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

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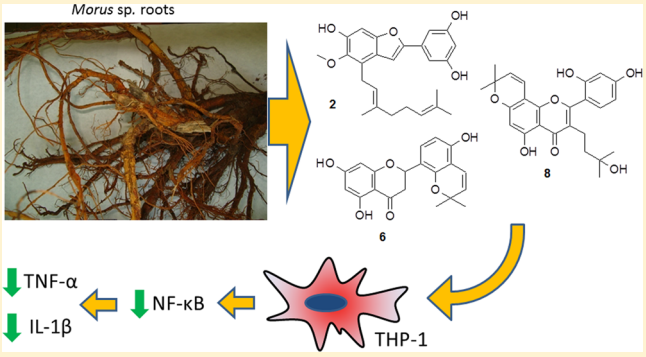
ABSTRACT: Chromatographic separation of root extracts of *Morus alba* and *M. nigra* led to the identification of the 2-arylbenzofurans moracin C (**1**), mulberrofuran Y (**2**), and mulberrofuran H (**3**), and the prenylated flavonoids kuwanon E (**4**), kuwanon C (**5**), sanggenon H (**6**), cudraflavone 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]

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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 dichroism 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.

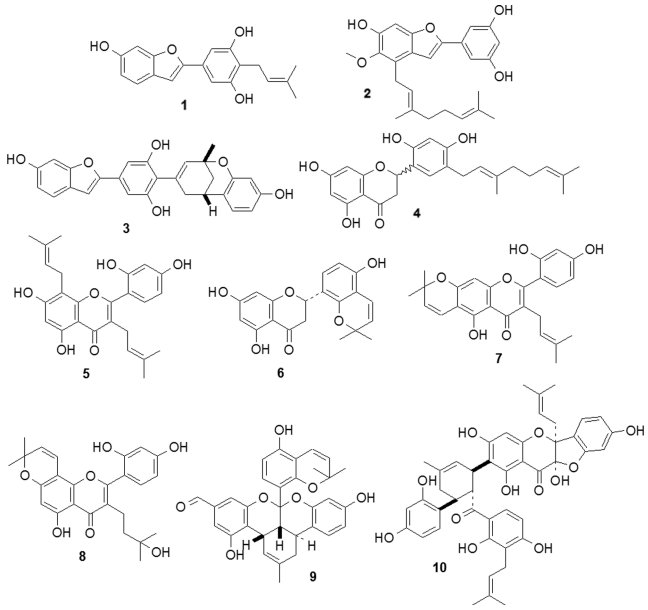
RESULTS AND DISCUSSION

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 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

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.

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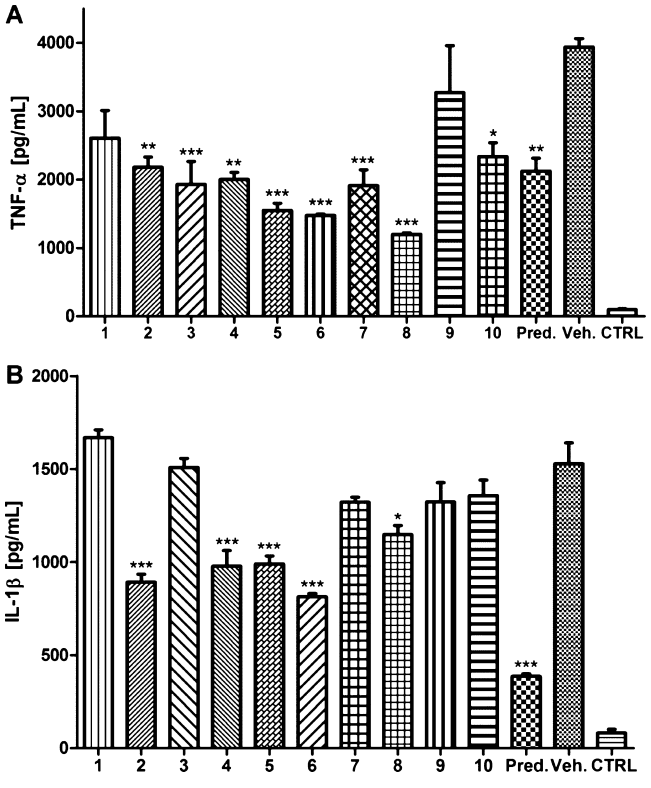

Figure 1. Effects of compounds isolated from mulberry root bark and prednisone on the LPS-induced secretion of TNF-α (A) and IL-1β (B). The cells were pretreated with compounds **1**–**10** (1 μM), prednisone (**Pred.**, 1 μM), or the vehicle (**Veh.**, DMSO) only. After 1 h of incubation, the inflammatory 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

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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**, **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]

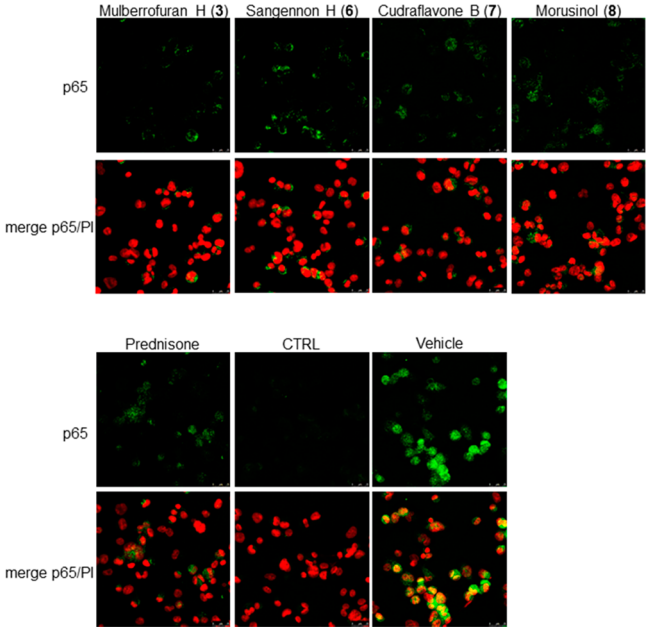

Figure 2. Effect of compounds **3**, **6**, **7**, and **8** on the nuclear translocation of NF-κB (p65) as determined by immunohistochemistry (green color). The cells were counterstained with propidium iodide (PI; red color) to visualize the nuclei. The colocalization of the fluorescence-conjugated 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.

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_6H_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_6H_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_6H_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_6H_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_6H_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_6H_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.

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