

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

Jan Hošek,^{*,†} Milan Bartos,^{†,‡} Stanislav Chudík,[†] Stefano Dall'Acqua,[§] Gabbriella Innocenti,[§] Murat Kartal,[⊥] Ladislav Kokoška,^{||} Peter Kollár,[∇] Zsófia Kutil,^{||,○} Přemysl Landa,[○] Radek Marek,[◇] Veronika Závalová,[∇] Milan Žemlička,[†] and Karel Šmejkal^{*,†}

[†]Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1-3, Brno, 612 42, Czech Republic

[‡]Genex CZ, Brno, Czech Republic

[§]Department of Pharmaceutical Sciences, Natural Product Laboratory, University of Padua, Via Marzolo 5, Padua, 351 31, Italy

[⊥]Department of Pharmacognosy, Faculty of Pharmacy, Ankara University, Tandogan, 06100, Ankara, Turkey

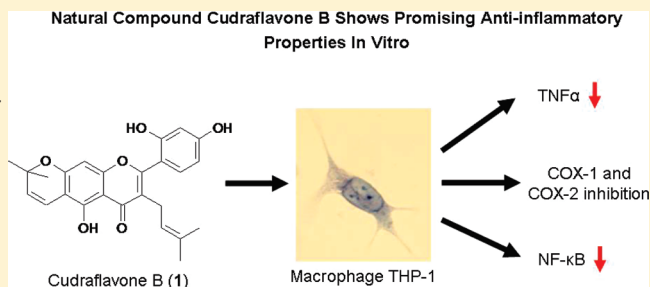
^{||}Department of Crop Sciences and Agroforestry, Institute of Tropics and Subtropics, Czech University of Life Sciences Prague, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic

[∇]Department of Human Pharmacology and Toxicology, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackého 1-3, Brno, 612 42, Czech Republic

[○]Laboratory of Plant Biotechnologies, Joint Laboratory of the Institute of Experimental Botany of the Academy of Science of the Czech Republic and Research Institute of Crop Production, Rozvojová 263, 165 02, Prague, Czech Republic

[◇]National Center for Biomolecular Research, Faculty of Science, Masaryk University, Kamenice 5/A4, CZ-625 00 Brno, Czech Republic

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 (TNF α) gene expression and secretion by blocking the translocation of nuclear factor κ B (NF- κ B) from the cytoplasm to the nucleus in macrophages derived from a THP-1 human monocyte cell line. The NF- κ B activity reduction resulted in the inhibition of cyclooxygenase 2 (COX-2) gene 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 a 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 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 E₂ (PGE₂) 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-823¹³ and mouse melanoma cells B16,¹⁴ moderate

inhibitory effects on mouse brain monoamine oxidase (MAO),¹⁵ and antiproliferative activity caused by the down-regulation 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 ZFP36. This protein binds to the AU-rich regions of some pro-inflammatory mRNAs (such as

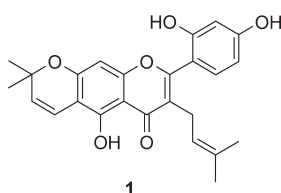
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TNF α), destabilizes them, and consequently decreases their production.¹⁸

Nuclear factor κ B (NF- κ B) (in the present paper “NF- κ B” 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 (TNF α) or CCL2, and enzymes, such as cyclooxygenase 2 (COX-2) and inducible NO-synthase (iNOS or NOS2).^{19,20} NF- κ B 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.



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 ¹H and ¹³C 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 μ M) of this compound for 24 h, stained for viability, and counted. The final LD₅₀ value for **1** was determined to be 47.6 μ M. To compare the cytotoxicity of **1** with a clinically used anti-inflammatory agent, increasing concentrations of indomethacin (1–100 μ M) were added to THP-1 cells. The LD₅₀ value for indomethacin derived from this data was assessed as >100 μ M.

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 paper²⁴) showed that **1** is a potent inhibitor of TNF α 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 μ M, decreased significantly the transcription of TNF α 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 TNF α by a factor of 5.8 in comparison with vehicle-only treated cells. Cudraflavone B (**1**) decreased TNF α transcription more effectively than indomethacin (Figure 1). The inhibition of TNF α transcription caused by **1** was in concert with the inhibited TNF α 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.

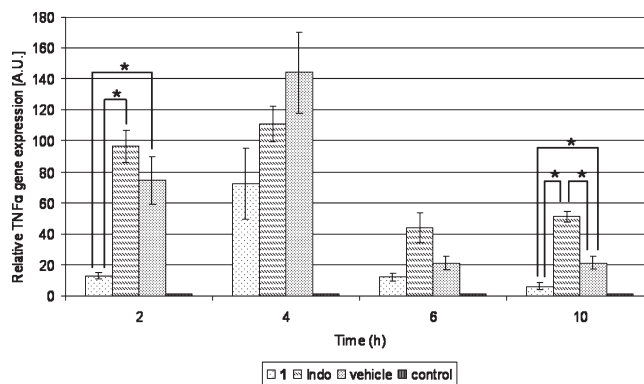


Figure 1. Effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced TNF α gene expression. Cells were pretreated with **1** (10 μ M), indomethacin (10 μ M), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results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

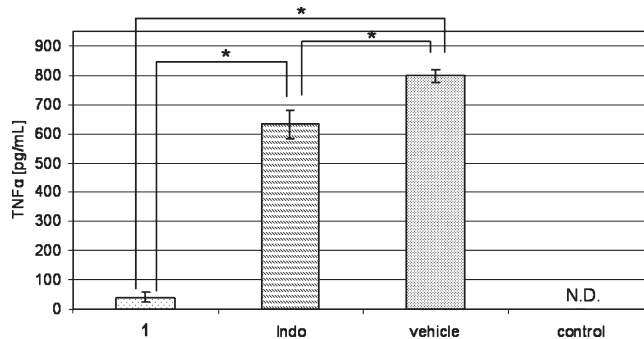


Figure 2. Effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced TNF α protein synthesis. Cells were pretreated with **1** (10 μ M), indomethacin (10 μ M), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are means \pm SE for three independent experiments. N.D. = not detected. * p < 0.05.

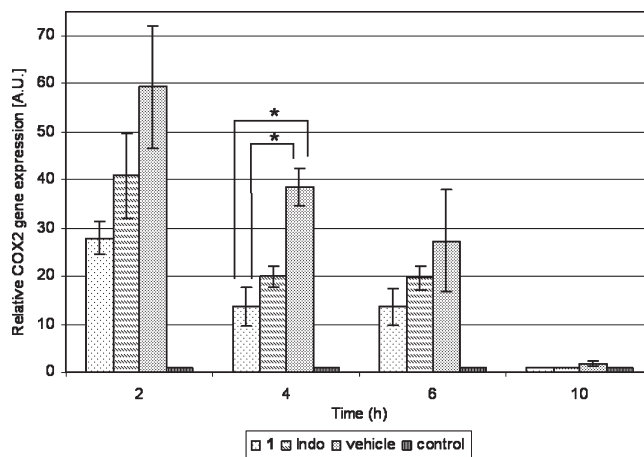


Figure 3. Comparison of the effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced COX-2 gene expression. Cells were pretreated with **1** (10 μ M), indomethacin (10 μ M), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control cells). Results are means \pm SE for three independent experiments. A.U. = arbitrary unit. * p < 0.05.

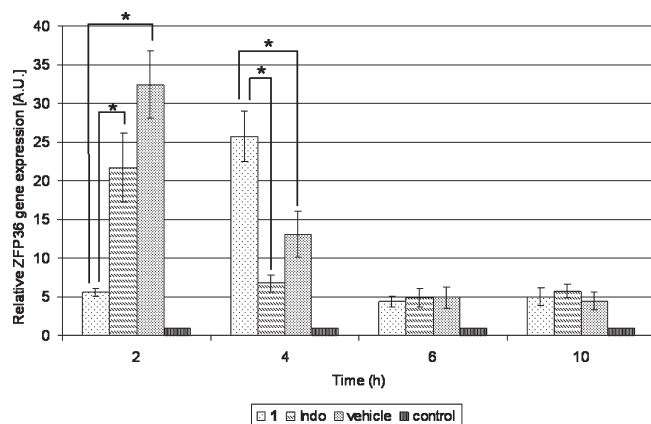


Figure 4. Comparison of the effects of cudraflavone B (**1**) and indomethacin (Indo) on LPS-induced ZFP36 gene expression. Cells were pretreated with **1** ($10 \mu\text{M}$), indomethacin ($10 \mu\text{M}$), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS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

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 $2.5 \pm 0.89 \mu\text{M}$ (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 $1.9 \pm 0.61 \mu\text{M}$ (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 $1.5 \pm 0.65 \mu\text{M}$ (mean \pm SE for four independent experiments with at least two replicates). For indomethacin, the COX-1 IC_{50} value was equal to $0.3 \pm 0.14 \mu\text{M}$ (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 pro-inflammatory genes, its influence on the activity of the transcription factor NF- κ B, which controls the production of the relevant mRNA, was evaluated. The inactive form of NF- κ B is kept in the cytoplasm, whereas the active form is located in the nucleus.²¹ The ratio between the cytoplasmic and the nuclear NF- κ B 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- κ B in their nuclei 3.2 times lower than in vehicle-only treated cells. Similar results were observed for indomethacin-treated cells.

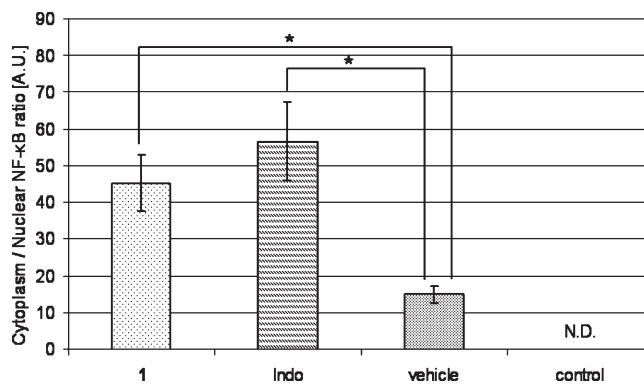


Figure 5. Effect of cudraflavone B (**1**) and indomethacin on LPS-induced NF- κ B nuclear translocation. Cells were pretreated with **1** ($10 \mu\text{M}$), indomethacin ($10 \mu\text{M}$), or the vehicle only. After 1 h of incubation, the inflammatory response was induced by LPS (except for the control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

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 anti-inflammatory 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 $100 \mu\text{M}$ 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 $90 \mu\text{M}$) than were used here ($10 \mu\text{M}$) 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 TNF α was decreased

significantly by **1** on both transcriptional and translational levels. Previously, a decrease in the level of TNF α transcription has been observed for extracts obtained from white mulberry bark²⁹ 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- κ B at micromolar levels. Cudraflavone B (**1**) decreased the transcription of the chemokine CCL2 and inducible NO-synthase (iNOS), which are also under the transcriptional control of NF- κ B, but without any statistical significance (data not shown). It has been observed in previous studies that *M. alba* aqueous leaf³⁰ and bark²⁹ extracts, which could also contain **1**, are able to ameliorate NF- κ B 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 E₂ (PGE₂), a COX-2 product. This effect is comparable to that of the typical COX inhibitor indomethacin (COX-2 IC₅₀ = 2.5 \pm 0.89 μ M for **1** and 1.9 \pm 0.61 μ M for indomethacin), showing that **1** is a strong COX-2 inhibitor, but **1** also exhibited COX-1 inhibitory activity (COX-1 IC₅₀ = 1.5 \pm 0.65 μ M). In addition to **1**, several other compounds with COX-2 inhibitory activity have been found in mulberry root bark, but their IC₅₀ values were around 40–50 μ M.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 TNF α .

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 (¹H) and 75.48 MHz (¹³C). The spectra were measured in DMSO-*d*₆ or CDCl₃ at 303 K. The ¹H and ¹³C NMR chemical shifts (δ in ppm) were referenced to the signal of the solvent [2.50 (¹H) and 39.43 (¹³C) for DMSO-*d*₆ and 7.26 (¹H) and 77.00 (¹³C) for CDCl₃]. 2D NMR, gs-COSY, gs-HSQC, and gs-HMBC were used to assign the individual ¹H and ¹³C 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 μ L, 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 \times 4.6 mm i.d., particle size 3 μ m).

RPMI 1640 medium, penicillin–streptomycin mixture, and trypsin 170 U/mL supplemented with EDTA 200 μ g/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 TNF α , 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- κ B p65 transcription factor kit (Thermo Scientific, Rockford, IL) was utilized to detect NF- κ B. An AlphaLISA TNF α kit (PerkinElmer, Boston, MA) was used to evaluate the production of TNF α . The EIA kit used to measure the concentration of prostaglandin E₂ 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 CHCl₃ extract was separated using reversed-phase preparative HPLC (Supelcosil ABZ+Plus, 250 \times 21.2 mm i.d., particle size 5 μ m). 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 μ g/mL streptomycin in a humidified atmosphere containing 5% CO₂. The medium was changed twice a week, when cells had reached a concentration of 5–7 \times 10⁵ 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% CO₂ 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 LD₅₀ value, the concentration of the compound lethal for 50% of the cells, as calculated from the dose–response curves obtained. The cytotoxic LD₅₀ 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 LD₅₀ data derived from the values plotted on the graph.

Drug Treatment and Induction of Inflammation. Differentiated macrophages were pretreated for 1 h with 10 μ M **1** dissolved in DMSO. For comparison with a conventional drug, 10 μ M 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 μ g/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- κ B 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 TNF α , 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 $\Delta\Delta\text{C}_T$ method.³³ Transcription of the control cells was set as 1, and other experimental groups were multiples of this value.

Determination of Nuclear NF- κ B 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. Cytoplasmic and nuclear protein fractions were isolated from collected cells using a PARIS kit. The presence of NF- κ B subunit p65 was measured in both fractions by an EZ-Detect NF- κ B p65 transcription factor kit, and the cytoplasm/nuclear ratio was calculated. The lower detection limit was 25 pg/mL.

Evaluation of TNF α 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 TNF α was measured using an AlphaLISA TNF α 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 Bauer³⁴ 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 μ M porcine hematin, 18 mM L-epinephrine, and 50 μ M 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 PGE₂, the main product of the reaction, was determined by using a PGE₂ EIA kit based on a competitive enzyme immunoassay. The intensity of the yellow color generated, which is inversely proportional to the concentration of PGE₂ in the sample, was measured on a Tecan Infinite M200 microplate reader (Tecan Group, Männedorf, Switzerland) at 405 nm. IC₅₀ 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.

AUTHOR INFORMATION

Corresponding Author

*Tel: +420 541562836. Fax: +420 541 240 606. E-mail: hosek@vfucz.cz (J.H.) or karel.mejkal@post.cz (K.Š.).

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