However, the other assay showed that low concentrations of water extracts from black tea and some other medicinal plants (rich in phenolics) can stimulate the formation of *E. coli* biofilm. The same was shown for low concentrations of quercetin. Stimulatory effect of the extracts and pure polyphenols on biofilm formation was probably related to their pro-oxidant properties, as the H_2O_2 formation and chelating activity was evaluated and found stimulated (Samoilova *et al.*, 2014b).

The mechanism of anti-oxidant action of polyphenols on living cells is not limited to ROS scavenging, but includes upregulation of anti-oxidant and detoxification enzymes, modulation of cellular redox thiols, and modulation of cell signaling and gene expression (Eberhardt and Jeffery, 2006). Both oxidants and anti-oxidants can increase the ability of organism to kill invading microorganisms or to improve the resistance to infection *via* immunomodulation. As it was described above, flavonoids are well known anti-oxidant, but on the other hand, their pro-oxidative potential was also described. At the same time it has been demonstrated that under certain conditions polyphenols may take part in generationof reactive oxygen species (ROS) and acts as pro-oxidants (Smith *et al.*, 2003; Tang and Halliwell, 2010).

Therefore, compounds modifying the oxidative status of organism, resp. oxidative status of affected tissue can be useful in improvement of antibacterial defense of organism, but it requires further investigations.

Later, it may be of a significant practical importance for complex therapy of bacterial infections.

Inflammation, ROS/RNS and Flavonoids

Reactive oxygen species (ROS) possess dual role during the inflammation – beneficial and detrimental. Higher amounts of ROS are produced by phagociting cells to kill captured bacteria or viruses. Low concentration of ROS also serves as signaling agents in cells. On the other hand, when the production of ROS exceeds the anti-oxidant capacity of cells, they can damage different biomolecules, especially proteins, unsaturated lipids and DNA (Valko *et al*., 2007). Oxidative stress itself could also trigger the inflammatory response in tissue and thus contribute to acceleration of some stages of inflammation (Gutowski and Kowalczyk, 2013; Saeidnia and Abdollahi, 2013). However, it is still not clear whether higher ROS production is cause or consequence of inflammation (Valko *et al*., 2007). Although this dilemma is not still solved, many therapeutic approaches are coming to be based on the elimination of these potentially dangerous molecules.

The fate and response of cells on oxidative stress is strongly dependent on the ROS concentration (Fig. 4). The low ROS level activates Nrf2 (nuclear-erythroid 2-p45-related factor 2) transcription factor, which positively regulates the expression of anti-oxidant enzymes. This leads to protection of cells against further oxidative stress. Higher ROS concentration contributes to activation of NF-κB (nuclear factor κB) and AP-1 (activator protein 1) and hence sustains the inflammation. Very high concentrations of ROS result in collapse of mitochondrial membrane potential, irreversible damage of cell structures and lead to apoptosis or necrosis (Gloire *et al*., 2006).

Many experiments were performed to describe an anti-oxidant potential of flavonoids connected with their anti-inflammatory effect, but limited number of studies is focusing on the pro-oxidant role of flavonoids during modulation of inflammatory response. From this point of view, the most studied are green tea catechins. Pro-oxidant effect of epigallocatechin galate (EGCG) is known for a long time, but its influence on inflammation remains more or less unclear. Ho *et al*. (2014) showed that consummation of green tea (rich source of catechins) by healthy volunteers led to increasing of anti-inflammatory protein heme oxygenase 1 (HO-1) (Ho *et al*., 2014). The expression of this protein is regulated by changing of cellular redox potential. Hence, pro-oxidant effect of catechins could trigger production of HO-1 and thus contribute to overall anti-oxidant and anti-inflammatory capacity of cells. EGCG is able to retain the pro-oxidant and anti-inflammatory activities even after biotransformation *in vivo* (Lambert *et al*., 2010). Pro-oxidant and concurrently anti-inflammatory effect was also
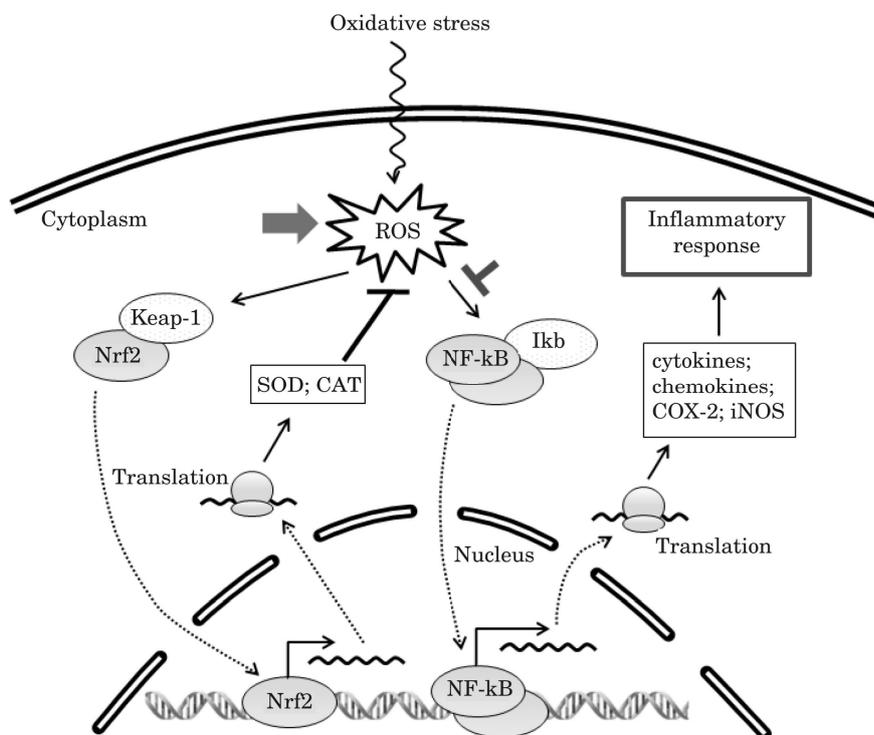
Fig. 4: Mechanisms of redox action of flavonoids. Full arrows mean direct interaction, dashed arrows indicate translocation. Thick red **T** marks show points, at which flavonoids inhibit; the thick green arrow indicates the pro-oxidant effect of flavonoids. *CAT* catalase; *COX* cyclooxygenase; *IκB* inhibitor of κB; *Keap-1* Kelch-like ECH-associated protein 1; *NF-κB* nuclear factor κB; *Nrf2* nuclear factor erythroid 2-related factor 2; *ROS* reactive oxygen species; *SOD* superoxide dismutase.

proven for cudraflavone B and osajin, but the direct causality between higher production of ROS and lower activation of NF-κB signaling pathway was not determined (Hošek *et al*., 2013).

The pro-oxidant effect of flavonoids on the attenuation of inflammation is a big challenge for the research. Flavonoids possess both anti-oxidant and pro-oxidant activities, which are concentration and time dependent (Kim *et al*., 2004). It is apparent from the available results that mild oxidation stress, caused by pro-oxidant activity of flavonoids, could be beneficial as prophylactic treatment rather than curative therapy. This justifies long-term consumption of flavonoid-rich food as a prevention of inflammatory disorders. On the other hand, different anti-phlogistic mechanisms of flavonoids are involved in the solving of acute or chronic inflammatory response (Kim *et al*., 2004).

PRO-OXIDANT ACTIVITY OF FLAVONOIDS, CYTOTOXICITY AND CANCER

Many flavonoids are found to be cytotoxic. Several reviews bring the overview of their direct cytotoxic effect in cell systems (López-Lázaro *et al.*, 2002; Imperato, 2009; Smejkal, 2014). The cytotoxicity of flavonoids could be at least partially selective against cancer cells in comparison to normal cells (Sakagami *et al.*, 2007).

Interesting is an ability of some flavonoids to generate substantial and therefore cytotoxic amounts of hydrogen peroxide or in other ways to stimulate formation of ROS (Miura *et al.* 1998). This effect is observed also *in vitro* after adding some flavonoids (quercetin and EGCG) into culture media (in relatively high concentration). The cancer cells lines (Caco-2, HT-29) or rat non-transformed intestinal crypt cells (IEC-6) showed strong condensation of chromatin as a marker of apoptosis. Further, quercetin has shown many other effects on the inhibition of cell proliferation, cell cycle, caspases activation etc., but it is questionable, if these effects are connected with its ability to induce H_2O_2 formation, pro-oxidant or anti-oxidative action (Gibellini *et al.*, 2011). Similarly, apigeninand other flavonoids (including prenylated substances) proved ability to induce apoptosis characterized by DNA fragmentation and caspase activation, but the connection of these effects with hydrogen peroxide production was not proved; however, the formation of radicals was observed by EPR (Sakagami *et al.*, 2000). Sergediené *et al.* (1999) showed that several dietary flavonoids exhibited a dose-dependent toxicity against human promyelocytic leukemia cells (HL-60). Their action was accompanied by malondialdehyde formation, which showed a pro-oxidant character of the cytotoxicity. Also other study confirmed the importance of single-electron oxidation of flavonoids and formation of the reactive oxygen species in their cytotoxicity (Nemeikaite-Ceniene *et al.*, 2005). Interestingly, the pro-oxidant cytotoxic potential can be affected and diminished by combination of different flavonoid substances (Dickancaité *et al.*, 1998).

The results obtained in cell cultures are not unequivocal and it must be taken into account, that many of studies of this type can be affected by the ability of compounds tested to oxidize in culture media (Halliwell, 2008).

Some reports also show that pro-oxidative effects of high concentration of flavonoids can be connected with pro-apoptotic signaling in cancer cells (Wätjen *et al.*, 2007a, 2007b). There is evident link between oxidative stress and cancerogenesis. Long-lasting exposition to elevated ROS concentration could lead to irreversible DNA damage connected with mutagenesis and neoplastic formation (Valko *et al.*, 2006). This observation has led to use anti-oxidants to protect genetic material against damage and hence to forestall the cancerogenesis. Anti-oxidant activities of flavonoids explain their preventive effect against cancer development. However, this effect is not sufficient for explaining their curative potential in the case of developed

tumors. Overall flavonoids' anti-oxidant capacity *in vivo* is considerably lower than anti-oxidant capacity of endogenous anti-oxidants, e.g. vitamin C. Pro-oxidant capability of flavonoids is considered as their key feature for cancer treatment (not prevention) (Galati and O'Brien, 2004).

Two main mechanisms of anti-cancer activity of flavonoids connected with their pro-oxidant properties are suggested. The first one is based on the elevated concentration of copper ions in tumors. The higher amount of this metal ion has been described in many types of cancer (Khan *et al*., 2012). Especially in the nucleus, the copper represents promising target of anti-cancer therapy. Flavonoids can interact with Cu(II) in Fenton's reaction and generate ROS. This is typical for molecules bearing catechol or pyrogallol moiety on the B rings (Galati and O'Brien, 2004). Because copper is located in significant concentration close to DNA, the elevated concentration of ROS can lead to irreversible damaging of DNA structure causing cell death. Health cells do not possess so high cupper concentrations and remain relatively resistant to pro-oxidant action of flavonoids. Higher concentrations of Cu(II) and naturally greater level of ROS in tumors in comparison with normal cells can explain the preferential cytotoxicity of flavonoids to cancer cells (Khan *et al*., 2012). The second mechanism of an anticancer effect of flavonoids is a dissipation of the mitochondrial membrane potential ($\Delta\psi_m$). Flavonoid phenoxyl radicals are able to trigger the release of apoptogenic cytochrome *c* by altering $\Delta\psi_m$ (Galati and O'Brien, 2004). Both above mentioned anti-cancer effects are beneficial during tumor therapy, but also represent potential risk of out-of proportion oxidative stress for health population during overuse of food supplements rich on flavonoids and other natural phenolic compounds. Nevertheless, regular daily intake of flavonoid rich foodstuffs and beverages can increase the protection against cancer development. This hypothesis was proven by Ho *et al*. (2014), who described that pro-oxidant effect of green tea. Phenolic compounds (also flavonoids) were able to decrease the DNA damage in health volunteers after consummation of green tea. This could be caused by higher activity of oxoguanine glycosylase 1, which contribute to DNA reparation after its oxidation damaging (Ho *et al*., 2014).

CONCLUSIONS

It is estimated that the average intake of flavonoids innormal humandiet is 1-2 g per day. Such a high consumption of relatively unknown compounds is a good reason for deep analysisand possible revision of the research efforts in the fields of flavonoid toxicology and nutrition. Large attention has been focused on highly toxic compounds in low concentration, but little attention has been given to the massive intake of weak toxins. The anti-oxidant and pro-oxidant effects, and protection and damage of DNA can be in principle caused by the same flavonoid substance, and the effect must be strongly

influenced by the dose used, by the cell and tissue type and by presence or absence of specific factors. Difference between physiological and pathological conditions can contribute to this situation. Therefore, we need to improve our knowledge of these effects to better understand to the potential of flavonoids in therapy.

REFERENCES

Amiæ, D., Davidoviè-Amiæ, D., Bešlo, D., Rastija, V., Luèiæ, B. and Trinajstiæ, N. (2007). SAR and QSAR of the antioxidant activity of flavonoids. *Curr. Med. Chem*., 14: 827–45.

Andersen, O.M. and Markham, K.R. (2005). Flavonoids: Chemistry, Biochemistry, Applications. CRC Press.

Apak, R., Gorinstein, S., Böhm, V., Schaich, K.M., Özyürek, M. and Güçlü, K. (2013). Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report). *Pure. Appl. Chem*., 85: 957–98.

Arango, D., Parihar, A., Villamena, F.A., Wang, L., Freitas, M.A., Grotewold, E. *et al*. (2012). Apigenin induces DNA damage through the PKCä-dependent activation of ATM and H2AX causing down-regulation of genes involved in cell cycle control and DNA repair. *Biochem. Pharmacol.*, 84: 1571–80.

Awad, H.M., Boersma, M.G., Boeren, S., Vervoort, J., van Bladeren, P.J. and Rietjens, I.M.C.M. (2002). The regioselectivity of glutathione adduct formation with flavonoid quinone/quinone methides is pH-dependent. *Chem. Res. Toxicol*., 15: 343–51.

Bodewes, T.C.F., Luttikhold, J., van Stijn, M.F.M., Visser, Ma., van Norren, K., Vermeulen Mechteld, A.R. *et al*. (2011). Antioxidative properties of flavonoids. *Curr. Org. Chem*., 15: 2616–26.

Bruneton, J. (1999). Pharmacognosy, Phytochemistry, Medicinal Plants. 2nd Ed. Lavoisier.

Buss, G.D., Constantin, J., de Lima, L.C., Teodoro, G.R., Comar, J.F., Ishii-Iwamoto, E.L. *et al.* (2005). The action of quercetin on themitochondrial NADH to NAD(+) ratio in the isolated perfused rat liver. *Planta Med.*, 71: 1118–22.

Canada, A.T., Giannella, E., Nguyen, T.D. and Mason, R.P. (1990). The production of reactive oxygen species by dietary flavonols. *Free Radic. Biol. Med*., 9: 441–9.

Cao, G. and Prior, R.L. (1998). Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin. Chem.*, 44: 1309–15.

Cao, G., Sofic, E. and Prior, R.L. (1997). Antioxidant and prooxidant behavior of flavonoids: Structure–activity relationships. *Free Radic. Biol. Med*., 22: 749–60.

Chan, T., Galati, G. and O'Brien, P.J. (1999). Oxygen activation during peroxidase catalyzed metabolism of flavones or flavanones. *Chem. Biol. Interact.*, 122: 15–25.

Constantin, R.P., Constantin, J., Pagadigorria, C.L., Ishii-Iwamoto, E.L., Bracht, A., Castro, C.V. *et al.* (2011). Prooxidant activity of fisetin: Effects on energy metabolism in the rat liver. *J. Biochem. Mol. Toxicol*., 25: 117–26.

Cushnie, T.P.T., Hamilton, V.E.S., Chapman, D.G., Taylor, P.W. and Lamb, A.J. (2007). Aggregation of *Staphylococcus aureus* following treatment with the antibacterial flavonol galangin. *J. Appl. Microbiol*., 103: 1562–7.

Cushnie, T.P. and Lamb, A.J. (2005). Antimicrobial activity of flavonoids. *Int. J. Antimicrob*. *Agents,* 26: 343–56.

Cushnie, T.P. and Lamb, A.J. (2011). Recent advances in understanding the antibacterial properties of flavonoids. *Int. J. Antimicrob. Agents,* 38: 99–107.

Cushnie, T.P.T. and Lamb, A.J. (2005). Detection of galangin-induced cytoplasmic membrane damage in *Staphylococcus aureus* by measuring potassium loss. *J. Ethnopharmacol.*, 101: 243–8.

Dickancaité, E., Nemeikaitè, A., Kalvelytè, A. and Cènas, N. (1998). Prooxidant character of flavonoid cytotoxicity: Structure-activity relationships. *Biochem. Mol. Biol. Int.*, 45: 923–30.

Duthie, S.J., Johnson, W. and Dobson, V.L. (1997). The effect of dietary flavonoids on DNA damage (strand breaks and oxidised pyrimdines) and growth in human cells. *Mutat. Res.,* 390: 141–51.

Dzoyem, J.P., Hamamoto, H., Ngameni, B., Ngadjui, B.T. and Sekimizu, K. (2013). Antimicrobial action mechanism of flavonoids from *Dorstenia species*. *Drug Discov. Ther.*, 7: 66–72.

Eberhardt, M.V. and Jeffery, E.H. (2006). Perspective. When dietary antioxidants perturb the thiol redox. *J. Sci. Food Agric.*, 86: 1996–98.

El Hajji, H., Nkhili, E., Tomao, V. and Dangles, O. (2006). Interactions of quercetin with iron and copper ions: Complexation and autoxidation. *Free Radic. Res.*, 40: 303–20.

Galati, G., Chan, T., Wu, B. and O'Brien, P.J. (1999). Glutathione-dependent generation of reactive oxygen species by the peroxidase-catalyzed redox cycling of flavonoids. *Chem. Res. Toxicol.*, 12: 521–5.

Galati, G., Moriani, M.Y., Chan, T.S. and O´Brien, P.J. (2001). Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: Glutathione oxidation and conjugation. *Free Radic. Biol. Med.*, 30: 370–82.

Galati, G., Sabzevari, O., Wilson, J.X. and O'Brien, P.J. (2002). Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology*, 177: 91–104.

Galati, G. and O'Brien, P.J. (2004). Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. *Free Radic. Biol. Med.*, 37: 287–303.

Gibellini, L., Pinti, M., Nasi, M., Montagna, J.P., De Biasi, S., Roat, E., Bertoncelli, L., Cooper, E.L. and Cossarizza, A. (2011). Quercetin and cancer chemoprevention. *Evid. Based Complement. Alternat. Med.,* 591356. doi: 10.1093/ecam/neq053.

Gloire, G., Legrand-Poels, S. and Piette, J. (2006). NF-kappaB activation by reactive oxygen species: Fifteen years later. *Biochem. Pharmacol.*, 72: 1493–505.

Gomes, A., Fernandes, E., Garcia, M.B., Silva, A.M., Pinto, D.C., Santos, C.M. and Cavaleiro, J.A. (2008). Cyclic voltammetric analysis of 2-styrylchromones: Relationship with the antioxidant activity. *Bioorg. Med. Chem.*, 16: 7939–43.

Gutowski, M. and Kowalczyk, S. (2013). A study of free radical chemistry: Their role and pathophysiological significance. *Acta Biochim. Pol.*, 60: 1–16.

Hadi, S.M., Bhat, S.H., Azmi, A.S., Hanif, S., Shamim, U. and Ullah, M.F. (2007). Oxidative breakage of cellular DNA by plant polyphenols: A putative mechanism for anticancer properties. *Semin. Cancer Biol.,* 17(5): 370–6.

Hanasaki, Y., Ogawa, S. and Fukui, S. (1994). The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radic. Biol. Med.*, 16: 845–50.

Halliwell, B. (2008). Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and *in vivo* studies? *Arch. Biochem. Biophys.*, 476: 107–12.

Hassett, D.J. and Imlay, J.A. (2007). Bactericidal antibiotics and oxidative stress: A radical proposal. *ACS Chem. Biol.*, 2: 708–710

Haraguchi, H., Tanimoto, K., Tamura, Y., Mizutani, K. and Kinoshita, T. (1998). Mode of antibacterial action of retrochalcones from *Glycyrrhiza inflata*. *Phytochemistry*, 48: 125–9.

Havsteen, B.H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacol. Ther.*, 96 :67–202.

Heim, K.E., Tagliaferro, A.R. and Bobilya, D.J. (2002). Flavonoid antioxidants: Chemistry, metabolism and structure–activity relationships. *J. Nutr. Biochem.*, 13: 572–84.

Hernández, I., Alegre, L., van Breusegem, F. and Munné-Bosch, S. (2009). How relevant are flavonoids as antioxidants in plants? *Trends Plant Sci.*, 14: 125–32.

Ho, C.K., Choi, S.W., Siu, P.M. and Benzie, I.F.F. (2014). Effects of single dose and regular intake of green tea (*Camellia sinensis*) on DNA damage, DNA repair and heme oxygenase-1 expression in a randomized controlled human supplementation study. *Mol. Nutr. Food Res.*, 58: 1379–83.

Hodnick, W.F., Duval, D.L. and Pardini, R.S. (1994). Inhibition of mitochondrial respiration and cyanide-stimulated generation of reactive oxygen species by selected flavonoids. *Biochem. Pharmacol.*, 47: 573–80.

Hodnick, W.F., Kalyanaraman, B., Pritsos, C.A. and Pardini, R.S. (1989). The production of hydroxyl and semiquinone free radicals during the autoxidation of redox active flavonoids. *In*: Simic, M.G., Taylor, K.A., Ward, J.F. and von Sonntag, C. *Eds.*, Oxygen radicals in biology and medicine. New York: Plenum, pp. 149–52.

Hodnick, W.F., Kung, F.S., Roettger, W.J., Bohmont, C.W. and Pardini, R.S. (1986). Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. A structure-activity study. *Biochem. Pharmacol.*, 35: 2345–57.

Hodnick, W.F., Milosavljeviæ, E.B., Nelson, J.H. and Pardini, R.S. (1988). Electrochemistry of flavonoids. Relationships between redox potentials, inhibition of mitochondrial respiration and production of oxygen radicals by flavonoids. *Biochem. Pharmacol.*, 37: 2607–11.

Hošek, J., Toniolo, A., Neuwirth, O. and Bolego, C. (2013). Prenylated and geranylated flavonoids increase production of reactive oxygen species in mouse macrophages but inhibit the inflammatory response. *J. Nat. Prod.*, 76: 1586–91.

Imperato, F. (2009). New cytotoxic flavonoids from plants. *In*: Recent Advances in Phytochemistry, *Ed.* Imperato, F., pp. 133-59.

Izzi, V., Masuelli, L., Tresoldi, I., Sacchetti, P., Modesti, A., Galvano, F. *et al.* (2012). The effects of dietary flavonoids on the regulation of redox inflammatory networks. *Front. Biosci.*, 17: 2396–2418.

Johnson, M.K. and Loo, G. (2000). Effects of epigaloocatechin gallate and quercetin on oxidative damage to cellular DNA. *Mutat. Res.*, 459: 211–8.

Jomova, K. and Valko, M. (2011). Advances in metal-induced oxidative stress and human disease. *Toxicology*, 283: 65–87.

Juranek, I., Nikitovic, D., Kouretas, D., Hayes, A., Wallace, T. and Aristidis, M. (2013). Biological importance of reactive oxygen species in relation to difficulties of treating pathologies involving oxidative stress by exogenous antioxidants. *Food Chem. Toxicol.*, 61: 240–7.

Kanakis, C.D., Tarantilis, P.A., Polissiou, M.G., Diamantoglou, S. and Tajmir-Riahi, H. (2005). DNA interaction with naturally occurring antioxidant flavonoids quercetin, kaempferol and delphinidin. *J. Biomol. Struct. Dyn.*, 22: 719–24.

Kim, H.P., Son, K.H., Chang, H.W. and Kang, S.S. (2004). Anti-inflammatory plant flavonoids and cellular action mechanisms. *J. Pharmacol. Sci.*, 96: 229–45.

Khan, H.Y., Zubair, H., Ullah, M.F., Ahmad, A. and Hadi, S.M. (2012). A prooxidant mechanism for the anticancer and chemopreventive properties of plant polyphenols. *Curr. Drug Targets*, 13: 1738–49.

Kussmaul, L. and Hirst, J. (2006). The mechanism of superoxide production by NADH: Ubiquinone oxidoreductase (complex I) from bovine heart mitochondria. *PNAS,* 103: 7607–12.

Labuda, J., Bucková, M., Heilerová, L., Silhár, S. and Stepánek, I. (2003). Evaluation of the redox properties and anti/pro-oxidant effects of selected flavonoids by means of a DNA-based electrochemical biosensor. *Anal. Bioanal. Chem.*, 376: 168–73.

Lambert, J.D., Sang, S., Hong, J. and Yang, C.S. (2010). Anticancer and anti-inflammatory effects of cysteine metabolites of the green tea polyphenol, (-)-epigallocatechin-3-gallate. *J. Agric. Food Chem.*, 58: 10016–9.

Laughton, M.J., Halliwell, B., Evans, P.J., Robin, J. and Hoult, S. (1989). Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin: Effects of lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem. Pharmacol.*, 38: 2859–64.

Lin, C.N., Chen, H.L. and Yen, M.H. (2008). Flavonoids with DNA strand-scission activity from *Rhus javanica* var. *roxburghiana*. *Fitoterapia*, 79: 32–6.

Litescu, S.C., Eremia, S. and Radu, G.L. (2010). Methods for the determination of antioxidant capacity in food and raw materials. *In*: Bio Farms for nutraceuticals: Functional food and safety control by biosensors, *Ed*s., Giardi, M.T., Rea, G. and Berra, B. Landes Bioscience and Springer Science+Business Media.

López-Lázaro, M., Gálvez, M., Martín-Cordero, C. and Ayuso, M.J. (2002). Cytotoxicity of flavonoids on cancer cell lines. Structure-activity relationship. *Stud. Nat. Prod. Chem.*, 27: 891–932.

Mariniæ, M., Piantanida, I., Rusak, G. and Žiniæ, M. (2006). Interactions of quercetin and its lanthane complex with double stranded DNA/RNA and single stranded RNA: Spectrophotometric sensing of poly G. *J. Inorg. Bioch.*, 100: 288–98.

Michels, G., Haenen, G.R., Wätjen, W., Rietjens, S. and Bast, A. (2004). The thiol reactivity of the oxidation product of 3,5,7-trihydroxy-4H-chromen-4-one containing flavonoids. *Toxicol. Lett.*, 151: 105–11.

Miura, Y.H., Tomita, I., Watanabe, T., Hirayama, T. and Fukui, S. (1998). Active oxygens generation by flavonoids. *Biol. Pharm. Bull.*, 21: 93–6.

Narayana, K.R., Reddy, M.S., Chaluvadi, M.R. and Krishna, D.R. (2001). Bioflavonoids classification, pharmacological, biochemical effects and therapeutic potential. *Indian J. Pharmacol.*, 33: 2–16.

Nemeikaite-Ceniene, A., Imbrasaite, A., Sergediene, E. and Cenas, N. (2005). Quantitative structure-activity relationships in prooxidant cytotoxicity of polyphenols: Role of potential of phenoxyl radical/phenol redox couple. *Arch. Biochem. Biophys.*, 441: 182–90.

Neta, P. and Grodkowski, J. (2005). Rate constants for reactions of phenoxyl radicals in solutions. *J. Phys. Chem.*, 34: 109–99.

Nijveldt, R.J., van Nood, E., van Hoorn, D.E., Boelens, P.G., van Norren, K. and van Leeuwen, P.A. (2001). Flavonoids: A review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.*, 74: 418–25.

Niki, E. (2010). Assessment of antioxidant capacity *in vitro* and *in vivo*. *Free Radic. Biol. Med.*, 49: 503–15.

Ohshima, H., Yoshie, Y., Auriol, S. and Gilibert, I. (1998). Antioxidant and pro-oxidant actions of flavonoids: Effects on DNA damage induced by nitric oxide, peroxynitrite and nitroxyl anion. *Free Radic. Biol. Med.*, 25: 1057–65.

Oktyabrsky, O., Vysochina, G., Muzyka, N., Samoilova, Z., Kukushkina, T. and Smirnova, G. (2009). Assessment of anti-oxidant activity of plant extracts using microbial test systems. *J. Appl. Microbiol.*, 106: 1175–83.

Pietta, P.G. (1998). Flavonoids in medicinal plants. *In*: Rice-Evans, C.A. and Packer, L. (*Eds*.,) Flavonoids in health and disease. New York: Dekker, pp. 61–110.

Pietta, P.G. (2000). Flavonoids as antioxidants. *J. Nat. Prod.*, 63: 1035–42.

Prochazkova, D., Boušová, I. and Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*, 82: 513–523.

Rapta, P., Misík, V., Stasko, A. and Vrábel, I. (1995). Redox intermediates of flavonoids and caffeic acid esters from propolis: An EPR spectroscopy and cyclic voltammetry study. *Free Radic. Biol. Med.*, 18: 901–8.

Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends Plant Sci.*, 2: 152–9.

Rusak, G., Piantanida, I., Mašiæ, L., Kapuralin, K., Durgo, K. and Kopjar, N. (2010). Spectrophotometric analysis of flavonoid-DNA interaction and DNA damaging/

protecting and cytotoxic potential of flavonoids in human peripheral blood lymphocytes. *Chem. Biol. Interact*., 188: 181–89.

Saeidnia, S. and Abdollahi, M. (2013). Toxicological and pharmacological concerns on oxidative stress and related diseases. *Toxicol. Appl. Pharmacol.*, 273: 442–55.

Sahu, S.C. and Gray, G.C. (1996). Pro-oxidant activity of flavonoids: Effects on glutathione and glutathione S-transferase in isolated rat liver nuclei. *Cancer Lett.*, 104: 193–6.

Sahu, S.C. and Gray, G.C. (1994). Kaempferol-induced nuclear DNA damage and lipid peroxidation. *Cancer Lett*., 85: 159–164.

Sahu, S.C. and Gray, G.C. (1997). Lipid peroxidation and DNA damage induced by morin and naringenin in isolated rat liver nuclei. *Food Chem. Toxicol*., 35: 443–7.

Sakano, K., Mizutani, M., Murata, M., Oikawa, S., Hiraku, Y. and Kawanishi, S. (2005). Procynidin B2 has anti- and pro-oxidant effects on metal-mediated DNA damage. *Free Rad. Biol. Med.*, 39: 1041–9.

Sakagami, H., Kobayashi, M., Chien, C.H., Kanegae, H. and Kawase, M. (2007). Selective toxicity and type of cell death induced by various natural and synthetic compounds in oral squamous cell carcinoma. *In Vivo*, 21: 311–20.

Sakagami, H., Jiang, Y., Kusama, K., Atsumi, T., Ueha, T., Toguchi, M. *et al*. (2000). Induction of apoptosis by flavones, flavonols (3-hydroxyflavones) and isoprenoid-substituted flavonoids in human oral tumor cell lines. *Anticancer Res*., 20: 271–7.

Samoilova, Z., Smirnova, G., Muzyka, N. and Oktyabrsky, O. (2014a). Medicinal plant extracts variously modulate susceptibility of *Escherichia coli* to different antibiotics. *Microbiol. Res*., 169: 307–13.

Samoilova, Z., Muzyka, N., Lepekhina, E., Oktyabrsky, O. and Smirnova, G. (2014). Medicinal plant extracts can variously modify biofilm formation in *Escherichia coli*. *Anton. Leeuw,* 105: 709–22.

Senthilmohan, R. and Kettle, A.J. (2006). Bromination and chlorination reactions of myeloperoxidase at physiological concentrations of bromide and chloride. *Arch. Biochem. Biophys*, 445: 235–44.

Sergediene, E., Jönsson, K., Szymusiak, H., Tyrakowska, B., Rietjens, I.M. and Cenas, N. (1999). Prooxidant toxicity of polyphenolic antioxidants to HL-60 cells: Description of quantitative structure-activity relationships. *FEBS Lett.*, 462: 392–6.

Simiæ, A., Manojloviæ, D., Segan, D. and Todoroviæ, M. (2007). Electrochemical behavior and antioxidant and prooxidant activity of natural phenolics. *Molecules*, 12: 2327–40.

Smejkal, K. (2014). Cytotoxic potential of C-prenylated flavonoids. *Phytochem. Rev.,* 13: 245–275.

Smirnova, G.V., Samoylova, Z.Y., Muzyka, N.G. and Oktyabrsky, O.N. (2009). Influence of polyphenols on *Escherichia coli* resistance to oxidative stress. *Free Radic. Biol. Med*., 46: 759–68.

Smirnova, G., Samoylova, Z., Muzyka, N. and Oktyabrsky, O. (2012). Influence of plant polyphenols and medicinal plant extracts on antibiotic susceptibility of *Escherichia coli*. *J. Appl. Microbiol*., 113: 192–9.

Smith, A.H., Imlay, J.A. and Mackie, R.I. (2003). Increasing the oxidative stress response allows *Escherichia coli* to overcome inhibitory effects of condensed tannins. *Appl. Environ. Microbiol*., 69: 3406–11.

Spencer, J.P.E., Rice-Evans, C.A. and Schroeter, H. (2003). Cytoprotective and cytotoxic effects of flavonoids. *In*: Flavonoids in Health and Disease. 2nd edition, *Ed*s., Rice Evans, C.A. and Packer, L. CRC Press, pp. 309–32.

Sugihara, N., Arakawa, T., Ohnishi, M. and Furuno, K. (1999). Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linolenic acid. *Free Radic. Biol. Med*., 27: 1313–23.

Sun, C., Fu, J., Chen, J., Jiang, L. and Pan, Y. (2010). On-line HPLC method for screening of antioxidants against superoxide anion radical from complex mixtures. *J. Sep. Sci.*, 33: 1018–23.

Tang, S.Y. and Halliwell, B. (2010). Medicinal plants and antioxidants: What do we learn from cell culture and *Caenorhabditis elegans* studies? *Biochem. Biophys. Res. Commun.,* 394: 1–5.

Teixeira, S., Siquet, C., Alves, C., Boal, I., Marques, M.P., Borges, F. *et al.* (2005). Structure-property studies on the antioxidant activity of flavonoids present in diet. *Free Radic. Biol. Med.,* 39: 1099–8.

Torres, J.L., Lozano, C. and Maher, P. (2005). Conjugation of catechins with cysteine generates antioxidant compounds with enhanced neuroprotective activity. *Phytochemistry,* 66: 2032–7.

Tsai, Y.C., Wang, Y.H., Liou, C.C., Lin, Y.C., Huang, H. and Liu, Y.C. (2012). Induction of oxidative DNA damage by flavonoids of propolis: Its mechanism and implication about antioxidant capacity. *Chem. Res. Toxicol.*, 25: 191–6.

Utrera, M. and Estévez, M. (2013). Impact of trolox, quercetin, genistein and gallic acid on the oxidative damage to myofibrillar proteins: The carbonylation pathway. *Food Chem.*, 141: 4000–9.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. and Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.*, 39: 44–84.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. and Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem. Biol. Interact.*, 160: 1–40.

Wassmann, S., Wassmann, K. and Nickenig, G. (2004). Modulation of oxidant and antioxidant enzyme expression and function in vascular cells. *Hypertension*, 44: 381–86.

Wätjen, W., Chovolou, Y., Kampkötter, A. and Kahl, R. (2007a). Antiapoptotic and proapoptotic effects of plant polyphenols. *In*: New Cell Apoptosis Research, *Ed.*, Valentino, R.G. New York: Nova Science Publishers Inc., pp. 161–91.

Wätjen, W., Chovolou, Y., Kampkötter, A. and Kahl, R. (2007). Anti- and prooxidative effects of flavonoids. *In*: Leading edge antioxidants research, *Ed.*, Panglossi, H.V. New York: Nova Science Publishers Inc., pp. 1–16.

Wilms, L.C., Kleinjans, J.C., Moonen, E.J. and Briedé, J.J. (2008.) Discriminative protection against hydroxyl and superoxide anion radicals by quercetin in human leucocytes *in vitro*. *Toxicol. In Vitro,* 22: 301–7.

Wood, L.G., Gibson, P.G. and Garg, M.L. (2006). A review of the methodology for assessing *in vivo* antioxidant capacity. *J. Sci. Food Agric.*, 86: 2057–66.

Yamashita, N., Tanemura, H. and Kawanishi, S. (1999). Mechanism of oxidative DNA damage induced by quercetin in the presence of Cu(II). *Mutat. Res.*, 425: 107–15.

Yen, G.C., Duh, P.D., Tsai, H.L. and Huang, S.L. (2003). Pro-oxidative properties of flavonoids in human lymphocytes. *Biosci. Biotechnol. Biochem.*, 67: 1215–22.

Yoshino, M., Haneda, M., Naruse, M. and Murakami, K. (1999). Prooxidant activity of flavonoids: Copper-dependent strand breaks and the formation of 8-hydroxy-2´-deoxyguanosine in DNA. *Mol. Genet. Metab.*, 68: 468–72.

Anti-inflammatory Activity of Natural Geranylated Flavonoids: Cyclooxygenase and Lipoxygenase Inhibitory Properties and Proteomic Analysis

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S *Supporting Information*

ABSTRACT: Geranyl flavones have been studied as compounds that potentially can be developed as anti-inflammatory agents. A series of natural geranylated flavanones was isolated from *Paulownia tomentosa* fruits, and these compounds were studied for their anti-inflammatory activity and possible mechanism of action. Two new compounds were characterized [paulownione C (**17**) and tomentodiplacone O (**20**)], and all of the isolated derivatives were assayed for their ability to inhibit cyclooxygenases (COX-1 and COX-2) and 5-lipoxygenase (5-LOX). The compounds tested showed variable degrees of activity, with several of them showing activity comparable to or greater than the standards used in COX-1, COX-2, and 5-LOX assays. However, only the compound tomentodiplacone O (**20**) showed more selectivity against COX-2 versus COX-1 when compared with ibuprofen. The ability of the test compounds to interact with the above-mentioned enzymes was supported by docking studies, which revealed the possible incorporation of selected test substances into the active sites of these enzymes. Furthermore, one of the COX/LOX dual inhibitors, diplacone (**14**) (a major geranylated flavanone of *P. tomentosa*), was studied in vitro to obtain a proteomic overview of its effect on inflammation in LPS-treated THP-1 macrophages, supporting its previously observed anti-inflammatory activity and revealing the mechanism of its anti-inflammatory effect.
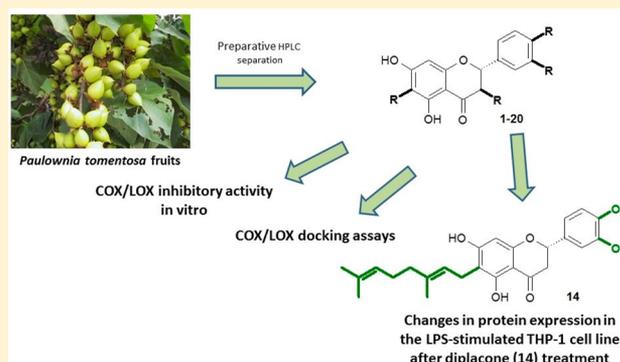
P revious experimental in vitro and in vivo studies have revealed many biological activities of flavonoid substances. These phytochemicals show antibacterial, antiviral, hypolipidemic, and cytotoxic effects.[1] Furthermore, their antioxidant and anti-inflammatory properties have been studied intensively.[2,3] Nevertheless, the elucidation of the structure–activity relationships for the anti-inflammatory effects of a group of flavonoids is complicated because pleiotropic effects targeting multiple cellular proteins can lead to different mechanisms of action.

Flavonoids can modulate a broad spectrum of inflammatory regulatory keypoints. Their antiphlogistic action probably derives from simultaneous actions on different molecular targets.

Several pathways can mediate the antiphlogistic action of flavonoids: their antioxidant and pro-oxidant effects, inhibition of the expression of inflammation-related genes, interaction with signaling pathways, and last but not least, interaction with pro-inflammatory proteins, including the inhibition of specific enzymes.[3]

Modulation of the activity of pro-inflammatory enzymes is one of the most important mechanisms of action for flavonoids. Pro-inflammatory enzymes, such as cytosolic phospholipase A2 (cPLA2), cyclooxygenases (COX), lipoxygenases (LOX),

and NO synthase (NOS), produce very potent inflammatory mediators, and therefore their inhibition contributes to the overall antiphlogistic potential of flavonoids. Special attention should be paid to enzymes metabolizing arachidonic acid (AA), which is oxidized to different eicosanoids in a process mediated by the COX and LOX enzymes. Flavonoids can diminish COX activity, but they usually inhibit COX-1 preferentially over COX-2. Only a few observations of selective anti-COX-2 effects of flavonoids have been reported. Most of the LOX-inhibition experiments on prenylated flavonoids have been performed using 5-LOX and 12-LOX, and information about any effects on other isoforms of LOX is limited.[4,5]

Flavonoids are also known to interact with intracellular signal transduction pathways and thereby to regulate the expression of inflammation-related genes. The transcription nuclear factor κB (NF-κB) is one of the most prominent pathways involved in inflammation. Many studies have confirmed the ability of flavonoids to diminish the activity of NF-κB by inhibiting events upstream, e.g., reducing the phosphorylation of IκB kinase (IKK), which leads to reduced degradation of the inhibitor of κB (IκB) or attenuation of the DNA-binding capability of NF-κB. The activator protein (AP)-1 is also an important pro-inflammatory transcription factor, with its activity regulated by upstream mitogen-activated protein kinases (MAPKs). The three most frequently studied MAPKs are extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK). These kinases not only influence AP-1 but also modulate the activity of NF-κB. Flavonoids are able to inhibit the phosphorylation of all of the above-mentioned MAPKs and thereby lower the transcription of the target genes. Flavonoids are able also to positively regulate the activity of several other signaling pathways, such as those connected with the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Together with its negative regulator, Kelch-like ECH-associated protein 1 (Keap-1), Nrf2 is redox sensitive. Flavonoids possessing a redox potential are therefore able to trigger the activation of Nrf2, which subsequently leads to the transcription of genes coding antioxidant enzymes, e.g., superoxide dismutase (SOD), which modulate the inflammatory response. Transcription factors from the peroxisome proliferator-activated receptor (PPAR) family are also activated by several flavonoids. The activation of this pathway attenuates the activity of NF-κB.[4−6] Since the transcription of inflammation-related genes is regulated by multiple transcription factors, the exact mechanism of flavonoid action still remains unclear. It is safe to say that these phytochemicals interfere with the intracellular signaling network on several different targets and thereby contribute to the overall anti-inflammatory effect.

Paulownia tomentosa Steud. (Paulowniaceae), a rich source of biologically active secondary metabolites, is used traditionally in Chinese herbal medicine. Flavonoids, lignans, phenolic glycosides, quinones, terpenoids, glycerides, phenolic acids, and other miscellaneous compounds have been isolated from different parts of *P. tomentosa*.[7] Recent interest in this species has been focused on isolating and identifying prenylated flavanones, which exhibit potent antioxidant, antibacterial, and antiphlogistic activities and inhibit SARS coronavirus papain-like protease. They show cytotoxic activity against various human cancer cell lines and inhibit the effects of human cholinesterase, butyrylcholinesterase, and bacterial neuraminidases.[8−13]

Our recent studies have shown the potential of *P. tomentosa* C-geranylated flavanones to affect inflammatory conditions in vitro in THP-1 cells stimulated by lipopolysaccharide (LPS) and in vivo in rats with dextran sulfate sodium (DSS)-induced colitis.[13,14] Therefore, we have continued with the isolation of compounds from the *P. tomentosa* extract investigated earlier to discover two new geranylated flavonoids (**17** and **20**), together with several known structures (**1−16**, **18**, and **19**). Owing to the known anti-inflammatory potential of several prenylated and geranylated flavonoids, it was aimed to gain a deeper understanding of the mechanism of action lying behind this anti-inflammatory effect, using assays for COX-1, COX-2, and 5-LOX direct inhibition in vitro, and also to analyze the potential for interaction of these compounds with the above-mentioned enzymes in silico, by docking the compounds into the active sites of these enzymes. Furthermore, one identified COX/LOX inhibitor, diplacone (**14**), previously shown to be antiphlogistic in vivo, was selected for a deeper proteomic analysis in LPS-treated THP-1 macrophages in vitro.

■ RESULTS AND DISCUSSION

Compound Isolation and Structure Elucidation. The chromatographic separation of the crude *P. tomentosa* extract using column chromatography on silica gel, preparative TLC on silica gel, and reversed-phase preparative HPLC led to the isolation of several lipophilic prenylated flavanones [tomentodiplacones L and M (**1** and **2**), 3′,4′-O-dimethyl-5′-hydroxydiplacone (**3**), mimulones F and G (**4** and **5**), tomentodiplacone N (**6**), tomentone (**7**), 3′-O-methyldiplacone (**8**), tomentodiplacones G and B (**9** and **10**), 6-prenyl-3′-O-methyleriodyctiol (**11**), mimulone (**12**), 3′-O-methyl-5′-methoxydiplacone (**13**), diplacone (**14**), 3′-O-methyldiplacol (**15**), paulownione C (**17**), 3′-O-methyl-5′-hydroxydiplacone (**18**), bonanniol A (**19**), and tomentodiplacone O (**20**)] and one geranylchromone (**16**).[13] Two of the compounds (**17** and **20**) that were isolated showed HPLC retention times and spectroscopic data that did not correspond to those previously obtained for *Paulownia* flavonoids.[13] These compounds were determined to be new flavanone derivatives, each with a cyclized geranyl chain, as their UV spectra showed maximum values at 230 and 275 nm and shoulder maxima at 292 and 360 nm, respectively. Comparison with the UV spectra of compounds previously isolated from *P. tomentosa* (**7**, **10**)[13,15] suggested the presence of a pyranoflavanone structure. The IR spectra displayed absorption bands typical for prenylated flavonoids: ν_{max} at 3500−3200 cm^{-1} (hydrogen bonds of phenols), 3000−2850 cm^{-1} (alkanes), 1650−1600 cm^{-1} (ketones), 1600−1500 cm^{-1} (aromatics), and 1260−1100 cm^{-1} (ethers).[13,15]

Compound **17** was obtained as an amorphous, yellow substance. HRMS analysis in the positive mode revealed the presence of a [M + Na]$^+$ ion at m/z 489.1869, corresponding to the molecular formula $C_{27}H_{30}O_7$ (calcd for $C_{27}H_{30}O_7Na$, 489.1889). Its structure was determined by NMR spectroscopy using ^1H NMR, HSQC, HMBC, COSY, and NOESY experiments. The presence of the double bond of the pyran ring was confirmed by the observation of a pair of doublets at δ_H 6.65 and 5.53 (J = 10.1 Hz, 1H each), both correlating with a quaternary carbon at δ_C 80.3 (C-3″) bearing oxygen and a methyl group (δ_C 26.2). The suspected cyclized geranyl chain was then confirmed by comparing the NMR data with those of tomentodiplacol B and tomentone, previously isolated from *P. tomentosa*.[13] An AMX pattern comprising two broad doublets

equal methoxy groups at δ_H 3.89 were assigned to ring B, and using HMBC and NOESY correlations, a 3′,5′-dimethoxy-4′-hydroxyphenyl arrangement was deduced. Compound **17** was named paulownione C, and the structure was assigned as shown.

Compound **20** was isolated as an amorphous, yellow substance. Its molecular formula was determined by HRMS to be $C_{26}H_{28}O_6$ on the basis of the $[M + Na]^+$ ion at m/z 459.1766 (calcd for $C_{26}H_{28}O_6Na$, 459.1778). The NMR data were closely comparable with those of compound **17**, with the only difference being the presence of three signals in the aromatic region (instead of the one singlet in the proton spectrum of **17**) corresponding to a 3′,4′-disubstituted ring B. HMBC and NOESY experiments then helped in assigning a methoxy group to this ring, and a 3′-methoxy-4′-hydroxyphenyl unit, typical for *P. tomentosa* flavonoids, was defined. Compound **20** (tomentodiplacone O) was proposed structurally as shown.

The absolute configurations of compounds **17** and **20** were determined by analyzing their electronic circular dichroism (ECD) spectra. Like the previously isolated paulowniones A and B,[13] paulownione C (**17**) also displayed no discernible Cotton effects, and it was therefore considered to be a racemic mixture of 2*S* and 2*R* enantiomers. On the other hand, tomentodiplacone O (**20**) showed a positive Cotton effect for the n → π* electronic transition (320−340 nm) and a negative Cotton effect for the π → π* electronic transition (270−290 nm) and was thus assigned as a 2*S* flavanone.[13,15] The configuration of another chiral center at C-3″ is unclear, and due to the presence of two distinguishable signals of H-4″ in the ¹H NMR spectrum, it is likely that more than one stereoisomer is present simultaneously.

Inhibition of COX and LOX. Compounds **1−20** were studied for inhibition of COX-2 at a concentration of 20 μM in order to compare the effects of each of the compounds and to select the most promising active substance, using ibuprofen as a positive control. Compounds **3**, **12**, **14**, **18**, and **20** showed significant effects on COX-2 inhibition, comparable with the reference compound. Therefore, further analyses of **3**, **12**, **14**, **18**, and **20** were carried out to determine the IC$_{50}$ values against both COX-2 and COX-1 and also to assess the selectivity of each compound (Table 1). Compounds **3**, **12**, **14**, and **18** showed activity comparable to or better than the reference inhibitor, ibuprofen. However, only compound **20**

	R¹	R²	R³	R⁴	R⁵
2	H	OH	OCH₃	H	
3	OH	OCH₃	OCH₃	H	
4	H	OH	H	H	
5	H	OH	H	H	
8	H	OH	OCH₃	H	
9	H	OH	OCH₃	H	
10	H	OH	OCH₃	H	
11	H	OH	OCH₃	H	
12	H	OH	H	H	
13	OCH₃	OH	OCH₃	H	
14	H	OH	OH	H	
15	H	OH	OCH₃	OH	
18	OH	OH	OCH₃	H	
19	H	OH	H	OH	

at δ_H 5.38 (*J* = 12.4 Hz) and 2.76 (*J* = 17.0 Hz) and one doublet of doublets at δ_H 3.20 (*J* = 12.4, 17.0 Hz) was assigned to ring C of a flavanone. Two equal singlets at δ_H 6.80 and two

Table 1. IC$_{50}$ Values for COX-1, COX-2, and 5-LOX and the COX-1/COX-2 Selectivity Ratio

compound	COX-1 IC$_{50}$ [μM]	COX-2 IC$_{50}$ [μM]	5-LOX IC$_{50}$ [μM]	COX-1/COX-2 selectivity ratio
3	4.2 ± 1.5	6.4 ± 2.2	−[b]	0.66
7	−[b]	−[b]	0.35 ± 0.13	−[a]
12	3.7 ± 1.0	6.0 ± 1.9	−[b]	0.62
13	−[b]	−[b]	0.38 ± 0.19	−[a]
14	1.8 ± 0.9	4.2 ± 1.0	0.05 ± 0.019	0.43
16	−[b]	−[b]	2.46 ± 0.96	−[a]
17	−[b]	−[b]	0.37 ± 0.13	−[a]
18	3.3 ± 1.0	10.6 ± 4.9	0.06 ± 0.03	0.31
20	26.3 ± 6.4	9.5 ± 3.8	−[b]	2.8
ibuprofen	6.3 ± 2.3	4.2 ± 1.9	−[b]	1.5
zileuton	−[b]	−[b]	0.35 ± 0.22	−[a]

[a]Not counted. [b]Not determined.

showed greater selectivity against COX-2 than ibuprofen, which is a relatively nonselective COX inhibitor (see Table 1).

The same procedure was used to determine the 5-LOX inhibitory activity. Compounds were tested at a concentration of 20 μM to screen the active derivatives. After this, active compounds **7**, **13**, **14**, **16**, **17**, and **18** were selected for the determination of IC$_{50}$ values (Table 1). Flavanones **14** and **18** showed activities almost 10 times greater than zileuton, which was used as a standard.

The number of papers evaluating the COX/LOX inhibitory activity of prenylated flavonoids is rather limited, with only two papers describing the effect of such compounds systematically for a series of prenylated and geranylated flavanones, flavones, and isoflavanones, tested to determine their ability to inhibit COX-1, COX-2, 5-LOX, and 12-LOX.[16,17] Several prenylated flavanones were tested,[16] revealing the importance of the C-6 prenylation of the flavanone skeleton for in vitro inhibitory potential against both COX-1 and COX-2. A C-4′ methoxy group at this position diminished the activity in comparison to the presence of a hydroxy group.[16] Considering the flavanones that correspond structurally to the compounds tested herein, sophoraflavanone G and kurarinone were found to inhibit the production of PGE$_2$ by direct inhibition of COX-1.[17] Sophoraflavanone G, with a C-8 lavandulyl substituent, also inhibited 5-LOX.[17] Interestingly, sophoraflavanone D (with an unmodified C-6 geranyl chain), the compound most closely related to the compounds tested in this study, did not show discernible anti 5-LOX activity. Further, this prior study revealed that the prenylated compounds tested were relatively weak COX-2 inhibitors.[17] Later, it was shown that sophoraflavanone also inhibited the production of PGE$_2$ in RAW 264.7 cells, although this was caused by the inhibition of COX-2 expression.[18,19] Similarly, artocarpesin [a prenylated flavone obtained from the fruits of *Artocarpus heterophyllus* (Moraceae)] suppressed the production of PGE$_2$ through the down-regulation of COX-2 expression in LPS-stimulated RAW 264.7 cells.[20] Our previous study pointed to the potential of diplacone (**14**) to down-regulate COX-2 expression.[21] Some compounds, such as glabridin (a prenylated flavan obtained from *Glycyrrhiza glabra*), are considered to be dual LOX/COX inhibitors, and some dual activity against 5-LOX and microsomal prostaglandin synthase 1 (mPGES-1) was also observed for 8-prenylnaringenin isolated from hops.[22,23]

From these data, it is clear that previous studies dealing with a structural variety of compounds and using a different methodology of biological testing do not permit generally valid conclusions to be made about the structure−activity relationships (SAR) of anti-inflammatory prenylated flavanones. The present study, therefore, had a primary aim in evaluating a relatively large set of structurally related prenylated flavanones in order to evaluate the crucial SAR characteristics of COX/LOX inhibition. Comparison of the experimental results obtained with the literature enabled the conclusion that prenylation is an important factor for the COX inhibitory activity of flavanones. Further, no tested flavanone with a geranyl side chain modified by oxidation or the formation of a five-membered furan ring showed a significant COX inhibitory effect, and an unmodified side chain is therefore crucial for such activity. The length of the chain also plays a role, as compound **11** showed a much lower effect in comparison with compounds with a geranyl moiety and the same oxidative modification of ring B. The activity is related to the presence of ring B itself, as shown by the low activity of

5,7-dihydroxy-6-geranylchromone (**16**). The substitution of ring B also affects the activity, and, in fact, diplacone (**14**, 3′,4′-dihydroxy), mimulone (**12**, 4′-hydroxy), 3′-*O*-methyl-5′-hydroxydiplacone (**18**, 3′-*O*-methyl, 4′,5′-dihydroxy), and 3′,4′-*O*-dimethyl-5′-hydroxydiplacone (**3**, 3′,4′-*O*-dimethyl, 5′-hydroxy) showed greater effects than 3′-*O*-methyl-5′-methoxy-diplacone (**13**), with a 3′-methoxy, 5′-methoxy, and 4′-hydroxy substitution. The 3-hydroxy group also influences the activity, as was exemplified clearly by the active mimulone (**12**) and the nonactive bonanniol A (**19**). Further, when the activities of compounds **6** and **20** are compared, it can be deduced that the addition of a molecule of water to the double bond of the pyran ring formed from the geranyl group diminishes the resultant activity. To confirm this observation, docking studies were performed on the COX isoforms.

Complex information about the LOX inhibitory effect of prenylated flavanones is also missing. Some information about the LOX inhibitory properties of flavonoids was presented in a review of Mladěnka et al.[24] Synthetic alkoxy flavonoids generally do not inhibit 5-LOX.[25] A large study by Vasquez-Martinez et al.[26] revealed some connections between the structure and activity of a large series of flavonoids, but did not distinguish flavanones or prenylated flavanones. Little or no activity was observed for simple nonprenylated flavanones (naringenin, hesperetin) against 15-LOX in a large study comparing different classes of flavonoids.[27−29] Some individual studies describe the LOX inhibitory effects of different types of prenylated flavonoids, but the results are difficult to compare due to the different LOX enzymes tested and the small number of compounds in the sampling sets. As an example, carpachromene together with prenylated pterocarpans weakly inhibits 15-LOX.[30] Several prenylated flavonoids and chalcones were tested against 15-LOX, but none of the structures matched the present set of samples.[31] Chi et al.[17] tested only compounds similar to *Paulownia* flavanones, but kurarinone and sophoraflavanone D showed no ability to inhibit 5-LOX. However, sophoraflavanone G, with a geranyl side chain at position C-8, displayed inhibitory data comparable to those of quercetin and nordihydroguaiaretic acid (NDGA) used as standards.

The present study is the first to compare a large set of prenylated flavanones and a set of geranylated flavanones to show their ability to inhibit 5-LOX in relative equivalence to their COX inhibitory potential. In particular, compounds **14** and **18** showed high activity; both have two hydroxy groups on ring B and an unmodified geranyl side chain.

Molecular Docking Studies. A docking study was performed exclusively on the COX enzymes because the compounds did not dock into the substrate-binding site of AA in 5-LOX. The available crystal structure of 5-LOX (3o8y) was crystallized in a closed conformation, allowing the solvent no access to the substrate channel.[32] It is possible to dock smaller compounds into a closed enzyme conformation, but larger compounds, such as those analyzed in this study, presumably require an open conformation structure to accurately simulate their binding.

The docking simulation on COX-1 and COX-2 did, however, help elucidate a structure−activity relationship for the compounds analyzed. The anti-COX-active compounds **3**, **12**, **14**, and **18** are all *C*-geranylated flavanones and displayed very clear activity patterns (Figure 1). This pose shows the crucial binding pattern for the active *C*-geranylated flavanones. A hydrogen bond partner for Tyr355 is needed, and the area
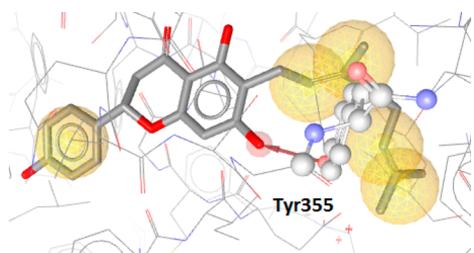
Figure 1. Compound **12** shown within the binding pocket of COX-1. A hydrogen bond acceptor feature (red arrow) connects at Tyr355, while the geranyl moiety fills the hydrophobic pocket.

at the opening of the binding site must be hydrophobic. In the case of compound **18** an additional hydrogen bond with Tyr385 is formed on the other side of the pocket (Figure 2).
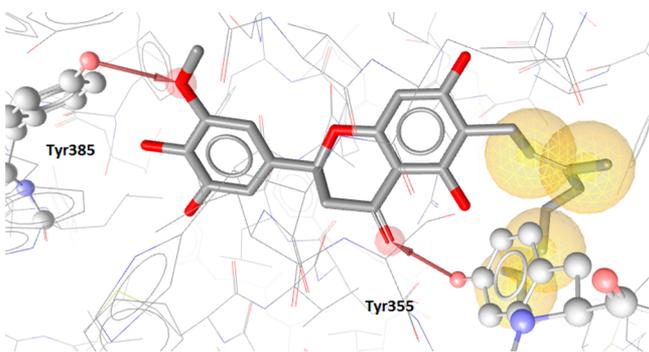
Figure 2. Compound **18** shown within the binding pocket of COX-1. An additional hydrogen bond is formed between the methoxy group and Tyr385 (red arrow).

A similar pattern was observed for COX-2. The core is sometimes flipped so that the keto group interacts with Tyr 355 (Figure 2). Both of these constellations occur in both subtypes.

There were other inactive geranylated flavanones in the data set. During the analysis of the docking poses, it became obvious that compounds that contain a hydroxy group within the polar alkyne moiety (such as **2**, **4**, **5**, **9**, and **10**) lose their activity. This corresponds to the hydrophobic nature of the binding pocket in this area.

Flavanone **20** was the only structure from a different scaffold that was active. Although closely related structurally to flavanones **7** and **17**, it is the only active compound with a condensed system of three rings. Only one of the *meta*-positions of the phenyl moiety can be substituted; otherwise the system would then get too large. In the docking simulation, only compound **20** could assume a pose that allowed the hydrophobic alkyne remainder to point toward the exit of the binding pocket (Figure 3).

Proteomic Analysis of Diplacone (14). A definitive effect on gene expression has been reported for diplacone (**14**),[11,21] and this compound has shown antiphlogistic activity in vivo.[12] As shown earlier, in assays of the COX/LOX inhibition and in further docking analysis, **14** also showed a significant inhibitory effect on the COX and LOX enzymes. This information provided the motivation for the analysis of proteomic changes in THP-1 LPS-stimulated macrophages in the presence of diplacone (**14**) at a nontoxic concentration.

Only proteins for which the expression was present in all experimental groups and was 1.5 times higher or lower after LPS stimulation were assigned for analysis (Figure 4
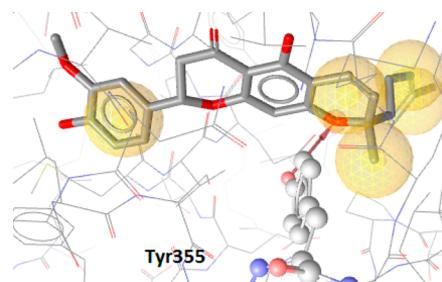
Figure 3. Compound **20** shown within the binding pocket of COX-2. The oxygen of the third pyran ring acts as a hydrogen bond acceptor for Tyr355 (red arrow).

and Figure S1, Supporting Information). Table S1 shows all of the proteins detected in the LC-MS/MS analysis. Not surprisingly, it was mainly several cytokines (e.g., TNF-α, IL-1) and chemokines (CCL1, CCL5, CXCL1), which are under the transcription control of NF-κB, that were up-regulated after LPS stimulation. Only a few proteins were down-regulated by LPS, e.g., inositol hexakisphosphate and diphosphoinositol-pentakisphosphate kinase 2 (VIP2). Diplacone (**14**) reversed the effect of LPS; that is, it attenuated the expression of up-regulated proteins and enhanced the production of down-regulated ones. These findings correlated with previous observations, where diplacone reduced the expression of pro-inflammatory TNF-α or MCP-1 and augmented the production of anti-inflammatory ZFP36 in the LPS-stimulated macrophage-like cell line THP-1 in vitro.[11,19] Interestingly, although diplacone (**14**) possesses significant anti-COX-2 activity, it slightly increased the expression of this enzyme, by a factor of 1.55. This could be caused by the dysregulation of negative feedback, which has been described for some nonsteroidal anti-inflammatory drugs (NSAIDs).[33,34] However, in contrast to previous studies, diplacone (**14**) did not change the COX-2 level in nonstimulated cells (Table S3 Supporting Information). This effect is dependent on the cell type; for this reason it was not observed in the murine macrophage cell line J774A.1, where diplacone (**14**) nonsignificantly diminished the COX-2 level.[35] However, the inhibition of COX-2 expression was noted for an in vivo model of inflammatory bowel disease.[14] The most overexpressed protein in the diplacone-treated LPS-stimulated cells was tubulin α 1A (TUBA1A). The reason for this effect remains unclear. In comparison with prednisone, diplacone (**14**) showed lower activity against LPS stimulation, but the mode of action of diplacone (**14**) was found to be more pleiotropic and complex than that of prednisone. For example, compound **14** is also able to inhibit COX-2 activity.

As detected, diplacone (**14**) did not influence the expression of proteins in nonstimulated cells (Table S3, Supporting Information). The only exceptions were the moderate up-regulation of RING finger protein 207 (RNF207) by a factor of 2.093 and the down-regulation of Tax1-binding protein 3 (TX1B3) by a factor of 1.876.

In summary, a series of geranylated flavanones was isolated from *P. tomentosa* fruits and studied for their anti-inflammatory activity and possible mechanism of action. Two new compounds, paulownione C (**17**) and tomentodiplacone O (**20**), were characterized structurally. All of the isolated derivatives (**1**−**20**) were assayed for their ability to inhibit cyclooxygenases (COX-1 and COX-2) and 5-lipoxygenase (5-LOX). The compounds tested showed variable degrees of activity; however, only tomentodiplacone O (**20**) showed more selectivity against COX-2
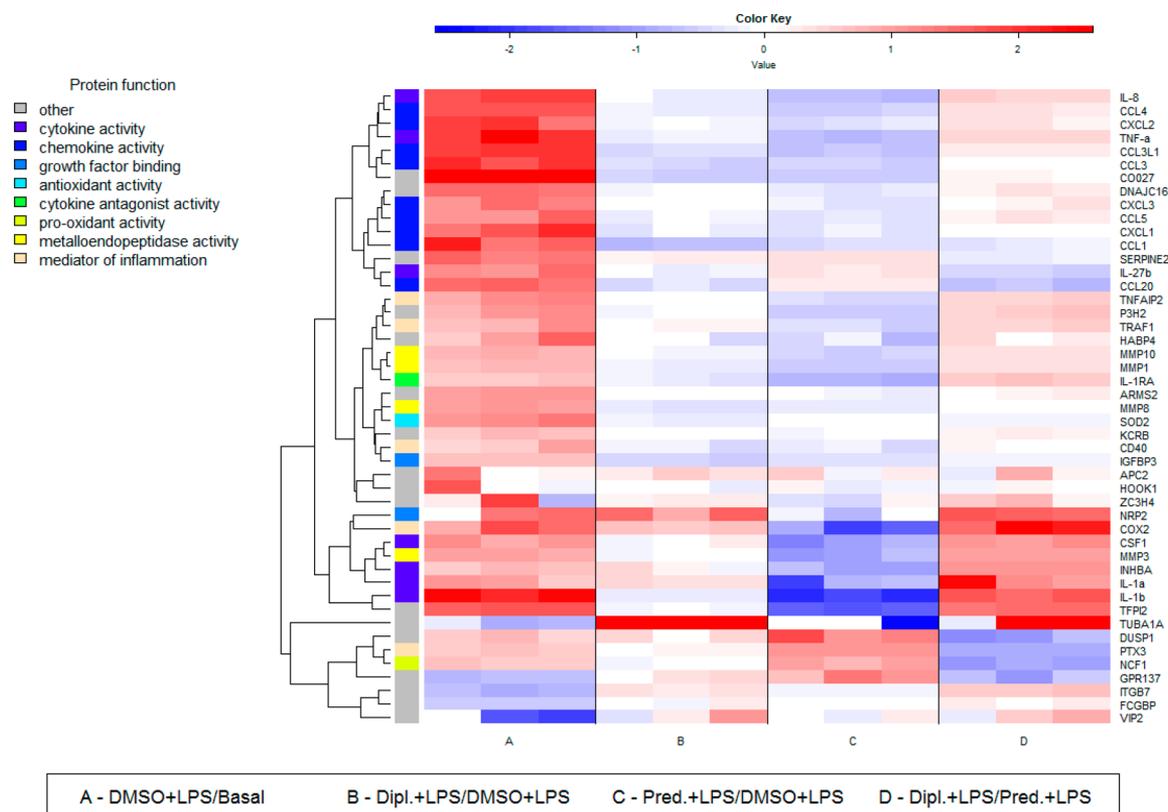
Figure 4. Changes in protein expression in the LPS-stimulated THP-1 cell line. The heat map shows ratios of protein expression between differently treated cells. (A) DMSO + LPS vs untreated (basal); (B) diplacone (**14**) 2 μM + LPS vs DMSO + LPS; (C) prednisone 2 μM + LPS vs DMSO + LPS; (D) diplacone (**14**) 2 μM + LPS vs prednisone 2 μM + LPS. Only proteins for which the expression was changed by a factor of more than 1.5 after LPS stimulation in (A) were evaluated. Each column in each group represents an independent LC-MS/MS analysis.

versus COX-1 when compared with ibuprofen. The ability of the test compounds to interact with the above-mentioned enzymes was supported by molecular docking studies, which revealed the possible incorporation of the test substances selected into the active sites of these enzymes. Furthermore, one of the COX/LOX dual inhibitors, diplacone (**14**) (a major geranylated flavanone of *P. tomentosa*), was studied in vitro to obtain a proteomic overview of its effect on inflammation in LPS-treated THP-1 macrophages, supporting its previously observed anti-inflammatory activity and revealing the mechanism of its anti-inflammatory effect. The results of the present study confirmed the anti-inflammatory potential of *P. tomentosa* flavonoids. However, further in vivo studies need to be carried out to fully elucidate the antiphlogistic potential of these geranylated flavanones.

■ EXPERIMENTAL SECTION

General Experimental Procedures. UV and circular dichroism (CD) spectra were obtained using a JASCO J-815 polarimeter (JASCO, Easton, MD, USA). IR spectra were measured with a Nicolet Impact 400D FT-IR instrument (Thermo Scientific, Waltham, MA, USA) using the ATR technique. 1D and 2D NMR spectra were obtained on a Bruker Avance III 400 spectrometer (Bruker, Billerica, MA, USA) with tetramethylsilane as the internal standard. HRESIMS data were recorded using an Orbitrap spectrometer (Thermo Scientific) in the positive-ion mode. Analytical HPLC measurements were obtained on an Agilent 1100 chromatographic system with a 1100 Series diode array detector (Agilent Technologies, Santa Clara, CA, USA). Preparative RP-HPLC was performed with a YL 9100 HPLC System (Young Lin, The Republic of Korea) with a FOXY R2 fraction collector (Teledyne Isco, Lincoln, NE, USA).

The exact masses were measured using an LTQ Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ion source. The mobile phase consisted of methanol–water (4:1), flow rate, 30 μL/min. The sample was dissolved in methanol, diluted with the mobile phase, and injected using a 2 μL loop. The mass spectra of the positively charged ions were calibrated internally using protonated or sodiated diisooctyl phthalate as the lock mass.

Compounds were separated using column chromatography with silica gel 40–63 μm (Merck, Billerica, MA, USA) and preparative RP-HPLC using an Ascentis RP-Amide, 25 cm × 10 mm, particle size, 5 μm, semipreparative column (Sigma-Aldrich, St. Louis, MO, USA). For analytical purposes, silica gel 60 F254, 20 × 20 cm, 200 μm TLC plates (Merck) and an Ascentis Express RP-Amide, 10 cm × 2.1 mm, particle size, 2.7 μm, analytical HPLC column (Sigma-Aldrich) were used.

Plant Material. *Paulownia tomentosa* fruits were collected during October and November of 2010 (as a mixture of immature and mature fruits) on the grounds of the University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic. A voucher specimen was deposited in the herbarium of the Department of Natural Drugs, UVPS Brno (no. PT102010).

Extraction and Isolation. The plant extraction and the isolation procedures of compounds **1–16**, **18**, and **19** have been described in previous work.[13] The separation of fraction PT4-12 using column chromatography yielded subfraction PT4-12-C, which was subjected to semipreparative RP-HPLC (with a gradient of redistilled acetonitrile and 0.2% HCOOH) to isolate compound **17** (126 mg). An analogous separation using semipreparative RP-HPLC was used for subfraction PT4-3 to yield compound **20** (7 mg). The purity of the isolated compounds was evaluated using HPLC-DAD analysis and exceeded 95% in all cases.

Paulownione C (17): amorphous, yellow substance; UV (CH$_3$OH) λ_{max} (log ε) 275 (4.38), 295 (4.07), 356 (3.86) nm; IR (ATR) ν_{max} 3273, 2921, 2351, 1611, 1519, 1451, 1337, 1293, 1214, 1151, 1109, 827 cm^{-1}; ^{1}H NMR (CD$_3$OD, 400 MHz) δ_H 6.80 (2H, s, H-2′, H-6′), 6.65 (1H, d, J = 10.1 Hz, H-1″), 5.94 (1H, s, H-8), 5.53 (1H, d, J = 10.1 Hz, H-2″), 5.38 (1H, br d, J = 12.4 Hz, H-2), 5.11 (1H, t, J = 7.0 Hz, H-7″), 3.89 (6H, s, MeO-3′, MeO-5′), 3.20 (1H, dd, J = 12.4, 17.0 Hz, H-3β), 2.76 (1H, br d, J = 17.0 Hz, H-3α), 2.09 (2H, m, H-6″), 1.72 (2H, m, H-5″), 1.67 (3H, s, H-9″), 1.58 (3H, s, H-10″), 1.413 and 1.406 (1H, s, H-4″); ^{13}C NMR (CD$_3$OD, 400 MHz) δ_C 196.6 (C-4), 162.6 (C-9), 161.7 (C-7), 158.7 (C-5), 147.7 (C-3′, C-5′), 135.5 (C-4′), 131.2 (C-8″), 129.3 (C-1′), 124.6 (C-2″), 123.5 (C-7″), 115.3 (C-1″), 103.6 (C-2′, C-6′), 102.2 (C-6), 102.1 (C-10), 95.0 (C-8), 80.3 (C-3″), 79.6 (C-2), 55.2 (MeO-3′, MeO-5′), 42.8 (C-3), 41.4 (C-5″), 26.2 (C-4″), 24.4 (C-9″), 22.1 (C-6″), 16.2 (C-10″); HRESIMS (positive) m/z 489.1869 [M + Na]$^+$ (calcd for C$_{27}$H$_{30}$O$_7$Na$^+$, 489.1889).

Tomentodiplacone O (20): amorphous, yellow substance; UV (CH$_3$OH) λ_{max} (log ε) 230 (4.12), 270 (4.42), 300 (sh) (4.01), 360 (3.54) nm; IR (ATR) ν_{max} 3731, 3358, 2970, 1589, 1519, 1445, 1350, 1270, 1153 cm^{-1}; ^{1}H NMR (CD$_3$OD, 400 MHz) δ_H 7.09 (1H, br s, H-2′), 6.94 (1H, br d, J = 8.0 Hz, H-6′), 6.84 (1H, d, J = 8.0 Hz, H-5′), 6.65 (1H, d, J = 10.0 Hz, H-1″), 5.92 (1H, s, H-8), 5.53 (1H, d, J = 10.0 Hz, H-2″), 5.39 (1H, br d, J = 12.5 Hz, H-2), 5.12 (1H, t, J = 7.4 Hz, H-7″), 3.90 (3H, s, MeO-3′), 3.19 (1H, dd, J = 12.5, 17.0 Hz, H-3β), 2.76 (1H, br d, J = 17.0 Hz, H-3α), 2.10 (2H, q, J = 7.4 Hz, H-6″), 1.75 (2H, m, H-5″), 1.66 (3H, s, H-9″), 1.58 (3H, s, H-10″), 1.41 and 1.40 (1H, s, H-4″); ^{13}C NMR (CD$_3$OD, 400 MHz) δ_C 195.9 (C-4), 162.3 (C-9), 161.8 (C-7), 158.5 (C-5), 147.7 (C-3′), 146.5 (C-4′), 130.8 (C-8″), 129.9 (C-1′), 124.8 (C-2″), 123.6 (C-7″), 119.1 (C-6′), 115.1 (C-1″), 114.6 (C-5′), 109.8 (C-2′), 102.2 (C-6), 101.8 (C-10), 95.2 (C-8), 80.5 (C-3″), 79.3 (C-2), 55.0 (MeO-3′), 42.7 (C-3), 41.4 (C-5″), 26.2 (C-4″), 24.3 (C-9″), 22.2 (C-6″), 16.1 (C-10″); HRESIMS (positive) m/z 459.1766 [M + Na]$^+$ (calcd for C$_{26}$H$_{28}$O$_6$Na$^+$, 459.1778).

Cell Cultivation and Stimulation. The THP-1 human monocytic leukemia cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 medium containing stabilized 2 mM L-glutamine (Biosera, France) supplemented with antibiotics [100 U/mL penicillin and 100 mg/mL streptomycin (Biosera)] and 10% fetal bovine serum (HyClone, UT, USA). Cells were kept in an incubator at 37 °C in a water-saturated atmosphere of air containing 5% CO$_2$.

To differentiate the THP-1 monocytes to macrophages, cells were split into six-well microtiter plates at a concentration of 500 000 cells/mL (3 mL of cell suspension per well) and stimulated by phorbol myristate acetate, as described previously.[13] Before adding the test compounds, the differentiated macrophages were cultivated for 24 h in 2 mL of serum-free medium. After this period, cells were pretreated with diplacone (**14**) or prednisone dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 2 μM (this concentration lacks any cytotoxic effect[21]) or with DMSO alone for 1 h. Then, the inflammatory-like reaction was triggered by adding 1.0 μg/mL of LPS isolated from *Escherichia coli* 0111:B4 (Sigma-Aldrich). Twenty-four hours after LPS stimulation, the cell samples were collected and processed for proteome analysis. All experimental groups were prepared as pentaplicates.

COX-1 and COX-2 Assays. Enzymatic in vitro assays using COX-1 from ram seminal vesicles or human recombinant COX-2 (both Sigma-Aldrich) were performed to test the inhibitory activities of the test compounds. COX-1 (1 unit/reaction) or COX-2 (0.5 unit/reaction) was added to 180 μL of the incubation mixture consisting of 100 mM Tris buffer (pH 8.0), 5 μM porcine hematin, 18 mM L-epinephrine, and 50 μM Na$_2$EDTA. The test substances were then dissolved in DMSO and added (10 μL), with the mixture incubated for 5 min at room temperature. Pure DMSO was used as a blank and (S)-(+)-ibuprofen (Sigma-Aldrich) as a reference inhibitor. Then, 5 μL of 10 μM arachidonic acid was added, and the reaction was incubated for 20 min at 37 °C. The reaction was terminated by adding 10 μL of 10% (v/v) formic acid. Prostaglandin E$_2$ (PGE$_2$), the main product of

the reaction, was quantified using a Prostaglandin E$_2$ ELISA kit (Enzo Life Sciences, NY, USA). Samples were diluted 1:15 in assay buffer (provided in the kit), and then the incubation was performed according to the manufacturer's instructions. The absorbance at 405 nm was measured using a Tecan Infinite M200 microplate reader (Tecan Group, Switzerland). The inhibitory activity was expressed as the percent inhibition compared to the blank. Experiments were repeated at least three times with two technical replicates for screening as well as to determine the IC$_{50}$ values.

5-LOX Assay. An enzymatic in vitro assay using human recombinant 5-LOX (Cayman Chemical, MI, USA) was performed. 5-LOX (1 unit/reaction) was added to 180 μL of the incubation mixture consisting of phosphate buffer saline (pH 7.4), 1 mM Na$_2$EDTA, and 200 μM ATP. After the addition of the test substances (10 μL) dissolved in DMSO, the mixture was incubated for 10 min at 4 °C. Pure DMSO was used as a blank and zileuton (Farmak a.s., Czech Republic) as reference inhibitor. Then, 5 μL of 80 mM CaCl$_2$ and 5 μL of 800 μM arachidonic acid were added, and the mixture was incubated for 10 min at 37 °C. The reaction was terminated by adding 10 μL of 10% (v/v) formic acid. Leukotriene B$_4$ (LTB$_4$), the main product of reaction, was quantified using a Leukotriene B$_4$ ELISA kit (Enzo Life Sciences). Samples were diluted 1:15 in assay buffer (provided in the kit), and the incubation was then performed according to the manufacturer's instructions. The measurement of the absorbance and the determination of the inhibitory activity were the same as for the COX assays.

Docking. All 20 compounds (**1−20**) were docked into the binding sites of COX-1 (using the pdb entry 1EQH cocrystallized with flurbiprofen) and COX-2 (using the pdb entry 3pgh, also cocrystallized with flurbiprofen). The docking was performed in GOLD 5.2 running on a Windows 7 machine. The binding site was defined by the location of the cocrystallized ligand in a radius of 6 Å. All water molecules were deleted from the binding site. CHEMPLP was selected as a scoring function to evaluate the quality of the individual poses.

Preparation of Proteins for Proteomic Analysis. Protein samples for proteome analysis were prepared as described previously.[36] Briefly, after LPS stimulation, the cell medium was aspirated, lyophilized (1 mbar, −50 °C), and redissolved in 0.2 mL of TRI Reagent (Sigma-Aldrich). When attached on plates, the cells were treated directly with 0.3 mL of TRI Reagent. Each cell lysate was mixed with its redissolved medium, and the total proteins were isolated from this mixture of secreted and intracellular proteins. Dissolved proteins from the organic phase of the TRI Reagent were precipitated with acetone. The protein pellets were washed three times with 0.3 M guanidine hydrochloride (Sigma-Aldrich) in 95% ethanol and 2.5% glycerol. The dried protein pellets were dissolved in 250 μL of 8 M urea (Sigma-Aldrich) and processed using a filter-aided sample preparation method that employed a Vivacon 500 MWCO 10 kDa filter (Sartorius Stedim Biotech, Germany). The dissolved proteins were then washed twice with 100 μL of 8 M urea and reduced using 100 μL of 10 mM dithiotreitol (Sigma-Aldrich). After reduction, the proteins were incubated with 100 μL of 50 mM iodoacetamide (Sigma-Aldrich) dissolved in 25 mM triethylammonium bicarbonate buffer (TEAB; Sigma-Aldrich) and washed twice with 100 μL of 25 mM TEAB. Trypsin (Promega, WI, USA) was used at 1:50 ratio (w/w), and the digestion proceeded for 16 h at 30 °C.

The peptide concentration for each sample was determined as the absorbance at 280 nm, using a NanoDrop 2000c UV−vis spectrophotometer (Thermo Scientific), and biological replicates were then pooled to obtain a single representative sample for each group. Samples were then labeled using iTRAQ 4-Plex isobaric tags (Applied Biosystems, CA, USA) according to the manufacturer's recommendations. The labeled samples were combined, and three fractions were prepared using Oasis MCX extraction cartridges (Waters, MA, USA), desalted on Empore SPE C18 extraction cartridges (Sigma-Aldrich), and concentrated in a SpeedVac (Thermo Scientific).

Mass Spectrometry and Data Analysis. LC-MS/MS analyses of each fraction were performed on an UltiMate 3000 RSLCnano system (Dionex, MA, USA) connected to an Orbitrap Velos Pro mass spectrometer (Thermo Scientific). Chromatographic separation was

performed on an EASY-Spray C_{18} separation column (50 cm × 75 μm, 3 μm particles) using a 4 h gradient. The mass spectrometer was operated in data-dependent manner, using the top 10 precursors for isolation and HCD fragmentation at a normalized collision energy of 40 V. Each sample fraction was analyzed three times.

Raw LS-MS/MS data were analyzed using Proteome Discoverer v.1.4 (Thermo Scientific). MS/MS spectra were identified by the SEQUEST algorithm, using *Homo sapiens* Swiss-Prot sequences as a database. The mass tolerances for the searches were 10 ppm and 0.1 Da for the precursor and fragment, respectively.[36] Only peptides with FDR ≤ 0.05 were considered for analysis.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b01011.

Table S1 and figures illustrating the NMR spectra (PDF)
Table S2 (XLSX)
Table S3 (XLSX)
Figures S1−S10 (DOCX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Havsteen, B. H. *Pharmacol. Ther.* **2002**, *96*, 67−202.
(2) Treml, J.; Šmejkal, K. *Compr. Rev. Food Sci. Food Saf.* **2016**, *15*, 720−738.
(3) Hošek, J.; Šmejkal, K. In *Compendium of Inflammatory Diseases*; Parnham, M. J., Ed.; Birhauser Basel: Switzerland, 2016; pp 1−17.
(4) Kim, P. K.; Son, K. H.; Chang, H. W.; Kang, S. S. *J. Pharmacol. Sci.* **2004**, *96*, 229−245.
(5) García-Lafuente, A.; Guillamón, E.; Villares, A.; Rostagno, M. A.; Martínez, J. A. *Inflammation Res.* **2009**, *58*, 537−552.
(6) Costa, G.; Francisco, V.; Lopes, M. C.; Cruz, M. T.; Batista, M. T. *Curr. Med. Chem.* **2012**, *19*, 2876−2900.
(7) Schneiderová, K.; Šmejkal, K. *Phytochem. Rev.* **2015**, *14*, 799−833.
(8) Cho, J. K.; Curtis-Long, M. J.; Lee, K. H.; Kim, D. W.; Ryu, H. W.; Yuk, H. J.; Park, K. H. *Bioorg. Med. Chem.* **2013**, *21*, 3051−3057.
(9) Lee, Y.; Ryu, Y. B.; Youn, H.-S.; Cho, J. K.; Kim, Y. M.; Park, J.-Y.; Lee, W. S.; Park, K. H.; Eom, S. H. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2014**, *D70*, 1357−1365.
(10) Šmejkal, K.; Svačinová, J.; Šlapetová, T.; Schneiderová, K.; Dall'Acqua, S.; Innocenti, G.; Závalová, V.; Kollár, P.; Chudík, S.; Marek, R.; Julínek, O.; Urbanová, M.; Kartal, M.; Csöllei, M.; Doležal, K. *J. Nat. Prod.* **2010**, *73*, 568−572.
(11) Šmejkal, K.; Grycová, L.; Marek, R.; Lemiére, F.; Jankovská, D.; Forejtníková, H.; Vančo, J.; Suchý, V. *J. Nat. Prod.* **2007**, *70*, 1244−1248.
(12) Šmejkal, K.; Chudík, S.; Klouček, P.; Marek, R.; Cvačka, J.; Urbanová, M.; Julínek, O.; Kokoška, L.; Šlapetová, T.; Holubová, P.; Zima, A.; Dvorská, M. *J. Nat. Prod.* **2008**, *71*, 706−709.
(13) Hanáková, Z.; Hošek, J.; Babula, P.; Dall'Acqua, S.; Václavík, J.; Šmejkal, K. *J. Nat. Prod.* **2015**, *78*, 850−863.
(14) Vochyánová, Z.; Bartošová, L.; Bujdáková, V.; Fictum, P.; Husník, R.; Suchý, P.; Šmejkal, K.; Hošek, J. *Fitoterapia* **2015**, *101*, 201−207.
(15) Navrátilová, A.; Schneiderová, K.; Veselá, D.; Hanáková, Z.; Fontana, A.; Dall'Acqua, S.; Cvačka, J.; Innocenti, G.; Novotná, J.; Urbanová, M.; Pelletier, J.; Čížek, A.; Žemličková, H.; Šmejkal, K. *Phytochemistry* **2013**, *89*, 104−113.
(16) Jang, D. S.; Cuendet, M.; Hawthorne, M. E.; Kardono, L. B. S.; Kawanishi, K.; Fong, H. H. S.; Mehta, R. G.; Pezzuto, J. M.; Kinghorn, A. D. *Phytochemistry* **2002**, *61*, 867−872.
(17) Chi, Y. S.; Jong, H. G.; Son, K. H.; Chang, H. W.; Kang, S. S.; Kim, H. P. *Biochem. Pharmacol.* **2001**, *62*, 1185−1191.
(18) Kim, D. W.; Chi, Y. S.; Son, K. H.; Chang, H. W.; Kim, J. S.; Kang, S. S.; Kim, H. P. *Arch. Pharmacal Res.* **2002**, *25*, 329−335.
(19) Wun, Z. Y.; Lin, C. F.; Huang, W. C.; Xu, P. Y.; Chang, W. T.; Wu, S. J.; Liou, C. J. *Food Chem. Toxicol.* **2013**, *62*, 255−261.
(20) Fang, S. C.; Hsu, C. L.; Yen, G. C. *J. Agric. Food Chem.* **2008**, *56*, 4463−4468.
(21) Hošek, J.; Závalová, V.; Šmejkal, K.; Bartoš, M. *Folia Biol.* **2010**, *56*, 124−130.
(22) Chandrasekaran, C. V.; Deepak, H. B.; Thiyagarajan, P.; Kathiresan, S.; Sangli, G. K.; Deepak, M.; Agarwal, A. *Phytomedicine* **2011**, *18*, 278−84.
(23) Forino, M.; Pace, S.; Chianese, G.; Santagostini, L.; Werner, M.; Weinigel, C.; Rummler, S.; Fico, G.; Werz, O.; Taglialatela-Scafati, O. *J. Nat. Prod.* **2016**, *79*, 590−597.
(24) Mladěnka, P.; Zatloukalová, L.; Filipský, T.; Hrdina, R. *Free Radical Biol. Med.* **2010**, *49*, 963−975.
(25) Sobottka, A. M.; Werner, W.; Blaschke, G.; Kiefer, W.; Nowe, U.; Dannhardt, G.; Schapoval, E. E.; Schenkel, E. P.; Scriba, G. K. *Arch. Pharm.* **2000**, *333*, 205−210.
(26) Vasquez-Martinez, Y.; Ohri, R. V.; Kenyon, V.; Holman, T. R.; Sepulveda-Boza, S. *Bioorg. Med. Chem.* **2007**, *15*, 7408−7425.
(27) Lee, J.-H.; Kim, G.-H. *J. Food Sci.* **2010**, *78*, H212−217.
(28) Sadik, C. D.; Sies, H.; Schewe, T. *Biochem. Pharmacol.* **2003**, *65*, 773−781.
(29) Ribeiro, D.; Freitas, M.; Tomé, S. M.; Silva, A. M.; Porto, G.; Cabrita, E. J.; Marques, M. M.; Fernandes, E. I. *Eur. J. Med. Chem.* **2014**, *72*, 137−145.
(30) Togola, A.; Hedding, B.; Theis, A.; Wangensteen, H.; Rise, F.; Smestad Paulsen, B.; Diallo, D.; Egil Malterud, K. *Planta Med.* **2009**, *75*, 1168−1170.
(31) Dzoyem, J. P.; Nkuete, A. H.; Ngameni, B.; Eloff, J. N. *Arch. Pharmacal Res.* **2015**, 1−6.
(32) Gilbert, N. C.; Bartlett, S. G.; Waight, M. T.; Neau, D. B.; Boeglin, W. E.; Brash, A. R.; Newcomer, M. E. *Science* **2011**, *331*, 217−219.
(33) Tanaka, Y.; Takizawa, M.; Igimi, S.; Amano, F. *Biol. Pharm. Bull.* **2004**, *27*, 985−991.
(34) Pang, L. H.; Nie, M.; Corbett, L.; Knox, A. J. *J. Immunol.* **2003**, *170*, 1043−1051.
(35) Hosek, J.; Toniolo, A.; Neuwirth, O.; Bolego, C. *J. Nat. Prod.* **2013**, *76*, 1586−1591.
(36) Wang, L.; Ladurner, A.; Latkolik, S.; Schwaiger, S.; Linder, T.; Hošek, J.; Palme, V.; Schilcher, N.; Polanský, O.; Heiss, E. H.; Stangl, H.; Mihovilovic, M. D.; Stuppner, H.; Dirsch, V. M.; Atanasov, A. G. *J. Nat. Prod.* **2016**, *79*, 1651−1657.

Prenylated flavonoid morusin protects against TNBS-induced colitis in rats

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Abstract

Morusin is a prenylated flavonoid isolated from the root bark of *Morus alba*. Many studies have shown the ability of flavonoids to act as anti-inflammatory agents. The aim of this study was to evaluate the effect of morusin on experimentally colitis induced by 2,4,6-trinitrobenzensulfonic acid in Wistar rats and to compare it with sulfasalazine, a drug conventionally used in the treatment of inflammatory bowel disease. Morusin was administered by gavage at doses of 12.5, 25, or 50 mg/kg/day for five days. The colonic tissue was evaluated macroscopically, histologically, and by performing immunodetection and zymographic analysis to determine the levels of antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)], interleukin (IL)-1β, and transforming growth factor (TGF)-β1 and the activities of matrix metalloproteinases (MMP) 2 and 9. The tissue damage scores were significantly reduced with increasing dose of morusin, however efficacy was not demonstrated at the highest dose. At the dose of 12.5 mg/kg, morusin exerted therapeutic effectivity similar to that of sulfasalazine (50 mg/kg). This was associated with significant reduction of TGF-β1 levels and MMP2 and MMP9 activities, and slight reduction of IL-1β. Our results suggest that morusin possesses therapeutic potential for the treatment of chronic inflammatory diseases.

Introduction

Flavonoids are a class of secondary plant metabolites showing a wide spectrum of biological activities, of which the anti-inflammatory and antioxidant effects draw the most attention. The arrangement of functional groups on the flavonoid skeleton necessary for enhancement of the anti-inflammatory activity has been reported previously [1]. Generally, the presence of a double bond between C-2 and C-3 is essential for the anti-inflammatory activity and it appears to

be required for the inhibition of phospholipase A2 and 5- and 12-lipoxygenases [2]. Another study showed that the presence of a carbonyl function group at position C-4 was important for the optimal inhibition of the expression of the tumor necrosis factor (TNF)-α induced intercellular adhesion molecule (ICAM)-1 [3].

Prenylated flavonoids represent an interesting subgroup of flavonoids because they combine the hydrophilic properties of the basic flavonoid skeleton with lipophilic side chains of C5 isoprene units possessing different lengths and modifications. Prenylation may increase bioactivity by accelerating absorption, reducing cell efflux, and enhancing the affinity for biological structures [4]. These features can make prenylated flavonoids leading compounds for the treatment of chronic inflammatory diseases, such as inflammatory bowel disease (IBD) or rheumatoid arthritis.

Numerous prenylated flavonoids have been isolated from the root bark of *Morus alba* L. (Moraceae). One is morusin, a prenylated flavone with two prenyl units, an unmodified one at position 3 and one forming an additional dimethylpyrane ring at position 8 (Fig 1). Some flavonoids that inhibit cyclooxygenase (COX)-2 activity have been studied and these all possessed a C-3 prenyl residue in their structure [2]. An investigation of the cyclization of a prenyl group attached at C-8 or C-6 found that cyclization reduced the cytotoxicity of the compounds studied but not their anti-inflammatory activity [5]. Morusin showed in previous *in vivo* studies beneficial effect on chemically induced acute bronchitis [6] and glomerulonephritis [7]. It also exerted neuroprotective effect associated with reduction of oxidative stress in brain [8].

Several *in vitro* studies describe the anti-inflammatory and cytoprotective properties of its structural derivative cudraflavone B. This flavone is able to attenuate the lipopolysaccharide (LPS)-stimulated secretion of TNF-α and the translocation of nuclear factor (NF)-κB, and inhibit the degradation of IκB, and the expression of COX-2 in macrophages [9, 10]. Hepatoprotective [11] and neuroprotective effects [12] of cudraflavone B on chemically induced cell damage have been ascribed to antioxidant activity. However, its treatment increased production of reactive oxygen species (ROS) [10]. All of these activities may be dose-dependent and could play important roles in the treatment of chronic inflammatory diseases. The 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced model of colitis in rats has been established to investigate the activity of morusin *in vivo*. Three different doses of morusin were administered to rats to observe its effectivity and involvement in the inhibition of inflammatory processes and the destruction of tissue and to compare its activity with that of the drug sulfasalazine (SAS) used clinically. For graphical summary of this study see S1 Fig.

Materials and methods

Plant material

The roots of *M. alba* were collected on the ground of the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic in April 2011. The plant material was identified by Associate Professor K. Šmejkal. A representative specimen (No. MA-11A) has been deposited in herbarium of the Department of Natural Drugs, UVPS Brno.

Extraction and isolation

Morusin was isolated from the chloroform extract of *M. alba* root bark using different chromatographic methods. Twenty-two kg of dry root bark were extracted three times in ethanol and the liquid-liquid extraction of ethanolic extract was carried out with a gain of 218 g of crude chloroform fraction. TLC was done on Merck aluminum foils with silica gel 60 F254 (20 × 20 cm, 200 μm), further separations were performed using Merck silica gel for column chromatography (40–63 μm, Darmstadt, Germany). Chloroform fraction was repeatedly
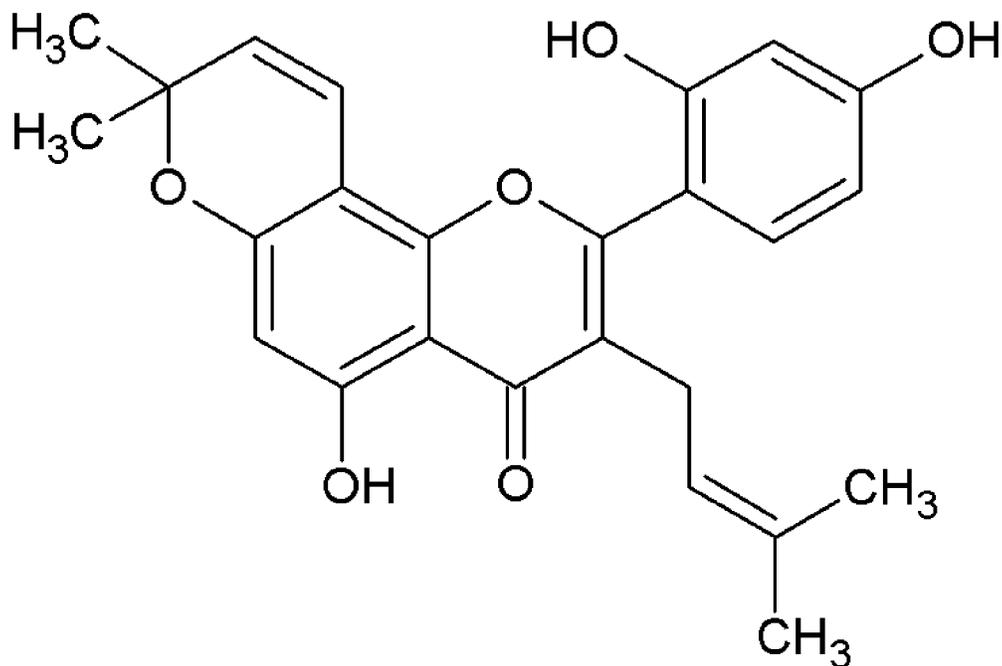
Fig 1. Chemical structure of morusin.

chromatographed over a silica gel column using mixtures of benzene/CHCl₃/MeOH with increasing polarity to afford 42 fractions. The fraction MA2-III (28.7 g) was chosen for the final separation and divided into 11 sub-fractions. The 10 g of sub-fraction MA2-III-C-4 was purified using reverse-phase preparative HPLC (Ascentis® RP-Amide, 5 μm, 250 mm × 10 mm, Supelco; Dionex Ultimate 3000 UHPLC, Thermo Scientific, Waltham, USA). Gradient elution employed 0.2% HCOOH and MeCN, the initial composition of 80% MeCN increased to final 100% MeCN after 30 min. Method was performed at a flow rate of 5 mL/min, detection wavelength 254 nm, injection volume 20 μL, and column temperature 40˚C. Collecting the fraction with a HPLC tR 15.50–18.50 has led to obtaining of yellow amorphous powder with the total weight of 2,400 mg. The purity of isolated compound was determined to be nearly 99% using HPLC DAD analysis (HPLC Agilent 1100 Series with DAD UV/Vis, Agilent Technologies, Santa Clara, USA) with an analytical column Ascentis® Express RP-Amide 2,7 μm, 150 mm × 4,6 mm, Supelco, Bellefonte, USA. The structure of morusin was characterized by UV spectrum (UV-Vis spectrometer Lambda 25, PerkinElmer, Waltham, USA), IR spectrum (Nicolet Impact 400D FT-IR spectrometer, Thermo Nicolet Corporation, Waltham, USA) and NMR spectra were measured in DMSO-d_6 and recorded using a Bruker Avance 300 spectrometer at frequencies 300.13 MHz ^{1}H and 75.48 MHz ^{13}C (Bruker, Billerica, USA). Its identity was confirmed by comparing with the spectroscopic data of morusin isolated previously [13]. For 1D and 2D NMR spectra see Supplemental Data (S2–S9 Figs).

Experimental animals

Male Wistar rats (180–220 g) were supplied by the Laboratory Animal Breeding and Experimental Facility of Masaryk University (Brno, Czech Republic). They were kept under standard conditions (22 ± 2˚C, 50 ± 10% relative humidity), alternating 12 hour light/dark cycles. The animals had access to a standard diet and water *ad libitum*. The experimental protocol was approved by the Experimental Committee for the Welfare of Experimental Animals of the

University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic (Approval No. 19–2015). To minimize the suffering of the laboratory animals, the number of pharmacological interventions was limited to the necessary minimum.

Experimental design

After one week period of acclimation the rats were divided into six groups (n = 8). All of the animals were fasted for 24 h prior to the induction of colitis. Colitis was induced by a single dose of TNBS (50 mg/kg; Sigma-Aldrich, Steinheim, Germany) dissolved in ethanol (50% v/v; total instilled volume: 1 mL/kg of rat body weight). TNBS was administered rectally using a rubber catheter inserted 8 cm proximal to the anus under light isoflurane anesthesia [14]. The rats were kept in a head-down position until they recovered from the anesthesia to prevent leakage of the instillation. The intact group received 0.9% saline instead of TNBS solution. Administration of the test compounds began one day after the induction of colitis and continued every 24 hours for five consecutive days. The compounds were suspended in a 10% gel of polyvinylpyrrolidone (PVP K30; Sigma-Aldrich) and were administered by gastric gavage at bolus doses of 12.5, 25, or 50 mg/kg of morusin and 50 mg/kg of sulfasalazine (Sigma-Aldrich). Rats in the untreated group received the vehicle (PVP K30) only. The animals were killed on the seventh day of the experiment by using an overdose of the veterinary euthanasia drug T61 (Intervet International B. V., Boxmeer, Netherlands).

Macroscopic and microscopic evaluation

After dissection, the colon was removed, cut longitudinally, cleaned with cold saline (0.9%), measured, and weighed. Each colon was scored for macroscopically visible damage according to the criteria of Minaiyan et al. (2014) with the following modifications: no ulcerations (0 points), hyperemia only (1 point), mild mucosal edema (2 points), moderate edema with erosions (3 points), severe ulceration < 5 mm (4 points), severe ulceration > 5 mm (5 points). Samples about 0.5 cm long for histological analysis were obtained from the area of colon with visible ulceration or inflammation; when no grossly visible inflammation was present, the samples were excised from the region 1 cm proximal to the anus [14]. Tissue samples were fixed in 10% neutral buffered formalin and then embedded in paraffin. Three micrometer thick sections were stained with haematoxylin-eosin. The histological damage was scored by a veterinary pathologist and graded 0–3 for the severity of inflammation and infiltration of immune cells, 0–3 for the extent of inflammation (mucosa, submucosa, transmural layers), and 0–4 for crypt damage. The total histological score was the sum of all of the parameters evaluated [15].

Preparation of colonic tissue homogenizates

Frozen colonic tissue was homogenized in lysis buffer [50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M saccharose] with protease inhibitors (Roche, Mannheim, Germany) as previously described [16]. The protein concentration was measured using a Bradford's method assay kit according to the manufacturer's instructions (Amresco, Cleveland, USA).

Western blot analysis

The samples were denatured in the presence of β-mercaptoethanol and SDS at 70˚C for 5 min. Proteins in the amount of 120 μg were separated onto 12% and 15% SDS-polyacrylamide gel, blotted to a polyvinylidene fluoride (PVDF) membrane with 0.2 μm pores (Bio-Rad, Hercules, USA), and then blocked with 5% bovine serum albumin (BSA) (SERVA, Heidelberg,

Germany) in TBST buffer [10 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 0.1% (v/v) Tween 20] for 1h. The membrane was incubated with the primary antibody [rabbit anti-IL-1β 1:2500 (AbCam, Cambridge, UK; product No. ab9722), mouse anti-CAT 1:1000 (Sigma-Aldrich; product No. C0979), rabbit anti-SOD2 1:1000 (Sigma-Aldrich; product No. HPA001814), mouse anti-TGF-β1 1:2000 (AbCam; product No. ab64715), rabbit anti-TNF-α 1:1000 (AbCam; product No. ab6671), or mouse anti-β-actin 1:5000 (AbCam; product No. ab8226)] at 4˚C overnight followed by washing and incubation with the secondary antibody [anti-mouse IgG (Sigma-Aldrich; product No. A0168) or anti-rabbit IgG (Sigma-Aldrich; product No. A0545) at a dilution of 1:2000] at room temperature for 1 h. Bands were visualized using a chemiluminescent kit (Bio-Rad) and a PXi Syngene Chemiluminescent Imaging System (Syngene, Cambridge, UK) and quantified by optical densitometry (GeneTools Software 4.03, Syngene).

Zymography

Zymography was used to evaluate the activity of matrix metalloproteinases (MMP) 2 and 9. Twenty micrograms of native proteins was loaded onto 10% SDS-polyacrylamide gel impregnated with 0.1% gelatin. After separation, the gel was washed twice in 2.5% (v/v) Triton X-100 and subsequently incubated in the development buffer [50 mM Tris-HCl (pH 8.8), 5 mM calcium chloride, 3mM sodium azide, 0.5% (v/v) Triton X-100] for 15 min at room temperature and then overnight at 37˚C. The gel was then stained with Coomasie blue for 2 h and destained until the bands were clearly visible. The intensity of the bands was evaluated by densitometric analysis using Gene Tools Software 4.03 (Syngene). Results were normalized to a standard control [1% fetal bovine serum (FBS), Sigma-Aldrich].

Statistical analysis

All results are expressed as the mean with error bars representing SEM. Parametric data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Mann-Whitney test was performed to evaluate the score of macroscopic damage and histological score. GraphPad Prism 6.01 software (GraphPad Software, San Diego, USA) was used for the analysis. Values of $p < 0.05$ were considered to be statistically significant.

Results

Morusin reduces macroscopic and microscopic damage

TNBS-induced colitis was characterized by transmural inflammation of the colon with signs of hyperemia, mucosal edema, and ulcerations. Adhesions to adjacent organs were visible in the untreated group. A 5-day treatment with morusin *via* gavage at doses of 12.5 mg/kg ($p < 0.05$) and 25 mg/kg ($p < 0.01$) significantly reduced the severity of colonic damage, by about 41% and 50% respectively, compared to the untreated animals (Fig 2A, Table 1). No significant reduction in the score was observed after the administration of 50 mg/kg of morusin; the animals in this group showed extensive intestinal damage. The animals treated by sulfasalazine showed non-significant reduction of score ($p = 0.0587$). The weight/length ratio of colon did not differ among the groups (S10 Fig).

Histological evaluation (Fig 2B, Table 1) of the test animals showed moderate to severe inflammation affecting the mucosa, submucosa or transmural layers, with greater infiltration of the immune cells and significant crypt damage (Fig 3B), as compared to the intact group (Fig 3A). Morusin reduced the extent of inflammation, infiltration, and crypt damage in a dose-dependent manner (Fig 3C and 3D). The microscopic damage score was reduced at the
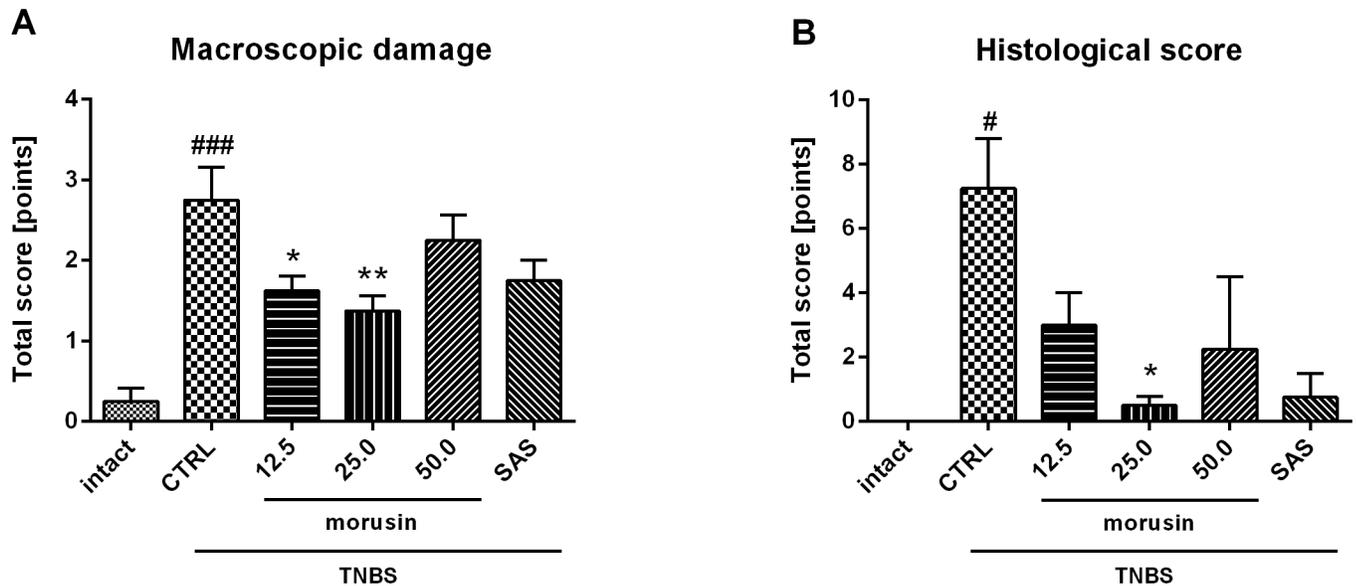
Fig 2. Effect of different doses of morusin (in mg/kg) on TNBS-induced colitis in rats, compared to the intact group, the untreated group (CTRL), and the group treated with sulfasalazine 50 mg/kg (SAS). (A) Score of macroscopically visible damage; (B) Histological assessment. The scoring system and parameters evaluated are described in Materials and methods. The results are expressed as the mean, with error bars representing SEM. CTRL vs. intact group: # $p < 0.05$, #### $p < 0.0001$; CTRL vs. treated groups: * $p < 0.05$, ** $p < 0.01$.

dose of 12.5 mg/kg ($p = 0.0571$) and after sulfasalazine treatment ($p = 0.0571$; Fig 3G) and significantly reduced at the dose of 25 mg/kg ($p < 0.05$). Consistent with the macroscopic evaluation, the damage score was the most variable in the group treated with the dose of 50 mg/kg (Fig 3E and 3F).

Table 1. Score of macroscopically visible damage; Histological assessment.

	Macroscopic damage	Histological score			
	mean ± SEM	Severity of inflammation and infiltration of immune cells (mean ± SEM)	Inflammation extent (mean ± SEM)	Crypt damage (mean ± SEM)	Total (mean ± SEM)
Intact	0.25 ± 0.15	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CTRL	2.75 ± 0.39###	2.25 ± 0.48#	2.25 ± 0.48#	2.75 ± 0.63#	7.25 ± 1.55#
Morusin 12.5	1.63 ± 0.17*	1.25 ± 0.25	1.50 ± 0.50	0.25 ± 0.25	3.00 ± 1.00
Morusin 25.0	1.38 ± 0.17**	0.50 ± 0.29	0.00 ± 0.00*	0.00 ± 0.00*	0.50 ± 0.29*
Morusin 50.0	2.25 ± 0.29	0.75 ± 0.75	0.75 ± 0.75	0.75 ± 0.75	2.25 ± 2.25
SAS	1.75 ± 0.23	0.25 ± 0.25	0.50 ± 0.50	0.00 ± 0.00*	0.75 ± 0.75

Effect of different doses of morusin (in mg/kg) on TNBS-induced colitis in rats, compared to the intact group, the untreated group (CTRL), and the group treated with sulfasalazine 50 mg/kg (SAS). The scoring system and the parameters evaluated are described in Materials and methods. CTRL vs. intact group:

# $p < 0.05$,

$p < 0.001$;

CTRL vs. treated groups:

* $p < 0.05$,

** $p < 0.01$.
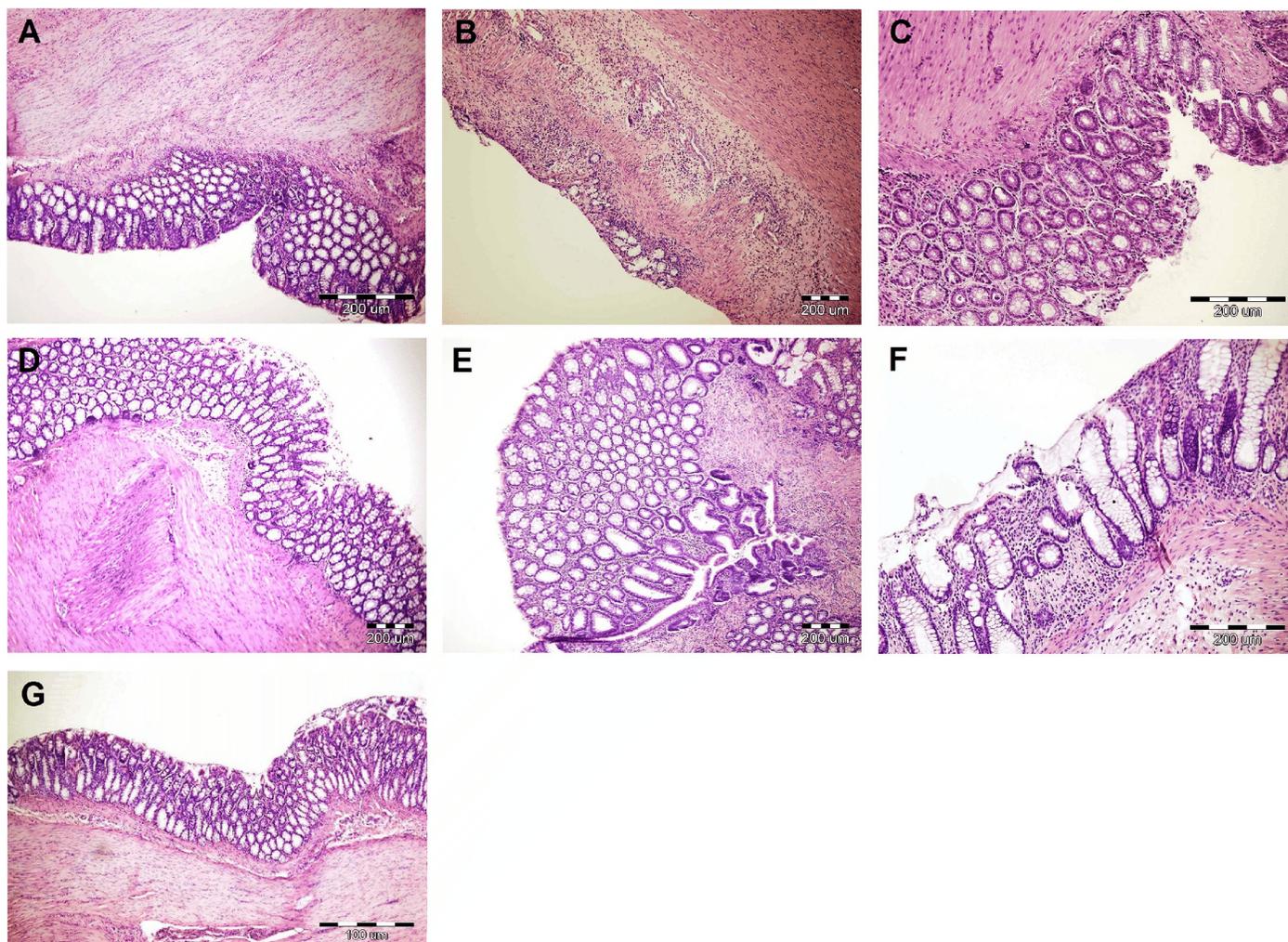
Fig 3. Histological findings (H&E). (A) Colon from the intact group, without induced colitis. (B) Colon after a single administration of TNBS, untreated group; tissue injury is characterized by severe crypt damage, inflammatory cell infiltration, and inflammation affecting transmural layers. Colon after induction of colitis and 5-day treatment with morusin at doses of 12.5 mg/kg (C) and 25 mg/kg (D) showing reduced extent of inflammation, infiltration and crypt damage. The 50 mg/kg dose of morusin displayed the greatest variability in tissue damage: the sample with the lowest (E) and the highest microscopic scores (F). Colon after induction of colitis and 5-day treatment with sulfasalazine 50 mg/kg (G).

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Effect of morusin on protein expression

As shown in Fig 4A, western blot analysis did not reveal significant differences in the level of IL-1β among the groups, although IL-1β was elevated after the administration of TNBS and slightly reduced after treatment with morusin. The 12.5 mg/kg dose of morusin was most effective in preventing an increase in this pro-inflammatory cytokine.

The highest level of profibrogenic factor TGF-β1 was found in untreated rats (Fig 4B). Morusin reduced its expression at all of the doses tested, significantly at 12.5 ($p < 0.05$) and 50 mg/kg ($p < 0.001$), as well as sulfasalazine ($p < 0.001$). The expression was also reduced below the level of the intact group.

The administration of TNBS also resulted in significantly greater expression of the antioxidant enzyme CAT ($p < 0.01$ compared to the intact group; Fig 4D). A significant decrease was
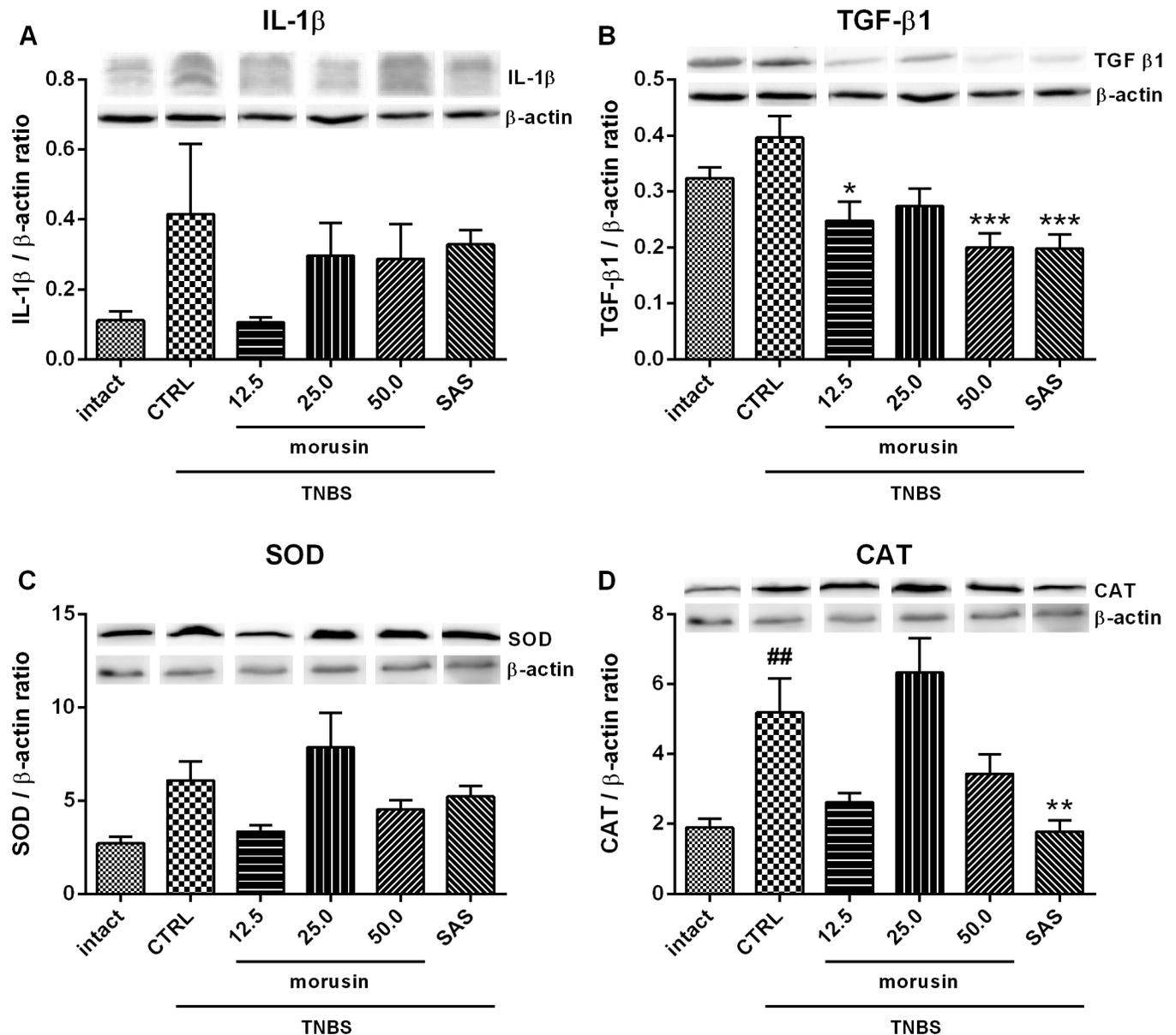
Fig 4. Effect of a 5-day treatment with morusin (in mg/kg) on (A) interleukin 1β, (B) transforming growth factor β1, (C) superoxide dismutase and (D) catalase levels in TNBS-induced colitis, compared to the intact group, the untreated group (CTRL), and the group treated with sulfasalazine 50 mg/kg (SAS). Values were obtained from Western blot analysis, quantified by densitometry, and normalized to the β-actin level, as described in Materials and methods. Representative blots are shown. The results are expressed as the mean, with error bars representing SEM. CTRL vs. intact group: ## $p < 0.01$; CTRL vs. treated groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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observed after sulfasalazine treatment ($p < 0.01$). Oral treatment with morusin reduced the levels of the enzyme at doses of 12.5 and 50 mg/kg. On the other hand, the 25 mg/kg dose caused an increase in the expression of CAT. The expression of SOD corresponded to the levels of CAT (Fig 4C).

In this model, TNBS did not influence TNF-α expression, however, morusin at the dose of 25 mg/kg non-significantly decreased its level (S11 Fig).

Effect of morusin on MMP activity

To evaluate the inflammation and tissue degradation, the activity of matrix metalloproteinases was observed (Fig 5). After administration of TNBS, the total activities of both MMP2 and MMP9 were markedly increased compared to the intact group ($p < 0.0001$ and $p < 0.01$, respectively), but no changes in the activity of the mature form of MMP2 were observed. As shown in Fig 5, all doses of the test compound significantly reduced the activity of both MMPs comparable to sulfasalazine ($p < 0.0001$). The MMP9 activity was reduced by 57–71% after treatment with morusin. The total MMP2 activity was decreased by about 39% after dose of 12.5 mg/kg and by 56% following the dose of 50 mg/kg. The activity of the mature form of MMP2 was strongly inhibited only at doses of 25 ($p < 0.001$) and 50 mg/kg ($p < 0.0001$), respectively. The ratio pro-MMP2/MMP2 increased with increasing dose of morusin, significantly so in the group treated with morusin at the dose of 50 mg/kg ($p < 0.01$) or sulfasalazine ($p < 0.05$).

Discussion

Morusin is a flavone substituted with two prenyl chains which can distinctly modify its activity. This compound can be isolated in relatively large amounts from the root bark of *M. alba* by combining normal and reversed-phase chromatography, and can be obtained commercially in large amount. *Cortex mori radicis* (Sang-Bai-Pi) is a plant material used in Traditional Chinese Medicine to cure different diseases associated with inflammation; its traditional uses have been validated by many studies dealing with anti-inflammatory activity. Morusin previously showed the protective effect in different chemically induced animal models of inflammation [6, 7]. In this study, the effect of morusin on TNBS-induced colitis, an acute intestinal inflammation with predominantly Crohn's disease-like features due to the transmural character of the inflammation, was demonstrated. According to macroscopic and histological evaluations of the colon, the greatest therapeutic effect was seen at a dose of 25 mg/kg. Whereas a lower dose also reduced the macroscopically visible damage, a higher one did not uniformly affect all of the animals in group. Studies using experimental models of bowel inflammation have usually evaluated extracts containing flavonoids, whereas analyses of the effects of the pure isolated compounds have been less frequent. Only two studies have focused on flavonoids substituted with isoprene units. Icariin, a prenylated flavonol glycoside, attenuated the progression of disease in the DSS model of inflammation [17]. In the same model, geranylated flavanones diplacone and mimulone reduced the symptoms of colitis and delayed their onset [16].

As a general marker of inflammatory response, the expression of IL-1β after morusin treatment was evaluated in this study. Its level was increased following the administration of TNBS and only the lowest dose of morusin reduced it to the level of the intact group. A previous study also showed that the structural derivative of morusin, cudraflavone B, was able to slightly reduce an elevated level of IL-1β in the presence of LPS in THP-1-derived macrophages *in vitro* [18].

The antioxidant activity of potential drugs is substantiated in IBD therapy because the oxidative damage to tissue caused by reactive oxygen species (ROS) is involved in the pathogenesis of inflammatory disorders [19]. Some of previous studies ascribed the cytoprotective effect to possible antioxidant properties of structurally similar cudraflavone B. An et al. (2006) showed a significant hepatoprotective effect against tacrine-induced cytotoxicity in human liver hepatocellular carcinoma cell line Hep G2, for which toxicity involves ROS and lipid peroxidation [11]. Lee et al. (2014) described inhibition of ROS and a neuroprotective effect against glutamate-induced neurotoxicity in mouse hippocampal HT22 cells through Nrf2
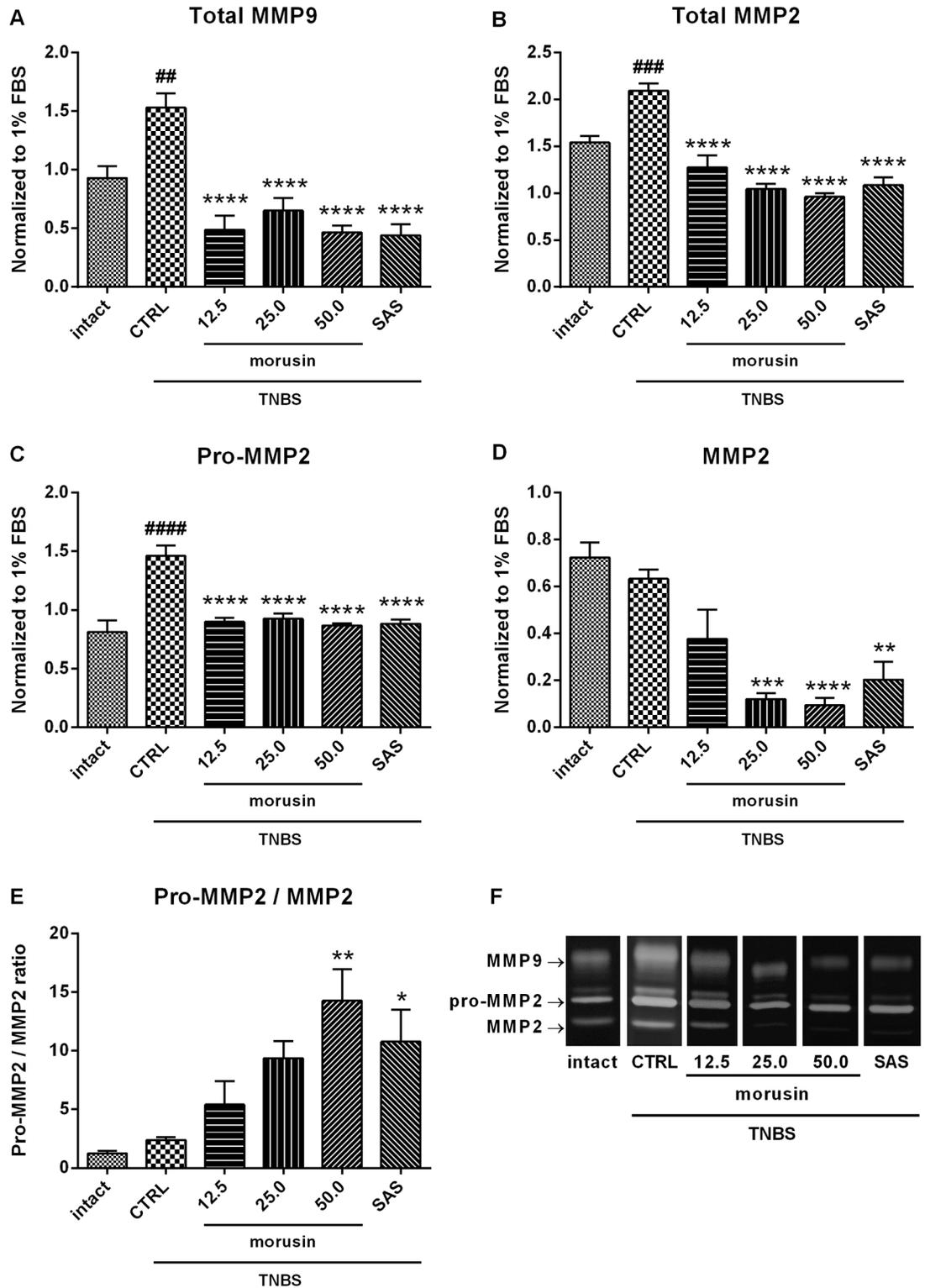
Fig 5. Effect of a 5-day treatment with morusin B (in mg/kg) on the activity of matrix metalloproteinases in TNBS-induced colitis, compared to the intact group, the untreated group (CTRL), and the group treated with sulfasalazine 50 mg/kg (SAS). (A) MMP9 activity, (B) total MMP2 activity, (C) activity of the pro-form of MMP2, (D) activity of the mature form of MMP2, (E) pro-MMP2/MMP2 ratio. The activity of MMPs was detected by zymography, quantified by densitometry, and normalized to a standard control (1% FBS). A representative zymogram is shown (F).

The results are expressed as the mean, with error bars representing SEM. CTRL vs. intact group: ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$; CTRL vs. treated groups: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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(nuclear factor-E2-related factor 2) [12]. On the other hand, Hošek et al. (2013) have reported, that cudraflavone B increased the production of ROS in the murine macrophage cell line J774. A1 [10]. To observe the antioxidant capacity in intestinal tissue, the expression of enzymes involved in the conversion of ROS (SOD, CAT), was evaluated in this study. Their expression and activity depend on the amounts of the substrates superoxide and hydrogen peroxide generated by phagocytes in inflamed tissue. This is confirmed by the higher levels of both of the enzymes evaluated in this study. Lower levels of CAT and SOD were achieved after morusin treatment at doses of 12.5 or 50 mg/kg and could imply a low level of oxidative stress. However, at a dose of 25 mg/kg the level of antioxidant enzymes was markedly increased. Such a stimulating effect on the SOD activity has previously been described for other flavonoids, such as tea catechins [20] or amentoflavone [21] with different experimental colitis models.

Intestinal fibrosis resulting from chronic transmural inflammation is a common and severe complication of IBD, especially Crohn's disease [22]. An important profibrogenic factor, TGF-β1, is a cytokine expressed in the inflammatory condition in response to injury and related to the initiation of wound healing [23]. An increased level of it after TNBS treatment in this study may indicate the onset of development of fibrosis. Greater production of colonic TGF-β had previously been observed in both acute [24] and chronic model of TNBS-induced colitis [25]. Like sulfasalazine, morusin was able to decrease the level of TGF-β1. This reduction is consistent with the histological findings, inasmuch the intestine was not affected transmurally after morusin or sulfasalazine treatment. The expression of TGF-β was also found to be inhibited after treatment of TNBS-induced colitis with *Scutellaria baicalensis* extract, which is rich in flavones such as baicalein, baicalin, and wogonin [26].

Matrix metalloproteinases (MMP) are involved in tissue remodelation as well. The shifted balance between MMPs and their tissue inhibitors may result in mucosal membrane injury, inflammation, and tissue destruction [27]. The expression and activities of MMP2 and MMP9 were increased in different models of experimental colitis [27], moreover, MMPs were overexpressed in the inflamed tissue of patients with ulcerative colitis [28]. Morusin was distinctly able to inhibit the activities of both MMP2 and MMP9 after a 5-day oral treatment. Inhibition of the activities of both MMP2 and MMP9 in TNBS-induced colitis after administration of the anti-inflammatory drugs sulfasalazine and prednisolone has also been described by Witaicenis et al. (2012) [29]. The other parameter observed in this work, the increasing ratio of pro-MMP2 to mature MMP2 after morusin treatment, is associated with significant inhibition of the mature form. Similar results have also been described after administration of the other prenylated flavonoids diplacone and mimulone in DSS-induced colitis [16]. Inhibitors of MMPs are studied intensively as new therapeutic targets with anti-inflammatory and cytostatic indications [30]. An increased risk of developing carcinoma is associated with IBD, in ulcerative colitis particularly.

In conclusion, morusin at the dose of 25 mg/kg shows a therapeutic effect similar to or greater than that of sulfasalazine (50 mg/kg), the conventional drug used in treating IBD. The interesting mechanism of action appears to be the inhibition of the activity of matrix metalloproteinases and the levels of TGF-β1. The results suggest that morusin can be considered a promising cytoprotective agent with possible targeting to prevent complications of chronic inflammation such as fibrosis or cancer. However, this study indicates that the protective effect of morusin is limited by dose, and further studies, especially long-term treatment conditions are necessary to confirm its therapeutic potential for managing chronic inflammation.

Supporting information

S1 Fig. Graphical summary of study.
(TIF)

S2 Fig. ^{1}H-NMR spectrum of morusin.
(TIF)

S3 Fig. ^{1}H-NMR spectrum of morusin (detail 1).
(TIF)

S4 Fig. ^{1}H-NMR spectrum of morusin (detail 2).
(TIF)

S5 Fig. ^{13}C-NMR spectrum of morusin.
(TIF)

S6 Fig. HMBC spectrum of morusin.
(TIF)

S7 Fig. HMBC spectrum of morusin (detail).
(TIF)

S8 Fig. COSY spectrum of morusin.
(TIF)

S9 Fig. NOESY spectrum of morusin.
(TIF)

S10 Fig. Effect of morusin (in mg/kg) 5-day treatment on weight/length ratio in TNBS-induced colitis, compared to the intact group, the untreated group (CTRL), and the group treated with sulfasalazine 50 mg/kg (SAS). The results are expressed as the mean, with error bars representing SEM.
(TIF)

S11 Fig. Effect of morusin (in mg/kg) 5-day treatment on TNF-α in TNBS-induced colitis, compared to the intact group, the untreated group (CTRL), and the group treated with sulfasalazine 50 mg/kg (SAS). Values were obtained from Western blot analysis of pooled samples, quantified by densitometry, and normalized to the β-actin level, as described in Materials and methods. Representative blots are shown.
(TIF)

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Supervision: Karel Šmejkal, Jan Hošek.

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Visualization: Zora Vochyánová.

Writing – original draft: Zora Vochyánová, Marie Pokorná.

Writing – review & editing: Zora Vochyánová, Marie Pokorná, Karel Šmejkal, Jan Hošek.

References

1. Hošek J, Šmejkal K. Flavonoids as anti-inflammatory agents. In: Parnham MJ, editor. Compendium of Inflammatory Diseases. Basel: Birkhäuser Springer; 2016.

2. Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory plant flavonoids and cellular action mechanisms. J Pharmacol Sci. 2004; 96(3):229–245. https://doi.org/10.1254/jphs.CRJ04003X PMID: 15539763

3. Benavente-García O, Castillo J. Update on uses and properties of citrus flavonoids: New findings in anticancer, cardiovascular, and anti-inflammatory activity. J Agric Food Chem. 2008; 56(15):6185–6205. https://doi.org/10.1021/jf8006568 PMID: 18593176

4. Terao J, Mukai R. Prenylation modulates the bioavailability and bioaccumulation of dietary flavonoids. Arch Biochem Biophys. 2014; 559:12–16. https://doi.org/10.1016/j.abb.2014.04.002 PMID: 24736381

5. Zelova H, Hanakova Z, Cermakova Z, Smejkal K, Dall'Acqua S, Babula P, et al. Evaluation of Anti-Inflammatory Activity of Prenylated Substances Isolated from Morus alba and Morus nigra. J Nat Prod. 2014; 77(6):1297–1303. https://doi.org/10.1021/np401025f PMID: 24901948

6. Lee HJ, Ryu J, Park SH, Woo ER, Kim AR, Lee SK, et al. Effects of Morus alba L. and natural products including morusin on in vivo secretion and in vitro production of airway MUC5AC mucin. Tuberc Respir Dis. 2014; 77(2):65–72. https://doi.org/10.4046/trd.2014.77.2.65 PMID: 25237377

7. Fukai T, Satoh K, Nomura T, Sakagami H. Antinephritis and radical scavenging activity of prenylflavonoids. Fitoterapia. 2003; 74(7–8):720–724. PMID: 14630182

8. Gupta G, Chellappan DK, Dua K, Ashwathanarayana M. Pharmacological evaluation of the recuperative effect of morusin against aluminum trichloride (AlCl3)—induced memory impairment in rats. Centr Nerv Sys Agents Med Chem. 2016: Forthcoming.

9. Hošek J, Bartos M, Chudík S, Dall'Acqua S, Innocenti G, Kartal M, et al. Natural Compound Cudraflavone B Shows Promising Anti-inflammatory Properties in Vitro. J Nat Prod. 2011; 74(4):614–619. https://doi.org/10.1021/np100638h PMID: 21319773

10. Hošek J, Toniolo A, Neuwirth O, Bolego C. Prenylated and Geranylated Flavonoids Increase Production of Reactive Oxygen Species in Mouse Macrophages but Inhibit the Inflammatory Response. J Nat Prod. 2013; 76(9):1586–1591. https://doi.org/10.1021/np400242e PMID: 23947936

11. An R-B, Sohn D-H, Kim Y-C. Hepatoprotective Compounds of the Roots of Cudrania tricuspidata on Tacrine-Induced Cytotoxicity in Hep G2 Cells. Biol Pharm Bull. 2006; 29(4):838–840. https://doi.org/10.1248/bpb.29.838 PMID: 16595932

12. Lee D-S, Ko W, Kim D-C, Kim Y-C, Jeong G-S. Cudarflavone B Provides Neuroprotection against Glutamate-Induced Mouse Hippocampal HT22 Cell Damage through the Nrf2 and PI3K/Akt Signaling Pathways. Molecules. 2014; 19(8):10818. https://doi.org/10.3390/molecules190810818 PMID: 25061726

13. Sun Q, Wang D, Li FF, Yao GD, Li X, Li LZ, et al. Cytotoxic prenylated flavones from the stem and root bark of Daphne giraldii. Bioorg Med Chem Lett. 2016; 26(16):3968–3972. https://doi.org/10.1016/j.bmcl.2016.07.002 PMID: 27400887

14. Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology. 1989; 96:795–803. PMID: 2914642

15. Minaiyan M, Ghannadi A, Asadi M, Etemad M, Mahzouni P. Anti-inflammatory effect of Prunus arme-niaca L. (Apricot) extracts ameliorates TNBS-induced ulcerative colitis in rats. Res Pharm Sci. 2014; 9 (4):225–231. PMID: 25657793

16. Vochyanova Z, Bartosova L, Bujdakova V, Fictum P, Husnik R, Suchy P, et al. Diplacone and mimulone ameliorate dextran sulfate sodium-induced colitis in rats. Fitoterapia. 2015; 101:201–207. https://doi.org/10.1016/j.fitote.2015.01.012 PMID: 25623260

17. Tao F, Qian C, Guo W, Luo Q, Xu Q, Sun Y. Inhibition of Th1/Th17 responses via suppression of STAT1 and STAT3 activation contributes to the amelioration of murine experimental colitis by a natural flavonoid glucoside icariin. Bioch Pharmacol. 2013; 85(6):798–807. https://doi.org/10.1016/j.bcp.2012.12.002 PMID: 23261528

18. Kollar P, Bárta T, Hošek J, Souček K, Závalová VM, Artinian S, et al. Prenylated flavonoids from Morus alba L. cause inhibition of G1/S transition in THP-1 human leukemia cells and prevent the lipopolysac-charide-induced inflammatory response. Evid Based Complement Alternat Med. 2013; 2013:350519. https://doi.org/10.1155/2013/350519 PMID: 23762124

19. Ahn BO, Ko KH, Oh TY, Cho H, Kim WB, Lee KJ, et al. Efficacy of use of colonoscopy in dextran sulfate sodium induced ulcerative colitis in rats: the evaluation of the effects of antioxidant by colonoscopy. Int J Colorectal Dis. 2001; 16(3):174–181. PMID: 11459291

20. Brückner M, Westphal S, Domschke W, Kucharzik T, Lügering A. Green tea polyphenol epigallocate-chin-3-gallate shows therapeutic antioxidative effects in a murine model of colitis. J Crohns Colitis. 2012; 6(2):226–235. https://doi.org/10.1016/j.crohns.2011.08.012 PMID: 22325177

21. Sakthivel KM, Guruvayoorappan C. Amentoflavone inhibits iNOS, COX-2 expression and modulates cytokine profile, NF-κB signal transduction pathways in rats with ulcerative colitis. Int Immunopharma-col. 2013; 17(3):907–916. https://doi.org/10.1016/j.intimp.2013.09.022 PMID: 24126114

22. Yamaguchi H, Suzuki K, Nagata M, Kawase T, Sukumaran V, Thandavarayan RA, et al. Irsogladine maleate ameliorates inflammation and fibrosis in mice with chronic colitis induced by dextran sulfate sodium. Med Mol Morphol. 2012; 45(3):140–151. https://doi.org/10.1007/s00795-011-0550-7 PMID: 23001296

23. Kisseleva T, Brenner DA. Fibrogenesis of Parenchymal Organs. Am Thor Soc. 2008; 5(3):338–342. https://doi.org/10.1513/pats.200711-168DR PMID: 18403330

24. Medina C, Santos-Martinez MJ, Santana A, Paz-Cabrera MC, Johnston MJ, Mourelle M, et al. Trans-forming growth factor-beta type 1 receptor (ALK5) and Smad proteins mediate TIMP-1 and collagen synthesis in experimental intestinal fibrosis. J Pathol. 2011; 224(4):461–472. https://doi.org/10.1002/path.2870 PMID: 21465486

25. Loeuillard E, Bertrand J, Herranen A, Melchior C, Guerin C, Coeffier M, et al. 2,4,6-trinitrobenzene sul-fonic acid-induced chronic colitis with fibrosis and modulation of TGF-beta1 signaling. World J Gastro-enterol. 2014; 20(48):18207–18215. https://doi.org/10.3748/wjg.v20.i48.18207 PMID: 25561788

26. Latella G, Sferra R, Vetuschi A, Zanninelli G, D'Angelo A, Catitti V, et al. Prevention of colonic fibrosis by Boswellia and Scutellaria extracts in rats with colitis induced by 2,4,5-trinitrobenzene sulphonic acid. Eur J Clin Invest. 2008; 38(6):410–420. https://doi.org/10.1111/j.1365-2362.2008.01955.x PMID: 18489401

27. Mao J-W, He X-M, Tang H-Y, Wang Y-D. Protective role of metalloproteinase inhibitor (AE-941) on ulcerative colitis in rats. World J Gastroenterol. 2012; 47(18):7063–7069.

28. von Lampe B, Barthel B, Coupland S, Riecken E, Rosewicz S. Differential expression of matrix metallo-proteinases and their tissue inhibitors in colon mucosa of patients with inflammatory bowel disease. Gut. 2000; 47(1):63–73. https://doi.org/10.1136/gut.47.1.63 PMID: 10861266

29. Witaicenis A, Luchini AC, Hiruma-Lima CA, Felisbino SL, Garrido-Mesa N, Utrilla P, et al. Suppression of TNBS-induced colitis in rats by 4-methylesculetin, a natural coumarin: Comparison with prednisolone and sulphasalazine. Chem Biol Interact. 2012; 195(1):76–85. https://doi.org/10.1016/j.cbi.2011.11.004 PMID: 22119283

30. Marshall DC, Lyman SK, McCauley S, Kovalenko M, Spangler R, Liu C, et al. Selective allosteric inhibi-tion of MMP9 is efficacious in preclinical models of ulcerative colitis and colorectal cancer. PLoS ONE. 2015; 10(5):e0127063. https://doi.org/10.1371/journal.pone.0127063 PMID: 25961845

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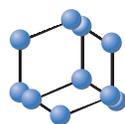
Anti-inflammatory Natural Prenylated Phenolic Compounds - Potential Lead Substances

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Abstract: ***Background***: Natural phenolics are secondary plant metabolites, which can be divided into several categories with the common structural feature of phenolic hydroxyl. The biological activity of phenolics is often modified and enhanced by prenylation by prenyl and geranyl; higher terpenoid chains are rare. The type of prenyl connection and modification affects their biological activity.

Objective: This review summarizes information about prenylated phenols and some of their potential sources, and provides an overview of their anti-inflammatory potential *in vitro* and *in vivo*.

Method: The literature search was performed using SciFinder and keywords *prenyl*, *phenol*, and *inflammation*. For individual compounds, an additional search was performed to find information about further activities and mechanisms of effects.

Result: We summarized the effects of prenylated phenolics *in vitro* in cellular or biochemical systems on the production and release of inflammation-related cytokines; their effects on inhibition of cyclooxygenases and lipoxygenases; the effects on production of nitric oxide, antiradical and antioxidant activity; and the effect on the inhibition of the release of enzymes and mediators from neutrophils, mast cells and macrophages. The information about the antiphlogistic potential of prenylated phenolics is further supported by a review of their action in animal models.

Conclusion: Almost 400 prenylated phenols were reviewed to overview their anti-inflammatory effect. The bioactivity of several prenylated phenols was confirmed also using *in vivo* assays. A pool of natural prenylated phenols represents a source of inspiration for synthesis, and prenylated phenols as components of various medicinal plants used to combat inflammation could be their active principles.

1. INTRODUCTION

Nature has been a source of therapeutics for thousands of years. Medicinal plants were commonly used for the treatment of various diseases, but their use was more instinctive and based on experience rather than on science. Medical procedures involving the use of medicinal plants were created gradually throughout history and were developed from empirical procedures, and, before the advent of chemical drugs, they constituted practically the only way to cure. The first mention of plants and their use is found in a Sumerian record on clay tablets dating back to approximately 4000 BC. Other valuable sources of information on the use of plants are from Ancient China, India and the medicine of ancient Greece and Rome.

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We know especially the works of the physician Hippocrates (Hippocratic Corpus, 5th cent. BC) and Dioscorides (De Materia Medica, 1st century AD), wherein the authors described over 600 species of plants and their medicinal uses. Later studies showed that traditional European 'materia medica' was based on a Dioscorides tradition that had lasted through the 19th century, and with little variation only. With the gradual development of science and technology accelerating the development of synthetic drugs, herbal treatment had been pushed to the sidelines. In recent years, however, plants have again gained considerable interest as potential sources of new drugs. With the possibility of obtaining scientific confirmation of the therapeutic effect and mechanisms, the value of herbal medicines is increasing in popularity, and they are often used as an effective alternative to standard therapy or as a source of lead compounds.

A number of compounds of plant origin have been experimentally proven to relieve pain in the human body, and some of these have been proposed to be the agents responsible for the action of traditional herbal remedies associated with the reduction of pain, fever, and inflammation [1]. Probably the best known anti-inflammatory compound of plant origin is salicin, isolated from the bark of the white willow (*Salix alba* L.) back in 1828 [2]. Today, its synthetic derivative acetylsalicylic acid (aspirin) is one of the most widely used nonsteroidal anti-inflammatory drugs (NSAID) in the world, with an estimated 40,000 tons of it being consumed each year. It has been previously clarified that the inhibition of arachidonic acid (AA) metabolism is the main mechanism responsible for the anti-inflammatory action of aspirin [3]. Following this, a number of studies confirmed the significant inhibitory effect of various plant-derived constituents on AA metabolite pathways, such as baicalein (*Scutellaria baicalensis* Georgi), boswellic acids (*Boswellia serrata* Triana and Planch.), curcumin (*Curcuma longa* L.), quercetin, resveratrol (*Vitis vinifera* L.), or rutin [4]. Inflammatory conditions modulation was also comprehensively described for compounds present for example in *Cannabis* L., which are included into group of so-called prenylated phenols [5].

We previously demonstrated the anti-inflammatory activity of several prenylated phenolics; therefore, we decided to gather the overall information about these interesting compounds and try in this way to attract attention to research of these compounds. The literature search was performed using scientific database SciFinder and keywords *prenyl*, *phenol*, and *inflammation*. For individual compounds, obtained by the prima-

ry literature screening, an additional search was performed to find information about further activities and mechanisms of effects. Later, the activities observed for prenylated phenolics were grouped into separate chapters to provide an overview of possible anti-inflammatory effects.

Prenylated phenolic compounds (PPC) are a unique class of plant secondary metabolites [6-8]. These natural products represent a combination of aromatic skeleton with terpenoid elements. Considering the length of the prenyl chain, different types of prenyl residues are presented in PPC, such as C_5 isopentenyl, C_{10} geranyl, C_{10} lavandulyl, C_{15} farnesyl or C_{20} geranylgeranyl substituent. PPC containing the isopentenyl and the geranyl side chains are more abundant in nature in comparison to other types of prenylation [6, 9]. PPCs are classified into several groups according to the phenolic moiety: *e.g.*, prenylated coumarins, prenylated flavonoids, prenylated chalcones and prenylated cinnamic acids [6, 8, 9]. PPCs occur mainly in plant families such as Fabaceae, Moraceae, Cannabaceae, Clusiaceae, Umbelliferae and Rutaceae [8], and their occurrence is chemotaxonomically limited also to several other species belonging to Euphorbiaceae, Asteraceae, Apiaceae, Boraginaceae and Piperaceae [6, 8].

Plants containing PPC are traditionally used as medicinal plants in many countries. They show a wide range of biological effects, such as anti-inflammatory, antioxidant, antimicrobial or anti-tumor activities [6, 8]. The addition of a prenyl moiety to an aromatic skeleton may contribute to the enhancement of the biological activity of such as phenolic compounds [8]. This review is focused on the PPC with reported anti-inflammatory effects. These compounds inhibit the production and release of certain pro-inflammatory enzymes and mediators, interfere with different pro-inflammatory signaling pathways or show significant antioxidant effect. As further displayed, their activity was proved also *in vivo*. For the comprehensiveness of the text, the activity of some other specific compounds like aromatic diterpenes containing the aromatic part of the skeleton and lipophilic side chains is mentioned. The prenylated compounds obtained during the literature search with previously mentioned keywords have been grouped into comprehensive tables (Figs. **1-27**) according to the basic skeleton or structural relationship of single substances. As visible from the overview, the search brought information about wide spectrum of different prenylated phenolic compounds with some potential to be anti-inflammatory active; however, the groups of prenylated coumarins (including some furanocoumarins and pyranocoumarins, Fig. **1-4**),

	R$_1$	R$_2$	R$_3$	R$_4$
Osthole (**1**)		OCH$_3$	H	H
Osthenol (**2**)		OH	H	H
Omphalocarpin (**3**)		OCH$_3$	H	OCH$_3$
Tamarin (**4**)	H	OCH$_3$		H
Ulopterol (**5**)	H	OCH$_3$		H
Phebalosin (**6**)		OCH$_3$	H	H
Meranzin (**7**)		OCH$_3$	H	H
Murracarpin (**8**)		OCH$_3$	H	H
Microminutin (**9**)		OCH$_3$	H	H
Micromarin A (**10**)		OCH$_3$	H	H
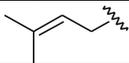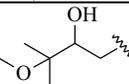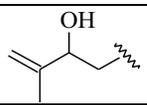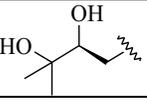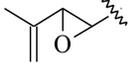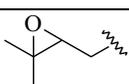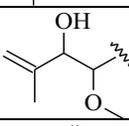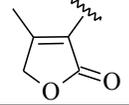
	R$_1$	R$_2$
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Mammea E/BD (**12**)		
Mammea B/BA (**13**)		
Mammea B/BB (**14**)		
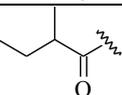
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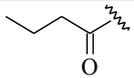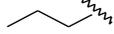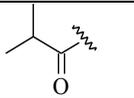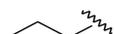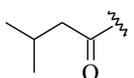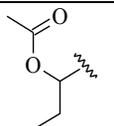
Mammea B/BC (**15**)		
Mammea B/BD (**16**)		
Mammea E/BA (**17**)		
Mammea E/BB (**18**)		
Fukanemarin B (**19**)		
Subcoriacin (**20**)		
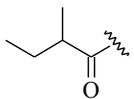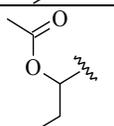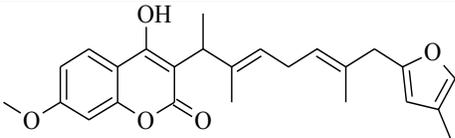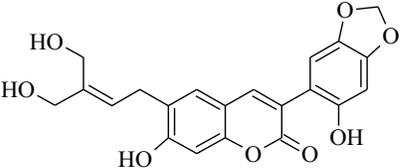
Fig. (1). Structures of C-prenylated simple coumarins.

	R₁	**R₂**	**R₃**	**R₄**
Auraptene (**21**)	H		H	H
Collinin (**22**)	OCH₃		H	H
6-Hydroxy-7-[(*E,E*)-3′,7′-dimethyl-2′,4′,7′-octatrienyloxy] coumarin (**23**)	H		OH	H
6-Hydroxy-7-[(*E*)-3′,7′-dimethyl-5′-hydroxy-2′,6′-octadienyloxy] coumarin (**24**)	H		OH	H
6-hydroxy-7-[(*E,E*)-3′,7′-dimethyl-7′-hydroxy-2′,5′-octadienyloxy] coumarin (**25**)	H		OH	H
Umbelliprenin (**26**)	H		H	H
8-Acetoxy-5-hydroxy-umbelliprenin (**27**)	H		H	H
5-Hydroxyumbelliprenin (**28**)	H		H	H
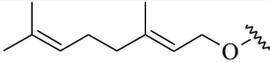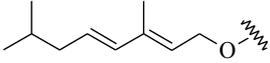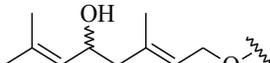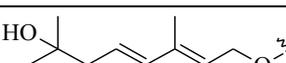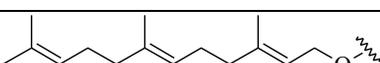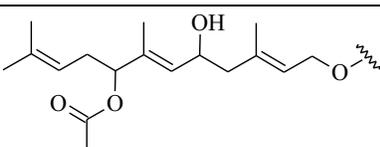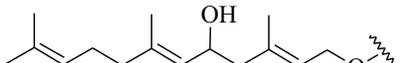
Fig. (2). contd….

Compound				
7-Isopentenyloxycoumarin (**29**)	H		H	H
Lacinartin (**30**)	OCH₃		H	H
7-Prenyloxy-6-methoxycoumarin (**31**)	H		OCH₃	H
6-isopentenyloxy-7-methoxycoumarin (**32**)	H	OCH₃		H
8-isopentenyloxy-7-methoxycoumarin (**33**)		OCH₃	H	H
Prenyletin (**34**)	H		OH	H
Haplopinol (**35**)	H		OH	H
Armenin (**36**)		OH	H	H
Isoarmenin (**37**)	OH		H	H
Lacarol (**38**)		OCH₃	H	OH
Deoxylacarol (**39**)		OCH₃	H	H
(*R*)-(+)-7-(2′,3′-epoxy-3′-methylbutoxy)-coumarin (**40**)	H		H	H
Methylgalbanate (**41**)	H		H	H
4-Isopentenyloxy-5-methylcoumarin (**42**)				

Fig. (2). Structures of *O*-prenylated simple coumarins.

chalcones (Fig. **5**), flavonoids (of different type, like flavones, flavanones, or isoflavones, see Figs. **6-11**), arylbenzofurans (Fig. **15**) and different acylphloroglucinols (Figs. **23-26**) are the most numerous.

Inflammation is a protective response of body tissues that occurs in reaction to any type of infectious agent, irritation or injury, characterized by the activation of the innate nonspecific immune system [10]. The classical signs of inflammation are redness, heat, swelling, pain and dysfunction of the organs [11]. These signs can be explained by increased blood flow, vasodilatation, release of inflammatory mediators, elevated cellular metabolism and cellular influx of various immune-system cells [10]. The migrated and activated immune cells generate and release a variety of mediators that control the progression and resolution of inflammatory reaction. Among the numerous inflammatory mediators are very important cytokines and lipid mediators [11]. Under normal conditions, inflammation is self-limiting, but, in some cases, it becomes continuous, and chronic inflammatory disease can develop subsequently [10]. Inflammation contributes to chronic diseases including diabetes, cardiovascular diseases, certain cancers and bowel diseases, arthritis, osteoporosis, and neurodegenerative diseases [12]. An inflammatory process has very specific characteristics, whether

	R₁	R₂
Imperatorin (**43**)	H	
Isoimperatorin (**44**)		H
Cnidicin (**45**)		
Oxypeucedanin hydrate (**46**)		H
Oxypeucedanin (**47**)		H
Isogospherol (**48**)	H	
Prangenin (**49**)	H	
Heraclenol (**50**)	H	
Byakangelicol (**51**)	OCH₃	
Byakangelicin (**52**)	OCH₃	
Methoxy-8-(3-hydroxymethyl-but-2-enyl oxy)-psoralen (**53**)	OCH₃	
Clausemarin A (**54**)	H	
Wampetin (**55**)	H	
Bergamottin (**56**)		H
Dihydroxybergamottin (**57**)		H
8-Geranyloxypsoralen (**58**)	H	
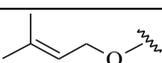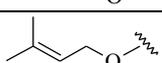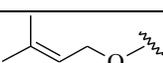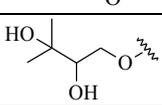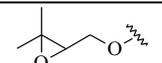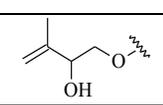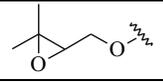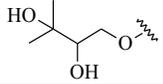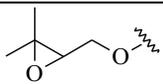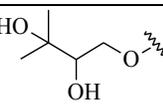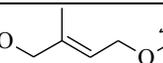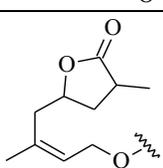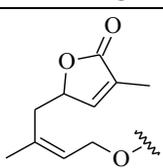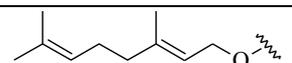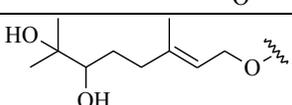
Fig. (3). contd….

	R
Deltoin (**59**)	
Marmesin isovalerate (**60**)	
Nodakenetin (**61**)	

	R₁	R₂
	R₁	**R₂**
Columbianadin (**62**)		H
Columbianetin (**63**)		H
8,9-Dihydro-8-(2-hydroxypropan-2-yl)-2-oxo-2*H*-furo[2,3-*h*]chromen-9-yl-3-methylbut-2-enoate (**64**)		
Majurin (**65**)		H

Microminutinin (**66**)	

	R₁	R₂	R₃	R₄
	R₁	**R₂**	**R₃**	**R₄**
Fukanefuromarin E (**67**)	OCH₃	H		
Fukanefuromarin F (**68**)	OCH₃	H		
Fukanefuromarin G (**69**)	OCH₃		H	
2,3-Dihydro-7-methoxy-2*S*,3*R*-dimethyl-2-[4-methyl-5-(4-methyl-2-furyl)-3(*E*)-pentenyl]-furo[3,2-c]coumarin (**70**)	OCH₃		H	
2,3-Dihydro-7-hydroxy-2S,3R-dimethyl-2-[4-methyl-5-(4-methyl-2-furyl)-3(*E*)-pentenyl]-furo[3,2-c]coumarin (**71**)	OH		H	

Fig. (3). Structures of prenylated furanocoumarins.

	R₁	R₂
Anomalin (**72**)		
Praeruptorin A (**73**)		
Praeruptorin C (**74**)		
Praeruptorin D (**75**)		
Praeruptorin E (**76**)		
Selinidin (**77**)	H	
Visnadin (**78**)		
Pyranocoumarin dimer (**79**) (*dimmer in original literature*)		
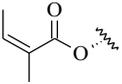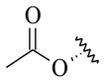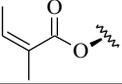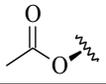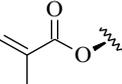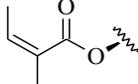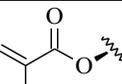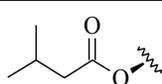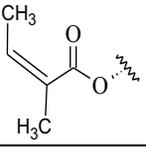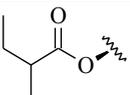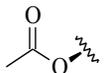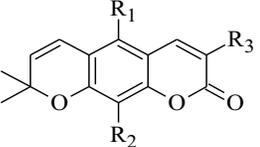
	R₁	R₂	R₃
Xanthyletin (**80**)	H	H	H
Nordentatin (**81**)	OH		H
Clausarin (**82**)	OH		
Ponfolin (**83**)			H

Fig. (4). contd….

Clausenidin (**84**)	
Cedrecoumarin A (**85**)	
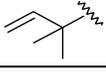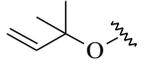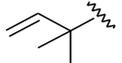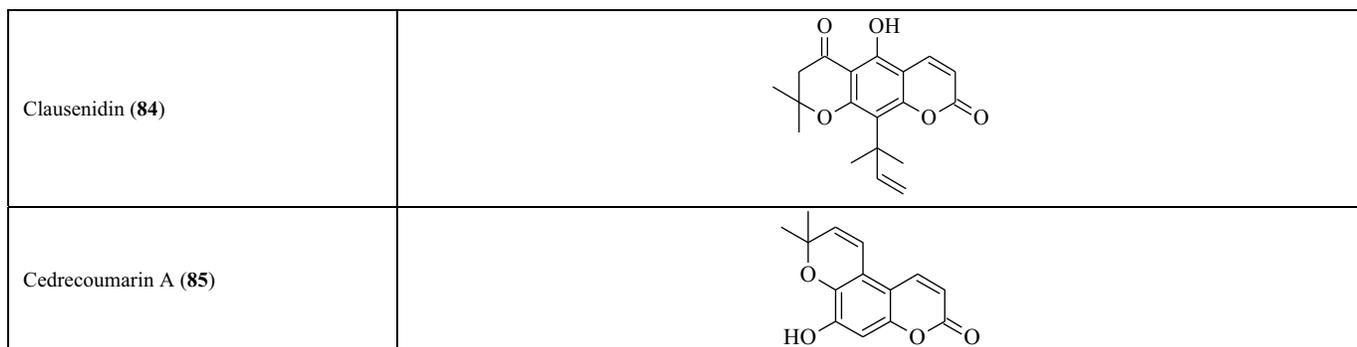
Fig. (4). Structures of prenylated pyranocoumarins.

	R₁	R₂	R₃	R₄
	R_1	R_2	R_3	R_4
Xanthohumol (**86**)	OCH₃	H	OH	
Xanthogalenol (**87**)	OH	H	OCH₃	
4′-O-Methylxanthohumol (**88**)	OCH₃	H	OCH₃	
2′,4′,6′,4-tetrahydroxy-3′-prenylchalcone (**89**)	OH	H	OH	
Tetrahydroxanthohumol (**90**)	OCH₃	H	OH	
5′-Prenylxanthohumol (**91**)	OCH₃		OH	
4-Hydroxyderricin (**92**)	H	H	OCH₃	
Isobavachalcone (**93**)	H	H	OH	
Xanthoangelol D (**94**)	H	H	OCH₃	
Xanthoangelol E (**95**)	H	H	OCH₃	
Xanthohumol D (**96**)	OCH₃	H	OH	
Xanthohumol H (**97**)	OCH₃	H	OH	
2′,4′,6′,4-Tetrahydroxy-3′-geranylchalcone (**98**)	OH	H	OH	
Xanthoangelol (**99**)	H	H	OH	

Fig. (5). contd….

Xanthoangelol F (**100**)	H	H	OCH₃	
Xanthoangelol B (**101**)	H	H	OH	
2´,4´,4-trihydroxy-3´-[2-hydroxy-7-methyl-3-methylene-6-octaenyl]chalcone (**102**)	H	H	OH	
Xanthokeismin A (**103**)	H	H	OH	
Gemichalcone B (**104**)	H	H	OH	
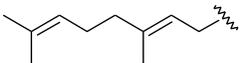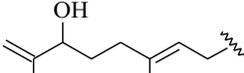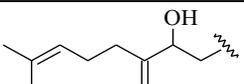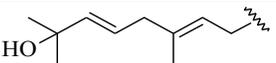
Xanthohumol B (**105**)	

	R₁	R₂
Morachalcone A (**106**)	H	
Kuraridin (**107**)	OCH₃	
Kuraridinol (**108**)	OCH₃	

	R₁	R₂	R₃	R₄	R₅
Broussochalcone (**109**)	H		OH	OH	H
Licoagrochalcone A (**110**)	H	H		OH	H
Abyssinone D (**111**)	H	H		OH	OCH₃
2´,4´,6´-trihydroxy-3´-prenylchalcone (**112**)	OH		H	H	H
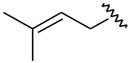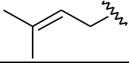
Fig. (5). contd….

	R₁	R₂	R₃
Mallotophilippen C (**113**)	H	H	
Mallotophilippen D (**114**)	OH	H	
Mallotophilippen E (**115**)	OH		H

	R	
Anthyllisone (**116**)		
3-*O*-Methylabyssinone A (**117**)	OCH₃	

	R₁	R₂
Xanthohumol C (**118**)	OCH₃	H
3′′,3′′-dimethylpyrano[3′,4′]2,4,2′-trihydroxychalcone (**119**)	H	OH

	R₁	R₂
Boesenbergin A (**120**)	CH₃	
Heliteretifolin (**121**)		H

Fig. (5). contd….

	R₁	R₂	R₃	R₄	R₅
Dihydroxanthohumol (**122**)	(prenyl group)	H	OCH₃	H	H
Trihydroxy-3´-[6-hydroxy-3,7-dimethyl-2(E)-7-octadienyl]chalcone (**123**)	(hydroxy-dimethyl-octadienyl group)	H	H	H	H
3,4,2´,4´-Tetrahydroxy-3´-geranyl-dihydrochalcone (**124**)	(geranyl group)	H	H	H	OH
4,2´,4´-Trihydroxy-5´-geranyl-dihydrochalcone (**125**)	H	(geranyl group)	H	H	H
3,4,2´,4´-Tetrahydroxy-2-geranyl-dihydrochalcone (**126**)	H	H	H	(geranyl group)	OH

Fig. (5). Structures of prenylated chalcones.

	R₁	R₂	R₃	R₄	R₅	R₆
8-Prenylnaringenin (**127**)	H	(prenyl group)	H	H	OH	H
6-Prenylnaringenin (**128**)	(prenyl group)	H	H	H	OH	H
6,8-Diprenylnaringenin (**129**)	(prenyl group)	(prenyl group)	H	H	OH	H
Tomentosanol D (**130**)	H	(hydroxy prenyl group)	H	H	OH	H
6,8-Diprenyleriodictyol (**131**)	(prenyl group)	(prenyl group)	H	OH	OH	H
Sigmoidin A (**132**)	H	H	(prenyl group)	OH	OH	(prenyl group)
Sigmoidin B (**133**)	H	H	H	OH	OH	(prenyl group)
Leachianone G (**134**)	H	(prenyl group)	OH	H	OH	H
Kushenol E (**135**)	(prenyl group)	(prenyl group)	OH	H	OH	H
Glabranin (**136**)	H	(prenyl group)	H	H	H	H
Isoglabranin (**137**)	(prenyl group)	H	H	H	H	H
Euchestaflavanone B (**138**)	(prenyl group)	H	OH	H	OH	(prenyl group)

Fig. (6). contd….

Euchrenon a7 (**139**)					
	R₁	**R₂**	**R₃**	**R₄**	**R₅**
Isoxanthohumol (**140**)	OCH₃	H	OH		OH
5,7-dihydroxy-4′′-methoxy-8-(3-methylbut-2-enyl)favanone (**141**)	OH	H	OH		OCH₃
5,7-dihydroxy-4′-methoxy-8-(2-hydroxy-3-methylbut-3-enyl)favanone (**142**)	OH	H	OH		OCH₃
7-Methoxyisoglabranin (**143**)	OH		OCH₃	H	H

	R	
Sanggenon H (**144**)		
Sanggenon F (**145**)		
5-hydroxy-4′-methoxy-2′′,2′′-dimethylpyrano-7,8:6′′,5′′flavanone (**146**)		
5,4′-dihydroxy[2′′-(1-hydroxy-1-methylethyl)dihydrofurano]-7,8:5′′,4′′flavanone (**147**)		
Lupinifolinol (**148**)		

Fig. (6). contd….

	R₁	R₂
Euchrestaflavanone C (**149**)		H
(2S)-5,7,7′-trihydroxy-8-(3-hydroxy-3-methylbutyl)-2′,2′-dimethyl-[2,6′-Bi-2H-1-benzopyran]-4(3H)-one (**150**)	H	

	R₁	R₂	R₃	R₄	R₅
8-Prenylquercetin (**151**)	OH	H		H	OH
5,7,4′-trihydroxy-8,3′-diprenylflavone (**152**)	H	H		H	
Kuwanon C (**153**)		H		OH	H
Albanin A (**154**)		H	H	OH	H
Artocarpesin (**155**)	H		H	OH	H
Isolicoflavonol (**156**)	OH	H	H	H	
2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-6,8-bis(3-methyl-2-buten-1-yl)-4H-1-benzopyran-4-one (**157**)	H			H	OH
Cannflavin B (**158**)	H		H	H	OCH₃
Kuwanon T (**159**)		H	H	OH	
Epimedonin D (**160**)					
Papyriflavonol A (**161**)					
Artocarpin (**162**)					

Fig. (6). contd….

Abyssinone V-4-methyl ether (**163**)	

	R
	R
Cudraflavone B (**164**)	
Cycloartocarpesin B (**165**)	H

	R₁	**R₂**	**R₃**	**R₄**	**R₅**
Atalantoflavon (**166**)	H	H	OH	H	CH₃
Sanggenol L (**167**)	H	OH	OH	H	
Artoflavone A (**168**)		OH	OCH₃	OH	CH₃
Morusin (**169**)		OH	OH	H	CH₃
Morusinol (**170**)		OH	OH	H	CH₃
Hydroxyartoflavone A (**171**)		OH	OH	OH	CH₃

Mornigrol G (**172**)	
Dorsmanin F (**173**)	

Fig. (6). contd….

	R₁	R₂
Mornigrol H (**174**)		H
Kuwanon A (**175**)		
Kuwanon G (**176**)		

Fig. (6). Structures of prenylated flavonoids.

	R₁	R₂	R₃
Mimulone (*syn.* 6-geranylnaringenin) (**177**)	H	OH	H
Diplacone (**178**)	OH	OH	H
6-geranyl-5,7-dihydroxy-3′,4′-dimethoxyflavanone (**179**)	OCH₃	OCH₃	H
3′-*O*-methyldiplacone (**180**)	OCH₃	OH	H
3′-*O*-methyl-5′-hydroxydiplacone (**181**)	OCH₃	OH	OH
3′-*O*-methyl-5′-*O*-methyldiplacone (**182**)	OCH₃	OH	OCH₃
Schizolaenone C (**183**)	OH	H	OH

	R₁	R₂	R₃
Kuwanon E (**184**)	H		OH
Tomentodiplacone (**185**)		OCH₃	H
Tomentodiplacone B (**186**)		OCH₃	H
Mimulone B (**187**)		H	H

Fig. (7). contd….
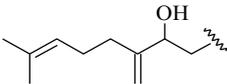
Mimulone H (**188**)		H	H
Tomentodiplacol B (**189**)			
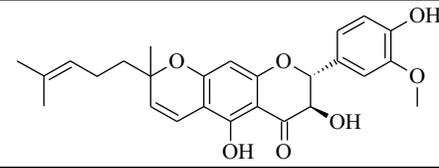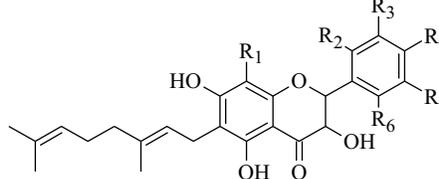
	R₁	R₂	R₃	R₄	R₅	R₆
	R_1	R_2	R_3	R_4	R_5	R_6
3′-*O*-methyldiplacol (**190**)	H	H	OCH₃	OH	H	H
Sophoraflavanone D (**191**)	H	OH	H	OH	H	OH
Kenusanone C (**192**)		OH	H	OCH₃	H	OH
3′-*O*-methyl-5′-hydroxydiplacol (**193**)	H	H	OCH₃	OH	OH	H
Diplacol (**194**)	H	H	OH	OH	H	H
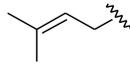
Tomentodiplacone N (**195**)	
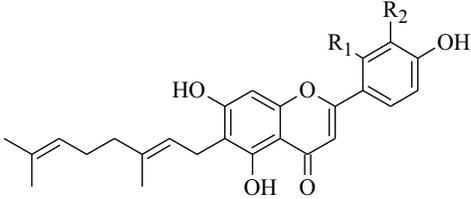
	R₁	R₂
	R_1	R_2
Albanin E (**196**)	OH	H
Cannflavin A (**197**)	H	OCH₃
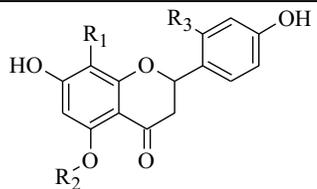
	R₁	R₂	R₃
	R_1	R_2	R_3
8-Geranylnaringenin (**198**)		H	H
Sophoraflavanone G (**199**)		H	OH
Kurarinone (**200**)		CH₃	OH
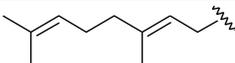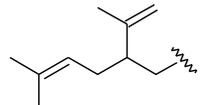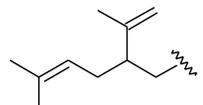
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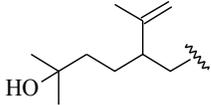
Kurarinol (**201**)		CH$_3$	OH
Kushenol C (**202**)			
Dorsmanin C (**203**)			
8-Geranyl-3-(hydroxyprenyl)isoetin (**204**)			
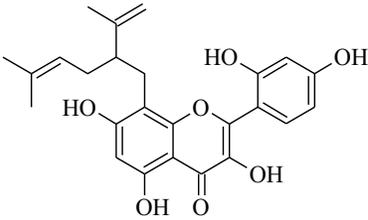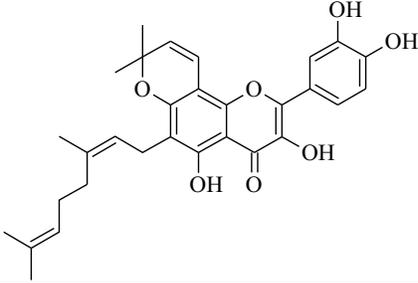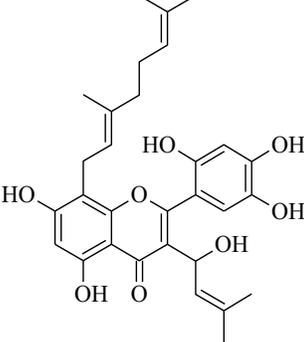
Fig. (7). Structures of geranylated and levandulylated flavonoid derivatives.

acute or chronic. Infiltration of innate immune system cells, specifically neutrophils and macrophages, characterizes acute inflammation, while infiltration of T lymphocytes and plasma cells are features of chronic inflammation. Macrophages play a central role in both, contributing to the final consequence of chronic inflammation which is represented by the loss of tissue function due to fibrosis [10]. The inflammation process involves a number of endogenous mediators, including vasoactive amines histamine and serotonin, the AA metabolites, the complement system, the kinin system, the fibrinolytic system, platelet-activating factor (PAF), neuropeptides, hydrolytic enzymes, adhesion molecules, reactive oxygen species (ROS) and inflammatory cytokines. All of these mediators manifest a broad amount of overlapping actions, cross-reactions, redundancies and synergistic effects [11, 12].

Inflammatory responses could be regulated at three levels: 1) signal-specific level, when intracellular signal pathways are regulated; 2) gen-specific level, when

transcription of particular genes is regulated; and 3) cell-specific level, when cell differentiation, activation, and cell-cell signaling is regulated. However, the main control point lies on the transcription level [13]. The regulation of gene expression is a complex system of transcription factors, RNA-binding proteins and chromatin-modifying proteins. A specific expression profile is created by their synchronization and leads to the fine tuning of inflammatory response on the presence of pathogen. With small modifications, this system is applicable on autoimmune inflammatory response or on tissue damaging.

In mammals, leukocytes play one of the most important roles during inflammation. Leukocytes are mediators of innate immune response. They are derived from precursors in the bone marrow [14]. Leukocytes are traditionally grouped into three major categories: granulocytes, monocytes, and lymphocytes [15]. Granulocytes, also known as polymorphonuclears (PMNs), are represented by neutrophils, eosinophils, and

	R₁	R₂
Sanggenon B (**205**)		OH
Sanggenon D (**206**)		H
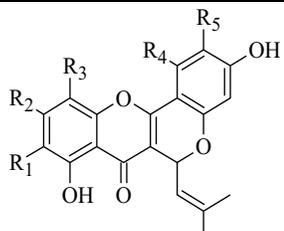
	R₁	R₂	R₃	R₄	R₅
Norartocarpetin (**207**)	H	H	OH	OH	H
Dihydroisocycloartomunin (**208**)	H	OH		H	OCH$_3$
Dihydrocycloartomunine (**209**)	H	OCH$_3$		H	OH
Cyclocommunine (**210**)		OH	H	H	H
Artelastin (**211**)		OH		H	H
Artogomezianone (**212**)			OCH$_3$	OH	H
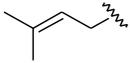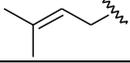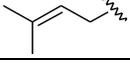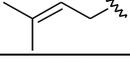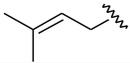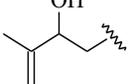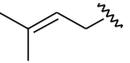
Cyclomorusin (**213**)	
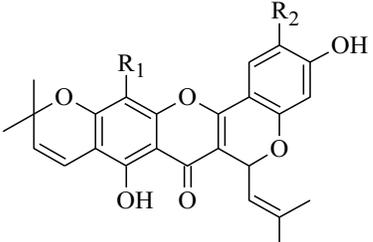
Fig. (8). contd….

	R₁	R₂
Cycloheterophyllin (**214**)		OH
Cudraflavone A (**215**)	H	H
Isocycloartobiloxanthone (**216**)		
Artonin B (**217**)		
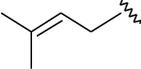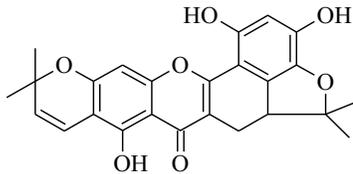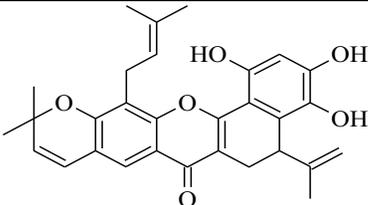
Fig. (8). Structures of prenylated polycyclic flavonoid derivatives.
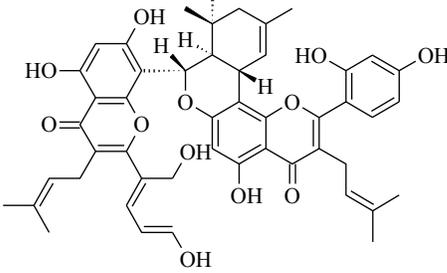
Mongolicin E (**218**)	
Guangsangon H (**219**)	
Guangsangon I (**220**)	
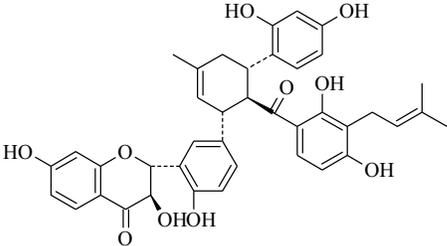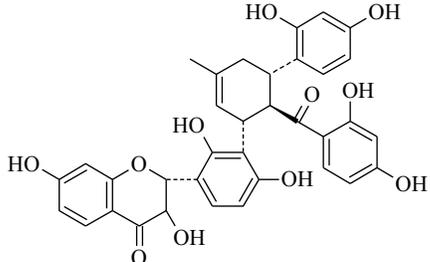
Fig. (9). Structures of miscelenous prenylated flavonoids.

basophils. They circulate in the blood unless recruited to act as effector cells at site of inflammation [14]. Neutrophils are the most common type of granulocytes. They phagocyte and destroy microorganisms, mainly bacteria, and thus have a key role in innate immunity to bacterial infection. Basophils secrete histamine to help mediate inflammatory reactions. Eosinophils help to destroy parasites and modulate allergic inflammatory responses [15]. Mast cells contain numerous granules and secrete an abundance of soluble inflammatory mediators such as cytokines, histamine and serotonin. They recruit basophils and eosinophils from the bloodstream [16]. When monocytes leave the bloodstream, they mature into macrophages, which phagocyte bacteria, and recruit other phagocytic cells, the neutrophils, from the blood [14, 15]. Monocytes also give rise to

	R₁	R₂
(2*S*)-7,4′-dihydroxy-8-prenylflavan (**221**)		
Kazinol B (**222**)	H	
Glabridin (**223**)		

Fig. (10). Structures of flavan and isoflavan prenylated derivatives.

	R₁	R₂	R₃	R₄	R₅
Sophoraisoflavanone A (**224**)	H	OH	OCH₃		H
Echinoisosophoranone (**225**)	H	OCH₃	OCH₃	H	
Isosophoranone (**226**)		OH	OCH₃		H
Kenusanone A (**227**)	H	OH	OH	H	
Echinoisoflavanone (**228**)					

	R₁	R₂	R₃	R₄
Neobavaisoflavone (**229**)	H	H	H	
6,8-Diprenylorobol (**230**)	OH			OH
6,8-Diprenylgenistein (**231**)	OH			H

Fig. (11). contd….
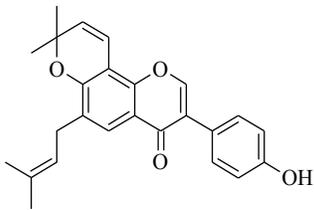
	R
Osajin (**232**)	H
Pomiferin (**233**)	OH
Isopomiferin (**234**)	
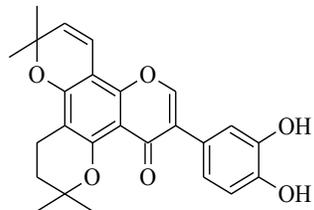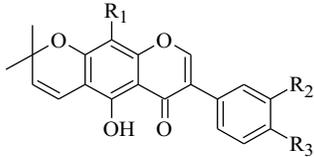
	R₁	R₂	R₃
	R$_1$	R$_2$	R$_3$
Alpinumisoflavone (**235**)	H	H	OH
4′-*O*-Methylalpinumisoflavone (**236**)	H	H	OCH$_3$
Scandenone (**237**)		H	OH
Auriculasin (**238**)		OH	OH
Griffonianon D (**239**)			
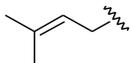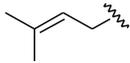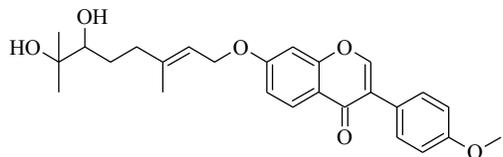
Fig. (11). Structures of prenylated isoflavones and isoflavanones.

dendritic cells, which are migratory cells that can ingest foreign substances and organisms, and have a crucial role as presenters of antigens to lymphocytes to trigger an immune response [15]. There are two major types of lymphocytes: B lymphocytes, which mature in the bone marrow and T lymphocytes, which mature in the thymus [14]. Natural killer (NK) cells are lymphocyte-like cells that kill some types of tumour cells and virus-infected cells [15].

This review will provide an overview on all important control points of inflammation and summarise how PPCs modulate the inflammatory response both *in vitro* and *in vivo*.

2. ANTI-INFLAMMATORY ACTIVITIES OF PRENYLATED PHENOLIC COMPOUNDS

2.1. Modulation of production and release of inflammation-related cytokines

Cytokines are small secreted polypeptides that display specific influence on the interactions and communication between cells. Cytokines are released by cells and may act in an autocrine, paracrine or endocrine manner [17]. Pro-inflammatory cytokines are involved in the up-regulation of inflammatory reactions. The main control point of not-only cytokine expression during inflammation is the regulation of their transcription [13]. The crucial role among transcription factors

Glyceollin A (**240**)	
Glyceollin B (**241**)	
Glyceollin C (**242**)	
Erycristagallin (**243**)	
Psoralidin (**244**)	

Fig. (12). Structures of prenylated pterocarpanes.

	R_1	R_2	R_3	R_4
Lepidotol A (**245**)				=O
Mammea A/AA (**246**)			H	OH

Fig. (13). Structures of prenylated neoflavonoids.

responsible for the inflammatory response play nuclear factor κB, interferon regulatory factors (IRFs), signal transducers and activators of transcription (STAT), and activator protein 1 (AP-1) [18]. Intracellular signaling pathways leading to the activation of these transcription factors create the net, which orchestrates and fine-tunes the expression of hundreds of genes contributing to an inflammatory reaction, where genes for cytokines represent a significant part. Simplified intracellular inflammatory-related signaling pathways are shown in Fig. (**28**). There are also marked points, where the inhibitory activity of PPC was proven.
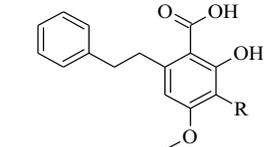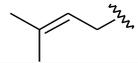
	R
Amorfrutin A (**247**)	
Amorfrutin B (**248**)	
Cajaninstilbene acid (**249**)	
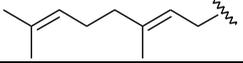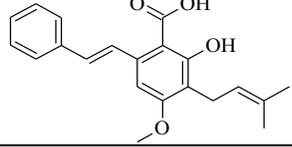
Fig. (14). Structures of prenylated stilbenes.

	R₁	**R₂**	**R₃**	**R₄**
Moracin C (**250**)	H	OH	H	
Mulberrofuran Y (**251**)		OCH₃	OH	H
	R₁	**R₂**	**R₃**	
Moracin R (**252**)	OH		H	
Artoindonesianin O (**253**)	OCH₃		H	
Alabafuran A (**254**)	OH	H		
Mornigrol D (**255**)	OH	H		
Mulberrofuran H (**256**)	OH		H	
Mongolicin C (**257**)	OH		H	
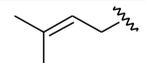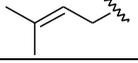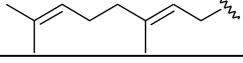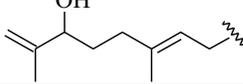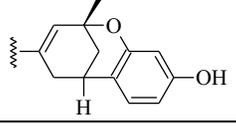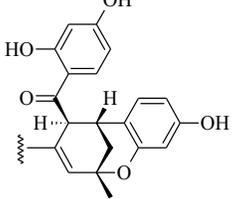
Fig. (15). contd….

Moracin D (**258**)				
	R₁	**R₂**	**R₃**	**R₄**
3′,5′-dihydroxy-6-methoxy-7-prenyl-2-arylbenzofuran (**259**)		OCH₃	H	H
Moracin N (**260**)	H	OH		H
Moracin S (**261**)		OH	H	H
Moracin T (**262**)	H	OH	OCH₃	
Regiafuran A (**263**)	H	OH		OCH₃
Regiafuran B (**264**)	H	OCH₃		OCH₃
Regiafuran C (**265**)	H	OH		OCH₃
Mulberrofuran L (**266**)		OH	H	H
Moracin O (**267**)				
	R₁		**R₂**	
Moracin P (**268**)	OH		OH	
Moracin Q (**269**)	OCH₃		OCH₃	
Moracin U (**270**)				

Fig. (15). Structures of prenylated arylbenzofurans.
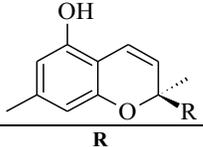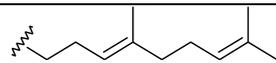
	R
Confluentin (**271**)	
Daurichromen A (**272**)	
Daurichromen B (**273**)	
Daurichromen C (**274**)	
Daurichromen D (**275**)	
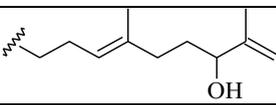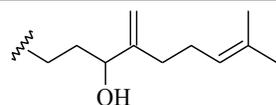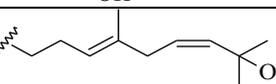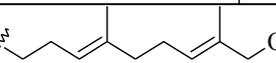
Fig. (16). Structures of prenylated chromenes.

	R₁	R₂	R₃	R₄	R₅	R₆	R₇	R₈
α-Mangostin (**276**)	H	OH	OCH$_3$	prenyl	OH	prenyl	OH	H
γ-Mangostin (**277**)	H	OH	OH	prenyl	OH	prenyl	OH	H
Bangangxanthon B (**278**)	H	H	H	OH	OH	prenyl	H	OH
Afzeliixanthone A (**279**)	H	OH	H	OH	OCH$_3$	prenyl	H	OH
1,3,5,7-tetrahydroxy-8-isoprenylxanthone (**280**)	OH	H	OH	prenyl	OH	H	OH	H
1,5,8-trihydroxy-3-methoxy-2-(3-methyl-2-buten-1-yl)xanthone (**281**)	OH	H	H	OH	OH	prenyl	OCH$_3$	H
Garciniaxanthone H (**282**)	OH	H	H	OH	OCH$_3$	OH	H	prenyl
Symphoxanthone (**283**)	OH	OH	H	H	OH	OH	H	prenyl
Gartanin (**284**)	OH	H	H	OH	OH	prenyl	OH	prenyl
Nuijangexanthone A (**285**)	OH	OH	prenyl	prenyl	OH	OH	OCH$_3$	prenyl
Garcinone D (**286**)	H	OH	OCH$_3$	HO-prenyl	OH	prenyl	OH	H
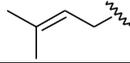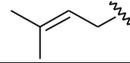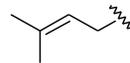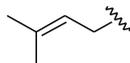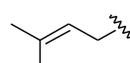
Fig. (17). contd….

Afzeliixanthone B (**287**)	H	H	OCH₃	H	OCH₃		H	OH
Virgaxanthone A (**288**)	OH	OH	H		OH	H	OH	geranyl
Smeathxanthone A (**289**)	OH	H	H	OH	OH	geranyl	OH	H
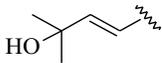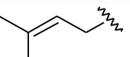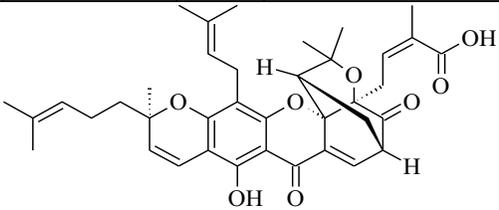
Gambogic acid (**290**)	
Hyperxanthone E (**291**)	
6-Methoxy-bispyranoxanthone (**292**)	
Virgaxanthone B (**293**)	
Bangangxanthon A (**294**)	
Garciniaxanthone D (**295**)	
	R
Garciniaxanthone F (**296**)	
Garciniaxanthone G (**297**)	H
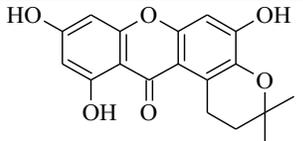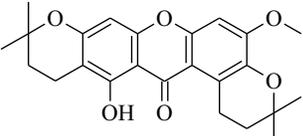
Fig. (17). Structures of prenylated xanthones.

Divaricatol (**298**)	
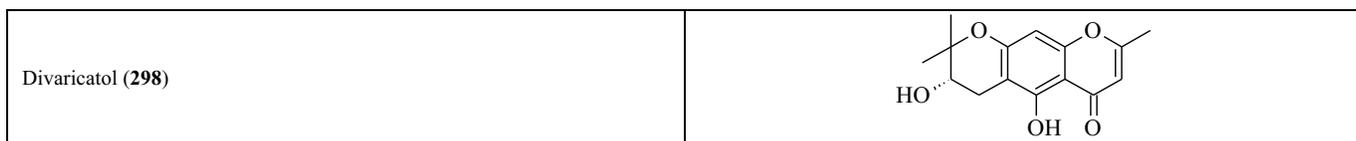
Fig. (18). Structure of prenylated pyrones.

	R₁	R₂
Pseudopterosin Q (**299**)	OH	
Pseudopterosin S (**300**)	OH	
Pseudopterosin T (**301**)	OH	
Pseudopterosin U (**302**)	OH	
Pseudopterosin G (**303**)		OH
Pseudopterosin P (**304**)	OH	
10-acetoxy-9-hydroxy-amphilecta-8,10,12,14-tetraene (**305**)	OH	
9-acetoxy-10-hydroxy-amphilecta-8,10,12,14-tetraene (**306**)		OH
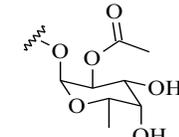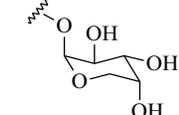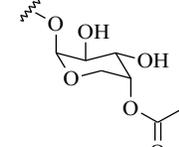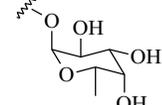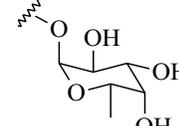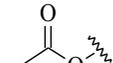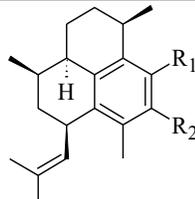
Fig. (19). contd….

	R$_1$	R$_2$
Pseudopterosin A (**307**)		OH
Pseudopterosin E (**308**)	OH	
Pseudopterosin K (**309**)		
	R	
Secopseudopterosin J (**310**)		
Secopseudopterosin K (**311**)		
Amphilectosin A (**312**)		
Amphilectosin B (**313**)		
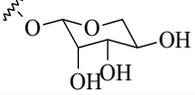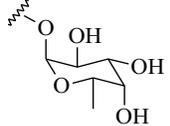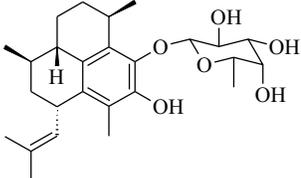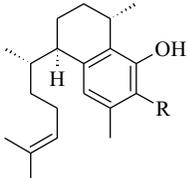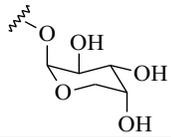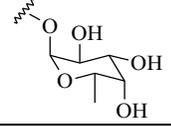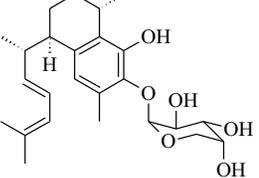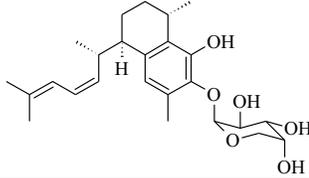
Fig. (19). Structures of pseudopterosins.

One of the most-studied and well-known cytokines is the tumor necrosis factor (TNF-α). It plays an important function in the initiation of inflammation and in the navigating of immune cells from bloodstream to site of inflammation by the expression of adhesion molecules ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) in the endothelium [14, 19]. The production of TNF-α is transcriptionally regulated by transcription factors nuclear factor kappa B (NF-κB), c-Jun, or AP-1. TNF-α is produced by a variety of cells, including activated mac-rophages, fibroblasts, mast cells, T cells and NK cells [20, 21]. The efficacy of anti-TNF-α antibodies and administration of soluble TNF-α receptors (TNFRs) in controlling ailment activity in different inflammatory conditions emphasizes the importance of TNF-α in inflammation process [22]. TNF-α also induces fever, either directly *via* stimulation of prostaglandin E$_2$ (PGE$_2$) synthesis by the vascular endothelium of the hypothalamus, or indirectly by inducing release of interleukin 1 (IL-1) [21].
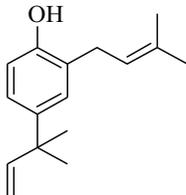
4-(1,1-dimethyl-2-propenyl)-2-(3-methyl-2-butenyl)phenol (**314**)	
Grifolin (**315**)	
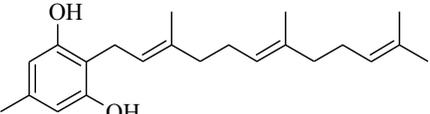
Fig. (20). Structures of prenylated phenol and resorcinol derivatives.

	R₁	R₂
	R₁	**R₂**
1-*O*-β-glucopyranosyl-1,4-dihydroxy-2-(3,3-dimetylallyl)benzene (**316**)		
1-*O*-β-glucopyranosyl-1,4-dihydroxy-2-(3-hydrohy,3-dimetylallyl)benzene (**317**)		
1-*O*-(4′-*O*-caffeoyl)-ß-glucopyranosyl-1,4,-dihydroxy-2-(3′,3′-dimetylallyl)benzene (**318**)		
1-methoxy-4-hydroxy-2-(3′,7′-dimethyl)-2′*E*,6′-octadienylbenzene (**319**)	CH₃	
1,4-dihydroxy-2-(3′,7′-dimethyl-1′-oxo-octa-2′-*E*-6′-dienyl)benzene (**320**)	H	
5-(1,1-dimethylprop-2-enyl)-2-(3-methylbut-2-enyl)cyclohexa-2,5-diene-1,4-dione (**321**)		
2,2-dimethyl-7-(1,1-dimethylprop-2-enyl)-2*H*-chromen-6-ol (**322**)		
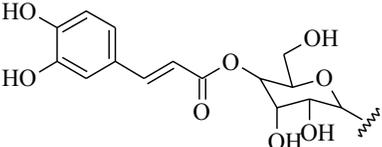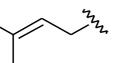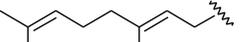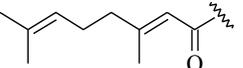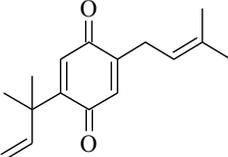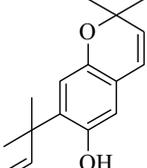
Fig. (21). Structures of prenylated hydroquinone derivatives.

Burmanicumol C (**323**)	

	R
Illifrognone E (**324**)	
Illifrognone G (**325**)	
Illioliganone B (**326**)	

	R₁	**R₂**	**R₃**
Artepillin C (**327**)		OH	
4´-Geranyloxyferulic acid (**328**)	OCH₃		H

Fig. (22). Structures of prenylated phenylpropanoids.

Garcimultiflorone E (**329**)	
Oliveridepsidone A (**330**)	
Oliveridepsidone B (**331**)	

Fig. (23). contd….

Oliveridepsidone C (**332**)	
Oliveridepsidone D (**333**)	
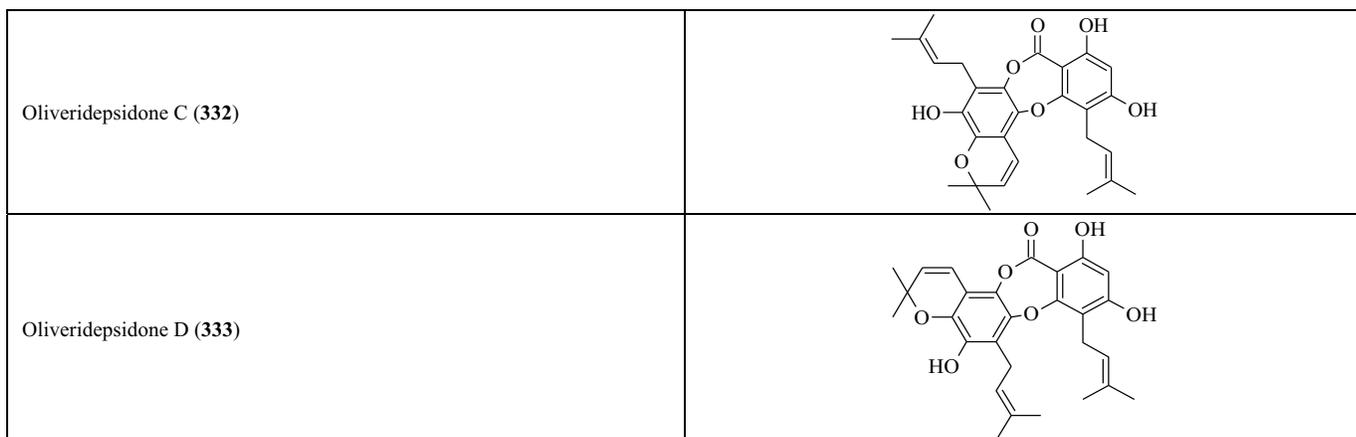
Fig. (23). Structures prenylated phloroglucinol derivatives.

	R
4-Geranyloxy-2,6-dihydroxybenzophenone (**334**)	
4-Geranyloxy-1-(2-methylpropanoyl)-phloroglucinol (**335**)	
4-Geranyloxy-1-(2-methylbutanoyl)-phloroglucinol (**336**)	
4-(1′-Geranyloxy)-2,6,-trihydroxyacetophenone (**337**)	
2-(1′-Geranyloxy)-4,6,-trihydroxyacetophenone (**338**)	

	R₁	**R₂**	**R₃**
2,4,6-Trihydroxy-3-geranylacetophenone (**339**)	OH		
Humudifucol (**340**)	OH		
Panduratin A (**341**)	OCH₃	H	
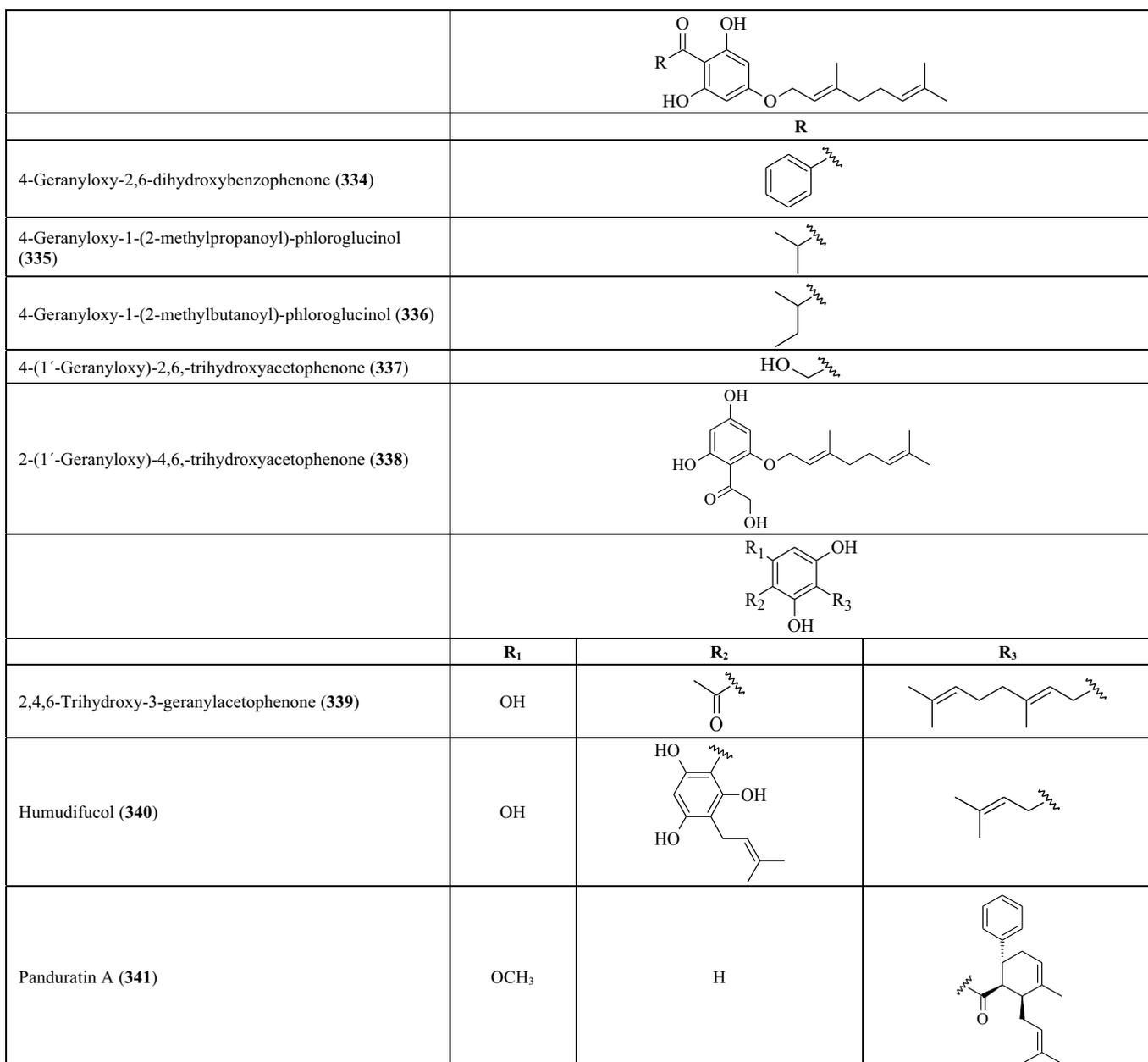
Fig. (24). contd….

4-Hydroxypanduratin (**342**)	OH	H	
1-(2,6-Dihydroxy-4-methoxyphenyl)-3-methylbutan-1-one (**343**)	OCH₃	H	

	R
Humulone (**344**)	OH
Lupulone (**345**)	

4-Hydroxycolupulone (**346**)	

	R₁	**R₂**	**R₃**
Cascadone (**347**)			OH
Lupulon D (**348**)			
Lupulon E (**349**)			
5-Deprenyllupulonol C (**350**)			H

Fig. (24). contd….
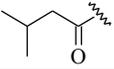
	R
Lupulone C (**351**)	
Colupox A (**352**)	
	R
Sampsonol C (**353**)	
Sampsonol F (**354**)	
Garcinol (**355**)	
7-Epiclusianone (**356**)	
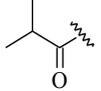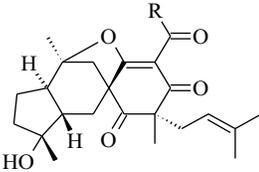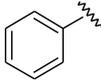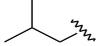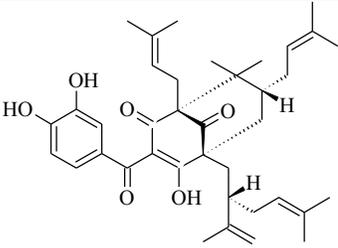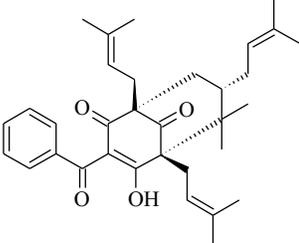
Fig. (24). Structures of prenylated phloroglucinol derivatives.

The other typical member of pro-inflammatory cytokines is a group of IL-1 cytokines, which consists of two distinct proteins called IL-1α and IL-1β. They are mediators of the acute phase of inflammation inducing of local and systemic responses. Signal transduction by IL-1α and IL-1β requires the recruitment of the IL-1 receptor accessory protein finally leading to activation of NF-κB, the c-Jun N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) pathways [23]. The main cellular sources of IL-1α and IL-1β are monocytes, tissue macrophages, dendritic cells, fibroblasts, keratinocytes, and T and B lymphocytes, NK cells and epithelial cells [21, 24]. IL-1 induces the expression of adhesion molecules on endothelial cells, which are required for the infiltration of the stressed tissue by inflammatory cells [24]. IL-1β increases the production of substance P and PGE$_2$ in a number of neuronal and glial cells [25]. IL-1α and IL-1β can trigger fever by enhancing PGE$_2$ synthesis by the vascular endothelium of the hypothalamus. IL-1 evokes the release of histamine from mast cells at the site of inflammation. Histamine then triggers early vasodilation and increases vascular permeability [21].

The other often-studied cytokine IL-6 is produced by mononuclear phagocytes, T cells and fibroblasts. It acts as a growth factor for mature B cells and induces their final maturation. IL-6 stimulates IgM, IgG, and IgA production by activated B cells. In addition, IL-6 is engaged in T cell activation and differentiation [21, 26]. IL-6 is also involved in microglial and astrocytic activation as well as in regulation of neuronal neuropeptides expression [25]. IL-6 activates transcription factor STAT3 that plays critical role in helper T cells development, dendritic cell production, inhibition of macrophage inflammatory signaling, and regulation of steady state and emergency granulopoiesis [27].
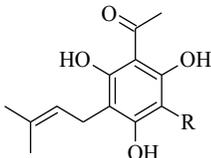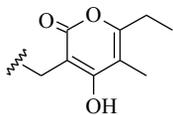
	R
Arzanol (**357**)	
Methylarzanol (**358**)	
Mallotojaponin (**359**)	
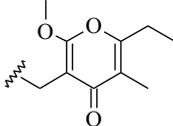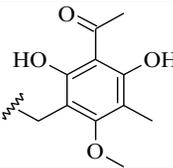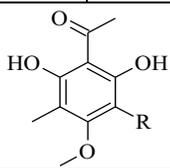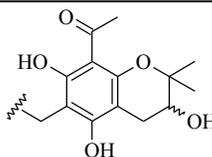
	R
Mallotochromanol (**360**)	
Butyrylmallotorelin (**361**)	
Isobutyrylmallotochromanol (**362**)	
Isomallotochromanol (**363**)	
Isomallotochromene (**364**)	
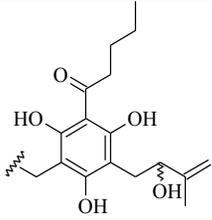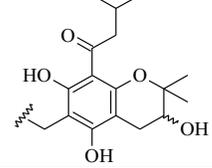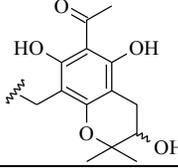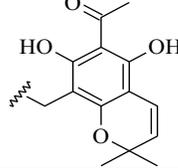
Fig. (25). contd….

Mallotochromene (**365**)	
	R
Mallotophilippen A (**366**)	
Mallotophilippen B (**367**)	CH₃

Fig. (25). Structures of prenylated acylphloroglucinols.

	R
Δ⁹-tetrahydrocannabinol (**368**)	H
Tetrahydrocannabinolic acid (**369**)	COOH
	R
Cannabidiol (**370**)	H
Cannabidiolic acid (**371**)	COOH
	R
Cannabigerol (**372**)	H
Cannabigerolic acid (**373**)	COOH
Cannabichromene (**374**)	

Fig. (26). Structures of cannabinoids.

Apart from the above-mentioned most-studied cytokines, many other pro-inflammatory cytokines and chemokines serve as a model for evaluation of the ability of PPC to modulate inflammatory response. This group includes, for example, C-C motif ligands (CCLs; *e.g.* CCL-2, CCL-5), IL-8, monocyte chemoattractant protein 1 (MCP-1), and many others.

On the other hand, there exists a group of so-called anti-inflammatory cytokines, which participate in the termination of the inflammatory response and keep the balance between innate and acquired immune responses [28]. Among typical anti-inflammatory cytokines could be listed, for example, IL-10 and IL-1 receptor antagonist (IL-1RA). Their up-regulation in model
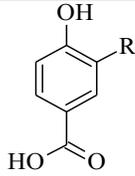
	R
4-Hydroxy-(3′,7′-dimethyl-1′- oxo-octa-2′-*E*-6′-dienyl)benzoic acid (**375**)	
4-Hydroxy-3-(3′,7′- dimethyl-3′-hydroxy-1′-oxo-6′-octenyl)benzoic acid (*syn*. crassinervic acid) (**376**)	
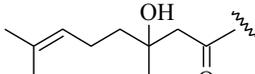
Fig. (27). Structures of prenylated benzoic acid derivatives.

systems can be a hallmark of the anti-inflammatory potential of investigated prenylated phenolic compounds.

2.1.1. Prenylated Coumarins

Osthole (**1**) is a prenylated coumarin that was obtained from the seeds of *Cnidium monnieri* (L.) CUSSON (Apiaceae). This prenylated coumarin dose-dependently decreases the production of TNF-α in lipopolysaccharide(LPS)-activated J774A.1 macrophages. **1** inhibits the phosphorylation of IKK(IκB kinase)-α, reduces IκB-α phosphorylation and inhibits NF-κB transcriptional activity in LPS-activated macrophages. Surprisingly, **1** increases IL-6 expression in LPS-activated J774A.1 macrophages. Further, it has been reported that **1** inhibits the phosphorylation levels of p38 and JNK1/2, and increases the phosphorylation levels of ERK1/2 in LPS-stimulated macrophages [29]. Moreover, osthole (**1**) suppresses the levels of IL-1β, IL-6, MCP-1 and IL-8 in HepG2 cells cultured in a differentiated medium from 3T3-L1 preadipocytes. **1** also decreases the phosphorylation of IκB-α and suppresses MAPKs phosphorylation [30]. **1** significantly suppresses an increased expression of TNF-α and IL-1β in the *in vivo* model of middle cerebral artery occlusion in rats [31].

Auraptene (**21**) (*syn*. 7-geranyloxycoumarin) is a simple oxyprenylated coumarin isolated from plants in the Rutaceae family, such as grapefruit. **21** significantly suppresses the release of TNF-α in a concentration-dependent manner in a murine macrophage line RAW264.7 [32]. **21** decreases, in a dose-dependent manner, the secretion of IL-6, IL-8, chemokine (C-C motif) ligand-5 (CCL-5) and MMP(matrix metalloproteinase)-2 secreted by *Aggregatibacter actinomycetemcomitans* LPS-stimulated oral epithelial cells [33]. **21**

reduces TNF-α and IL-8 secretion by a LPS-stimulated macrophage line [34]. **21** also suppresses MCP-1 and TNF-α secretion in RAW264.7 macrophages activated by a conditioned medium (CM) of hypertrophied 3T3-L1 adipocytes, or by LPS [35]. Lacinartin (**30**) (an oxy-isopentenylated coumarin) significantly inhibits TNF-α and IL-8 secretion in LPS-stimulated macrophages. **30** also supresses *Porphyromonas gingivalis* collagenase activity [34].

8-Acetoxy-5-hydroxyumbelliprenin (**27**) and 5-hydroxyumbelliprenin (**28**), two oxyprenyl coumarin derivatives, isolated from an acetonic extract of asafetida obtained from variety of *Ferula* species (Apiaceae), show inhibitory activity on the TNF-α-mediated activation of NF-κB in the 5.1 cell line. Compound **27** is more active than the corresponding deacetyl derivative (**28**) [36].

7-Prenyloxy-6-methoxycoumarin (**31**) has been isolated from *Polygala sabulosa* A.W. Bennett (Polygalaceae). This coumarin decreases the mRNA expression of TNF-α and IL-1β due to its ability to reduce activation of the NF-κB and p38MAPK pathways [37].

4-Isopentenyloxy-5-methylcoumarin (**42**), found in *Gerbera crocea* Kuntze and *G. serrate* Druce (Asteraceae), 6-isopentenyloxy-7-methoxycoumarin (**32**), obtained from *Haplophyllum pedicellatum* Bunge ex Boiss (Rutaceae), and 8-isopentenyloxy-7-methoxycoumarin (**33**) found in *Artemisia laciniata* Willd., *A. armeniaca* Lam., *A. armeniaca* Georgi., *Melampodium divaricatum* DC. (Asteraceae), *Coleonema calycinum* (Steud.) I. Williams, *Flindersia australis* (Rutaceae) and *Cyperus incompletes* Boeckeler (Cyperaceae) have been studied for their anti-inflammatory properties. It has been found that compounds **32** and **33** inhibit LPS-stimulated NF-κB acti-

vation in dose-dependent manner. Compound **42** is inactive [38].

Omphalocarpin (**3**), which has been isolated from *Radix Toddalie* (*Toddalia asiatica* (L.) Lam*.,* Asiaticae), the traditional Chinese medicine, dose-dependently suppresses LPS-stimulated TNF-α and IL-6 secretion in RAW264.7 macrophages. Omphalocarpin (**3**) also reduces phosphorylation of IκB-α and translocation of NF-κB p65 subunit to the nucleus in RAW264.7 cells induced by LPS [39, 40]. Significantly decreased production of TNF-α, IL-1β, IL-2 and IL-4 has further been observed for other prenylated coumarins osthole (**1**), tamarin (**4**), ulopterol (**5**), imperatorin (**43**), oxypeucedanin hydrate (**46**), oxypeucedanin (**47**), isogospherol (**48**), heraclenol (**50**) and majurin (**65**) obtained from roots of *Prangos pabularia* Lindl. (Apiaceae) [41-44].

Zosima absinthifolia (Vent.) Link (Apiaceae) extracts and two isolated coumarins deltoin (**59**) (linear) and columbianadin (angular) (**62**) show inhibitory effect on TNF-α production in the LPS-stimulated THP-1 human macrophages. The *n*-hexane root extract showed greater activity than both coumarins **59** and **62**, as well as two standard anti-inflammatory drugs: dexamethasone and acetylsalicylic acid. Compounds from the extract may act in a synergistic way, or some other compounds present in extract could be active in the lowering of TNF-α level [45]. Nodakenetin (**61**) and columbianadin (**62**), isolated from the roots of *Angelica decursiva* (Miquel) Franchet & Savatier (Apiaceae) inhibit IL-6 production in IL-1β-treated A549 human lung epithelial cell line [46].

Anomalin (**72**), a pyranocoumarin from *Saposhnikovia divaricate* (Turcz.) Schischk. (Apiaceae), decreases production of several pro-inflammatory cytokines including TNF-α and IL-6. This inhibition correlates with the down-regulation of the NF-κB signaling pathway [47]. Anomalin (**72**) also effectively blocks the sodium-nitroprusside-induced activation of the IKK α/β, IκB α, ERK1/2 and p38 MAPK pathways [48].

(+)-Praeruptorin A (**73**), isolated from the roots of *Peucedanum praeruptorum* Dunn (Apiaceae), suppresses the IL-1β, IL-6 and TNF-α release in LPS-stimulation of macrophages [49]. Praeruptorin C (**74**), D (**75**), and E (**76**), pyranocoumarins further isolated from *P. praeruptorum* significantly inhibit LPS-induced expression of TNF-α and IL-6. Compounds **75** and **76** exhibit greater anti-inflammatory activity than **74**. Praeruptorins show their anti-inflammatory activities in LPS-stimulated RAW264.7 macrophages

through the inhibition of NF-κB and STAT3 activation [50].

2.1.2. Prenylated Chalcones

The hop plant, *Humulus lupulus* L. (Cannabaceae), is a rich source of prenylated chalcones. Xanthohumol (**86**), xanthogalenol (**87**), 4′-*O*-methylxanthohumol (**88**), 2′,4′,6′,4-tetrahydroxy-3′-prenylchalcone (**89**), tetrahydroxanthohumol (**90**), 5′-prenylxanthohumol (**91**), 2′,4′,6′,4-tetrahydroxy-3′-geranylchalcone (**98**), and xanthohumol B (**105**) decrease the MCP-1 production in LPS-stimulated THP-1 cells and they further down-regulate IL-6 production. Tetrahydroxanthohumol (**90**) is the most potent inhibitor of IL-6 among the hop prenylated chalcones. Xanthohumol (**86**) inhibits both MCP-1 and IL-6 in dose-dependent manner [51]. Xanthohumol (**86**) also reduces the level of Toll like receptor 4 (TLR4) in LPS-activated THP-1 and RAW264.7 cells, resulting in the suppression of NF-κB activation. **86** significantly decreases the expression of TNF-α and IL-1β in a dose-dependent manner in the LPS-stimulated RAW264.7 macrophages. Moreover, **86** inhibits binding activity of STAT1α and IRF-1 in the IFN-γ-stimulated RAW264.7 cells [52]. **86** decreases mitogen/antigen-induced T cell proliferation, and Th1 cytokines production by inhibiting NF-κB *via* the suppression of IκBα phosphorylation [53].

4-Hydroxyderricin (**92**), xanthoangelol D (**94**), xanthoangelol E (**95**), xanthoangelol (**99**), xanthoangelol F (**100**), xanthoangelol B (**101**), 2′,4′,4-trihydroxy-3′-[2-hydroxy-7-methyl-3-methylene-6-octaenyl]chalcone (**102**) and xanthokeismin A (**103**) are prenylated chalcones, isolated from *Angelica keiskei* Koidzumi (Apiaceae). Xanthoangelol (**99**), xanthoangelol B (**101**) and compound **102** strongly inhibit IL-6 production in TNF-α stimulated MG-63 cell. Xanthoangelol B (**101**) and xanthoangelol E (**95**) significantly decrease the levels of IL-6 mRNA in activated RAW264.7 macrophages. Moreover, xanthoangelol B (**101**) strongly inhibits the transcription of IL-1β. Xanthoangelol B (**101**), xanthokeismin A (**103**), 4-hydroxyderricin (**92**) and xanthoangelol E (**95**) inhibit the activity of NF-κB through the inhibition of IKK. 4-Hydroxyderricin (**92**) and xanthoangelol (**99**) also decrease LPS-induced secretion of TNF-α [54-56].

Mallotophilippens C (**113**), D (**114**), and E (**115**) are the chalcone derivatives obtained from the fruits of *Mallotus philippinensis* Muell. Arg (Euphorbiaceae). These compounds inhibit the mRNA expression of the IL-1β and IL-6 in the LPS- and IFN-γ-activated RAW264.7 cells. The principal inhibitory mechanism of mallotophilippens is supposed to be the inactivation

of NF-κB [57, 58]. The geranyl derivatives isolated from the fruit of *Artocarpus communis* Forst. (Moraceae) suppress the transcription of TNF-α and IL-6 in S100 calcium-binding protein B (S100b) induced THP-1 monocytes. 4,2′,4′-Trihydroxy-3′-[6-hydroxy-3,7-dimethyl-2(*E*)-7-octadienyl]chalcone (**123**) exhibits moderate inhibitory effect, whereas 3,4,2′,4′-tetrahydroxy-3′-geranyldihydrochalcone (**124**) and 4,2′,4′-trihydroxy-5′-geranyldihydrochalcone (**125**) exerts more significant inhibitory activity [59].

Kuraridin (**106**), a lavandulyl chalcone isolated from *Sophora flavescens* Aiton (Fabaceae), decreases the expression of TNF-α, IL-1β and chemokine CCL-2 in LPS-stimulated RAW264.7 macrophages [60]. This supports the finding that a flavonoid-enriched fraction of the rhizomes of *S. flavescens* lowered production of TNF-α and IL-6 in the same cell system [61].

2.1.3. Prenylated Flavonoids

(2*S*)-7,4′-dihydroxy-8-prenylflavan (**221**) is a prenylated flavan isolated from the leaves of *Morus yunnanensis* Koidz. (Moraceae). It decreases the expression of inflammatory cytokine TNF-α in 3T3-L1 cells [62]. 8-prenylquercetin (**151**), found in *Desmodium caudatum* (Thunb.) DC. (Fabaceae), decreases significantly the level of IL-1, IL-6, IFN-γ, TNF-α, IL-3, IL-9, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), eotaxin, IL-17, granulocyte-colony stimulating factor (G-CSF), and MCP-1 in RAW264.7 cells. **151** directly targets and inhibits SEK1-JNK1/2 and MEK1-ERK1/2 signaling pathways [63].

Epimedonin D (**160**) and 5,7,4′-trihydroxy-8,3′-diprenylflavone (**152**) obtained from *Epimedium koreanum* Nakai. (Berberidaceae) decrease the level of IL-1β production in LPS-stimulated RAW264.7 cells [64]. Isoxanthohumol (**140**), 8-prenylnaringenin (**127**), 6-prenylnaringenin (**128**), 6,8–diprenylnaringenin (**129**), 8-geranylnaringenin (**198**) and 6-geranylnaringenin (**177**) (*H. lupulus*) have been tested for inhibitory effect on MCP-1 and IL-6 production in THP-1 cells. 8-geranylnaringenin (**58**) shows the most potent inhibitory effect on both MCP-1 and IL-6 expression. Isoxanthohumol (**140**) inhibits MCP-1 and IL-6 in dose-dependent manner, while 8-prenylnaringenin (**127**) and 6-prenylnaringenin (**128**) reduce only IL-6 production and do not inhibit MCP-1 expression [51]. Isoxanthohumol (**140**) decreases level of TNF-α and NF-κB in human aortic smooth muscle cells and human umbilical vein endothelial cells [65]. Highly **140** enriched hop extract obtained by pressurized hot water extraction decreases the production of TNF-α and IL-6 and in-

creases the production of anti-inflammatory cytokine IL-10 [66].

Diplacone (**178**), obtained from the extracts of *Paulownia tomentosa* Steud. (Paulowniaceae), down-regulates the expression of pro-inflammatory genes of TNF-α and MCP-1 and up-regulates mRNA of regulatory zinc finger protein 36 (ZFP36) in human monocyte-derived macrophages THP-1. The effect of this geranyl flavanone is in many ways similar to that of indomethacin [67]. 6-Geranyl-5,7-dihydroxy-3′,4′-dimethoxyflavanone (**179**), 3′-*O*-methyldiplacone (**180**), tomentodiplacone B (**186**), 3′-*O*-methyl-5′′-*O*-methyldiplacone (**182**), 3′-*O*-methyldiplacol (**190**), mimulone H (**188**), and tomentodiplacone N (**195**), all also isolated from fruits of *P. tomentosa*, decrease the secretion of TNF-α in LPS-activated macrophages as much as or more than the prednisone that was used as a positive control [68].

Sanggenon H (**144**), kuwanon C (**153**), cudraflavone B (**164**), morusinol (**170**), and kuwanon E (**184**) [prenylated flavonoids from root extracts of *Morus alba* L. and *M. nigra* L. (Moraceae)] have been efficient in significant reductions of the secretion of TNF-α in LPS-stimulated macrophages, but the production of this cytokine was in no case significantly lower than that of prednisone used as a positive control. Morusinol (**170**) has been found to be the most potent inhibitor of TNF-α [69]. Cudraflavone B (**164**) decreases the gene expression and secretion of TNF-α by blocking the translocation of NF-κB from the cytoplasm to the nucleus in macrophages derived from a THP-1 cell line, more effectively than indomethacin does [70]. Sangennon H (**144**), kuwanon C (**153**) and kuwanon E (**184**) greatly reduce the level of IL-1β. Morusinol (**170**) shows moderate inhibitory effect on IL-1β production, while cudraflavone B (**164**) does not significantly down-regulate production of IL-1β [69]. Kuwanon E (**184**) and kuwanon G (**176**) significantly decrease the production of IL-6 in lung epithelial cells A549 [71].

Sophoraflavanone G (**199**) and kurarinone (**200**) are two lavandulyl flavonoids isolated from the rhizomes of *S. flavescens*, which flavonoid-enriched fraction decreases the production of TNF-α and IL-6 in LPS-stimulated RAW264.7 cells [61]. Sophoraflavanone G (**199**) decreases the production of TNF-α, IL-1β and IL-6 in RAW264.7 macrophages through the interruption of the NF-κB and MAPK signaling pathways [72]. Kurarinone (**200**) inhibits the expression of TNF-α, IL-1β and chemokine CCL-2 in LPS-stimulated RAW264.7 macrophages [60].

2.1.4. Miscellaneous Prenylated Phenolics

Glabridine (**223**), a prenylated isoflavonoid of *Glycyrrhiza glabra* L. (Fabaceae), inhibits LPS-induced TNF-α and IL-1 release in murine macrophages and microglia cells. The down-regulation of NF-κB as well as down-regulation of AP-1 has also been observed. **223** shows to be an inhibitor of nuclear translocation of NF-κB p65/p50 and phosphorylation of elements in MAPKs signaling pathways [73]. Neobavaisoflavone (**229**), found in *Psoralea corylifolia* L. (Fabaceae), shows inhibitory properties in the production of TNF-α, IL-1β, IL-6, IL-12p40, IL-12p70 in activated RAW264.7 macrophages [74]. Prenylated pterocarpans glyceollins are members of a group of soybean phytoalexins whose biosynthesis is increased in response to various stress signals such as fungal infection. Glyceollin A (**240**), B (**241**) and C (**242**) exert an anti-inflammatory effect through the inhibition of NF-κB activation in LPS-activated RAW264.7 cells, resulting in the suppression of the production of IL-6, TNF-α and IL-1β. Glyceollins also inhibit the phosphorylation of IKK, the degradation of IκBα, and the formation of NF-κB-DNA binding complex in a dose-dependent manner [75, 76]. Lepidotol A (**245**), the neoflavonoid isolated from the fruits of *Mesua lepidota* (Calophyllaceae), significantly decreases the level of VCAM-1 and ICAM-1, both strongly inducible by TNF-α [77]. Gambogic acid (**290**), a prenylated xanthone obtained from *Garcinia hanburyi* Hook.f. and *G. morella* (Gaertn.) Desr. (Clusiaceae), decreases the secretion of IL-1 and TNF-α in the THP-1 cells [78]. Moracin C (**250**), mulberrofuran Y (**251**), and mulberrofuran H (**256**) are arylbenzofurans isolated from root extracts of *M. alba* and *M. nigra*. All three compounds reduce the secretion of TNF-α in LPS-stimulated macrophages [69].

Burmanicumol C (**323**), illifrognone E (**324**), illifrognone G (**325**), and illioliganone B (**326**), prenylated phenylpropanoids isolated from the stem bark of *Illicium burmanicum* Wils. (Schisandraceae), show moderate inhibitory effect on the NF-κB pathway [79]. Arzanol (**357**), the prenylated α-pyrone phloroglucinol isolated from *Helichrysum italicum* G. Don. (Asteraceae), decreases the NF-κB activation as well as the release of TNF-α, IL-1β, IL-6, IL-8 in the LPS-stimulated monocytes [80-82]. Mallotojaponin (**359**), mallotochromanol (**360**), butyrylmallotolerin (**361**), isobutyrylmallotochromanol (**362**), and isomallotochromanol (**363**) are phloroglucinols from the pericarp of *Mallotus japonicus* Mueller Arg. (Euphorbiaceae) [83, 84]. Isomallotochromene (**364**) is derived from

359 by cyclization of its side chain. Compounds **359**, **360**, **363** and **364** significantly reduce the secretion of TNF-α and IL-6 in LPS-stimulated RAW264.7 cells. Moreover, **359**, **360**, **362**, **363** and **364** are also active in human blood monocytes. Compounds **363** and **364** showing the *para*-cyclization are the greatest inhibitors. Phloroglucinol derivatives **363** and **364** from *M. japonicus* further inhibit the DNA-binding activity of NF-κB in the LPS-stimulated RAW 264.7 cells [83].

The two major cannabinoids present in *Cannabis sativa* L. (Cannabaceae), Δ⁹- tetrahydrocannabinol (THC) (**368**) and cannabidiol (CBD) (**370**) decrease the production and release of IL-1β, IL-6 and IFN-β from LPS-activated microglial cells. The anti-inflammatory action of cannabinoids not involves only their action on cannabinoid receptors CB1 and CB2 or the abn-CBD(abnormal cannabidiol)-sensitive receptors, but compound **370** also reduces the activity of NF-κB pathway, up-regulates the STAT3 activation, an element of homeostatic mechanism inducing anti-inflammatory events. Both compounds **368** and **370** decrease the activation of the LPS-induced STAT1 transcription factor, important in IFN-β-dependent pro-inflammatory processes [85].

2.2. Inhibition of the Release of Enzymes and Mediators from Neutrophils, Mast Cells and Macrophages

Granules of leukocytes store a mixture of cytotoxic molecules, including many enzymes and antimicrobial peptides that are released in response to stimuli during a process known as degranulation. Two types of granules are distinguished in neutrophils on the basis of their affinity to specific histologic staining dye. Specific granules are characterized as secretory granules that play important roles in initiating the inflammatory response, whereas azurophil granules are often viewed as lysosomes that are particularly active in the digestion of phagocytosed material. Azurophilic granules contain myeloperoxidase (MPO), β – glucuronidase, α₁-antitrypsin lysozyme, e.c. Specific granules are smaller and contain lysozyme, lactoferrin, collagenase, heparinase, gelatinase *etc.* [86, 87].

MPO is a well-known enzyme, mainly released by activated neutrophils. MPO plays an important role in the initiation and progression of acute and chronic inflammatory diseases, such as cardiovascular disease. MPO catalyzes the conversion of chloride and hydrogen peroxide to hypochlorite that is secreted during inflammatory conditions. It has been implicated in the oxidation of lipids contained within low-density
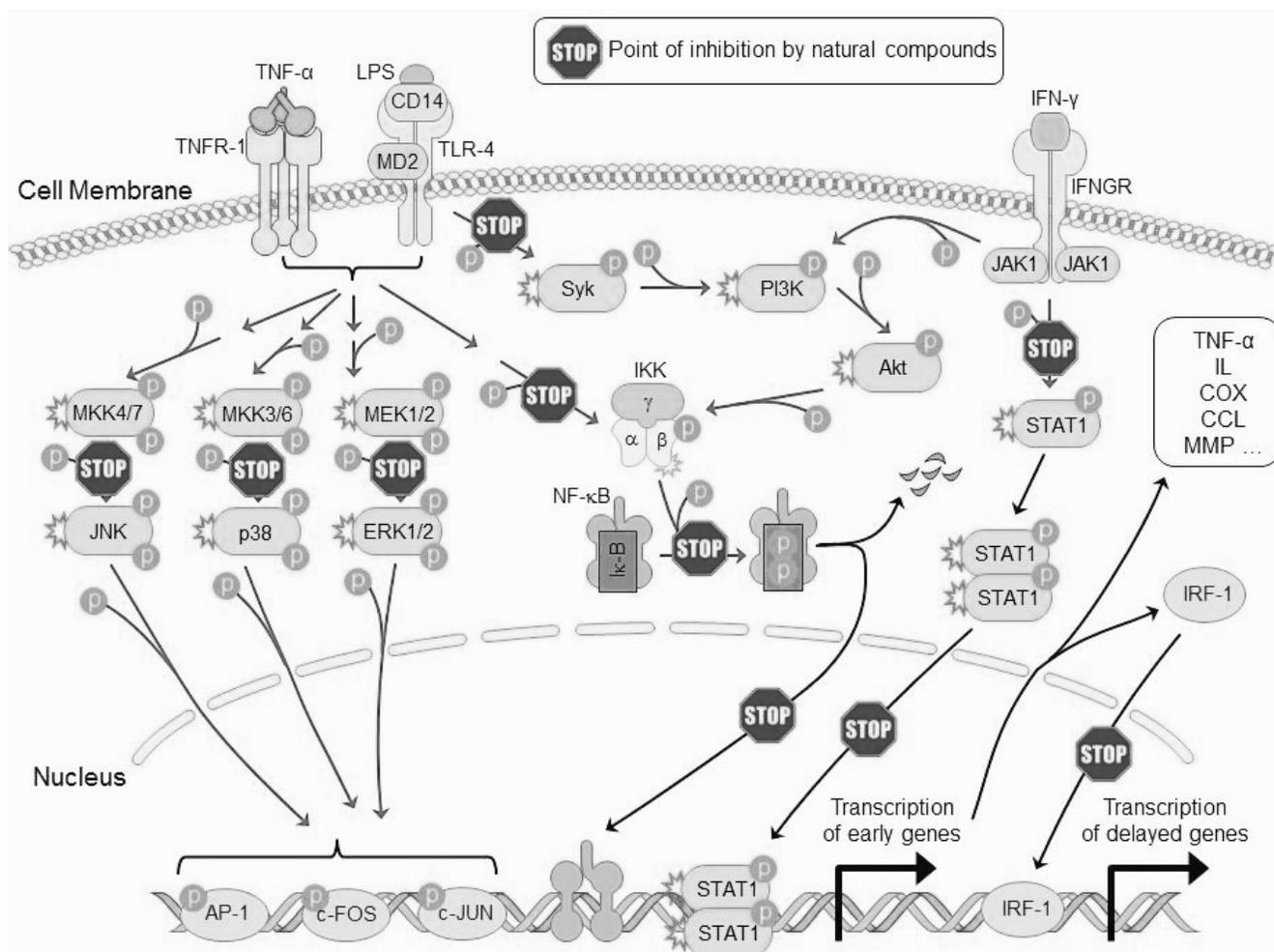
Fig. (28). Points in intracellular signaling pathways, where PPC inhibit the transmission of extracellular pro-inflammatory signal, are indicated as "Stop" mark.

lipoprotein (LDL) particles. In addition, MPO consumes endothelial-derived nitric oxide NO, thereby reducing NO bioavailability and impairing its vasodilating and anti-inflammatory properties [88, 89]. β - glucuronidase is a neutrophil-derived lysosomal acid hydrolase, stored in the azurophil granules and released in response to inflammation. This enzyme is active in the degradation of proteoglycans and components of the ground substances. The presence of β - glucuronidase is also an indicator of neutrophil influx into the inflammation site. The activity of β-glucuronidase is associated with the severity of inflammation in periodontal disease, thus making β - glucuronidase an important biochemical marker [90]. Matrix metalloproteinases (MMPs) are a large family of calcium-dependent zinc-containing endopeptidases, which are responsible for the tissue remodeling and degradation of the extracellular matrix (ECM), including collagens, elastins, gelatin, matrix glycoproteins, and proteoglycan. MMPs are usually minimally ex-

pressed in normal physiological conditions to maintain homeostasis [91].

Gelatinase B (MMP-9) and neutrophil collagenase (MMP-8) play a key role in the migration of immune cells to the site of inflammation. MMPs degrade basal membranes and extracellular matrix components and are therefore important effector molecules for migration [92]. Histamine, produced and released by basophils and mast cells, plays a central role in inflammatory process. Histamine is a vasodilator and a potent stimulant of vascular permeability, responsible for redness, wheal, flare and pruritus [93, 94].

2.2.1. Miscellaneous Prenylated Phenols

Mallotophilippens A (**366**) and B (**367**) decrease histamine release from rat peritoneal mast cells induced by compound 48/80 (*p*-methoxy-*N*-methylphenethylamine). Mallotophilippens may inhibit histamine release through their inhibitory effect on NO production caused by inhibition of iNOS [95]. Confluentin

(**271**), daurichromenes A (**272**), B (**273**), C (**274**), D (**275**) and grifolin (**315**), prenylated orcinol derivatives isolated from *Rhododendron dauricum* L. (Ericaceae), significantly inhibit compound 48/80-induced histamine release from rat peritoneal mast cells [96]. The same effect was also observed for oxypeucedanin hydrate (**46**), and byakangelicin (**52**) [97]. Dihydroisocycloartomunine (**208**), isolated from *Artocarpus communis* and *A. heterophyllus*, inhibits the release of histamine from compound 48/80-induced rat peritoneal mast cells [98].

Treatment with dihydroisocycloartomunine (**208**), as well as histamine, decreases the release of β - glucuronidase from the rat peritoneal mast-cells induced by compound 48/80 [98]. Further experiments using PAF as an inductor of *β*-glucuronidase release from rat polymorphonuclear leucocytes have shown inhibitory effects of moracin C (**250**) [69, 99], while from five PPC isolated from *Morus nigra* [namely mornigrol D (**255**), mornigrol G (**172**), mornigrol H (**174**), albanin A (**154**) and albanin E (**196**)], only mornigrol D (**255**) shows potent inhibitory activity [100]. The effects of mongolicin C (**257**) and mongolicin E (**218**) (isolated from *Morus mongolica* Schneid.), and guangsangon H (**219**) and I (**220**) isolated from *Morus macroura* Miq. on similar model of PAF-stimulated PMNs *β*-glucuronidase release has further been observed [101, 102].

Pseudopterosins are tricyclic diterpene glycosides isolated from the Caribbean Sea whip *Pseudopterogorgia elisabethae* Bayer (Gorgoniidae) [103]. Pseudopterosin Q (**299**), S (**300**), T (**301**) and U (**302**) are potent inhibitors of myeloperoxidase (MPO) release in PMNs. They exhibit greater inhibitory effects than dexamethasone and indomethacin. Pseudopterosin G (**303**) and seco-pseudopterosin K (**311**) are moderate inhibitors, while pseudopterosin K (**309**) and P (**304**) do not inhibit MPO release [104]. Auraptene (**21**) and lacinartin (**30**) both reduce neutrophil collagenase (MMP-8) and gelatinase B (MMP-9) secretion in LPS-stimulated macrophages [34]. Cnidicin (**45**), furanocoumarin found in *Angelica koreana* Maxim. (Apiaceae), dose-dependently decreases the release of β - hexosaminidase, a marker for mast cells degranulation in various allergic inflammation, in cultured RBL-2H3 cells [105]. 6,8-Diprenylorobol (*syn.* 5,7,3′,4′-tetrahydroxy-6,8-diprenylisoflavone) (**230**), isolated from the fruits of *Maclura tricuspidata* (*syn. Cudrania tricuspidata*) (Carriere) Bureau (Moraceae), attenuates the release of β-hexosaminidase from mast cells. Compound **230** suppresses mast cell degranulation and cal-

cium influx, and also inhibits mast cell activation *via* the inhibition of FcεRI-mediated Syk activation [106].

2.3. Inhibition of the Production of Prostaglandins and Leukotrienes

2.3.1. The Arachidonic Acid Pathway Constitutes

Cyclooxygenase (COX) and lipoxygenase (LOX) arachidonate products, including prostaglandins (PGs), leukotrienes (LTs), and hydroxyeicosatetraenoic acids (HETEs), are well-known modulators of inflammatory processes [107]. PGs and thromboxane A_2 (TXA_2), also known as prostanoids, are formed when AA, a 20-carbon unsaturated fatty acid, which is released from the plasmatic membrane by phospholipases (PLAs), is metabolized by the sequential actions of cyclooxygenases (COX), and respective synthases [108]. Secretory phospholipases A2 (sPLA2s) are a group of extracellular enzymes that release fatty acids at the sn-2 position of phospholipids. Different sPLA2s are considered as digestive, neurotoxic, myotoxic, and anticoagulant enzymes. The recovery of large quantities of IIA group of sPLA2 from inflammatory fluids and increased levels of this enzyme in plasma of patients with inflammatory diseases (such as septic shock, acute respiratory distress syndrome, acute pancreatitis) suggests that sPLA2s play an important role in inflammation. Eicosanoids generated from AA released by sPLA2 are potent mediators of inflammation by influencing vascular and bronchial responses and promoting inflammatory cell recruitment [109].

The cyclooxygenase (COX) enzymes catalyse a key step in the conversion of AA to prostaglandin H_2 (PGH_2), the immediate substrate for a series of cell-specific prostaglandin and thromboxane synthases [110]. There are two COX isoforms, constitutive COX-1 and inducible COX-2 (COX-3 probably also exists). Both isoforms of COX catalyze the production of prostanoids from AA [111]. COX-1, expressed constitutively in many tissues, is the dominant source of prostanoids that subserve housekeeping functions, such as gastric epithelial cytoprotection and homeostasis. COX-2 is induced by inflammatory stimuli, hormones and growth factors [108]. COX-2-induced production of prostanoids is implicated in inflammatory diseases, characterized by edema and tissue injury due to the release of many inflammatory cytokines and chemotactic factors, prostanoids and LTs [111]. Prostanoids derived from COX metabolism of AA regulate the changes in blood flow required for leukocytes to exit the postcapillary venules in the initiation phase of inflammation [112]. Expression of COX-2 mRNA and protein
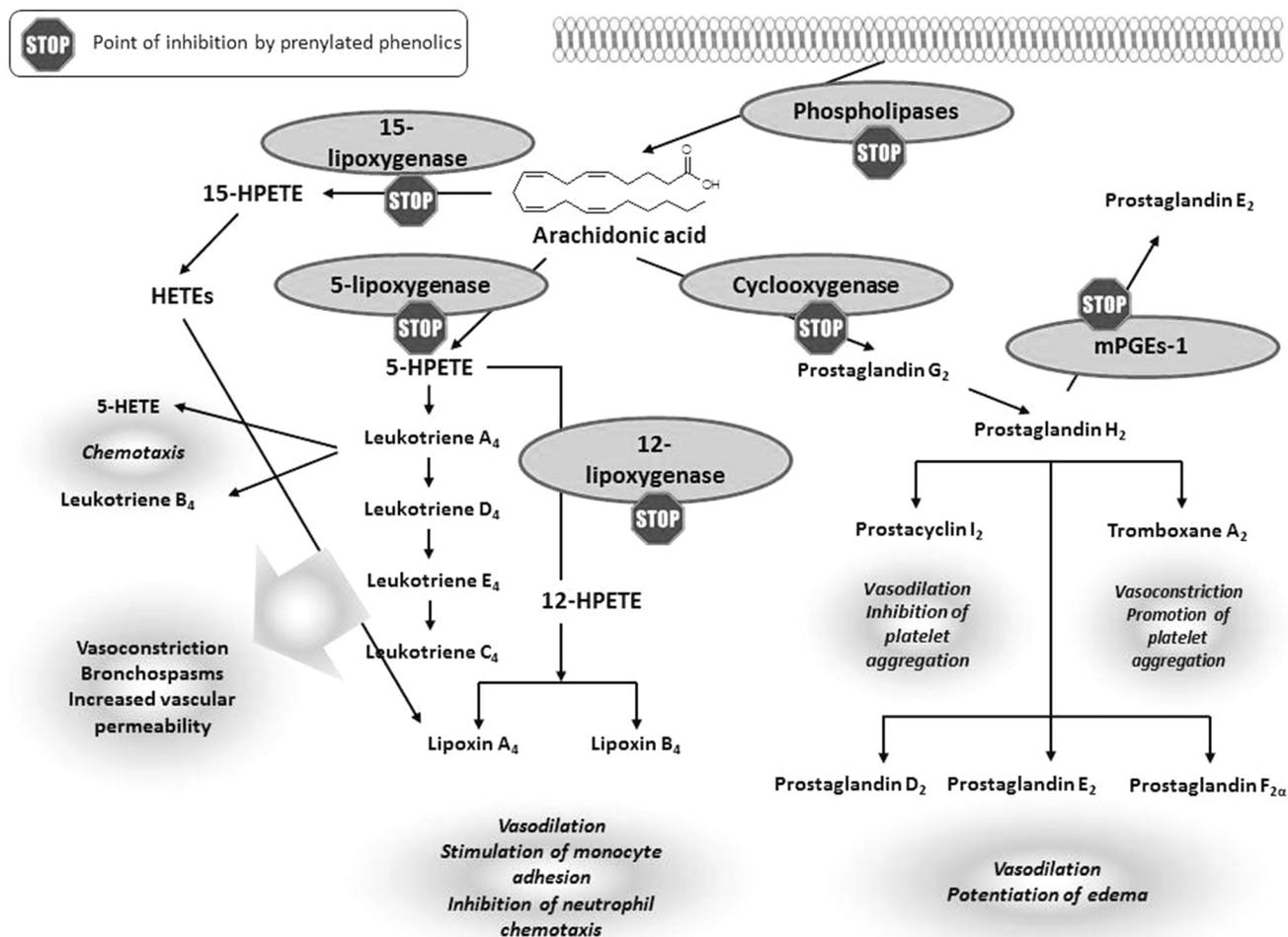
Fig. (29). Points in arachidonic acid metabolism pathway, where PPC inhibits the AA enzymes, indicated as "Stop" mark.

is often enhanced in various human cell types by inflammatory cytokines such as IL-1β and TNF [111]. LTs are important agents involved in the acute inflammation and asthma. Some, such as LTB₄, have a chemotactic effect on migrating neutrophils. LTs have also a powerful effect on bronchoconstriction and increase of vascular permeability [113]. 5-lipoxygenase (5-LOX), highly expressed in human leukocytes, is the key enzyme in the LTs biosynthesis. The first step in the 5-LOX pathway involves the conversion of AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE). 5-HPETE is metabolized into 5-hydroxyeicosatetraenoic acid (5-HETE), or could be dehydrated into LTA₄, which is enzymatically hydrolyzed to LTB₄. 12-LOX and 15-LOX convert AA to 12-HPETE and 15-HPETE, respectively. HPETEs are rapidly metabolized to 12-HETE and 15-HETE. LTB₄, 12-HETE and 15-HETE activate various blood cell types and stimulate their pro-inflammatory cytokine productions [114].

Prenylated phenolics can interact on several points of AA metabolism, as indicated, together with some effects of AA metabolites, on Fig. (**29**), and it is further explained in the following chapters of the text.

2.3.2. PLA2 Inhibitors

Diterpenic pseudopterosin A (**307**), found in marine gorgonian *Pseudopterogorgia elisabethae*, inhibits pancreatic PLA2, as well as production of PGE₂ and LTC₄ in zymosan-stimulated murine peritoneal macrophages in a dose-dependent manner. Pseudopterosin E (**308**), in comparison with compound **307**, is not so effective. Anti-inflammatory activity of both compounds could therefore be caused by interaction with COX and LOX pathways [115]. Moreover, compound **307** also inhibits the formation of phagosome [116].

Flavonol derivative papyriflavonol A (**161**), isolated from *Broussonetia papyrifera* (L.) Vent. (*Moraceae*), selectively inhibits recombinant human sPLA2s, as the inhibitory effect of **161** against human groups IIA and V sPLA2 has been shown to be strong, whereas against bovine group IB and the human group X sPLA2s activity is low. Furthermore, compound **161** potently inhib-

its the stimulus-induced production of leukotriene C_4 (LTC_4) in mouse bone marrow-derived mast cells [117, 118].

2.3.3. Inhibition of COX Expression and COX Inhibitors

Prenylated coumarin osthole (**1**) inhibits the COX-2 expression in LPS-stimulated RAW264.7 macrophages [29]. **1** significantly decreases mRNA and protein levels of COX-2 *in vivo* in MCAO rats [48]. Auraptene (**21**) exhibits concentration-dependent suppression of COX-2 expression and PGE_2 formation in RAW264.7 cells [31]. Furthermore, **21** shows a suppressive effect on LPS-stimulated COX-2 production without affecting IκB in the same cellular system [35]. In azoxymethane (AOM) and dextrane sodium sulphate (DSS)-induced colon carcinogenesis in mice **21** decreases COX-2 expression rates [119]. Auraptene (**21**) also suppresses the concentration of PGE_2 *via* decreasing the production of COX-2 protein by inhibition of the post-transcriptional expression [120], which was confirmed in another experiment on LPS-treated RAW264.7 cells reporting no suppression of COX-2 mRNA expression [121].

Other prenylated coumarin methylgalbanate (**41**), found in *Ferula szowitsiana* DC. slightly suppresses COX-2 mRNA expression in LPS-stimulated RAW264.7 macrophages [122]. Omphalocarpin (**3**) decreases the expression and enzymatic activity of COX-2 in RAW264.7 macrophages [39]. Imperatorin (**43**), isolated for example from *Angelica dahurica* (Apiaceae), shows significant inhibitory activity in the LPS-induced PGE_2 production and also inhibits LPS-induced expression of COX-2 and microsomal prostaglandin E synthase (mPGES) [123]. Byakangelicol (**51**), found in *Angelica* spp., *Citrus* spp., *Heracleum* spp., *Murraya koenigii* (L.) Sprengel (curry tree) and *Stauranthus perforates* Liemb., dose-dependently inhibits IL-1β-induced COX-2 expression and PGE_2 release in human pulmonary epithelial cell line A549. Byakangelicol (**51**) suppresses COX-2 activity selectively, without affecting that of COX-1 [42]. Anomalin (**72**) dose-dependently inhibits COX-2 mRNA and protein expression in LPS-stimulated RAW264.7 macrophages and in the sodium nitroprusside (SNP)-stimulated N2a cells. **72** effectively blocks the SNP-stimulated activation of the IKK α/β, IκB-α, ERK1/2 and p38 MAPK pathways. Moreover, **72** remarkably decreases the elevation in the SNP-induced pathway of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [47, 48].

Prenylated chalcones xanthoangelol (**99**), xanthoangelol B (**101**), xanthokeismin A (**103**), 4-hydroxyderricin (**92**), and xanthoangelol E (**95**) decrease the expression of COX-2 protein and mRNA in LPS-stimulated RAW264.7 cells, with **95** exerting the greatest effect [54]. The activity of **95** and **99** in RAW264.7 cells has been confirmed also in another study [56]. Also mallotophilippens C (**113**), D (**114**), and E (**115**) suppress COX-2 expression [57, 58]. Down-regulation of COX-2 mRNA has further been observed also for compounds **123-125** in S100b induced human THP-1 monocytes [59].

The expression of COX and its activity has also been affected by several prenylated flavonoids. The production of LPS-induced COX-2 protein in RAW264.7 macrophages is significantly suppressed by 8-prenylquercetin (**151**), as well as the PGE_2 production [63]. Extract obtained from the roots of *S. flavescens* containing sophoraflavanone G (**199**), kurarinone (**200**) and chalcone kuraridin (**107**) inhibits production of PGE_2 by direct inhibition of COX-2 enzyme activity [61]. **199** is a potent inhibitor of COX-2 induced PGE_2 production in LPS-stimulated RAW 264.7 cells [72]. Artocarpesin (**155**), a prenyl flavone from *Artocarpus heterophyllus* Lam (Moraceae), suppresses production of PGE_2 through the down-regulation of COX-2 expression in the LPS stimulated RAW264.7 cells [124].

Nine prenylated flavonoids, isolated from the leaves of *Macaranga conifera* (Rchb.f. & Zoll.) Müll. Arg. (Euphorbiaceae), have been evaluated for their inhibitory activity against COX-1 and COX-2. Three of these compounds, 5-hydroxy-4′-methoxy-2′′,2′′-dimethyl-pyrano-(7,8:6′′,5′′)-flavanone (**146**), 5,4′-dihydroxy [2′′-(1-hydroxy-1-methylethyl)dihydrofurano]-(7,8:5′′, 4′′)-flavanone (**147**), and 5,7-dihydroxy-4′′-methoxy-8-(3-methylbut-2-enyl)-flavanone (**141**), are inactive against both COX-1 and COX-2; inhibitory potency of lonchocarpol A (*syn*. 2*S*-6,8-diprenylnaringenin) (**129**) is higher than of sophoraflavanone B (*syn*. 2*S*-8-prenylnaringenin) (**127**) due to a prenylation at C-6. Replacement of a methoxyl at the C-4′ position, as observed in compound **142**, and with a hydroxyl group, as in tomentosanol D (**130**), leads to an increase of inhibitory activity against COX-2. Lupinifolinol (**148**) and especially isolicoflavonol (**156**) show significant inhibitory activities against both enzymes [125].

Cudraflavone B (**164**) inhibits the PGE_2 synthesis through the down-regulation of COX-2 transcription in LPS-stimulated THP-1 cells. Furthermore, **164** also possesses COX-1 and COX-2 direct inhibitory activity

with selectivity towards COX-2 greater than indomethacin [70]. Osajin (**232**) and pomiferin (**233**), isolated from *Maclura pomifera* (Rafin.) Schneider (Moraceae), together with cudraflavone B (**164**) and diplacone (**178**) have been tested on the ability to inhibit LPS-stimulated IκB-α degradation and COX-2 expression in J774.A1 cells. All these flavonoids are able to inhibit IκB-α degradation, but only diplacone (**178**) in parallel down-regulates COX-2 expression [126]. At the end of the list, sophoraisoflavanone A (**224**) selectively inhibits COX-1 obtained from the homogenates of bovine platelets and PMNs [127].

Glyceollins (**240-242**) markedly suppress the LPS-dependent expression of COX-2 in LPS-treated RAW264.7 cells [75, 76]. Prenylated phloroglucinols mallotojaponin (**359**), mallotochromanol (**360**), butyrylmallotolerin (**361**), isobutyrylmallotochromanol (**362**), and isomallotochromanol (**363**) exhibit an inhibitory effect against the production of PGE_2 in LPS-stimulated RAW264.7 cell line. Isomallotochromanol (**363**) is the most potent inhibitor of PGE_2 production; the effect is connected with inhibition of COX-2 induction *via* suppression of its transcription [84].

Two xanthones, α-mangostin (**276**) and γ-mangostin (**277**), isolated from the fruit hull of *Garcinia mangostana* L. (Guttiferae) significantly inhibit PGE_2 formation in LPS-stimulated RAW264.7 cells through inhibition of COX-2 activity and NO production. NO activates COX-1 and COX-2, which are rate-limiting enzymes for PGE_2 biosynthesis during the inflammatory process. Both compounds further inhibit COX-2 in C6 rat glioma cells [128].

4′-Geranyloxyferulic acid (**328**) is an oxyprenylated ferulic acid derivative isolated from the root and bark of *Acronychia baueri* Schott (Rutaceae). This compound dose-dependently suppresses COX-2 expression in human monocytes stimulated with LPS. Activity of COX-1 has not been affected [129]. Artepillin C (**327**), the major component of the Brazilian green propolis, obtained also from *Baccharis dracunculifolia* DC. (Asteraceae) shows anti-inflammatory effects mediated by down-regulation of PGE_2 production *via* NF-κB modulation [130]. Prenylated dihydrostilbenes amorfrutins A (**247**) and B (**248**), found in the fruits of *Amorpha fruticosa* L. (Fabaceae), and in roots of *Glycyrrhiza foetida* Desf. (Fabaceae), have been investigated for reduction of inflammation in colon cells. Amorfrutin A (**247**) strongly inhibits the expression of COX-2 both on the gene and the protein level (**248** was tested only for the inhibition of gene expression). In the case of the inhibition of gene expression of COX-2,

prenylated **247** shows greater effect than geranylated **248** [131]. 4-Geranyloxy-2,6-dihydroxybenzophenone (**334**), 4-geranyloxy-1-(2-methylpropanoyl)-phloroglucinol (**335**) and 4-geranyloxy-1-(2-methylbutanoyl)-phloroglucinol (**336**), obtained from *Hypericum densiflorum* Pursch. (Clusiaceae), show inhibitory effects against COX enzymes with greater selectivity towards COX-2 [105, 132].

The anti-inflammatory effect of several cannabinoids is mediated through the inhibition of COX enzymes. It has been evaluated by *in vitro* enzyme-based COX-1/COX-2 inhibition assay and a cell based PG production radioimmunoassay. Tetrahydrocannabinolic acid (Δ^9-THCA-A) (**369**), cannabidiolic acid (CBDA) (**371**), cannabigerol (CBG) (**372**) and cannabigerolic acid (CBGA) (**373**) show more than 30% inhibition on COX-1 in a dose-dependent manner. Compounds **369**, **372** and **373** express more than 30% inhibition of COX-2 enzyme. Compound **371** does not reach the 30% inhibition threshold for COX-2. In the both cases, the most significant inhibitors of the COX enzymes are **372** and **373**. Cannabinoids **368** and **370** do not affect the COX-1 and COX-2. All mentioned cannabinoids except the compound **371** decreased PGs production in TNF-α stimulated HT29 cells [133].

2.3.4. LOX Inhibitors

Farnesene-oxysubstituted coumarin umbelliprenin (**26**) shows significant and potent inhibition of soybean 5-LOX [134]. 3,4,2′,4′-Tetrahydroxy-2-geranyldihydrochalcone (**126**) isolated from *A. communis* is a significant selective 5-LOX inhibitor. This compound inhibits A23187-induced LTC_4 synthesis in the mice peritoneal cells [135]. Isobavachalcone (**93**) and diprenyleriodictyol (**131**), isolated from *Dorstenia* and *Polygonum* species, inhibit soybean 15-LOX. Inhibitory activity of isobavachalcone (**93**) was equal to the positive control quercetin [136].

Sigmoidins A (**132**) and B (**133**) are prenylated flavanones obtained from the extract of *Erythrina sigmoidea* Hua. (Fabaceae). Sigmoidin A (**132**) significantly inhibits LTB_4 production *via* 5-LOX inhibition in rat peritoneal leukocytes, whereas the same dose of sigmoidin B (**133**) possesses only half of **132** activity. The inhibitory effect towards COX-1 has not been confirmed [137]. Papyriflavonol A (**161**) and kenusanone A (**227**) inhibit 5-LOX and 12-LOX, whereas echinoisosophoranone (**225**) and isosophoranone (**226**) selectively down-regulate activity of 5-LOX. Sophoraflavanone D (**191**) and kenusanone C (**192**) show a strong 12-LOX inhibitory effect. Inhibitory activities have been tested on the homogenates of bovine platelets and

PMNs used as the enzyme source [127]. Papyriflavonol A (**161**) is a major constituent of the root bark of *Broussonetia papyrifera* (L.) Vent. (Moraceae), which ethanolic extract potently inhibits 5-LOX in A23187-treated rat basophilic leukemia (RBL-1) cells [138].

Erycristagallin (**243**) is a pterocarpane isolated from *Erythrina mildbraedii* Harms (Fabaceae). It inhibits the LTB_4 production *via* the interaction with 5-LOX pathway in PMNs obtained from the rat peritoneal cavity [139]. 1-*O*-β-glucopyranosyl-1,4-dihydroxy-2-(3′,3′-dimethylallyl)-benzene (**316**), 1-*O*-β-glucopyranosyl-1,4-dihydroxy-2-(3′-hydroxymethyl-3′-methylallyl)-benzene (**317**), and 1-*O*-(4′-*O*-caffeoyl)-β-glucopyranosyl-1,4-dihydroxy-2-(3′,3′-dimethylallyl)-benzene (**318**) are three prenylated hydroquinone glycosides isolated from *Phagnalon rupestre* (L.) DC. (Asteraceae). The production of LTB_4 has been significantly reduced by **318**, whereas compounds **316** and **317** are not active [140].

2.3.5. Dual COX/LOX Inhibitors

Psoralidin (**244**) inhibits the ionizing radiation (IR)-induced COX-2 expression and PGE_2 production through regulation of phosphoinositide 3-kinase (PI3K)/serine/threonine kinase (Akt) and NF-κB pathway. Direct interaction of **244** with 5-LOX activating protein in 5-LOX pathway leads to blockade of IR-induced LTB_4 production. **244** is not causing any significant changes in COX-1 expression. As the further effect, it is observed that psoralidin (**244**) attenuates IR-induced fibroblast migration [141].

A series of experiments carried out to analyze dual COX/LOX inhibitory effects of several prenylated chalcones, flavanones, flavones, flavonols and their Diels-Alder adducts, displayed the following results: the inhibition of COX-1 in order sophoraflavanone G (**199**) > indomethacin (standard) > kuraridin (**107**) = kurarinone (**200**) > sophoraisoflavanone A (**224**) > quercetin (non-prenylated flavonol). Against COX-2, only morusin (**169**), kuwanon C (**153**), sanggenon B (**205**), sanggenon D (**206**) and kazinol B (**222**) show some mild degree of inhibition. For the 5-LOX reaction, mojority of prenylated flavonoids from the compounds tested have been found to be active, with sequential order of inhibition: **199** > kenusanone A (**227**) = NDGA (nordihydroguaiaretic acid, standard of inhibition) > quercetin > psoralidin (**244**) > sanggenon D (**206**). The prenylated flavonoids tested have been generally less active inhibitors of 12-LOX than of 5-LOX. Further assays on RAW264.7 cells have shown that sophoraflavanone G (**199**) is, in difference to prenylated compounds tested, an inhibitor of COX-2 expression

[127, 142]. Glabridin (**223**) is a dual inhibitor of both COX and LOX enzymes. **223** shows inhibitory effect towards PGE_2 formation in J774A.1 murine macrophages stimulated by LPS, and TXB_2 formation in A23187-stimulated HL-60 cells. Furthermore, compound **223** inhibits A23187-induced production of LTB_4 in a dose-dependent manner in HL-60 cells. Whether the effect is connected with inhibition of enzyme expression or with direct inhibition of enzymatic activity is not fully stated [143].

The acylphloroglucinol derivative 2,4,6-trihydroxy-3-geranylacetophenone (**339**) isolated from *Melicope ptelefolia* Champ Ex. Benth (Rutaceae) displays a significant inhibitory effect against COX-2, and moderate inhibition of COX-1. **339** further significantly inhibits human 5-LOX and soybean 15-LOX [144, 145]. Arzanol (**357**) concentration-dependently inhibits PGE_2 formation either by interference with COX-2 or microsomal PGE_2 synthase mPGES-1. The assays on human monocytes and whole blood have not confirmed the effect of **357** on COX-2 or mPGES-1 protein expression; therefore, the effect could be caused by direct enzyme inhibition [81]. Other studies show that **357** potently inhibits the leukotriene formation in neutrophils *via* the inhibition of 5-LOX activity; it inhibits formation of COX-1-catalyzed synthesis of TXA_2 and also COX-2/mPGES-1-mediated PGE_2 biosynthesis in LPS-stimulated human monocytes [82, 146].

2.3.6. Dual mPGES/LOX Inhibitors

Prenylated flavones cannflavin A (**197**) and cannflavin B (**158**) are minor constituents of *C. sativa*. These compounds act as dual inhibitors of mPGES-1 and 5-LOX, with only a weak ability to inhibit COX-1 and COX-2 [147]. Xanthohumol (**86**), xanthohumol C (**118**), 8-prenylnaringenin (**127**), humulone (**344**), lupulone (**345**), 4-hydroxycolupulone (**346**), cascadone (**347**) and humudifucol (**340**) isolated from *H. lupulus*, have been evaluated for the inhibition of mPGES-1 and 5-LOX. Xanthohumol (**86**) and 4-hydroxycolupulone (**346**) are the most active from compounds tested in cell-free assays [148].

2.4. Inhibition of iNOS Expression and NO Release

2.4.1. The Role of Nitric Oxide in Inflammation

The nitric oxide (NO) is the one of the main inflammatory mediators. NO can be produced by three different isoforms of NO synthase (NOS), namely neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). nNOS and eNOS are constitutive forms that produce a small amount of NO upon the elevation of
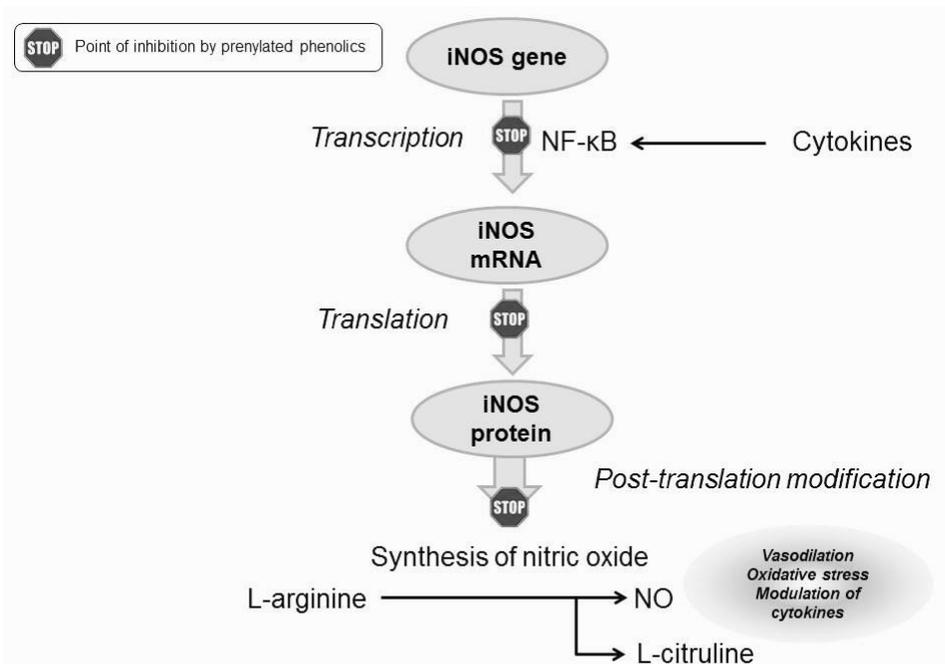
Fig. (30). Points in NO biosynthetic pathway, where PPC can inhibit its formation ("Stop" mark).

intracellular calcium concentration [149, 150]. The enzyme primarily responsible for the roles of NO in inflammatory processes is the inducible NOS (iNOS), which is not typically expressed in resting cells and must first be induced by certain cytokines or microbial products. iNOS remains very stable at both the mRNA and protein levels, and generates large amounts of NO over a period of days [151]. NO governs a wide spectrum of processes, such as upregulation of the cytokine cascade, recruitment and adhesion of leukocytes, increase of transendothelial cell migration of monocytes and enhancement of the proliferation of vascular smooth muscle cells [149, 152]. NO itself activates soluble guanylyl cyclases, which catalyze the synthesis of cyclic guanosin monophosphate. This activation of soluble guanylyl cyclase constitutes a common pathway in many processes, including vascular smooth muscle cell relaxation, inhibition of platelet activity, inhibition of neutrophil chemotaxis, and signal transduction in the central and peripheral nervous systems. The reaction of NO with ROS, for example with superoxide, results in the formation of reactive nitrogen oxygen species (RNOS), which can mediate a broad spectrum of physiological and also pathological effects [151]. Simplification of generation NO is showed in Fig. (**30**).

2.4.2. Prenylated Coumarins

Osthole (**1**) decreases LPS-stimulated NO production in RAW264.7 macrophages. Griess reaction has been used for measurement of the NO levels in the supernatant of RAW264.7 cells cultured with osthole (**1**) at various doses. Osthole (**1**) is not altering the background level of NO, but it inhibits the NO production in LPS-activated macrophages in a dose-dependent manner [29]. Moreover, suppression of iNOS expression by osthole (**1**) is observed [31]. Auraptene (**21**) decreases NO production in LPS-activated RAW264.7 macrophages in a dose-dependent manner. **21** also decreases NO production in macrophages activated by conditioned medium (CM) of hypertrophied 3T3-L1 adipocytes [35]. Bergamottin (**56**) and dihydroxybergamottin (**57**), found in nature in *Citrus*, *Ferulago* and *Glehnia* spp., show inhibition of NO generation induced by LPS or IFN-γ in RAW264.7 cells. Cnidicin (**45**), found in *Angelica coreana* Maxim. (Apiaceae), inhibits NO production and suppresses the expression of iNOS in activated RAW264.7 cells [105]. Omphalocarpin (**3**) down-regulates the NO overproduction through the suppression of the expression of iNOS and its enzymatic activity in LPS-activated macrophages [39]. Columbianadin (**62**) decreases NO production by the down-regulation of iNOS in lung epithelial cells A549 and cell line of alveolar macrophages (MH-S) [46]. Anomalin (**72**) dose-dependently suppresses iNOS in LPS-stimulated RAW264.7 macrophages. The suppression of iNOS mRNA and protein expression is responsible for the inhibitory effect of **72** on LPS-stimulated NO production [47]. **72** also reduces iNOS

mRNA gene expression in the SNP-induced N2a cells [48]. Praeruptorins C-E (**74-76**) suppress the mRNA and protein expression of iNOS in LPS-stimulated RAW264.7 cells [50].

Furthermore, the suppressive effect of several prenylcoumarins has been evaluated in a large study on both LPS- and IFN-γ-induced NO generation in RAW264.7 cells. Compounds from the group of structures with non-cyclized unmodified prenyl substituent including osthenol (**2**), umbelliprenin (**26**), 7-isopentenyloxycoumarin (**29**), nordentatin (**81**), clausarin (**82**), ponfolin (**83**), clausenidin (**84**) show significant inhibitory activity. Compounds **81**, **82**, **83** and **84** suppress NO generation by 63.6-91.2% at a concentration of 10 μM. Second group (with cyclized or oxidatively modified prenyl substituent) containing phebalosin (**6**), microminutin (**9**), micromarin-A (**10**), prangenin (**49**), columbianetin (**63**) and microminutinin (**66**) is more or less inactive, however prangenin (**49**) and micromarin-A (**10**) inhibit NO generation by 36.9 and 86.1 %, respectively, in the concentration of 50 μM [153]. Activity of umbelliprenin (**26**) on inhibition of NO production and iNOS expression has been confirmed in J774A.1 macrophages [154]. Imperatorin (**43**) and deltoin (**59**), isolated from the dried root of *Saposhnikovia divaricate* (Turcz.) Schischk (Apiaceae), inhibit the induction of iNOS in LPS-stimulated murine macrophages [155]. Divaricatol (**298**), and methoxy-8-(3-hydroxymethyl-but-2-enyloxy)-psoralen (**53**) show moderate inhibitory activity on NO production in LPS-induced RAW264.7 cells [156]. Psoralidin (**244**) suppresses NO production as well as iNOS expression in LPS-activated RAW264.7 macrophages. **244** decreases LPS-stimulated iNOS mRNA production in a dose-dependent manner, mechanistic study shows that this LPS-induced iNOS expression is inhibited *via* abolishing IKK phosphorylation, IκB degradation and NF-κB nuclear translocation without affecting MAPKs phosphorylation [157].

(+)-Praeruptorin A (**73**) down-regulates NO production in LPS-activated RAW264.7 cells [49]. Sesquiterpene coumarin derivatives, fukanemarin B (**19**), fukanefuromarin E (**67**), fukanefuromarin F (**68**), fukanefuromarin G (**69**), 2,3-dihydro-7-methoxy-2*S*,3*R*-dimethyl-2-[4-methyl-5-(4-methyl-2-furyl)-3(*E*)-pentenyl]-furo[3,2-c]coumarin (**70**), and 2,3-dihydro-7-hydroxy-2*S*,3*R*-dimethyl-2-[4-methyl-5-(4-methyl-2-furyl)-3(E)-pentenyl]-furo[3,2-c]coumarin (**71**), isolated from a 80% aqueous methanol extract of the roots of *Ferula fukanensis* K.M.Shen (Apiaceae), inhibit iNOS gene expression in LPS and IFN-γ stimulated RAW264.7 in a dose-dependent manner [158-160].

Xanthyletin (**80**) and nordentatin (**81**), the potentially anti-inflammatory compounds isolated from stems and root barks of *Citrus medica* L. var. *sarcodactylis* (Rutaceae) display potent NO-reducing activity in LPS-stimulated microglial cells [161].

2.4.3. Prenylated Chalcones

Broussochalcone A (**109**), isolated from *Broussonetia papyrifera* Vent. (Moraceae), suppresses NO production concentration-dependently in LPS-activated macrophages mediated by the suppression of IκBα phosphorylation, IκBα degradation, NF-κB activation, and iNOS expression [162]. Compounds isolated from *H. lupulus* are able to inhibit the production of NO and the expression of inducible iNOS. Xanthohumol (**86**) significantly lowers the level of NO production induced by LPS in RAW264.7 cells [52]. In another study, xanthohumol (**86**), xanthohumol D (**96**), xanthohumol B (**105**), and dihydroxanthohumol (**122**) strongly inhibit NO production at low concentrations without showing cytotoxic effects by the complete suppression of the expression of iNOS induced by LPS/IFN-γ in mouse macrophage RAW264.7 cells [163]. 4-Hydroxyderricin (**92**), and xanthoangelol E (**95**), xanthoangelol B (**101**) and xanthokeismin A (**103**) inhibit the production of NO and attenuate protein and mRNA levels of iNOS in LPS-activated RAW264.7 cells, while xanthoangelol D (**94**), xanthoangelol (**99**), and xanthoangelol F (**100**) shows only weak activity [54, 56]. This effect is also observed for mallotophilippens C-E (**113-115**) [58]. Also, prenylated chalcones licoagrochalcone A (**110**) and abyssinone D (**111**) from *Glycyrrhiza eurycarpa* P:C.Li, *Dalbergia stipulacea* Roxb., *Crotalaria orixensis* Willd., *C. medicaginea* Lam., *Glycyrrhiza glabra* and *Erythrina abyssinica* Lam. ex DC., and two pyranochalcones anthyllisone (**116**) and 3-*O*-methylabyssinone A (**117**) obtained from *Paratocarpus venezosa* Zoll, *Anthyllis hermanniae* L. and *Lonchocarpus nicou* (Aublet) DC., suppress the NO production in LPS-stimulated RAW264.7 cells [164]. Chalcones 3′′,3′′-dimethylpyrano[3′,4′]2,4,2′-trihydroxychalcone (**119**), isobavachalcone (**93**), morachalcone A (**106**), and gemichalcone B (**104**), isolated from *A. communis*, exhibit potent inhibitory activity towards NO production in LPS-activated cells RAW264.7 [165]. Kuraridin (**107**) [60], isobavachalcone (**93**) and diprenyleryodictyol (**131**) [136] inhibit NO production in LPS-stimulated RAW264.7 macrophages. Boesenbergin A (**120**) isolated from the rhizome of *Boesenbergia pandurata* Roxb. Schlecht. (Zingiberaceae) significantly decreases the NO production in IFN-γ/LPS-treated RAW264.7 cells [166].

2.4.4. Prenylated Flavonoids

Kazinol B (**222**), an isoprenylated flavan obtained from *Broussonetia kazinoki* Sieb. (Moraceae), concentration-dependently suppresses NO production in LPS-activated macrophages; however, the inhibition of NO production was weak, when **222** was added after the induction of iNOS by LPS for 18 h. The co-treatment of **222** with LPS resulted in significant inhibition. Therefore, this effect has not been the consequence of a direct inhibitory action on iNOS enzyme activity [167, 168]. 8-Prenylquercetin (**151**) significantly decreases the production of LPS-induced iNOS in RAW264.7 macrophages [63].

Artocarpesin (**155**) decreases LPS-induced NO production in RAW264.7 cells through the down-regulation of iNOS protein expression [124]. Artocarpin (**162**) and (2*S*)-euchrenone a7 (**139**) isolated from *A. communis* exhibit potent inhibitory activity on NO production in RAW264.7 LPS-activated cells [165]. Atalantoflavon (**166**) and lonchocarpol A (**129**), obtained from the stem and root barks of *Citrus medica* L. var. *sarcodactylis* (Rutaceae), show potent NO reducing activity in LPS-induced microglial cells [161]. Sanggenon F (**145**), kuwanon C (**153**), kuwanon T (**159**), sanggenol L (**167**), morusin (**169**), kuwanon A (**175**), kuwanon E (**184**) and sanggenon D (**206**) are prenylated flavonoids isolated from *M. alba*, which show significant inhibitory effect against NO production in RAW264.7 cells [168-170].

5,7,4′-Trihydroxy-8,3′-diprenylflavone (**152**) and epimedonin D (**160**) decrease the levels of LPS-induced NO production in RAW264.7 cells through the suppression of the transcription of the iNOS [64]. Kuwanon G (**176**) has been found to inhibit iNOS-catalyzed NO production in lung macrophages MH-S [71]. Sophoraflavanone G (**199**) significantly inhibits the release of NO and down-regulates iNOS protein expression in LPS-stimulated macrophages [72]. Prenylated flavonoid-enriched fraction of rhizomes of *S. flavescens* decrease iNOS-catalyzed NO production in LPS-treated RAW264.7 cells [61].

Artelastin (**211**), isolated from *Artocarpus elasticus* Reinw. ex Blume (Moraceae), shows a potent inhibitory effect on NO production in a J774 murine macrophage cell line. This effect is not irreversible, as macrophages have been able to restore their capacity to produce NO after the **211** removal. The disappearing of **211** activity happens probably due to its metabolization or association with macromolecules. A ten times higher concentration of **211** is necessary to inhibit NO production in macrophages exposed to **211** one hour before stimulation, when compared with the concentration needed when macrophages have been simultaneously exposed to **211** and LPS/IFN-γ stimulus. NO production is weakly inhibited by the addition of **211** 6 or 14 hours after stimulation. Therefore, compound **211** inhibits NO production by interfering with iNOS expression immediately after LPS/IFN-γ macrophages stimulation, which is supported by the fact that **211** is inactive in a cell-free model of NO scavenging [171].

Diplacone (**178**), 3′-*O*-methyldiplacol (**190**), 3′-*O*-methyl-5′-hydroxydiplacol (**193**), diplacol (**194**),3′-*O*-methyl-5′-hydroxydiplacone (**235**), and 6-geranyl-4′,5,7-trihydroxy-3′,5′-dimethoxyflavanone (**179**), obtained from the methanol extract of the flower of *Paulownia coreana* UYEKI, show potent inhibitory activities against LPS-induced NO production [172, 173]. Mimulone B (**187**) isolated also from *P. tomentosa*, with hydroxyl group at C-7′′ position of geranyl moiety, shows moderate inhibitory activity, suggesting that the hydroxylation of the geranyl group might be responsible for the loss of activity [172].

2.4.5. Miscellaneous Prenylated Phenolics

Echinoisoflavanon (**228**), isolated from *Echinosophora koreensis* Nakai, inhibits NO production as well as iNOS enzyme activity in LPS-induced RAW264.7 cells [168]. Glabridin (**223**) shows dose-dependent moderate inhibition in NO levels in the same cellular system [174], similar to glyceollins (**240-242**), which suppressed the LPS-dependent secretion of NO in LPS-activated murine RAW264.7 cells through the inhibition of iNOS expression [75, 76].

Arylbenzofurans isolated from *M. alba*, including artoindonesianin O (**253**), moracin C (**250**), mulberrofuran Y (**251**), moracin R (**252**), alabafuran A (**254**), moracin D (**258**), 3′,5′-dihydroxy-6-methoxy-7-prenyl-2-arylbenzofuran (**259**), mulberrofuran L (**266**), moracin O (**267**), and moracin P (**268**), significantly inhibit NO production in RAW264.7 cells [99, 169].

Garcimultiflorone E (**329**), 1,3,5,7-tetrahydroxy-8-isoprenylxanthone (**280**) and hyperxanthone E (**291**), isolated from *Garcinia esculenta* Y. H. Li (Clusiaceae), show potent inhibitory effect on IFN-γ/LPS-induced NO production in RAW264.7 cells [175]. α-Mangostin (**276**) and γ-mangostin (**277**) significantly inhibit NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner [128].

Prenylated phloroglucinol lupulone (**345**) and its three oxidative derivatives lupulones C (**351**), D (**348**) and E (**349**) have been investigated for the inhibitory effect on the production of NO and the expression of

iNOS in RAW264.7 cells; however, these phloroglucinol derivatives have shown a weaker effect in comparison with *H. lupulus* chalcones mentioned above. Oxidized derivatives (**348**, **349**, **351**) show weaker inhibitory activities on NO production than lupulone (**345**), and the oxidation may therefore reduce lupulone (**345**) anti-inflammatory potential [163].

Phloroglucinols from *M. japonicus* including isomallotochromanol (**363**), isomallotochromene (**364**), butyrylmallotolerin (**361**), isobutyrylmallotochromanol (**362**), mallotojaponin (**359**), mallotochromanol (**360**), and mallotochromene (**365**) inhibit production of NO in LPS/IFN-γ-induced RAW264.7 cells. Furthermore, their activity is based on the ability to significantly reduce both the induction of iNOS protein and iNOS mRNA expression [176]. Mallotophilippens A (**366**) and B (**367**) inhibit NO production and iNOS gene expression by a LPS/IFN-γ-activated RAW264.7 cells in a dose-dependent manner [95]. Panduratin A (**341**) and 4-hydroxypanduratin (**342**), obtained from rhizome of *B. pandurata*, strongly inhibit production of NO in LPS-treated RAW264.7 cells [166], similarly to sampsonols C (**353**) and F (**354**), prenylated phloroglucinol derivatives from *Hypericum sampsonii* Hance [177].

10-acetoxy-9-hydroxy-amphilecta-8,10,12,14-tetraene (**305**), 9-acetoxy-10-hydroxy-amphilecta-8,10,12,14-tetraene (**306**), pseudopterosins P (**304**) and T (**301**), isolated from *P. elisabethae,* inhibit NO release from J774 macrophages [104]. Methylgalbanate (**41**) significantly decreases NO production and iNOS mRNA expression in LPS/IFN-γ-stimulated RAW264.7 macrophages [114]. Artepillin C (**327**) decreases the NO level in RAW264.7 macrophages through the inhibition of iNOS expression [130].

2.5. Inhibition of ROS Release and Antioxidant Activity

2.5.1. Reactive Oxygen Species

ROS are signaling molecules that play a significant role in the development and progression of inflammatory process [178]. An enhanced ROS generation by PMNs at the inflammation site causes endothelial dysfunction and tissue injury [179]. ROS is a heterogeneous group of oxygen radicals and other strongly oxidizing molecules [180]. The widely studied and understood family members include superoxide anion (O_2^-), hydroxyl radical ($OH^•$), hydrogen peroxide (H_2O_2), and hypochlorous acid (HOCl) [179]. Mitochondrial respiration is one of the major sources of ROS in biological systems [180]. ROS are generated as byproducts of cellular metabolism through the electron transport chain (ETC) in mitochondria as well as *via* the cytochrome P450. The other major source, however where ROS are not produced as byproducts, are the NADPH oxidases (NOXs) that are present in a variety of cells, especially in the "professional" phagocytes and endothelial cells which are central to the genesis of the inflammatory response [179]. NOXs are a family of transmembrane oxidases that reduce molecular oxygen to O_2^- using energy derived from the oxidation of NADPH/NADH to NADP/NAD. The NOX2 complex is the first identified and, to date, probably best studied member of the NOX family. It is expressed in phagocytes (granulocytes, monocytes, dendritic cells) and its expression has also been reported in other cells of the immune system such as NK cells, B cells and mast cells. Somewhat lower levels of NOX2 have also been detected in T cells [180]. ROS also serve as signaling agents for inflammation. Transcription factors that could be directly activated by ROS include MAPK, NF-κB, AP-1, specificity protein 1 (Sp1), peroxisome proliferator-activated receptors (PPARs) and other members of the nuclear receptor superfamily [59, 181]. Interaction of advanced glycation endproducts (AGEs) with their receptor (RAGE) plays an important role in monocyte activation involved in diabetic inflammation. AGEs and S100b, a specific RAGE ligand, could intensify monocyte inflammatory responses and thus contribute to overproduction of ROS [182].

2.5.2. Prenylated Coumarins

Several prenylated coumarins are mentioned in the literature in relation to ROS production. Osthole (**1**) suppresses O_2^- generation in LPS-stimulated macrophages [29]. Furthermore, **1** has been proven to decrease ROS release in 1-methyl-4-phenylpyridinium ion-stimulated rat adrenal pheochromocytoma PC12 cells [183]. Armenin (**36**), isoarmenin (**37**), lacarol (**38**) and deoxylacarol (**39**), prenylated coumarins of *Artemisia armeniaca* Lam. (Asteraceae), show the significant free radical scavenging activity determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Lacarol (**38**) is found to be the most active one [184].

6-Hydroxy-7-[(*E,E*)-3′,7′-dimethyl-2′,4′,7′-octatrienyloxy] coumarin (**23**), prenyletin (**34**), haplopinol (**35**), 6-hydroxy-7-[(*E*)-3′,7′-dimethyl-5′-hydroxy-2′,6′-octadienyloxy] coumarin (**24**), and 6-hydroxy-7-[(*E,E*)-3′,7′-dimethyl-7′-hydroxy-2′,5′-octadienyloxy] coumarin (**25**), isolated from *Haplopappus multifolius* Reiche, display a moderate antioxidant activity in the DPPH assay [185]. A similar DPPH assay has been used for elucidation and confirmation of antiradical

activity of coumarins mammea E/BC (**11**), mammea E/BD (**12**), mammea A/AA (**246**), mammea B/BA (**13**), mammea B/BB (**14**), mammea B/BC (**15**), mammea B/BD (**16**), mammea E/BA (**17**), and mammea E/BB (**18**), isolated from the seeds of *Mammea americana* L. (Clusiaceae) [186]. DPPH radical scavenging assay has also shown moderate antioxidant activities of pyranocoumarin dimer (**79**) (dimmer in [187]) and (+)-8,9-dihydro-8-(2-hydroxypropan-2-yl)-2-oxo-2*H*-furo[2,3-*h*]chromen-9-yl-3-methylbut-2-enoate (**64**), obtained from aerial parts of *Angelica urumiensis* Mozaff. (Apiaceae) [187], subcoriacin (**20**), isolated from aerial parts of *Eysenhardtia subcoriacea* Pennell (Fabaceae), [188], and deltoin (**59**), prangenin (**49**) and marmesin isovalerate (**60**), obtained from the aerial parts of *Prangos uloptera* DC. (Apiaceae). However, the majority of these prenylated coumarins showed only low antioxidant properties [189].

Cedrecoumarin A (**85**), prenylated coumarin from *Cedrelopsis grevei* and *C. microfoliata* (Rutaceae), is an inhibitor of the luminol-induced chemiluminescence of ROS generated by human PMNs activated with opsonized zymosan and scavenger of O_2^- in a cell-free system [190]. Osthenol (**2**), imperatorin (**43**), isoimperatorin (**44**), clausemarin A (**54**), wampetin (**55**) and 8-geranyloxypsoralen (**58**), constituents of the roots of *Clausena lansium* Skeels (Rutaceae), exhibit strong inhibition of superoxide anion generation in cytochalasin B/N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP/CB) stimulated neutrophils [191, 192].

2.5.3. Prenylated Chalcones

Geranyl chalcones isolated from the fruit of *A. communis* (Moraceae) possess an inhibitory effect on intracellular ROS production. Compounds **123**, **124** and **125** significantly reduce ROS production in S100B-induced THP-1 monocytes. Compound **124** is the most powerful inhibitor of S100B-driven ROS production. These three compounds also inhibit the morphological characteristics of inflammation, exhibited in S100b-induced human THP-1 monocytes, through the inhibition of the mRNA expression of RAGE [59].

The radical scavenging activity of broussochalcone A (**109**) in DPPH test is greater than that of α-tocopherol. The antiradical activity of **109** is also proved by various other assays. **109** suppresses iron-induced lipid peroxidation in rat brain homogenate in a concentration-dependent manner. Its ability to scavenge O_2^- is attributed to an inhibition of cytochrome *c* reduction and only partially to its inhibition of xanthine oxidase [162]. Furthermore, broussochalcone A (**109**) inhibits oxygen consumption in fMLP- and phorbol

myristate acetate (PMA)-stimulated rat neutrophils in a concentration-dependent manner. In PMA-activated neutrophil particulary NADPH oxidase is inhibited by **109** and this attenuates O_2^- generation. The inhibitory effect of **109** on respiratory bursts in neutrophils is in general mediated by the suppression of protein kinase C (PKC) activity and its mediated effect on the NADPH oxidase complex [193].

Xanthoangelol (**99**) and isobavachalcone (**93**), isolated from *A. keiskei*, show antioxidant activity in the DPPH assay [194], similar to kuraridin (**107**) and kuraridinol (**108**) [195], chalcones isolated from *S. flavescens*. **107** and **108** have further been tested in 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and peroxynitrite scavenging (ONOO⁻) assays, and kuraridinol (**108**) shows significant inhibitory activity against intracellular ROS levels [195]. **107** significantly reduces LPS-induced ROS production in LPS-stimulated RAW264.7 cells [60]. Xanthohumol H (**97**) displays potent antioxidative activity in the oxygen radical absorbance (ORAC)-fluorescein assay [196]. Heliteretifolin (**121**), isoxanthohumol (**140**) and 2′,4′,6′-trihydroxy-3′-prenylchalcone (**112**), isolated from *Helichrysum teretifolium* (L.) D. Don (Asteraceae), show antioxidant activity in ORAC assay. Moreover, compounds **112** and **140** showed potent scavenging activity in the Trolox equivalent antioxidant capacity (TEAC) assay and moderate ferric reducing ability of plasma (FRAP) [197].

2.5.4. Prenylated Flavonoids

Mornigrols D (**255**), G (**172**) and H (**174**) as well as albanin A (**154**) and albanin E (**196**) isolated from *M. nigra* L. show antioxidant activity tested in the inhibition of malondialdehyde (MDA) formation [100]. Lonchocarpol A (**129**) inhibits NOX, the major ROS-producing enzyme in activated inflammatory cells [161]. Sigmoidin A (**132**) and B (**133**) have proven to be potent scavengers of DPPH radicals [137].

Prenylated flavonoids leachianone G (**134**), kushenol E (**135**), sophoraflavanone G (**199**), kushenol C (**202**), kurarinone (**200**) and kurarinol (**201**) obtained from *S. flavescens*, show antioxidant activities in ABTS and ONOO⁻ assays. Kushenol C (**202**) exhibits free radical scavenging activity also in DPPH assay and kurarinone (**200**) potent antioxidant effects against the radical generator 2,2-azo*bis*(2-amidinopropane) dihydrochloride (AAPH) in renal epithelial LLC-PK(1) cells [195], and another assay confirms **200** is also able to reduce LPS-induced ROS production in RAW264.7 cells [60].

Glabranin (**136**), isoglabranin (**137**) and 7-methoxyisoglabranin (**143**), isolated from *Helichrysum teretifolium* (L.) D. Don (Asteraceae), show antioxidant activity in ORAC, FRAP and TEAC assays [197]. DPPH scavenging activity, more potent than that of common antioxidant butylated hydroxytoluene (BHT) is reported for 6,8-diprenyleriodictyol (**131**), dorsmanin C (**203**) and dorsmanin F (**173**), isolated from *Dorstenia manii* Hook.f. These three compounds also suppress Cu^{2+}-mediated oxidation of human low-density lipoprotein (HLDL) [198].

Euchrestaflavanone C (**149**), (2*S*)-5,7,7´-trihydroxy-8-(3-hydroxy-3-methylbutyl)-2´,2´-dimethyl-[2,6´-Bi-2*H*-1-benzopyran]-4(3*H*)-one (**150**), euchestaflavanone B (**138**), cycloartocarpesin B (**165**) and cudraflavone B (**164**), prenylated flavonoids isolated from *Cudrania tricuspidata* (L.) D. Don (Asteraceae), display potent scavenging activity against OH· and against ABTS. All five compounds were inactive against DPPH [199]. Compounds mimulone (**177**), diplacone (**178**), 3´-*O*-methyldiplacone (**180**), tomentodiplacone (**185**), 3´-*O*-methyldiplacol (**190**), 3´-*O*-methyl-5´-OH-diplacone (**181**), 3´-*O*-methyl-5´-*O*-methyldiplacone (**182**), tomentodiplacone B (**186**), and schizolaenone C (**183**), isolated from *P. tomentosa*, show antioxidant properties in O_2^- scavenging assay, they inhibit Fenton reaction and are active in ABTS, DPPH, and FRAP assays [200].

Artoflavone A (**168**), hydroxyartoflavone A (**171**) and isocycloartobiloxanthone (**216**), isolated from *A. communis*, have been found to be significant DPPH scavengers. Isocycloartobiloxanthone (**216**) shows antioxidant activity in ABTS assay. The same ABTS assay, together with O_2^- scavenging test has confirmed activity of artogomezianone (**212**), norartocarpetin (**207**), artocarpin (**162**) and 8-geranyl-3-(hydroxyprenyl)isoetin (**204**) from *A. altilis* [201]. Test carried out on fMLP-stimulated rat neutrophils show antiradical activity of cycloheterophyllin (**214**) and artonin B (**217**) based on significant inhibition of O_2^- formation; however, cyclomorusin (**213**), dihydrocycloartomunin (**209**), cudraflavone A (**215**) and cyclocommunin (**210**) rather stimulate O_2^- generation [98]. Artelastin (**211**) is a strong inhibitor of ROS in human PMNs. PMA stimulation of PMNs directly activates PKC, leading to the activation of NADPH oxidase and O_2^- production. The decrease of O_2^- production could be related to an inhibition of NADPH oxidase activity or to a scavenging activity toward that radical. Artelastin (**211**) is also a weak inhibitor of MPO enzymatic activity [171].

The antioxidant activity of Diels-Alder adducts guangsangon H (**219**) analyzed in assay using detection of MDA released from microsomal lipids during ferrous-cysteine induced lipoperoxidation is greater than that of the vitamin E [102].

2.5.5. Miscellaneous Prenylated Phenolics

Many other miscellaneous compounds combining prenyl substitution with phenolic skeleton display antiradical activity. Assays based both on cellular systems and cell-free assays are used. Glabridin (**223**) displays antioxidant activity, compared to carotene, against AAPH-induced LDL oxidation [73]. Neobavaisoflavone (**229**) significantly inhibits the production of ROS and RNS in LPS/IFN-γ stimulated RAW264.7 macrophages [74]. Pomiferin (**233**), isopomiferin (**234**), and osajin (**232**) isolated from *Derris malaccensis* Prain (Fabaceae) and *Maclura pomnifera* (Moraceae) exhibit antioxidative activity in DPPH test, by inhibition of lipid peroxidation and ethoxyresorufin-*O*-deethylase (EROD) inhibitory activity. The most effective one is pomiferin (**233**) [202, 203]. 6,8-Diprenylorobol (**230**), 6,8-diprenylgenistein (**231**), alpinumisoflavone (**235**), 4´-*O*-methylalpinumisoflavone (**236**), osajin (**232**) and pomiferin (**233**), all isolated from the fruits of *Cudrania tricuspidata*, have been tested in assay using *bis*retinoid A2E as singlet oxygen source, **230** and **233** reducing A2E photo-oxidation in a dose dependent manner [204].

Two xanthones isolated from the stem bark of *Garcinia polyantha* Oliv. (Clusiaceae), bangangxanthone A (**294**) and B (**278**), possess DPPH radical scavenging activities. Compound **294** is a more potent scavenger than compound **278** [205]. Garciniaxanthones D (**295**), F (**296**), G (**297**), H (**282**) and symphoxanthone (**283**), antioxidative substances from the wood of *Garcinia subelliptica* Merr. (Clusiaceae), significantly inhibit lipid peroxidation. Compounds **282**, **283** and **295** show O_2^- scavenging activity. Moreover, symphoxanthone (**283**) displays scavenging activity of DPPH [206]. α-Mangostin (**276**), γ-mangostin (**277**), gartanin (**284**), garcinone D (**286**), and 6-methoxy-*bis*pyranoxanthone (**292**), isolated from the pericarp of *G. mangostana*, have been found to be potent DPPH scavengers [207]. Smeathxanthone A (**289**) and 1,5,8-trihydroxy-3-methoxy-2-(3-methyl-2-buten-1-yl) xanthone (**281**) isolated from the pericarp of *G. mangostana*, show strong antioxidative activity in FRAP assay [208]. Afzeliixanthone A (**279**) and B (**287**), two prenylated xanthones isolated from *Garcinia afzelii* ANGL., exhibit significant antioxidant effects, based on the scavenging of the stable DPPH free radical

[209]. Virgaxanthone A (**288**) and B (**293**), isolated from the stem bark of *G. virgata* Vieill. ex Guillaumin, exhibit potent antioxidant activities, based on the scavenging of the DPPH radical [210].

Moracin Q (**269**), moracin R (**252**), moracin S (**261**), moracin T (**262**) and moracin U (**270**) are prenylated arylbenzofurans isolated from *Morus mesozygia* Stapf. (*Moraceae*). Based on the results of DPPH test, moracins show significant antioxidant potential, with the activity of moracin T (**262**) close to that of vitamin E. On the other hand, **269** do not possess the activity in this test [211]. Arylbenzofurans regiafuran A-C (**263-265**), mulberrofuran Y (**251**), moracin N (**260**) and moracin O (**267**), isolated from *Chlorophora regia* A. Chev. (Moraceae), show antioxidant effects in the DPPH assay [212]. 4-Geranyloxy-2,6-dihydroxy-benzophenone (**334**), 4-geranyloxy-1-(2-methylpropanoyl)-phloroglucinol (**335**), and 4-geranyloxy-1-(2-methylbutanoyl)-phloroglucinol (**336**) inhibit lipid peroxidation by monitoring the fluorescence decay of Fe^{2+} induced oxidation of a model liposome system. Compound **334** was the most potent inhibitor [132].

1-(2,6-Dihydroxy-4-methoxyphenyl)-3-methylbutan-1-one (**343**), a major phloroglucinol from the flowers of *Callistemon citrinus* (Curtis) Skeels (Myrtaceae), shows remarkable free radical-scavenging activity in the ABTS assay, greater than in the DPPH assay [213]. Arzanol (**357**) exhibits a concentration-dependent reduction of DPPH with similar efficiency as L-cysteine or ascorbic acid [82]. Arzanol (**357**) also exerts a strong inhibition of *t*-butylhydroperoxide (TBH)-induced oxidative stress in VERO cells. Arzanol (**357**) and methylarzanol (**358**) show a remarkable scavenging of lipid peroxyl radicals. Compounds **357** and **358** also show scavenging activity in the cholesterol assay [214].

2-(1′-Geranyloxy)-4,6,-trihydroxyacetophenone (**338**) and 4-(1′-geranyloxy)-2,6,-trihydroxyacetophenone (**337**), obtained from the fruits of *Melicope semecarpifolia* (Merr.) T. G. Hartley (Rutaceace), show significant inhibitory activities in human neutrophil O_2^- generation and elastase release [215]. Garcinol (**355**), polyisoprenylated benzophenone isolated from the dried fruit of *Garcinia indica* Choisy (Clusiaceae), shows potent antioxidant activity in several tests. Garcinol (**355**) suppresses the generation of $OH^{\bullet}$ in the Fenton's reaction strongly than DL-R-tocopherol, and it suppresses O_2^- production in the hypoxanthine/ xanthine oxidase system and O_2^-, $OH^{\bullet}$, and methyl radical in the $H_2O_2/NaOH/DMSO$ system [216]. Oliveridepsidones A-D (**330-333**) from *Garcinia oliveri* Pierre (Clusiaceae) show antioxidant activity in DPPH assay [217].

Bis-prenylated phenols, 4-(1,1-dimethyl-2-propenyl)-2-(3-methyl-2-butenyl)phenol (**314**), 5-(1,1-dimethylprop-2-enyl)-2-(3-methylbut-2-enyl)cyclohexa-2,5-diene-1,4-dione (**321**) and 2,2-dimethyl-7-(1,1-dimethylprop-2-enyl)-2*H*-chromen-6-ol (**322**), isolated from the New Zealand brown alga *Perithalia capillaris* J. Agardh (Sporochnaceae), inhibit O_2^- production in human neutrophils *in vitro*. Compound **321** displays greater potency than compounds **314** and **322** [218]. Prenylated hydroquinones and 4-hydroxybenzoic acids isolated from the fruits of *Piper crassinervium* Kunth (Piperaceae) exhibit antioxidative activity. In DPPH assay 1-methoxy-4-hydroxy-2-(3′,7′-dimethyl)-2′*E*,6′-octadienylbenzene (**319**) and 1,4-dihydroxy-2-(3′,7′-dimethyl-1′-oxo-octa-2′-*E*-6′-dienyl) benzene (**320**) show antioxidant activity. Furthermore, **319** possessed the greatest hydrogen-donating capacity. Benzoic acids derivatives 4-hydroxy-(3′,7′-dimethyl-1′-oxo-octa-2′-*E*-6′-dienyl)benzoic acid (**375**) and 4-hydroxy-3-(3′,7′-dimethyl-3′-hydroxy-1′-oxo-6′-octenyl)benzoic acid (**376**) have been inactive as antioxidants in this model. Prenylated hydroquinones **319** and **320** are potent luminescence inhibitors compared to benzoic acid derivatives. Compound **320** decreases lipoperoxidation using a $Fe^{3+}/EDTA$ and ascorbic acid induced peroxidation in liposomes formed from phosphatidylcholine [219]. Cajaninstilbene acid (**249**), obtained from *Cajanus cajan* (L.) Millsp. (Fabaceae), shows certain antioxidant properties in DPPH assay [220].

2.6. *In vivo* Assays Carried Out to Evaluate Prenylated Phenolics Anti-inflammatory Potential

Many different experimental models are used in preclinical *in vivo* studies. Generally, they can be divided into two main groups: a) models evaluating individual features of inflammation, *e.g.*, edema or pain; and b) models of particular inflammatory diseases, *e.g.*, rheumatoid arthritis or Crohn's disease (Fig. 31). This part is divided according to the models used for the evaluation of anti-inflammatory potential of PPCs.

2.6.1. *In Vivo* Edema Model

A widely favored *in vivo* model is the carrageenan-induced paw edema assay used in both rats and mice. Inflammation induced by carrageenan is acute, non-immune, well-researched, and highly reproducible. The inflammatory response is usually quantified by the increase in size of paw edema, which is maximal around 5 hours' post-carrageenan injection and is modulated by inhibitors of specific molecules within the inflammatory cascade [221]. Mouse ear edema inflammation
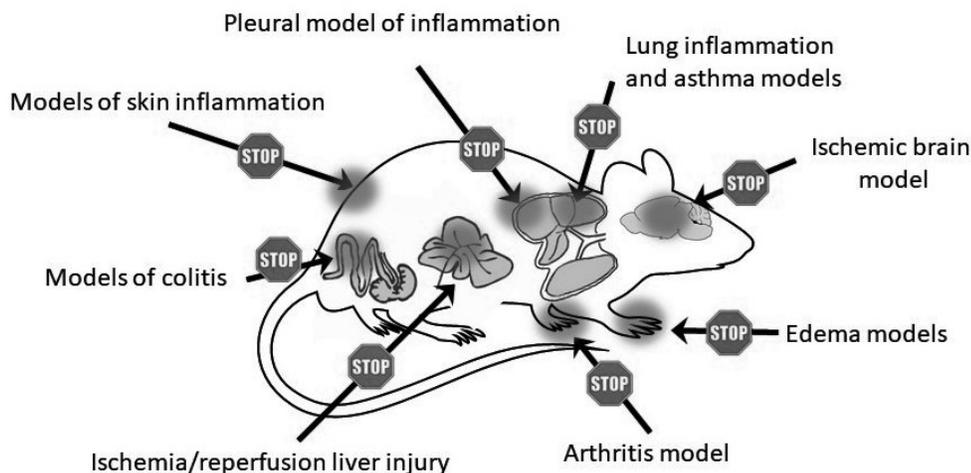
Fig. (31). *In vivo* models confirming PPC anti-inflammatory effects ("Stop" mark).

models are suitable for the evaluation of both topically and systemically administered substances. The ear model is a quick and simple method that requires small quantities of substances for testing, provides well-reproducible results and is accompanied by low possibilities of errors and gives rapid results. Ear edema can be induced by different substances, such as croton oil, cantharidin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), ethyl phenylpropiolate (EPP), dithranol, zymosan and other irritants [221].

Osthole (**1**), phebalosin (**6**), meranzin (**7**), murracarpin (**8**) are the compounds isolated from the leaves of *Murraya exotica* L. (Rutaceae). They inhibit the carrageenan-induced paw oedema in rats, murracarpin (**8**) shows the greatest potential [222]. Anomalin (**72**) has been evaluated in carrageenan-induced and complete Freund's adjuvant (CFA)-induced paw edema in acute and chronic models. Administration of anomalin (**72**) one hour before carrageenan and 40 min before CFA significantly reduces paw edema 2, 4, and 6 h after the triggering inflammation [223]. Umbelliprenin (**26**) significantly (39 %) inhibits the carrageenan-induced paw edema whereas indomethacin as a reference drug shows 47% inhibition [134, 154].

Abyssinone V-4′-methyl ether (**163**), isolated from *Erythrina droogmansiana* De Wild. & T. Durand (Fabaceae), significantly inhibits edema formation after carrageenan injection to the rat hind paw in a dose-dependent manner [224]. Prenylated isoflavones scandenone (*syn*. warangalon) (**237**) and auriculasin (**238**), isolated from the fruits of *Maclura pomifera* exhibit significant inhibition in carrageenan-induced hind paw edema model. Auriculasin (**238**) is less potent than scandenone (**237**) [225].

α-Mangostin (**276**) attenuates *in vivo* formation of carrageenan-induced paw edema in mice, while γ-mangostin (277) does not significant inhibit it [128]. A carrageenan-induced mouse paw edema test of the ethyl acetate extract obtained from *E. mildbraedii* has shown anti-inflammatory activity, and erycristagallin (**243**) was later isolated as the active principle [139]. 7-Epiclusianone (**356**), a benzophenone obtained from *Garcinia brasiliensis* Mart. (Clusiaceae), displays the reduction of paw edema induced by carrageenan [226]. Orally administered cannabidiol (**370**) significantly reduces acute inflammation and hyperalgesia induced by intraplantar injection of carrageenan into the rat paw. Detailed evaluation of markers has shown a decrease in PGE$_2$ plasma levels, tissue COX activity, production of ROS, and NO after administration of **370**. The lowering effect of **370** on NO depends on a lower expression of the endothelial isoform of NOS [227].

Anti-inflammatory activity of 8-prenylquercetin (**151**) was evaluated in LPS-induced paw edema in mice. Pretreatment with **151** reduced significantly LPS-induced paw thickness, compared with LPS treatment alone. The serum level of LPS-induced IL-6 has also been decreased by **151** treatment [63]. Cannabichromene (CBC) (**374**) and Δ^9-tetrahydrocannabinol (**368**) show dose-dependent anti-inflammatory effects in the LPS-induced paw edema model. These effects are enhanced when CBC (**374**) is given in combination with THC (**368**). These anti-inflammatory effects are not CB1 or CB2 receptor-mediated [228].

Sigmoidins A (**132**) and B (**133**) have been tested *in vivo* in PLA2-induced paw edema test. Sigmoidin B (**133**) is more a potent inhibitor of PLA2-induced ede-

ma than sigmoidin A (**132**) [137]. Griffonianone D (**239**) isolated from root bark of *Millettia griffoniana* (Fabaceae) shows an anti-inflammatory effect in PLA2-induced paw edema tests. Griffonianone D (**239**) inhibits edema formation by 42% at 30 min at a dose of 5 mg/kg (but shows no effect at 60 min) by modifying the proinflammatory vasoactive amine release from mast cells, together with having potential antioxidant effects [229]. Scandenone (**237**), obtained from the bark of *Erythrina addisoniae,* is effective as the standard drug cyproheptadine on the PLA2-induced paw edema at 60 min. The effectiveness of **237** in this test would arise from an antagonism of histamine or serotonin, in possible combination with some kind of membrane-stabilizing properties [230]. When erycristagallin (**243**) has been assayed against the PLA2-induced edema in mouse paws, edema formation was inhibited by 51% after 30 min at a dose of 5 mg/kg. The effect disappeared 60 min after application. The mechanism underlying this effect may be either an indirect inhibition of the enzyme or a blocking of the mastocyte degranulation [139].

In the test utilizing TPA-induced ear edema, sigmoidins A (**132**) and B (**133**) are effective at a dose of 0.25 mg/ear, decreasing the edema by 89% and 83%, respectively [137]. Griffonianon D (**239**) is effective when assayed against TPA-induced mouse ear edema. **239** acts by modifying the pro-inflammatory vasoactive amine release from mast cells, together with having potential antioxidant effects [229]. Scandenone (**237**) reduces swelling in the TPA-induced ear edema by 78 % when applied at a dose of 0.25 mg/ear. **237** markedly reduces the neutrophil efflux into the dermis, suppresses the edema and induces a moderate papillary fibrosis. No signals of hyperkeratosis, acanthosis or papillomatosis are noticed after **237** treatment. However, in a model of chronic dermatitis caused by repeated administration of TPA, the results were not so positive, since only a modest reduction in MPO activity was observed [230].

Erycristagallin (**243**) exhibits a strong effect when assayed in the TPA acute ear edema test. It also inhibits the neutrophils and leukocytes infiltration, hyperkeratosis, papillomatosis, and acanthosis [139]. Pseudopterosin A (**307**) and pseudopterosin E (**308**) reduce TPA-induced mouse ear edema when administered systemically. **307** suppresses edema more significantly than **308**. Both compounds are more active than indomethacin. Topically applied **308** is, in opposite, more potent than **307** [115]. Topical application of the fractions obtained from *P. elisabethae,* containing pseudopterosin Q (**299**), S (**300**) and U (**302**), fraction-2

containing amphilectosins A (**312**) and B (**313**), pseudopterosin G (**303**), K (**309**), P (**304**), and T (**301**), seco-pseudopterosin J (**310**) and seco-pseudopterosin K (**311**) exhibit an inhibition of the edema formation, and PMNs infiltration and degranulation in the TPA-induced edema, comparable to that shown by the indomethacin [104]. Glyceollins A-C (**240-242**) reduce TPA-induced ear inflammation in a mice model [75].

Treatment with topically applied double-dose TPA leads to a dramatic elevation of edema weight and H_2O_2 formation in the dermis and epidermis 1 h after the second TPA application. Double pretreatment with auraptene (**21**) suppresses TPA-induced skin edema and H_2O_2 generation. Single pre-treatment with **21** during the activation stage of inflammation remarkably decreases edema and H_2O_2 formation, the same application during the priming stage is less effective [32]. Eight PPC, isolated from the female inflorescence pellet extracts of *H. lupulus*, were evaluated against TPA-induced inflammation in mice, and the inhibitory effects were compared with that of indomethacin. Xanthohumol (**86**), isoxanthohumol (**140**), 8-prenylnaringenin (**127**), 6-prenylnaringenin (**128**), lupulone E (**349**), 5-deprenyllupulonol C (**350**), lupulone C (**351**), and colupox A (**352**) exhibit marked anti-inflammatory activities [231].

The ethanol extract of *M. exotica* leaves suppresses xylene-induced ear swelling in the mice with murracarpin (**8**) identified as a compound with the most significant inhibitory effect. Osthole (**1**) also exhibits a strong effect on inhibiting the ear edema in a dose-dependant manner. Phebalosin (**6**) and meranzin (**7**) possess mild inhibitory activity [222]. Omphalocarpin (**3**) exerts a remarkable dose-dependent inhibition of xylene-induced ear swelling development [39].

Tetrahydroxy-2-geranyldihydrochalcone (**126**) exerts strong inhibitory activity in AA-induced ear edema [135].

Anti-inflammatory activities of collinin (**22**), found in *Zanthoxylum schinifolium*, *Flindersia maculata* and *Haplophyllum alberti-regelli* (Rutaceae) and auraptene (**21**) have been evaluated by the inhibition of croton oil ear edema in mice. At the tested doses, auraptene (**21**) and its 8-methoxy derivative **22** are markedly active compounds inhibiting the edematous response by about 50 % [232]. (+)-Praeruptorin A (**73**), selinidin (**77**), visnadin (**78**) and (*R*)-(+)-7-(2´,3´-epoxy-3´-methylbutoxy)-coumarin (**40**), obtained from the aerial parts of *Ligusticum lucidum* Mill. subsp. *cuneifolium* (Guss.) Tammaro (Apiaceae), have been investigated for their anti-inflammatory activity by the inhibition of croton

oil-induced ear dermatitis in mice. All these coumarins reduce the edematous response to a certain extent at a dose of 0.3 μmol/cm². The most active compound is **40**, it induces a 68% edema reduction, exerting an effect higher than that of the same dose of indomethacin (58 %) [233]. Sophoraflavanone G (**199**) also exerts *in vivo* anti-inflammatory activity against mouse croton-oil induced ear edema. The potency of this activity is dependent on the route of administration. **199** shows a more potent inhibition by topical application than by oral treatment [142].

2.6.2. In Vivo Carragenan-Induced Pleural Model of Inflammation

Carragenan-induced rat pleural model of inflammation requires the harvesting of the entire exudate, allowing quantification of the fluid phase and enumeration of the different cell types that have migrated. Inflammatory irritants can be directly injected into a cavity to produce a non-immune inflammatory response [221]. Osthole (**1**) significantly inhibits pleural exudates formation and PMNs infiltration in carrageenan-induced pleurisy in rats. It also shows inhibition of MPO level in pleural exudates and decreases TNF-α and IL-1β levels in the lungs [234]. 7-prenyloxy-6-methoxycoumarin (**31**) reduces inflammation in the pleural cavity and lungs. **31** exerts its anti-inflammatory activity, at least partly, due to its ability to inhibit the NF-κB and p38 MAPK pathways activation which represent the main intracellular triggers of extensive inflammatory cascades [37]. Arzanol (**357**) reduces the inflammatory reaction in an *in vivo* model of acute inflammation, the carrageenan-induced rat pleurisy, with activity almost comparable to that of indomethacin. PGE₂ plays a central role in the early phase of carrageenan-induced pleurisy. Lowering PGE₂ by the inhibition of mPGES-1 may contribute to the anti-inflammatory properties of **357**. Arzanol (**357**) is less potent than indomethacin in the reduction of PGE₂ levels, but it is still efficiently able to suppress exudate formation and infiltration of inflammatory cells. In contrast to indomethacin, **357** also reduces the levels of LTB₄ in the pleural exudates [82].

2.6.3. In Vivo Lung Inflammation and Asthma Models

Columbianadin (**62**) has been examined in a mouse model of airway inflammation; LPS-induced acute lung injury. **62** strongly reduces the total cell numbers in the bronchoalveolar lavage fluid. The numbers of alveolar macrophages in the bronchoalveolar lavage fluid have been reduced more significantly in the group treated by **62** than in the dexamethasone-treated group. **62** mark-

edly decreases macrophage activation and stabilises the detachment of alveolar macrophages. It significantly reduces the numbers of dendritic cells and interstitial macrophages, as well [46].

Psoralidin (**244**) decreases ionizing radiation (IR)-induced expression of pro-inflammatory cytokines in IR-irradiated lung of BALB/c mouse. **244** inhibits the radiation-increased mRNA level of TNF-α, TGF-β and ICAM-1 by half, and suppresses the radiation-increased mRNA level of IL-6 and IL-1α/-1β by one quarter 12 h after irradiation. The inhibitory effects of **244** are more effective at 1 week after irradiation [141].

(±)-Praeruptorin A (**73**), obtained from *Peucedanum praeruptorum* Dunn. (Apiaceae), has been investigated using an ovalbumin-induced airway inflammation murine model. (±)-Praeruptorin A (**73**) reduces levels of IL-4, IL-5, IL-13 in bronchoalveolar lavage fluid and inhibits IκBα degradation, NF-κB nuclear translocation, and NF-κB DNA-binding activity [235]. Nujiangexanthone A (**285**), isolated from the leaves of *Garcinia nujiangensis* (Clusiaceae), inhibits the IL-4, IL-5, IL-13, and IgE levels in ovalbumin-induced asthma model and substantially inhibits ovalbumin-induced cellular infiltration and increases mucus production in the lung tissue [236].

2.6.4. In vivo Models of Skin Inflammation

Papyriflavonol A (**161**) shows potent anti-inflammatory activity in IgE-induced passive cutaneous anaphylaxis test provided on Sprague–Dawley rats [118].

Artocarpin (**162**) shows significant photoprotective effect on UVB-induced skin damage in hairless mice. **162** also decreases the levels of ROS and lipid peroxidation, levels of TNF-α and IL-1β of mice UVB-induced oxidative stress-mediated skin inflammation. Moreover, **162** downregulated the synthesis of cytosolic PLA2 and COX-2 [237].

2.6.5. In Vivo Murine Collagen-Induced Arthritis

Cannabidiol (**370**) exerts a dose-dependent suppressive action on the collagen-induced arthritis (CIA). A daily oral dose of **370** immediately after onset of arthritis results in the suppression of acute CIA. The 25 mg/kg dose has been used in a chronic experiment of CIA and has shown to effectively decrease progression of disease over a period of four weeks [238].

2.6.6. In Vivo Models of Colitis

Mimulone (**177**) and diplacone (**178**) have been evaluated *in vivo* on a model of colitis induced in Wistar rats by an oral administration of DSS. Both

compounds ameliorate and delay changes in the consistency of the stool and rectal bleeding. Diplacone (**178**) shows the greater therapeutic effect. The ability of both compounds to ameliorate colitis could be partly associated with their antioxidant activity [239].

2.6.7. In Vivo Warm Ischemia/Reperfusion Liver Injury

Xanthohumol (**86**) has been evaluated for anti-inflammatory and antioxidant activity in a model of warm ischemia/reperfusion (I/R) liver injury in BALB/c mice. Pretreatment with xanthohumol (**86**) almost completely blunts the I/R-induced Akt and NF-κB activation and the expression of the proinflammatory genes IL-1α, IL-6, MCP-1, and ICAM-1, which are known to play a crucial role in the subacute phase of I/R-induced liver damage [240].

2.6.8. In Vivo Rat Model of Middle of Cerebral Artery Occlusion

Osthole (**1**) has been tested in assay of middle cerebral artery occlusion (MCAO) in rats. It decreases the mRNA and protein levels of inflammatory factors including, TNF-α, IL-1β, COX-2, and iNOS in MCAO rat cerebral cortex ischemic penumbra. **1** displays a protective effect in abnormal conditions such as cortex inflammatory reactions observed during MCAO [31].

2.6.9. In Vivo Mice Ischemic Brain Model

Auraptene (**21**) decreases COX-2, and glial fibrillary acidic protein (GFAP; a marker of activated astrocytes) expression in astrocytes in mouse brain after ischemic injury. Moreover, **21** attenuates the expression of COX-2 in hippocampal tissue. **21** also suppresses the LPS-induced transcription of COX-2, and proinflammatory cytokines TNF-α, and IL-1β in isolated primary murine astrocytes [241].

CONCLUSION

Plant prenylated phenolics can be divided into several categories according to their basic structure. Typically, prenylated derivatives can be found between flavonoids, coumarins, arylbenzofurans and phloroglucinols; however, almost all plant phenolics can be substituted by some prenyl moiety. To obtain the structure-activity relationship of anti-inflammatory effect of such a broad group of compounds is therefore extremely difficult. Further important factor, making such a structure-activity relationship evaluation intricate, is the variability of assays available and used. As visible from the data introduced above, there are tens of different assays used for anti-inflammatory activity testing, including *in vitro* cell-free tests (inhibition of enzymatic

activity, like COX/LOX), cell-based assays with different types of inflammatory cells (macrophages, monocytes, glial cells and others) with different types of triggering of inflammatory reaction, and monitoring the development or progress of inflammation at different stage - gene expression, protein expression, end-point inflammation marker, and also *in vivo* assays using laboratory animals and various types of triggering inflammation and monitoring its progress.

As visible from the Table **S1** (Supporting info), compounds **1-85** showing some anti-inflammatory effect belong to prenylated coumarins. Waste majority of these compounds shows the effect in assays describing modulation of levels of inflammation-related cytokines, some interact with COX pathway, and inhibit iNOS expression and NO production. These compounds were very rarely found to be interacting with LOX. Activity of several prenylated coumarins was proved *in vivo*. The same situaton can be observed for chalcones **86-126**, however, the number of compounds interacting with COX is higher. On the other side, the *in vivo* prove of anti-inflammatory effects of prenylated chalcones is almost missing, with exception of well examined xanthohumol (**86**) and 3,4,2′,4′-tetrahydroxy-2-geranyldihydrochalcone (**126**) isolated from hop. Prenylated flavonoids **127-220** (with inclusion of flavans **221** and **222** are the largest group of prenylated phenolic compounds with some confirmation of possible anti-inflammatory activity. Flavonoids are often assigned to be antioxidants and this is compatible also for their prenylated forms. Similarly to prenyl coumarins, these compounds are commonly tested for their ability to inhibit COX metabolic pathways, they are tested both on direct COX inhibition and COX expression, with possible greater effect on gene expression; however, some compounds, like for example cudraflavone B (**164**) can be active in both ways. The activity *in vivo* was proved mainly for hop and *Paulownia* prenylated flavanones. Last bigger groups of anti-inflammatory active natural phenols are prenylated arylbenzofurans (**250-270**), xanthones (**276-297**), and phloroglucinols (**334-367**), which can include several cannabinoids (**368-374**). Arylbenzofurans were tested mainly as inhibitors of NO and ROS formation and prenylated xanthones as ROS inhibitors. Prenylated phloroglucinols (including cannabinoids) are structuraly very very heterogenous, however also here can be seen a trend to act as COX/LOX inhibitors and inhitors of iNOS expression or release. Some prenylated phloroglucinols (mainly cannabionoids and hop compounds) proved their effect *in vivo*. Interestingly, only

three prenylated stilbenoids were isolated to be tested in anti-inflammatory activity assays (**247-249**).

We tried to gather the information about clinical studies evaluating the anti-inflammatory therapeutic potential of prenylated phenols. However, such studies are not numerous, with exception of medicinal plants of Cannabaceae family – *Humulus* and *Cannabis*.

It is well known that cannabinoids interact with cannabinoid receptors, in which mainly CB_2 subtype has the importance for the correct function of immune system and control of inflammation [5, 242]. Several clinical studies showed the effect of cannabinoids (*Cannabis* or extracts from *Cannabis*) on the Crohn´s disease or ulcerative collitis [242-245]. The mechanism of the effect is not completely clear and may include not only the action on CB receptors, however, the interaction of cannabinoids with CB receptors is observed to affect leukocyte recruitment, migration of inflammatory cells and aopoptosis (and also gut motility and gut epithelial permeability) [242, 246]. Many studies *in vitro*/*in vivo* also showed cannabinoid receptor independent anti-inflammatory effects of cannabinoids, for example shown for cannabidiol (**370**), non-psychotropic cannabinoid with low affinity to CB receptor [247]. Despite numerous in vitro/in vivo studies, the clinical effect of **370** on inflammation was not evaluated [248].

Some effects of *iso*-alpha-acids (modified hop extract) were evaluated in pilot trial of clinical efficacy for knee osteoarthritis (open-label, single arm study), and the application of the extract led to significant decrease of pain and stiffness and physical function improvement [249].

This paper brings overview of almost four hundred prenylated phenolic substances and mechanisms, which influence process of inflammation. Many of these compounds can be prospective drugs or lead structure for the synthesis of other more active substances. As majority of experimental results were obtained from *in vitro* assays, additional studies especially *in vivo* are necessary for exact understanding of structure and activity relationship of anti-inflammatory prenylated phenolic compounds, their metabolism and their real therapeutical potential.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers web site along with the published article.

REFERENCES

[1]   Taylor, J.L.S.; Rabe, T.; McGaw, L.J.; Jäger, A.K.; van Staden, J. Towards the scientific validation of traditional medicinal plants. *Plant Growth Regul.*, **2001**, *34*, 23-27.

[2]   Bruneton, J. *Pharmacognosy: Phytochemistry, Medicinal Plants*. 2nd ed.; Andover: Intercept, **1999**.

[3]   Warner, T.D.; Mitchell, J.A. Cyclooxygenase-3 (COX-3): Filling in the gaps toward a COX continuum? *Proc. Natl. Acad. Sci. USA,* **2002**, *99*, 13371-13373.

[4]   Calixto, J.B.; Otuki, M.F.; Santos, A.R.S. Anti-inflamatory compounds of plant origin. Part I. Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa B (NF-kappaB). *Planta Med.,* **2003**, *69*, 973-983.

[5]   Nagarkatti, P.; Pandey, R.; Reider, S.A.; Hedge, V.L.; Nagarkatti, M. Cannabinoids as novel anti-inflammatory drugs. *Future Med. Chem.*, **2009**, *1*(7), 1333-1349.

[6]   Alhassan, M.A.; Abdullahi, M.I.; Uba, A.; Umar, A. Prenylation of aromatic secondary metabolites: A new frontier for development of novel drugs. *Trop. J. Pharm. Res.*, **2014**, *13*(2), 307-314.

[7]   Dewick, P.M *Medicinal Natural Products*. John Wiley & Sons: Chichester, **2002**.

[8]   Yazaki, K.; Sasaki, K.; Tsurumaru, Y. Prenylation of aromatic compounds, a key diversification of plant secondary metabolites. *Phytochemistry*, **2009**, *70*(15-16), 1739-1745.

[9]   Epifano, F.; Genovese, S.; Menghini, L.; Curini, M. Chemistry and pharmacology of oxyprenylated secondary plant metabolites. *Phytochemistry,* **2007**, *68*(7), 939-953.

[10]   Ferrero-Miliani, L.; Nielsen, O.H.; Andersen, P.S.; Girardin, S.E. Chronic inflammation: Importance of NOD2 and NALP3 in interleukin-1 beta generation. *Clin. Exp. Immunol.*, **2006**, *147*(2), 227-235.

[11]   Quinn, P.J.; Wang, X. *Lipids in Health and Disease*; Springer-Verlag: New York, **2008**.

[12]   Libby, P. Inflammatory mechanisms: The molecular basis of inflammation and disease. *Nutr. Rev.*, **2007**, *65*(12), 140-146.

[13]   Medzhitov, R.; Horng, T. Transcriptional control of the inflammatory response. *Nat. Rev. Immunol.*, **2009**, *9*(10), 692-703.

[14]   Janeway, Ch.A.; Travers, P.; Walport, M.; Shlomchik, M. J. *Immunobiology*; Garland Publishing: New York, **2001**.

[15]   Alberts, B. *Molecular biology of the cell*; Garland Science: New York, **2008**.

[16]   Polyzoidis, S.; Koletsa T.; Panagiotidou, S.; Ashkan, K.; Theoharides, T.C. Mast cells in meningiomas and brain inflammation. *J. Neuroinflammation*, **2015**, *12*, 170.

[17]   Schmitz, M.L.; Weber, A.; Roxlau, T.; Gaestel, M.; Kracht, M. Signal integration, crosstalk mechanisms and networks in the function of inflammatory cytokines. *Biochim. Biophys. Acta*, **2011**, *1813*, 2165-2175.

[18] Smale, S.T.; Natoli, G. Transcriptional control of inflammatory responses. *Cold Spring Harb. Perspect. Biol.*, **2014**, *6*(11), a016261. doi: 10.1101/cshperspect.a016261.

[19] Zelová, H.; Hošek, J. TNF-α signalling and inflammation: interactions between old acquaintances. *Inflamm. Res.*, **2013**, *62*(7), 641-651.

[20] Sedger, L.M.; McDermott, M.F. TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future. *Cytokine Growth Factor Rev.*, **2014**, *25*(4), 453-472.

[21] Feghali, C.A.; Wright, T.M. Cytokines in acute and chronic inflammation. *Front. Biosci.*, **1997**, *2*, d12-d26.

[22] Bradley, J.R. TNF-mediated inflammatory disease. *J. Pathol.*, **2008**, *214*(2), 149-160.

[23] Weber, A.; Wasiliew, P.; Kracht, M. Interleukin-1 (IL-1) pathway. *Sci. Signal.*, **2010**, *3*(105) cm1.

[24] Contassot, E.; Beer, H.-D.; French L.E. Interleukin-1, inflammasomes, autoinflammation and the skin. *Swiss Med. Wkly.*, **2012**, *142*, w13590.

[25] Frank, P.G.; Lisanti, M.P. ICAM-1: role in inflammation and in the regulation of vascular permeability. *Am. J. Physiol. Heart Circ. Physiol.*, **2008**, *295*(3), 926-927.

[26] Akira, S.; Hirano, T.; Taga, T.; Kishimoto, T. Biology of multifunctional cytokines. *FASEB J.*, **1990**, *4*(11), 2860-2867.

[27] Yang, X.O.; Panopoulos, A.D.; Nurieva, R.; Chang, S.H.; Wang, D.; Watowich, S.S.; Dong, Ch. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J. Biol. Chem.*, **2007**, *282*(13), 9358-9363.

[28] Opal, S.M.; DePalo, V. A. Anti-inflammatory cytokines. *Chest*, **2000**, *117*(4), 1162-1172.

[29] Liao, P.C.; Chien S.C.; Ho, C.L.; Wang, E.I.; Lee, S.C.; Kuo, Y.H.; Jeyashoke, N.; Chen, J.; Dong, W.C.; Chao, L.K.; Hua, K.F. Osthole regulates inflammatory mediator expression through modulating NF-κB, mitogen-activated protein kinases, protein kinase C, and reactive oxygen species. *J. Agric. Food Chem.*, **2010**, *58*(19), 10445-10451.

[30] Wu, S.J. Osthole attenuates inflammatory responses and regulates the expression of inflammatory mediators in HepG2 cells grown in differentiated medium from 3T3-L1 preadipocytes. *J. Med. Food*, **2015**, *18*(9), 972-979.

[31] Li, F.; Gong, Q.; Wang, L.; Shi, J. Osthole attenuates focal inflammatory reaction following permanent middle cerebral artery occlusion in rats. *Biol. Pharm. Bull.*, **2012**, *35*(10), 1686-1690.

[32] Murakami, A.; Nakamura, Y.; Tanaka, T.; Kawabata, K.; Takahashi, D.; Koshimizu, K.; Ohigashi, H. Suppression by citrus auraptene of phorbol ester- and endotoxin- induced inflammatory responses: role of attenuation of leukocyte activation. *Carcinogenesis*, **2000**, *21*(10), 1843-1850.

[33] La, V.D.; Zhao, L.; Epifano, F.; Genovese, S.; Greiner, D. Anti-inflammatory and wound healing potential of citrus auraptene. *J. Med. Food*, **2013**, *16*(10), 961-964.

[34] Marquis, A.; Genovese, S.; Epifano, F.; Grenier, D. The plant coumarins auraptene and lacinartin as potential multifunctional therapeutic agents for treating periodontal disease. *BMC Complement. Altern. Med.*, **2012**, *12*, 80.

[35] Lin, S.; Hirai, S.; Goto, T.; Sakamoto, T.; Takahashi, N.; Yano, M.; Sasaki, T.; Yu, R.; Kawada, T. Auraptene suppresses inflammatory responses in activated RAW264 macrophages by inhibiting p38 mitogen-activated protein kinase activation. *Mol. Nutr. Food Res.*, **2013**, *57*(7), 1135-1144.

[36] Appendino, G.; Maxia, L.; Bascope, M.; Houghton, P.J.; Sanchez-Duffhues, G.; Muñoz, G.; Sterner, O. A mero-terpenoid NF-kappaB inhibitor and drimane sesquiterpenoids from Asafetida. *J. Nat. Prod.*, **2006**, *69*(7), 1101-1104.

[37] Dos Santos Nascimento, M.V.; Arruda-Silva, F.; Luz, A.B.; Venzke, D.; Queiroz, G.S.; Mendes, B.G.; Fernandes-Ribeiro, E.R.; Fröde, T.S.; Pizzolatti, M.G.; Dalmarco, E.M. 7-prenyloxi-6-methoxycoumarin from *Polygala sabulosa* A.W. Bennett regulates p38 MAPK and NF-kB pathways inhibiting the inflammation induced by carrageenan in the mouse model of pleurisy. *Inflamm. Allergy Drug Targets*, **2015**, *14*(1), 37-46.

[38] Azelmat, J.; Fiorito, S.; Taddeo, V.A.; Genovese, S.; Epifano, F.; Grenier, D. Synthesis and evaluation of antibacterial and anti-inflammatory properties of naturally occurring coumarins. *Phytochem. Lett.*, **2015**, *13*, 399-405.

[39] Tong, L.; Chen, T.; Chen, Z.; Zhang, P.; Pi, H.; Ruan, H.; Wu, J. Anti-inflammatory activity of omphalocarpin isolated from Radix *Toddaliae* Asiaticae. *J. Ethnopharmacol.*, **2014**, *155*(3), 1553-1560.

[40] Kinoshita, T.; Wu, J.-B.; Ho, F.-Ch. Prenylcoumarins from *Murraya paniculata* var. *omphalocarpa* (Rutaceae): the absolute configuration of sibiricin, mexoticin and omphamurin. *Chem. Pharm. Bull.*, **1996**, *40*(6), 1208-1211.

[41] Tada, Y.; Shikishima, Y.; Takaishi, Y.; Shibata, H.; Higuti, T.; Honda, G.; Ito, M.; Takeda, Y.; Kodyhimatov, O.K.; Ashurmentov, O.; Ohmoto, Y. Coumarins and γ-pyrone derivatives from *Prangos pabularia*: antibacterial activity and inhibition of cytokine release. *Phytochemistry*, **2002**, *59*(6), 649-654.

[42] Curini, M.; Cravotto, G.; Epifano, F.; Giannone, G. Chemistry and biological activity of natural and synthetic prenyloxycoumarins. *Curr. Med. Chem.*, **2006**, *13*(2), 199-222.

[43] Ito, Ch.; Fujiwara, K.; Kajita, M.; Juichi, M.; Takemura, Y.; Suzuki, Y.; Tanaka, K.; Omura, M.; Furukawa, H. New coumarins from citrus plants. *Chem. Pharm. Bull.*, **1991**, *39*(10), 2509-2513.

[44] Lemmich, J.; Havelund, S. Constituents of umbelliferous plants. Part 21. Coumarin glycosides of *Seseli montanum*. *Phytochemistry,* **1978**, *17*(1), 139-141.

[45] Bahadir, O.; Çitoğlu, G.S.; Ozbek, H.; Dall'Acqua, S.; Hošek, J.; Šmejkal, K. Hepatoprotective and TNF-α inhibitory activity of *Zosima absinthifolia* extracts and coumarins. *Fitoterapia*, **2011**, *82*(3), 454-459.

[46] Lim, H.J.; Lee, J.H.; Choi, J.S.; Lee, S.K.; Kim, H.P. Inhibition of airway inflammation by the roots of *Angelica decursiva* and its constituent, columbianadin. *J. Ethnopharmacol.*, **2014**, *155*(2), 1353-1361.

[47] Khan, S.; Shin, E.M.; Choi, R.J.; Jung, J.H.; Kim, J.; Tosun, A.; Kim, Y.S. Suppression of LPS-induced inflammatory and NF-κB responses by anomalin in RAW 264.7 macrophages. *J. Cell. Biochem.*, **2011**, *112*(8), 2179-2188.

[48] Khan, S.; Choi, R.J.; Lee, J.; Kim, Y.S. Attenuation of neuropathic pain and neuroinflammatory responses by a pyranocoumarin derivative, anomalin in animal and cellular models. *Eur. J. Pharmacol.*, **2016**, *774*, 95-104.

[49] Lee, J.; Lee, Y.J.; Kim, J.; Bang, O.S. Pyranocoumarins from root extracts of *Peucedanum praeruptorum* Dunn with multidrug resistance reversal and anti-inflammatory activities. *Molecules*, **2015**, *20*(12), 20967-20978.

[50] Yu, P.J.; Jin, H.; Zhang, J.Y.; Wang, G.F.; Li, J.R.; Zhu, Z.G.; Tian, Y.X.; Wu, S.Y.; Xu, W.; Zhang, J.J.; Wu, S.G. Pyranocumarins isolated from *Peucedanum praeruptorum* Dunn suppress lipopolysaccharide-induced inflammatory response in murien macrophages through inhibition of NF-κB and STAT3 activation. *Inflammation*, **2012**, *35*(3), 967-977.

[51] Peluso, M.R.; Miranda, C.L.; Hobbs, D.J.; Proteau, R.R.; Stevens, J.F. Xanthohumol and related prenylated flavonoids inhibit inflammatory cytokine production in LPS-activated THP-1 monocytes: Structure-activity relationships

and *in silico* binding to myeloid differentiation protein-2 (MD-2). *Planta Med.*, **2010**, *76*(14), 1536-1543.

[52] Cho, Y.C.; Kim, H.J; Kim, Y.J.; Lee, K.Y.; Choi, H.J.; Lee, I.S.; Kang, B.Y. Differential anti-inflammatory pathway by xanthohumol in IFN-γ and LPS-activated macrophages. *Int. Immunopharmacol.*, **2008**, *8*(4), 567-573.

[53] Gao, X.; Deeb, D.; Liu, Y.; Gautam, S.; Dulchavsky, S.A.; Gautam, S.C. Immunomodulatory activity of xanthohumol: inhibition of T cell proliferation, cell-mediated cytotoxicity and Th1 cytokine production through suppression of NF-kappaB. *Immunopharmacol. Immunotoxicol.*, **2009**, *31*(3), 477-484.

[54] Chang, R.H.; Lee, H.J.; Ryu, J.H. Chalcones from *Angelica keiskei* attenuate the inflammatory responses by suppressing nuclear translocation of NF- κB. *J. Med. Food*, **2014**, *17*(12), 1306-1313.

[55] Shin, J.E.; Choi, E.J.; Jin, Q.; Jin, H.G.; Woo, E.R. Chalcones isolated from *Angelica keiskei* and their inhibition of IL-6 production in TNF-α-stimulated MG-63 cell. *Arch. Pharm. Res.*, **2011**, *34*(3), 437-442.

[56] Yasuda, M.; Kawabata, K.; Miyashita, M.; Okumura, M.; Yamamoto, N.; Takahashi, M.; Ashida, H.; Ohigashi, H. Inhibitory effects of 4-hydroxyderricin and xanthoangelol on lipopolysaccharide-induced inflammatory responses in RAW264 macrophages. *J. Agric. Food Chem.*, **2014**, *62*(2), 462-467.

[57] Nowakowska, Z. A review of anti-infective and anti-inflammatory chalcones. *Eur. J. Med. Chem.*, **2007**, *42*(2), 125-137.

[58] Daikonya, A.; Katsuki, S.; Kitanaka, S. Antiallergic agents from natural sources 9. Inhibition of nitric oxide production by novel chalcone derivatives from *Mallotus philippinensis* (Euphorbiaceae). *Chem. Pharm. Bull.*, **2004**, *52*(11), 1326-1329.

[59] Lin, J.A.; Wu, C.H.; Fang, S.C.; Yen, G.C. Combining the observation of cell morphology with the evaluation of key inflammatory mediators to assess the anti-inflammatory effects of geranyl flavonoid derivatives in breadfruit. *Food Chem.*, **2012**, *132*(4), 2118-2125.

[60] Han, J.M.; Jin, Y.Y.; Kim, H.Y.; Park, K.H.; Lee, W.S.; Jeong, T.S. Lavandulyl flavonoids from *Sophora flavescens* suppress lipopolysaccharide-induced activation of nuclear factor-kappaB and mitogen-activated protein kinases in RAW264.7 cells. *Biol. Pharm. Bull.*, **2010**, *33*(6), 1019-1023.

[61] Jin, J.H.; Kim, J.S.; Kang, S.S.; Son, K.H.; Chang, H.W.; Kim, H.P. Anti-inflammatory and anti-arthritic activity of total flavonoids of the roots of *Sophora flavescens*. *J. Ethnopharmacol.*, **2010**, *127*(3), 589-595.

[62] Ji, J.; Zhu, J.; Hu, X.; Wang, T.; Zhang, T.; Hou, A.J.; Wang, H. (2S)-7,4′-dihydroxy-8-prenylflavan stimulates adipogenesis and glucose uptake through p38MAPK pathway in 3T3-L1 cells. *Biochem. Biophys. Res. Commun.*, **2015**, *460*(3), 578-582.

[63] Hisanaga, A.; Mukai, R.; Sakao, K.; Terao, J.; Hou, D.X. Anti-inflammatory effects and molecular mechanisms of 8-prenyl quercetin. *Mol. Nutr. Food Res.*, **2016**, *60*(5), 1020-1032.

[64] Jin, Q.; Lee, C.; Lee, J.W.; Yeon, E.T.; Lee, D.; Han, S.B.; Hong, J.T.; Kim, Y.; Lee, M.K.; Hwang, B.Y. 2-Phenoxychromones and prenylflavonoids from *Epimedium koreanum* and their inhibitory effects on LPS-induced nitric oxide and interleukin-1β production. *J. Nat. Prod.*, **2014**, *77*(7), 1724-1728.

[65] Negrão, R.; Duarte, D.; Costa, R.; Soares, R. Isoxanthohumol modulates angiogenesis and inflammation *via* vascular endothelial growth factor receptor, tumor necrosis factor al-

pha and nuclear factor kappa B pathways. *Biofactors*, **2013**, *39*(6), 608-622.

[66] Gil-Ramírez, A.; Mendiola, J.A.; Arranz, E.; Ruíz-Rodríguez, A.; Reglero, G.; Ibáñez, E.; Marín, F.R. Highly isoxanthohumol enriched hop extract obtained by pressurized hot water extraction (PHWE). Chemical and functional characterization. *Innov. Food Sci. Emerg.*, **2012**, *16*, 54-60.

[67] Hošek, J.; Závalová, V.; Šmejkal, K.; Bartoš, M. Effect of diplacone on LPS-induced inflammatory gene expression in macrophages. *Folia Biol. (Praha)*, **2010**, *56*(3), 124-130.

[68] Hanáková, Z.; Hošek, J.; Babula, P.; Dall′Acqua, S.; Václavík, J.; Šmejkal, K. *C*-Geranylated flavanones from *Paulownia tomentosa* fruits as potential anti-inflammatory compounds acting *via* inhibition of TNF-α production. *J. Nat. Prod.*, **2015**, *78*(4), 850-863.

[69] Zelová, H.; Hanáková, Z.; Čermáková, Z.; Šmejkal, K.; Dall′Acqua, S.; Babula, P.; Cvačka, J.; Hošek, J. Evaluation of anti-inflammatory activity of prenylated substances isolated from *Morus alba* and *Morus nigra*. *J. Nat. Prod.*, **2014**, *77*(6), 1297-1303.

[70] Hošek, J.; Bartoš, M.; Chudík, S; Dall′Acqua, S.; Innocenti, G.; Kartal, M.; Kokoška, L.; Kollár, P.; Kutil, Z.; Landa, P.; Marek, R.; Závalová, V.; Žemlička, M.; Šmejkal, K. Natural compound cudraflavone B shows promising anti-inflammatory properties *in vitro*. *J. Nat. Prod.*, **2011**, *74*(4), 614-619.

[71] Lim, H.J.; Jin, H.G.; Woo, E.R.; Lee, S.K.; Kim, H.P. The root barks of *Morus alba* and the flavonoid constituents inhibit airway inflammation. *J. Ethnopharmacol.*, **2013**, *149*(1), 169-175.

[72] Wun, Z.Y.; Lin, C.F.; Huang, W.C.; Xu, P.Y., Chang, W.T.; Wu, S.J., Liou, C.J. Anti-inflammatory effect of sophoraflavanone G isolated from *Sophora flavescens* in lipopolysaccharide-stimulated mouse macrophages. *Food Chem. Toxicol.*, **2013**, *62*, 255-261.

[73] Simmler, C.; Pauli, G.F.; Chen, S.N. Phytochemistry and biological properties of glabridin. *Fitoterapia*, **2013**, *90*, 160-184.

[74] Szliszka, E.; Skaba, D.; Czuba Z.P.; Krol, W. Inhibition of inflammatory mediators by neobavaisoflavone in activated RAW 264.7 macrophages. *Molecules*, **2011**, *16*(5), 3701-3712.

[75] Kim, H.J.; Sung, M.K.; Kim, J.S. Antiinflammatory effects of glyceollins derived from soybean by elicitation with *Aspergillus sojae*. *Inflamm. Res.*, **2011**, *60*(10), 909-917.

[76] Yoon, E.K.; Kim, H.K.; Cui, S.; Kim, Y.H. Soybean glyceollins mitigate inducible nitric oxide synthase and cyclooxygenase-2 expression levels *via* suppression of the NF-κB signaling pathway in RAW 264.7 cells. *Int. J. Mol. Med.*, **2012**, *29*(4), 711-717.

[77] Rouger, C.; Derbré, S.; Charreau, B.; Pabois, A.; Cauchy, T.; Litaudon, M.; Awang, K.; Richomme, P. Lepidotol A from *Mesua lepidota* inhibits inflammatory and immune mediators in human endothelial cells. *J. Nat. Prod.*, **2015**, *78*(9), 2187-2189.

[78] Cascão, R.; Vidal, B.; Raquel, H.; Neves-Costa, A.; Figueiredo, N.; Gupta, V.; Fonseca, J.E.; Moita, L.F. Potent anti-inflammatory and antiproliferative effects of gamboic acid in a rat model of antigen-induced arthritis. *Mediators Inflamm.*, **2014**, *2014*, 195327.

[79] Huang, D.; Zhu, H.; Chen, Y.; Chen, W.; Xue, D.; Sun, L. Prenylated phenylpropanoid compounds from the stem bark of *Illicium burmanicum*. *Fitoterapia*, **2015**, *107*, 22-28.

[80] Appendino, G.; Ottino, M.; Marquez, N.; Bianchi, F.; Giana, A.; Ballero, M.; Sterner, O.; Fiebich, B.L.; Munoz, E. Arzanol, an anti-inflammatory and anti-HIV-1 phloroglucinol alpha-Pyrone from *Helichrysum italicum* ssp. *microphyllum*. *J. Nat. Prod.*, **2007**, *70*(4), 608-612.

[81] Kothavade, P.S.; Nagmoti, D.M.; Bulani, V.D.; Juvekar, A.R. Arzanol, a potent mPGES-1 inhibitor: Novel anti-inflammatory agent. *Sci. World J.*, **2013**, *2013*, 986429.

[82] Bauer, J.; Koeberle, A.; Dehm, F.; Pollastro, F.; Appendino, G.; Northoff, H.; Rossi, A.; Sautebin, L.; Werz, O. Arzanol, a prenylated heterodimeric phloroglucinyl pyrone, inhibits eicosanoid biosynthesis and exhibits anti-inflammatory efficacy *in vivo*. *Biochem. Pharmacol.*, **2011**, *81*(2), 259-268.

[83] Ishii, R.; Horie, M.; Saito, K.; Arisawa, M.; Kitanaka, S. Inhibition of lipoplysaccharide-induced pro-inflammatory cytokine expression *via* suppression of nuclear factor-κB activation by *Mallotus japonicus* phloroglucinol derivatives. *Biochim. Biophys. Acta*, **2003**, *1620*(1-3), 108-118.

[84] Ishii, R.; Horie, M.; Saito, K.; Arisawa, M.; Kitanaka, S. Prostaglandin E$_2$ production and induction of prostaglandin endoperoxide synthase-2 is inhibited in a murine macrophage-like cell line, RAW 264.7, by *Mallotus japonicus* phloroglucinol derivatives. *Biochim. Biophys. Acta,* **2002**, *1571*(2), 115-123.

[85] Kozela, E.; Pietr, M.; Juknat, A.; Rimmerman, N.; Levy, R.; Vogel, Z. Cannabinoids Δ^9-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-κB and interferon-*β*/STAT proinflammatory pathways in BV-2 microglial cells. *J. Biol. Chem.*, **2010**, *285*(3), 1616-1626.

[86] Rausch, P.B.; Moore T.G. Granule enzymes of polymorphonuclear neutrophils: A phylogenetic comparison. *Blood*, **1975**, *46*(6), 913-919.

[87] Borregaard, N.; Cowland, J.B. Granules of the human neutrophilic polymorphonuclear leukocytes. *Blood*, **1997**, *89*(10), 3503-3521.

[88] Loria, V.; Dato, I.; Graziani, F.; Biasucci, L.M. Myeloperoxidase: A new biomarker of inflammation in ischemic heart disease and acute coronary syndromes. *Mediators Inflamm.*, **2008**, *2008*, 135625.

[89] Olza, J.; Aguilera, C.M.; Gil-Campos, M.; Leis, R.; Bueno, G.; Martínez-Jiménez, M.D.; Valle, M.; Cañete, R.; Tojo, R.; Moreno, L.A.; Gil, A. Myeloperoxidase is an early biomarker of inflammation and cardiovascular risk in prepubertal obese children. *Diabetes Care*, **2012**, *35*(11), 2373-2376.

[90] Chowdhary, A.; Gayathri, G.V.; Metha, D.S. Comparative analysis of GCF β-glucuronidase level in diabetic and nondiabetic patients with chronic periodontitis: A clinicobiochemical study. *J. Indian. Soc. Periodontol.*, **2008**, *12*(1), 16-20.

[91] Verma, R.P.; Hansch, C. Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. *Bioorg. Med. Chem.*, **2007**, *15*(6), 2223-2268.

[92] Van Den Steen, P.E.; Wuyts, A.; Husson, S.J.; Proost, P.; Van Damme, J.; Opdenakker, G. Gelatinase B/MMP-9 and neutrophil collagenase/MMP-8 processthe chemokines human GCP-2/CXCL6, ENA-78/CXCL5 and mouseGCP-2/LIX and modulate their physiological activities. *Eur. J. Biochem.*, **2003**, *270*(18), 3739-3749

[93] Buddenkotte, J.; Maurer, M.; Worm, M.; Zuberbier, T.; Steinhoff, M. Histamine and antihistamines in atopic dermatitis. *Adv. Exp. Med. Biol.*, **2010**, *709*, 73-80.

[94] White, M. Mediators of inflammation and the inflammatory process. *J. Allergy. Clin. Immunol.*, **1999**, *103*, 378-381.

[95] Daikonya, A.; Katsuki, S.; Wu, J.B.; Kitanaka, S. Anti-allergic agents from natural sources (4): Anti-allergic activity of new phloroglucinol derivatives from *Mallotus philippensis* (Euphorbiaceae). *Chem. Pharm. Bull.*, **2002**, *50*(12), 1566-1659.

[96] Iwata, N.; Wang, N.; Yao, X.; Kitanaka, S. Structures and histamine release inhibitory effects of prenylated orcinol derivatives from *Rhododendron dauricum*. *J. Nat. Prod.*, **2004**, *67*(7), 1106-1109.

[97] Kimura, Y.; Okuda, H. Histamine-release effectors from *Angelica dahurica* var. *dahurica* Root. *J. Nat. Prod.*, **1997**, *60*, 249-251.

[98] Wei, B.L.; Weng, J.R.; Chiu, P.H.; Hung, C.F.; Wang, J.P; Lin, C.N. Antiinflammatory flavonoids from *Artocarpus heterophyllus* and *Artocarpus communis*. *J. Agric. Food Chem.*, **2005**, *53*(10), 3867-3871.

[99] Naik, R.; Harmalkar, D.S.; Xu, X.; Jang, K.; Lee, K. Bioactive benzofuran derivatives: moracins A-Z in medicinal chemistry. *Eur. J. Med. Chem.*, **2015**, *90*, 379-393.

[100] Wang, L.; Yang, Y.; Liu, C.; Chen, R.Y. Three new compounds from *Morus nigra* L. *J. Asian Nat. Prod. Res.*, **2010**, *12*(6), 431-437.

[101] Kang, J.; Chen, R.Y.; Yu, D.Q. Five new Diels-Alder type adducts rom the stem and root bark of *Morus mongolica. Planta Med.*, **2006**, *72*(1), 52-59.

[102] Dai, S.J.; Ma, Z.B.; Wu, Y.; Chen, R.Y.; Yu, D.Q. Guangsangons F-J, anti-oxidant and anti-inflammatory Diels-Alder type adducts, from *Morus macroura* Miq. *Phytochemistry*, **2004**, *65*(23), 3135-3141.

[103] Terracciano, S.; Aquino, M.; Rodriquez, M.; Monti, M.C.; Casapullo, A.; Riccio, R.; Gomez-Paloma, L. Chemistry and biology of anti-inflammatory marine natural products: Molecules Interfering with cyclooxygenase, NF-κB and other unidentified tragets. C*urr. Med. Chem.*, **2006**, *13*(16), 1947-1969.

[104] Correa, H.; Valenzuela, A.L.; Ospina, L.F.; Duque, C. Anti-inflammatory effects of the gorgonian *Pseudopterogorgia elisabethae* collected at the Islands of Providencia and San Andrés (SW Caribbean). *J. Inflamm.*, **2009**, *6*, 5.

[105] Genovese, S.; Curini, M.; Epifano, F. Prenyloxyphenylpropanoids as a novel class of anti-inflammatory agents. *Anti-Inflamm. Anti-Allergy. Agents Med. Chem.*, **2010**, *9*, 158-165.

[106] Lee, T.; Kwon, J.; Lee, D.; Mar, W. Effects of *Cudrania tricuspidata* fruit extract and its active compound, 5,7,3',4'-tetrahydroxy-6,8-diprenylisoflavone, on the high-affinity IgE receptor-mediated activation of Syk in mast cells. *J. Agric. Food Chem.*, **2015**, *63*(22), 5459-5467.

[107] Yang, P.; Chan, D.; Felix, E.; Madden, T.; Klein, R.D.; Shureiqi, I.; Chen, X.; Dannenberg, A.J.; Newman, R.A. Determination of endogenous tissue inflammation profiles by LC/MS/MS: COX- and LOX-derived bioactive lipids. *Prostaglandins Leukot. Essent. Fatty Acids*, **2006**, *75*(6), 385-395.

[108] Ricciotti, E.; FitzGerald, G.A. Prostaglandins and inflammation. *Arterioscler. Thromb. Vasc. Biol.*, **2011**, *31*(5), 986-1000.

[109] Triggiani, M.; Granata, F.; Oriente, A.; De Marino, V.; Gentile, M.; Calabrese, C.; Palumbo, C.; Marone, G. Secretory phospholipases A2 induce β-glucuronidase release and IL-6 production from human lung macrophages. *J. Immunol.*, **2000**, *164*, 4908-4915.

[110] Williams, Ch.S.; Mann M.; DuBois R.D. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene*, **1999**, *18*, 7908-7916.

[111] Kuwano, T.; Nakao, S.; Yamamoto, H.; Tsuneyoshi, M.; Yamamoto, T; Kuwano, M.; Ono, M. Cyclooxygenase 2 is a key enzyme for inflammatory cytokine-induced angiogenesis. *FASEB J.*, **2004**, *18*(2), 300-310.

[112] Buckley, Ch.D.; Gilroy, D.W.; Serhan, C.N. Proresolving lipid mediators and mechanisms in the resolution of acute inflammation. *Immunity*, **2014**, *40*(3), 315-327.

[113] Dahlén, S.E.; Björk, J.; Hedqvist, P.; Arfors, K.E.; Hammarström, S.; Lindgren, J.Á; Samuelsson, B. Leukotrienes promote plasma leakage and leukocyte adhesion in postca-

pillary venules: *In vivo* effect with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. USA*, **1981**, *78*(6), 3887-3891.

[114] Vincent, C.; Fiancette, R.; Donnard, M.; Bordessoule, D.; Turlure, P.; Trimoreau, F.; Denizot, Y. 5-LOX, 12-LOX and 15-LOX in immature forms of human leukemic blasts. *Leuk. Res.*, **2008**, *32*(11), 1756-1762.

[115] Mayer, A.M.; Jacobson, R.S.; Fenical, W.; Glaser, K.B. Pharmacological characterization of the pseudopterosins: novel anti-inflammatory natural products isolated from the Caribbean soft coral, *Pseudopterogorgia elisabethae. Life Sci.*, **1998**, *62*(26), 401-407.

[116] Moya, C.E.; Jacobs, R.S. Pseudopterosin A inhibits phagocytosis and alters intracellular calcium turnover in a pertussis toxin sensitive side in *Tetrahymena thermophila. Comp. Biochem. Physiol. C. Toxicol. Pharmacol.*, **2006**, *143*(4), 436-443.

[117] Son, K.H.; Kwon, S.J.; Chang, H.W.; Kim, H.P.; Kang, S.S. Papyriflavonol A, a new prenylated flavonol from *Broussonetia papyrifera. Fitoterapia*, **2001**, *72*(4), 456-458.

[118] Kwak, W.J.; Moon, T.C.; Lin, C.X.; Rhyn, H.G.; Jung, H.; Lee, E.; Kwon, D.Y.; Son, K.H.; Kim, H.P; Kang, S.S.; Murakami, M.; Kudo, I.; Chang, H.W. Papyriflavonol A from *Broussonetia papyrifera* inhibits the passive cutaneous anaphylaxis reaction and has a secretory phospholipase A$_2$-inhibitory activity. *Biol. Pharm. Bull.*, **2003**, *26*(3), 299-302.

[119] Genovese, S.; Epifano, F. Auraptene: A natural biologically active compound with multiple targets. *Curr. Drug Targets*, **2011**, *12*(3), 381-386.

[120] Yan, H.; Ma, Z.; Peng, S.; Deng, X. Anti-inflammatory effect of auraptene extracted from trifoliate orange (*Poncirus trifoliate*) on LPS-stimulated RAW 264.7 cells. *Inflammation*, **2013**, *36*(6), 1525-1532.

[121] Murakami, A.; Shigemori, T.; Ohigashi, H. Zingiberaceous and citrus constituents, 1´-acetoxychavicol acetate, zerumbone, auraptene, and nobiletin, suppress lipopolysaccharide-induced cyclooxygenase-2 expression in RAW264.7 murine macrophages through different modes of action. *J. Nutr.*, **2005**, *135*, 2987-2992.

[122] Kohno, S.; Murata, T.; Sugiura, A.; Ito, C.; Iranashi, M.; Hikita, K.; Kaneda, N. Methyl galbanate, a novel inhibitor of nitric oxide production in mouse macrophage RAW264.7 cells. *J. Nat. Med.*, **2011**, *65*(2), 353-359.

[123] Ban, H.S.; Lim, S.S.; Suyuki, K.; Jung, S.H.; Lee, S.; Lee, Y.S.; Shin, K.H.; Ohuchi, K. Inhibitory effects of furanocoumarins isolated from the roots of *Angelica dahurica* on prostaglandin E2 production. *Planta Med.*, **2003**, *69*(5), 408-412.

[124] Fang, S.C.; Hsu, C.L.; Yen, G.C. Anti-inflammatory effects of phenolic compounds isolated from the fruits of *Artocarpus heterophyllus. J. Agric. Food Chem.*, **2008**, *56*(12), 4463–4468.

[125] Jang, D.S.; Cuendet, M.; Hawthorne, M.E.; Kardono, L.B.; Kawanishi, K.; Fong, H.H.; Mehta, R.G.; Pezzuto, J.M.; Kinghorn, A.D. Prenylated flavonoids of the leaves of *Macaranga conifera* with inhibitory activity against cyclooxygenase-2. *Phytochemistry*, **2002**, *61*(7), 867-872.

[126] Hošek, J.; Toniolo, A.; Neuwirth, O.; Bolego, C. Prenylated and geranylated flavonoids increase production of reactive oxygen species in mouse macrophages but inhibit the inflammatory response. *J. Nat. Prod.*, **2013**, *76*(9), 1586-1591.

[127] Chi, Y.S.; Jong, H.G.; Son, K.H.; Chang, H.W.; Kang, S.S.; Kim, H.P. Effects of naturally occurring prenylated flavonoids on enzymes metabolizing arachidonic acid: cycooxygenases and lipoxygenases. *Biochem. Pharmacol.*, **2001**, *62*(9), 1185-1191.

[128] Chen, L.G.; Yang, L.L.; Wang, C.C. Anti-inflammatory activity of mangostins from *Garcinia mangostana. Food Chem. Toxicol.*, **2008**, *46*(2), 688-693.

[129] Epifano, F.; Fiorito, S.; Taddeo, V.A.; Genovese, S. 4´-Geranyloxyferulic acid: an overview of its potentialities as an anti-cancer and anti-inflammatory agent. *Phytochem. Rev.*, **2015**, *14*(4), 607-612.

[130] Paulino, N.; Abreu, S.R.; Uto, Y.; Koyama, D.; Nagasawa, H.; Hori, H.; Dirsch, V.M.; Vollmar, A.M.; Scremin, A.; Bretz, W.A. Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis. *Eur. J. Pharmacol.*, **2008**, *587*(1-3), 296-301.

[131] Fuhr, L.; Rousseau, M.; Plauth, A.; Schroeder, F.C.; Sauer, S. Amorfrutins are natural PPARγ agonists with potent anti-inflammatory properties. *J. Nat. Prod.*, **2015**, *78*(5), 1160-1164.

[132] Henry, G.E.; Campbell, M.S.; Zelinsky, A.A.; Liu, Y.; Bowen-Forbes, C.S.; Li, L.; Nair, M.G.; Rowley, D.C.; Seeram, N.P. Bioactive acylphloroglucinols from *Hypericum densiflorum. Phytoter. Res.*, **2009**, *23*(12), 1759-1762.

[133] Ruhaak, L.R.; Felth, J.; Karlsson, P.C.; Rafter, J.J.; Verpoorte, R.; Bohlin, L. Evaluation of the cyclooxygenase inhibiting effects of six major cannabinoids isolated from Cannabis sativa. *Biol. Pharm. Bull.*, **2011**, *34*(5), 774-778.

[134] Iranshahi, M.; Askari, M.; Sahebkar, A.; Hadjipavlou-Litina, D. Evaluation of antioxidant, anti-inflammatory and lipoxygenase inhibitory activities of the prenylated coumarin umbelliprenin. *DARU J. Pharm. Sci.*, **2009**, *17*(2), 99-103.

[135] Koshihara, Y.; Fujimoto, Y.; Inoue, H. A new 5-lipoxygenase selective inhibitor derived from *Artocarpus communis* strongly inhibits arachidonic acid-induced ear edema. *Biochem. Pharmacol.*, **1988**, *37*(11), 2161-2165.

[136] Dzoyem, J.P.; Nkuete, A.H.; Ngameni, B.; Eloff, J.N. Anti-inflammatory and anticholinesterase activity of six flavonoids isolated from *Polygonum* and *Dorstenia* species. *Arch. Pharm. Res.*, **2015**, doi:10.1007/s12272-015-0612-9.

[137] Njamen, D., Mbafor, J.T.; Fomum, Z.T.; Kamanyi, A.; Mbanya, J.C.; Recio, M.C.; Giner, R.M.; Máñez, S.; Ríos, J.L. Anti-inflammatory activities of two flavanones, sigmoidin A and sigmoidin B, from *Erythrina sigmoidea. Planta Med.*, **2004**, *70*(2), 104-107.

[138] Jin, J.H.; Lim, H.; Kwon, S.Y.; Son, K.H.; Kim, H.P. Anti-Inflammatory activity of the total flavonoid fraction from *Broussonetia papyrifera* in combination with *Lonicera japonica. Biomol. Ther.*, **2010**, *18*(2), 197-204.

[139] Njamen, D.; Talla, E.; Mbafor, J.T.; Fomum, Z.T.; Kamanyi, A.; Mbanya, J.C.; Cerdá-Nicolás, M.; Giner, R.M.; Recio, M.C.; Ríos, J.L. Anti-inflammatory activity of erycristagallin, a pterocarpene from *Erythrina mildbraedii. Eur. J. Pharmacol.*, **2003**, *468*(1), 67-74.

[140] Góngora, L.; Giner, R.M.; Máñez, S.; Recio Mdel, C.; Schinella, G.; Ríos, J.L. Effects of caffeoyl conjugates of isoprenyl-hydroquinone glucoside and quinic acid on leukocyte function. *Life Sci.*, **2002**, *71*(25), 2995-3004.

[141] Yang, H.J.; Youn, H.; Seong, K.M.; Yun, Y.J.; Kim, W.; Kim, Y.H.; Lee, J.Y.; Kim, C.S.; Jin, Y.W.; Youn, B. Psoralidin, a dual inhibitor of COX-2 and 5-LOX, regulates ionizing radiation (IR)-induced pulmonary inflammation. *Biochem. Pharmacol.*, **2011**, *82*(5), 524-534.

[142] Kim, D.W.; Chi, Y.S.; Son, K.H.; Chang, H.W.; Kim, J.S.; Kang, S.S.; Hyun, P.K. Effects of Sophoraflavanone G, a prenylated flavonoid from *Sophora flavescens*, on cyclooxygenase-2 and *in vivo* inflammatory response. *Arch. Pharm. Res.*, **2002**, *25*(3), 329-335.

[143] Chandrasekaran, C.V.; Deepak, H.B.; Thiyagarajan, P.; Kathiresan, S.; Sangli, G.K.; Deepak, M.; Agarwal, A. Dual inhibitory effect of Glycyrrhiza glabra (GutGard™) on

COX and LOX products. *Phytomedicine*, **2011**, *18*(4), 278-284.

[144] Shaari, K.; Suppaiah, V.; Wai, L.K.; Stanslas, J.; Tejo, B.A.; Israf, D.A.; Abas, F.; Ismail, I.S.; Shuaib, N.H.; Zareen, S.; Lajis, N.H. Bioassay-guided identification of an anti-inflammatory prenylated acylphloroglucinol from *Melicope ptelefolia* and molecular insights into its interaction with 5-lipoxygenase. *Bioorg. Med. Chem.*, **2011**, *19*(21), 6340-6347.

[145] Ng, C.H.; Rullah, K.; Aluwi, M.F.; Abas, F.; Lam, K.W.; Ismail, I.S.; Narayanaswamy, R.; Jamaludin, F.; Shaari, K. Synthesis and docking studies of 2,4,6-trihydroxy-3-geranylacetophenone analogs as potential lipoxygenase inhibitor. *Molecules*, **2014**, *19*(8), 11645-11659.

[146] Minassi, A.; Cicione, L.; Koeberle, A.; Bauer, J.; Laufer, S.; Werz, O.; Appendino, G. A multicomponent carba-betti strategy to alkylidene heterodimers–total synthesis and structure-activity relationships of arzanol. *Eur. J. Org. Chem.*, **2012**, *2012*(4), 772-779.

[147] Werz, O.; Seegers, J.; Schaible, A.M.; Weinigel, C.; Barz, D.; Koeberle, A.; Allagrone, G.; Pollastro, F.; Zampieri, L.; Grassi, G.; Appendino, G. Cannflavins from hemp sprouts, a novel cannabinoid-free hemp food product, target microsomal prostaglandin E2 synthase-1 and 5-lipoxygenase. *PharmaNutrition*, **2014**, *2*(3), 53-60.

[148] Forino, M.; Pace, S.; Chianese, G.; Santagostini, L.; Werner, M.; Weinigel, C.; Rummler, S.; Fico, G.; Werz, O.; Tagliatela-Scafati, O. Humudifucol and Bioactive Prenylated Polyphenols from Hops (*Humulus lupulus* cv. "Cascade"). *J. Nat. Prod.*, **2016**, 79(3), 590-597.

[149] Cassini-Vieira, P.; Araújo, F.A.; da Costa Dias, F.L.; Russo, R.C.; Andrade, S.P.; Teixeira, M.M.; Barcelos, L.S. iNOS activity modulates inflammation, angiogenesis, and tissue fibrosis in polyether-polyurethane synthetic implants. *Mediators Inflamm.*, **2015**, *2015*, 138461.

[150] Kobayashi, Y. The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation. *J. Leukoc. Biol*, **2010**, *88*(6), 1157-1162.

[151] Zamora, R.; Vodovotz, Y.; Billiar, T.R. Inducible nitric oxide synthase and inflammatory diseases. *Mol. Med.*, **2000**, *6*(5), 347-373.

[152] Widgerow, A.D., Kalaria, S. Pain mediators and wound healing - Establishing the connection. *Burns*, **2012**, *38*(7), 951-959.

[153] Murakami, A.; Gao, G.; Omura, M.; Yano, M.; Ito, C.; Furukawa, H.; Takahashi, D.; Koshimizu, K.; Ohigashi, H. 1,1-Dimethylallylcoumarins potently supress both lipopolysaccharide- and interferon-γ-induced nitric oxide generation in mouse macrophage RAW 264.7 cells. *Bioorg. Med. Chem. Lett.*, **2000**, *10*(1), 59-62.

[154] Shakeri, A.; Iranshahy, M.; Iranshahi, M. Biological properties and molecular targets of umbelliprenin--a mini-review. *J. Asian Nat. Prod. Res.*, **2014**, *16*(8), 884-889.

[155] Wang, C.C.; Chen, L.G.; Yang, L.L. Inducible nitric oxide synthase inhibitor of the Chinese herb I. *Saposhnikovia divaricata* (Turcz.) Schischk. *Cancer Lett.*, **1999**, *145*(1-2), 151-157.

[156] Chin, Y.W.; Jung, Y.H.; Chae, H.S.; Yoon, K.W.; Kim, J. Anti-inflammatory Constituents from the Roots of *Saposhnikovia divaricate*. *Bull. Kor. Chem. Soc.*, **2011**, *32*(6), 2132-2134.

[157] Chiou, W.F.; Don, M.J.; Liao, J.F.; Wei, B.L. Psoralidin inhibits LPS-induced iNOS expression *via* repressing Syk-mediated activation of PI3K-IKK-IκB signaling pathways. *Eur. J. Pharmacol.*, **2011**, *650*(1), 102-109.

[158] Motai, T.; Daikonya, A.; Kitanaka, S. Sesquiterpene coumarins from *Ferula fukanensis* and nitric oxide production inhibitry effects. *J. Nat. Prod.*, **2004**, *52*(10), 1215-1218.

[159] Motai, T.; Kitanaka, S. Sesquiterpene coumarins from *Ferula fukanensis* and nitric oxide production inhibitory effects. *Chem. Pharm. Bull.*, **2004**, *52*(10), 1215-1218.

[160] Isaka, K.; Nagatsu, A.; Ondognii, P.; Zevgeegiin, O.; Gombosurengyin, P.; Davgiin, K.; Ogihara, Y. Sesquiterpenoid derivatives from *Ferula ferulioides*. *Chem. Pharm. Bull.*, **2001**, *49*(9), 1072-1076.

[161] Chan, Y.Y.; Li, C.H.; Shen, Y.C.; Wu, T.S. Anti-inflammatory principles from the stem and root barks of *Citrus medica. Chem. Pharm. Bull.*, **2010**, *58*(1), 61-65.

[162] Cheng, Z.; Lin, C.; Hwang, T.; Teng, C. Broussochalcone A, a potent antioxidant and effective suppressor of inducible nitric oxide synthase in lipopolysaccharide-activated macrophages. *Biochem. Pharmacol.*, **2001**, *61*(8), 939-946.

[163] Zhao, F.; Nozawa, H.; Daikonnya, A.; Kondo, K.; Kitanaka, S. Inhibitors of nitric oxide production from hops (*Humulus lupulus* L.). *Biol. Pharm. Bull.*, **2003**, *26*(1), 61-65.

[164] Damodar, K.; Kim, J.K.; Jun, J.G. Synthesis and pharmacological properties of naturally occurring prenylated and pyranochalcones as potent anti-inflammatory agents. *Chin. Chem. Lett.*, **2016**, *27*(5), 698-702.

[165] Han, A.R.; Kang, Y.J.; Windono, T.; Lee; S.K.; Seo, E.K. Prenylated flavonoids from the heartwood of *Artocarpus communis* with inhibitory activity on lipopolysaccharide-induced nitric oxide production. *J. Nat. Prod.*, **2006**, *96*(4), 719-721.

[166] Chahyadi, A.; Hartati, R.; Wirasutisna, K.R.; Elfahmi. *Boesenbergia pandurata* Roxb., An Indonesian medicinal plant: phytochemistry, biological activity, plant biotechnology. *Procedia Chem.*, **2014**, *13*, 13-37.

[167] Wang, G.W.; Huang, B.K.; Qin, L.P. The genus *Broussonetia*: a review of its phytochemistry and pharmacology. *Phytother. Res.*, **2012**, *26*(1), 1-10.

[168] Cheon, B.S.; Kim, Y. H.; Son, K.S.; Chang, H.W.; Kang, S.S.; Kim, H.P. Effects of prenylated flavonoids and bioflavonoids on lipopolysaccharide-induced nitric oxide production from the mouse macrophage cell line RAW 264.7. *Planta Med.*, **2000**, *66*(7), 596-600.

[169] Yang, Z.G.; Matsuzaki, K.; Takamatsu, S.; Kitanaka, S. Inhibitory effects of constituents from *Morus alba* var. *multicaulis* on differentiation of 3T3-L1 cells and nitric oxide production in RAW 264.7 cells. *Molecules*, **2011**, *16*(7), 6010-6022.

[170] Qin, J.; Fan, M.; He, J.; Wu, X.D.; Peng, L.Y.; Su, J.; Cheng, X.; Li, Y.; Kong, L.M.; Li, R.T.; Zhao, Q.S. New cytotoxic and anti-inflammatory compounds isolated from *Morus alba* L. *Nat. Prod. Res.*, **2015**, *29*(18), 1711-1718.

[171] Cerqueira, F.; Cidade, H.; van Ufford, L.; Beukelman, C.; Kijjoa, A.; Nascimento, M.S. The natural prenylated flavone artelastin is an inhibitor of ROS and NO production. *Int. Immunopharmacol.*, **2008**, *8*(4), 597-602.

[172] Jin, Q.; Lee, C.; Lee, J.W.; Lee, D.; Kim, Y.; Hong, J.T.; Kim, J.S.; Kim, J.H.; Lee, M.K.; Hwang, B. Y. Geranylated flavanones from *Paulownia coreana* and their inhibitory effects on nitric oxide production. *Chem. Pharm. Bull.*, **2015**, *63*(5), 384-387.

[173] Nešuta, O.; López-Abán J.; Shariati, F.; Yepes, E.; Navrátilová, A.; Šmejkal, K.; Žemlička, M.; Muro, A. Polyphenols as inhibitors of NO production in LPS-stimulated rat macrophages. *J. Nat. Pharm.*, **2011**, *2*(1), 15-19.

[174] Thiyagarajan, P.; Chandrasekaran, C.V.; Deepak, H.B.; Agarwal, A. Modulation of lipopolysaccharide-induced pro-inflammatory mediators by an extract of *Glycyrrhiza glabra* and its phytoconstituents. *Inflammopharmacology*, **2011**, *19*(4), 235-241.

[175] Zhang, H.; Zhang, D.D.; Lao, Y.Z.; Fu, W.W.; Liang, S; Yuang, Q.H.; Yang, L.; Xu, H.X. Cytotoxic and anti-inflammatory prenylated benzoylphloroglucinols and xanthones from the twigs of *Garcinia esculenta. J. Nat. Prod.*, **2014**, *77*(7), 1700-1707.

[176] Ishii, R.; Horie, M.; Saito, K.; Arisawa, M.; Kitanaka, S. Inhibitory effects of phloroglucinol derivatives from *Mallotus japonicus* on nitric oxide production by a murine macrophage-like cell line, RAW 264.7, activated by lipopolysaccharide and interferon-gamma. *Biochim. Biophys. Acta*, **2001**, *1568*(1), 74-78.

[177] Xin, W.B.; Man, X.H.; Zheng, C.J.; Jia, M.; Jiang, Y.P.; Zhao, X. X.; Jin, G.L.; Mao, Z.J.; Huang, H.Q.; Qin, L.P. Prenylated phloroglucinol derivatives from *Hypericum sampsonii. Fitoterapia*, **2012**, *83*(8), 1540-1547.

[178] Closa, D.; Folch-Puy, E. Oxygen free radicals and the systemic inflammatory response. *IUBMB Life*, **2004**, *56*(4), 185-191.

[179] Mittal, M.; Siddiqui, M.R.; Tran, K.; Reddy, S.P.; Malik, A.B. Reactive oxygen species in inflammation and tissue injury. *Antioxid. Redox Signal.*, **2014**, *20*(7), 1126-1167.

[180] Kelkka, T. *Reactive oxygen species in inflammation*. Thesis, Turun Yliopisto University of Turku: Turku, **2013**.

[181] Lavrovsky, Y.; Chatterjee, B.; Clark, R.A.; Roy, A.K. Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp. Gerontol.*, **2000**, *35*(5), 521-532.

[182] Figarola, J.L.; Shanmugam, N.; Natarajan, R.; Rahbar, S. Anti-inflammatory effects of the advanced glycation end product inhibitor LR-90 in human monocytes. *Diabetes*, **2007**, *56*(3), 647-655.

[183] Liu, W.B.; Zhou, J.; Qu, Y.; Li, X.; Lu, C.T.; Xie, K.L.; Sun, X.L.; Fei, Z. Neuroprotective effect of osthole on MPP+-induced cytotoxicity in PC12 cells *via* inhibition of mitochondrial dysfunction and ROS production. *Neurochem. Int.*, **2010**, *57*(3), 206-215.

[184] Mojarrab, M.; Delazar, A.; Moghadam, S.B.; Nayemiyeh, H.; Nahar, L.; Kumarasamy, Y.; Asnaaashari, S.; Hadjiakhoondi, A.; Sarker, S.D. Armenin and isoarmenin-two prenylated coumarins from the aerial parts of *Artemisia armeniaca. Chem. Biodivers.*, **2011**, *8*(11), 2097-2103.

[185] Torres, R.; Faini, F.; Modak, B.; Urbina, F.; Labbé, C.; Guerrero, J. Antioxidant activity of coumarins and flavonols from the resinous exudate of *Haplopappus multifolius. Phytochemistry*, **2006**, *67*(10), 984-987.

[186] Yang, H.; Protiva, P.; Gil, R.R.; Jiang, B.; Baggett, S.; Basile, M.J.; Reynerston, K.A.; Weinstein, I.B.; Kenelly, E.J. Antioxidant and cytotoxic isoprenylated coumarins from *Mammea americana. Planta Med.*, **2005**, *71*(9), 852-860.

[187] Mohammadi, M.; Yousefi, M.; Habibi, Z.; Shafiee, A. Two new coumarins from the chloroform extract of *Angelica urumiensis* from Iran. *Chem. Pharm. Bull.*, **2010**, *58*(4), 546-548.

[188] Narváez-Mastache, J. M.; Novillo, F.; Delgado, G. Antioxidant aryl-prenylcoumarin, flavan-3-ols and flavonoids from *Eysenhardtia subcoriacea. Phytochemistry*, **2008**, *69*(2), 451-456.

[189] Razavi, S.M.; Nazemiyeh, H.; Hajiboland, R.; Yashodharan, K.; Delazar, A.; Nahar, L.; Sarker, S.D. Coumarins from the aerial parts of *Prangos uloptera* (Apiaceae). *Braz. J. Pharmacogn.*, **2007**, *18*(1), 1-5.

[190] Koorbanally, N.A.; Randrianarivelojosia, M.; Mulholland, D.A.; Quarles van Ufford, L.; van den Berg, A.J. Bioactive constituents of *Cedrelopsis microfoliata. J. Nat. Prod.*, **2002**, *65*(9), 1349-1352.

[191] Shen, D.Y.; Chan, Y.Y.; Hwang, T.L.; Juang, S.H.; Huang, S.C.; Kuo, P.C.; Thang, T.D.; Lee, E.J.; Damu, A.G.; Wu, T.S. Constituents of the roots of *Clausena lansium* and their potential anti-inflammatory activity. *J. Nat. Prod.*, **2014**, *77*(5), 1215-1223.

[192] Kostova, I.; Bhatia, S.; Grigorov, P.; Balkansky, S.; Parmar, V.S.; Prasad, A.K.; Saso, L. Coumarins as antioxidants. *Curr. Med. Chem.*, **2011**, *18*(25), 3929-3951.

[193] Wang, J.P.; Tsao, L.T.; Raung, S.L.; Lin, C.N. Investigation of the inhibitory effect of broussochalcone A on respiratory burst in neutrophils. *Eur. J. Pharmacol.*, **1997**, *320*(2-3), 201-208.

[194] Luo, L.; Wang, R.; Wang, X.; Ma, X.; Li, N. Compounds from *Angelica keiskei* with NQO1 induction, DPPH scavenging α -glucosidase inhibitory activities. *Food Chem.*, **2012**, *131*, 992-998.

[195] Jung, H.A.; Jeong, D.M.; Chung, H.Y.; Lim, H.A.; Kim, J.Y.; Yoon, N.Y.; Choi, J.S. Re-evaluation of the antioxidant prenylated flavonoids from the roots of *Sophora flavescens. Biol. Pharm. Bull.*, **2008**, *31*(5), 908-915.

[196] Vogel, S.; Heilmann, J. Synthesis, cytotoxicity, and antioxidative activity of minor prenylated chalcones from *Humulus lupulus. J. Nat. Prod.*, **2008**, *71*(7), 1237-1241.

[197] Popoola, O.K.; Marnewick, J.L.; Rautenbach, F.; Ammeer, F.; Iwuoha, E.I.; Hussein, A.A. Inhibition of oxidative stress and skin aging-related enzymes by prenylated chalcones and other flavonoids from *Helichrysum teretifolium. Molecules*, **2015**, *20*(4), 7143-7155.

[198] Dufall, K.G.; Ngadjui, B.T.; Simeon, K.F.; Abegaz, B.M.; Croft, K.D. Antioxidant activity of prenylated flavonoids from the West African medicinal plant *Dorstenia manii. J. Ethnopharmacol.*, **2003**, *87*(1), 67-72.

[199] Lee, B.W.; Lee, J.H.; Gal, S.W.; Moon, Y.H.; Park, K.H. Selective ABTS radical-scavenging activity of prenylated flavonoids from *Cudrania tricuspidata. Biosci. Biotechnol. Biochem.*, **2006**, *70*(2), 427-432.

[200] Zima, A.; Hošek, J.; Treml, J.; Muselík, J.; Suchý, P.; Pražanová, G.; Lopes, A.; Žemlička, M. Antiradical and cytoprotective activities of several *C*-geranyl-substituted flavanones from *Paulownia tomentosa* fruit. *Molecules*, **2010**, *15*(9), 6035-6049.

[201] Lan, W.C.; Tzeng, C.W.; Lin, C.C.; Yen, F.L.; Ko, H.H. Prenylated flavonoids from *Artocarpus altilis*: antioxidant activities and inhibitory effects on melanin production. *Phytochemistry*, **2013**, *89*, 78-88.

[202] Diopan, V.; Babula, P.; Shestivska, V.; Adam, V.; Žemlička, M.; Dvorská, M.; Hubálek, J.; Trnková, L.; Havel, L.; Kizek, R. Electrochemical and spectrometric study of antioxidant activity of pomiferin, isopomiferin, osajin and catalposide. *J. Pharm. Biomed. Anal.*, **2008**, *48*(1), 127-133.

[203] Veselá, D.; Kubínová, R.; Muselík, J.; Žemlička, M.; Suchý, V. Antioxidative and EROD activities of osajin and pomiferin. *Fitoterapia*, **2004**, *75*(2), 209-211.

[204] Uddin, G.M.; Lee, H.J.; Jeon, J.S.; Chung, D.; Kim, Ch.Y. Isolation of prenylated isoflavonoids from *Cudrania tricuspidata* fruits that inhibit A2E photooxidation. *Nat. Prod. Sci.*, **2011**, *17*(3), 206-211.

[205] Lannang, A.M.; Komguem, J.; Ngninzeko, F.N.; Tangmouo, J.G.; Lontsi, D.; Ajaz, A.; Choudhary, M.I.; Ranjit, R.; Devkota, K.P.; Sondengam, B.L. *Phytochemistry*, **2005**, *66*(19), 2351-2355.

[206] Minami, H.; Kuwayama, A.; Yoshizawa, T.; Fukuyama, Y. Novel prenylated xanthones with antioxidant property from the wood of *Garcinia subelliptica. Chem. Pharm. Bull.*, **1996**, *44*(11), 2103-2106.

[207] Xu, T.; Deng, Y.; Zhao, S.; Shao, Z. A new xanthone from the pericarp of *Garcinia mangostana. J. Chem. Res.*, **2016**, *40*, 10-11.

[208] Zhou, X.; Huang, R.; Hao, J.; Huang, H.; Fu, M.; Xu, Z.; Zhou, Y.; Li, X.; Qiu, S. X.; Wang, B. Two new prenylated

xanthones from the pericarp of *Garcinia mangostana* (Mangosteen). *Helv. Chem. Acta*, **2011**, *94*(11), 2092-2098.

[209] Kandem Waffo, A.F.; Mulholland, D.; Wansi, J.D.; Mbaze, L.M.; Powo, R.; Mpondo, T.N.; Fomum, Z.T.; König, W.; Nkengfack, A.E. Afzeliixanthones A and B, two prenylated xanthones from *Garcinia afzelii* ENGL. (Guttiferae). *Chem. Pharm. Bull.*, **2006**, *54*(4), 448-451.

[210] Merza, J.; Aumond, M.C.; Rondeau, D.; Dumontet, V.; Le Ray, A.M; Séraphin, D.; Richomme, P. Prenylated xanthones and tocotrienols from *Garcinia virgata. Phytochemistry*, **2004**, *65*(21), 2915-2920.

[211] Kapche, G.D.; Foying, C.D.; Donfack, J.H.; Fotso, G.W.; Amadou, D.; Tchana, A.N.; Bezabih, M.; Moundipa, P.F.; Ngadjui, B.T.; Abegaz, B.M. Prenylated arylbenzofuran derivatives from *Morus mesozygia* with antioxidant activity. *Phytochemistry*, **2009**, *70*(2), 216-221.

[212] Kyekyeku, J.O.; Kusari, S.; Adosraku, R.K.; Zühlke, S.; Spiteller, M. Prenylated 2-arylbenzofuran derivatives with potent antioxidant properties from *Chlorophora regia* (Moraceae). *Fitoterapia*, **2016**, *108*, 41-47.

[213] Radulović, N.S.; Randjelović, P.J.; Stojanović, N.M.; Cakić, N.D.; Bogdanović, G.A.; Živanović, A.V. Aboriginal bush foods: A major phloroglucinol from Crimson Bottlebrush flowers (*Callistemon citrinus*, Myrtaceae) displays strong antinociceptive and anti-inflammatory activity. *Food Res. Inst.*, **2015**, *77*(2), 280-289.

[214] Rosa, A.; Deiana, M.; Atzeri, A.; Corona, G.; Incani, A.; Melis, M.P.; Appendino, G.; Dessi, M.A. Evaluation of the antioxidant and cytotoxic activity of arzanol, a prenylated alpha-pyrone-phloroglucinol etherodimer from *Helichrysum italicum* subsp. *microphyllum. Chem. Biol. Interact.*, **2007**, *165*(2), 117-126.

[215] Chen, J.J.; Cho, J.Y.; Hwang, T.L.; Chen, I.S. Benzoic acid derivatives, acetophenones, and anti-inflammatory constituents from *Melicope semecarpifolia. J. Nat. Prod.*, **2008**, *71*(1), 71-75.

[216] Yamaguchi, F.; Saito, M.; Ariga, T.; Yoshimura, Y.; Nakazawa, H. Free radical scavenging activity and antiulcer activity of garcinol from *Garcinia indica* fruit rind. *J. Agric. Food. Chem.*, **2000**, *48*, 2320-2325.

[217] Ha, L.D.; Hansen, P.E.; Duus, F.; Pham, H.D.; Nguyen, L.H. Oliveridepsidones A-D, antioxidant depsidones from *Garcinia oliveri. Magn. Reson. Chem.*, **2012**, *50*(3), 242-245.

[218] Sansom, C.E.; Larsen, L.; Perry, N.B.; Berridge, M.V.; Chia, E.W.; Harper, J.L.; Webb, V.L. An antiproliferative bis-prenylated quinone from the New Zealand brown alga *Perithalia capillaris. J. Nat. Prod.*, **2007**, *70*(12), 2042-2044.

[219] Yamaguchi, L.F.; Lago, J.H.G.; Tanizaki, T.M.; Di Mascio, P.; Kato, M.J. Antioxidant activity of prenylated hydroquinone and benzoic acid derivatives from *Piper crassinervium* Kunth. *Phytochemistry*, **2006**, *67*, 1838-1843.

[220] Wu, N.; Fu, K.; Fu, Y.J.; Zu, Y.G.; Chang, F.R.; Chen, Y.H.; Liu, X.L.; Kong, Y.; Liu, W.; Gu, C.B. Antioxidant activities of extracts and main components of Pigeonpea [Cajanus cajan (L.) Millsp.] leaves. *Molecules*, **2009**, *14*(3), 1032-1043.

[221] Winyard, P.G.; Willoughby, D.A. *Inflammation protocols*. Humana Press Inc.: Totowa NJ, **2003**.

[222] Wu, L.; Li, P.; Wang, X.; Zhuang, Z.; Farzaneh, F.; Xu, R. Evaluation of anti-inflammatory and antinociceptive activities of *Murraya exotica. Pharm. Biol.*, **2010**, *48*(12), 1344-1353.

[223] Khan, S.; Shehzad, O.; Chun, J.; Kim, Y.S. Mechanism underlying anti-hyperalgesic and anti-allodynic properties of anomalin in both acute and chronic inflammatory pain models in mice through inhibition of NF-κB, MAPKs and

CREB signaling cascades. *Eur. J. Pharmacol.*, **2013**, *718*(1-3), 448-458.

[224] Sokeng, S.D.; Talla, E.; Jeweldai, V.; Yaja, A.J.G.; Koube, J.; Dongmo, F.; Goulimé, M.; Mbafor, J.T. Anti-inflammatory effect of abyssinone V-4′-methyl ether on acute and chronic inflammation models. *Hygeia: J. Drugs Med.*, **2013**, *5*(1), 121-128.

[225] Kupeli, E.; Orhan, I.; Toker, G.; Yesilada, E. Anti-inflammatory and antinociceptive potential of *Maclura pomifera* (Rafin.) Schneider fruit extracts and its major isoflavonoids, scandenone and auriculasin. *J. Ethnopharmacol.*, **2006**, *107*(2), 169-174.

[226] Santa-Cecília, F.V.; Freitas, L.A.; Vilela, F.C.; Veloso, C. de C.; da Rocha, C.Q.; Moreira, M.E.; Dias, D.F.; Giusti-Paiva, A.; dos Santos, M.H. Antinociceptive and anti-inflammatory properties of 7-epiclusianone, a prenylated benzophenone from *Garcinia brasiliensis. Eur. J. Pharmacol.*, **2011**, *670*(1), 280-285.

[227] Costa, B., Colleoni, M.; Conti, S.; Parolaro, D.; Franke, C.; Trovato, A.E.; Giagnoni, G. Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituens of cannabis, in acute carrageenan-induced inflammation in the rat paw. *Naunyn Schmiedebergs Arch. Pharmacol.*, **2004**, *369*(3), 294-299.

[228] DeLong, G.T.; Wolf, C.E.; Poklis, A.; Lichtman, A.H. Pharmacological evaluation of the natural constituent of *Cannabis sativa,* cannabichromene and its modulation by Δ^9-tetrahydrocannabinol. *Drug Alcohol Depend.*, **2010**, *112*(1-2), 126-133.

[229] Yankep, E.; Njamen, D.; Fotsing, M.T.; Fomum, Z.T.; Mbanya, J.C.; Giner, R.M.; Recio, M.C.; Máñez, S.; Ríos, J.L. Griffonianone D, an isoflavone with anti-inflammatory activity from the root bark of *Millettia griffoniana. J. Nat. Prod.*, **2003**, *66*(9), 1288-1290.

[230] Talla, E., Njamen, D.; Mbafor, J.T.; Fomum, Z.T.; Kamanyi, A.; Mbanya, J.C.; Giner, R.M.; Recio, M.C.; Máñez, S.; Ríos, J.L. Warangalolne, the isoflavonoid anti-inflammatory principle of *Erythrina addisoniae* stem bark. *J. Nat. Prod.*, **2003**, *66*(6), 891-893.

[231] Akazawa, H.; Kohno, H.; Tokuda, H.; Suzuki, N.; Yasukawa, K.; Kimura, Y.; Manosroi, A.; Manosroi, J.; Akihisa, T. Anti-inflammatory and anti-tumor-promoting effects of 5-deprenyllupulonol C and other compounds from Hop (*Humulus lupulus* L.). *Chem. Biodivers.,* **2012**, *9*(6), 1045-1054.

[232] Curini, M.; Epifano, F.; Maltese, F.; Marcotullio, M.C.; Tubaro, A.; Altinier, G.; Gonyales, S.P.; Rodriguez, J.C. Synthesis and anti-inflammatory activity of natural and semisynthetic geranyloxycoumarins. *Bioorg. Med. Chem. Lett.*, **2004**, *14*(9), 2241-2243.

[233] Menghini, L.; Epifano, F.; Genovese, S.; Marcotullio, M.C.; Sosa, S.; Tubaro, A. Antiinflammatory activity of coumarins from *Ligusticum lucidum* Mill. subsp. *cuneifolium* (Guss.) Tammaro (Apiaceae). *Phytother. Res.*, **2010**, *24*(11), 1697-1699.

[234] Li, Z.; Ji, X.; Song, X.; Hu, J.; Han, N.; Chen, N. Osthole attenuates the development of carrageenan-induced lung inflammation in rats. *Int. Immunopharmacol*, **2014**, *20*(1), 33-36.

[235] Xiong, Y.; Wang, J.; Wu, F.; Li, J.; Zhou, L.; Kong, L. Effects of (±)-praeruptorin A on airway inflammation, airway hyperresponsiveness and NF-κB signaling pathway in a mouse model of allergic airway disease. *Eur. J. Pharmacol.*, **2012**, *683*(1-3), 316-324.

[236] Lu, Y.; Cai, S.; Nie, J.; Li, Y.; Shi, G.; Hao, J.; Fu, W.; Tan, H.; Chen, S.; Li, B.; Xu, H. The natural compound nujiangexanthone A suppresses mast cell activation and allergic asthma. *Biochem. Pharmacol.*, **2016**, *100*, 61-72.

[237]   Lee, C.W.; Ko, H.H.; Lin, C.C.; Chai, C.Y.; Chen, W.T.; Yen, F.L. Artocarpin attenuates ultraviolet B-induced skin damage in hairless mice by antioxidant and anti-inflammatory effect. *Food Chem. Toxicol.*, **2013**, *60*, 123-129.

[238]   Malfait, A.M.; Gallily, R.; Sumariwalla, P.F.; Malik, A.S.; Andreakos, E.; Mechoulam, R.; Feldmann, M. The nonpsy-choactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc. Natl. Acad. Sci. USA*, **2000**, *97*(17), 9561-9566.

[239]   Vochyánová, Z.; Bartošová, L.; Bujdáková, V.; Fictum, P.; Husník, R.; Suchý, P.; Šmejkal, K.; Hošek, J. Diplacone and mimulone ameliorate dextran sulfate sodium induced colitis in rats. *Fitoterapia,* **2015**, *101,* 201-207.

[240]   Dorn, C.; Massinger, S.; Wuzik, A.; Heilmann, J.; Helle-brand, C. Xanthohumol suppresses inflammatory response to warm ischemia-reperfusion induced liver injury. *Exp. Mol. Pathol.*, **2013**, *94*(1), 10-16.

[241]   Okuyama, S.; Morita, M.; Kaji, M.; Amakura, Y.; Yoshi-mura, M.; Shimamoto, K.; Ookido, Y.; Nakajima, M.; Fu-rukawa, Y. Auraptene acts as an anti-Inflammatory agent in the mouse brain. *Molecules*, **2015**, *20*(11), 20230-20239.

[242]   Pertwee, R.G. Pharmacological and therapeutic targets for Δ9 tetrahydrocannabinol and cannabidiol. *Euphytica*, **2004**, *140*, 73-82.

[243]   Lahat, A.; Lagn, A.; Ben-Horin, S. Impact of cannabis treatment on the quality of life, weight and clinical disease activity in inflammatory bowel disease patients: A pilot prospective study. *Digestion*, **2012**, *85*(1), 1-8.

[244]   Ravikoff Allegretti, J.; Courtwright, A.; Lucci, M.; Korzen-ik, J.R.; Levine J. Marijuana use patterns among patients with inflammatory bowel disease. *Inflamm. Bowel Dis.,* **2013**, *19*(13), 2809-2814.

[245]   Naftali, T.; Bar-Lev Schleider, L.; Dotan, I.; Lanski, E. P.; Sklerovski Benjaminov, F.; Konikoff, F.M. Cannabis in-duces a clinical response in patients with Crohn's disease: a prospective placebo-controlled study. *Clin. Gastroenterol. Hepatol.,* **2013**, *11*(10), 1276-1280.e1

[246]   Schicho, R.; Storr, M. Cannabis finds its way into treatment of crohn's disease. *Pharmacology,* **2014**, *93*, 1-3.

[247]   Alhouayek, M.; Muccioli, G.G. The endocannabinoid sys-tem in inflammatory bowel diseases: from pathophysiology to therapeutic opportunity. *Trends Mol. Med.,* **2012**, *18*(10), 615-625.

[248]   Burstein, S. Cannabidiol (CBD) and its analogs: a review of their effects on inflammation. *Bioorg. Med. Chem.,* **2015**, *23*(7), 1377-1385.

[249]   Hall, A.J.; Babish, J.G.; Darland G.K.; Carroll, B.J.; Konda, V.R.; Lerman, R.H.; Bland, J.S.; Tripp, M.L. Safety, effica-cy and anti-inflammatory activity of rho iso-alpha-acids from hops. *Phytochemistry,* **2008**, *69*(7), 1534-1547.