

***Petasites hybridus* Extracts *in vitro* Inhibit COX-2 and PGE₂ Release by Direct Interaction with the Enzyme and by Preventing p42/44 MAP Kinase Activation in Rat Primary Microglial Cells**

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Abstract

Rhizomes of butterbur, *Petasites hybridus* L. (Asteraceae), have been used since ancient times for the treatment of inflammatory diseases. In the present study, the effects of lipophilic extracts from rhizomes of *Petasites hybridus* on the formation and release of prostaglandin E₂ were investigated. The extracts had different contents of petasin and isopetasin: **A**: 2.1 % and 0.4 %, **B**: 0.2 % and 0.1 %, **C**: 12.1 % and 6.1 % and **D**: 21.9 % and 9.4 %, respectively. Direct inhibition of cyclooxygenase (COX) -1 and -2 isoenzymes and inhibition of the expression of COX-2 and p42/44 MAP kinase in rat primary microglial cells were tested. All extracts were found to be only weak direct inhibitors of COX-1 (IC₅₀ > 400 µg/mL). However, most extracts revealed a strong inhibitory activity against the inducible isoform COX-2 (**A**: IC₅₀ = 30.4 µg/mL; **B**: IC₅₀ = 60.6 µg/mL; **C**: IC₅₀ = 22.6 µg/mL; **D**: IC₅₀ = 20.0 µg/mL). This activity was not correlated to the content of petasin and isopetasin. Pure petasin and isopetasin neither inhibited COX-1 nor COX-2 (IC₅₀ > 400 µM for both compounds and enzymes). *Petasites* extracts dose-dependently inhibited LPS-induced and thus COX-2-mediated PGE₂ release in primary rat microglial cells (**A**: IC₅₀ = 2.4 µg/mL; **C**: IC₅₀ = 5.8 µg/mL and **D**: IC₅₀ = 4.6 µg/mL). Also this effect was independent from the pe-

tasin and isopetasin content. COX-2 synthesis in microglia was totally blocked with 5 µg/mL of **C** whereas COX-1 synthesis was not influenced. **C** and **D** did not affect the LPS-induced activation of p38 MAPK and IκBα, but they prevented the LPS-induced activation of p42/44 MAPK. Therefore, these *Petasites hybridus* extracts can be regarded as natural selective inhibitors of COX-2 and its expression, an effect which is independent from the petasin content.

Key words

Inflammation · microglia · *Petasites hybridus* · Asteraceae · cyclooxygenase · petasin · isopetasin

Abbreviations

COX: cyclooxygenase
IκBα: inhibitory protein kappaBα
iNOS: inducible nitric oxide synthase
LOX: lipoxygenase
NF-κB: nuclear factor-kappa B
PG: prostaglandins
MAPK: mitogen activated protein kinase

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Introduction

The rhizomes of butterbur (*Petasites hybridus* L., Asteraceae) have been used medicinally since ancient times. In the 17th century they were applied against intestinal spasms, asthma and as a mucolytic. Modern research has given evidence for new indications, like migraine prophylaxis [1], [2], tension headache, spasms of the urogenital tract [3] and bile duct, dysmenorrhoea and hay fever [4]. Spasmolytic and anti-inflammatory effects have already been demonstrated for methanolic extracts of fresh roots [5], for a dried *Petasites* extract from a commercial source and for two petroleum ether extracts [6]. Several petroleum ether extracts of ground roots and leaves from different sources were also found to inhibit leukotriene biosynthesis [7]. Three isomeric “oxopetasan esters”, petasin and isopetasin were identified as the main compounds of these extracts and “oxopetasan esters” and isopetasin were regarded as the main inhibitory components [7]. Scheidegger et al. tested defined extracts from petasin and furanopetasin varieties of *Petasites hybridus* and *Petasites albus* and an ethanolic “spissum” extract from a commercial source as well as purified compounds extracted with dichloromethane for inhibition of prostaglandin synthesis in cultured skin fibroblasts and for effects on leukotriene synthesis in isolated human peripheral leukocytes [8]. All extracts stimulated the synthesis of PGE₂ in a dose- and time-dependent manner. Petasin, isopetasin, an epimeric mixture of two 8*H*-eremophilanolides, 8β-hydroxyeremophilanolide and 2-(*Z*)-methylthioacryloyl-8α*H*-eremophilanolide were only weak stimulators of PGE₂ synthesis. The petasin and eremophilane lactone containing extracts and their components petasin, isopetasin and 8β-hydroxyeremophilanolide showed to be highly effective inhibitors of LTC₄ synthesis and release [8]. Sesquiterpene esters (petasins) inhibit the intracellular release of calcium from the endoplasmic reticulum and thereby the activation of 5-lipoxygenase and also 5-lipoxygenase directly. In addition, the release of ECP (eosinophil cationic protein) from eosinophils was reduced [9], [10]. Petasins were also shown to exert a relaxing effect on the isolated guinea pig trachea [11] and [12].

Clinical studies proved that COX-2 is up-regulated in migraine patients. Thereby this enzyme catalyzes the synthesis of inflammatory prostaglandins like PGE₂ and generates a large amount of oxygen radicals [13].

We now present evidence for a further pharmacological effect of special lipophilic butterbur extracts, which seems not to be correlated to the petasin content, namely the inhibition of cyclooxygenase-2 via direct interaction with the enzyme and via prevention of p42/44 MAP kinase activation in rat primary microglial cells [14] and [15].

Materials and Methods

Extracts and tested compounds

Petasites extracts and isopetasin were provided by Weber&Weber GmbH & Co. KG Biologische Arzneimittel, Inning a. A., Germany. The extraction was performed as follows: subterranean plant material (Rhizoma petasitidis), identified by Weber&Weber (voucher specimen deposited at Weber&Weber with the

numbers VZX 269 for **A**, VZX 272 for **B**, VZX 269/270/274 for **C** and VZX 274 for **D**) was crushed (2000 g) and extracted with supercritical carbon dioxide. The extracts were obtained as solvent-free spissum extracts. They were characterized by their gas chromatograms (Fig. 1A–D) and by thin layer chromatography (Fig. 2) and could be divided according to the content of petasin and isopetasin, which was 2.1 % and 0.4 % in extract **A**, 0.2 % and 0.1 % in extract **B**, 12.1 % and 6.1 % in extract **C** and 21.9 % and 9.4 % in extract **D**, respectively.

Isopetasin was gained from *Petasites* extracts rich in isopetasin by preparative column chromatography with a purity of > 95 %. Petasin was isolated by preparative and semi-preparative HPLC with a purity of > 98 %.

The quantification of petasin and isopetasin was carried out by gas chromatography (HP 5890); 175.0 mg of each sample were dissolved in 25.0 mL of *n*-hexane:dichloromethane, 1:1. 20.0 mg of the internal standard tricosanoic acid methyl ester were dissolved in 2 mL dichloromethane and diluted with 8 mL *n*-hexane. 1.0 mL of each sample and internal standard solution were mixed and 0.5 µL injected onto a 30 m column HP-5MS (cross-linked 5 % PH ME siloxane), ID 0.25 mm, dF 0.5 µm. Helium was used as carrier gas. The injection temperature was 260 °C and a temperature gradient mode was applied from 150 to 280 °C with 8 °C/min, followed by 24 min isothermic elution. Detection was performed by an FID at 300 °C.

For precision the coefficient of variation was 1.3 %. For accuracy the mean recovery rate was 99.5 % and the coefficient of variation was 1.1 %. The analytical method is linear from 0.20 to 1.61 mg isopetasin/mL with a coefficient of correlation ($n = 6$) $r = 0.9997$.

For identification of the compounds a GC HP 5890 Series II plus was used, connected with a mass-selective detector HP 5989B. Injection and detection temperature as well as the elution mode were the same as described for the quantification of the petasin and isopetasin contents.

For thin layer chromatography 15 µL of each extract (1 % ethanol p.a.) were applied on a silica plate 60 F₂₅₄, Merck, Germany, and developed in a Camag normal chamber 20 × 20 cm with 50 mL of toluene/ethyl acetate (93:7, v/v) without chamber saturation to a distance of 10 cm. Detection was performed with anisaldehyde/sulfuric acid reagent [anisaldehyde (0.5):100% acetic acid (10):methanol (85):conc. sulfuric acid (5)] and heating at 160 °C for 1½ min (VIS and UV 365 nm) [16].

For the COX-1 and COX-2 assays all samples were dissolved in ethanol. Extracts were used in the following test concentrations: 5 µg/mL, 10 µg/mL, 25 µg/mL and 50 µg/mL. The test concentrations of isopetasin were 5 µM, 10 µM, 25 µM and 50 µM in ethanol, respectively. Petasin was tested at 400 µM. For studying the effects in microglial cells, the samples were dissolved in DMSO at a concentration of 100 µg/mL and subjected to the cells in doses of between 0.1 and 10 µg/mL.

COX-1 and COX-2 assays

The assays were performed in a microtiter scale with purified PGHS-1 (COX-1) from ram seminal vesicles and purified PGHS-2

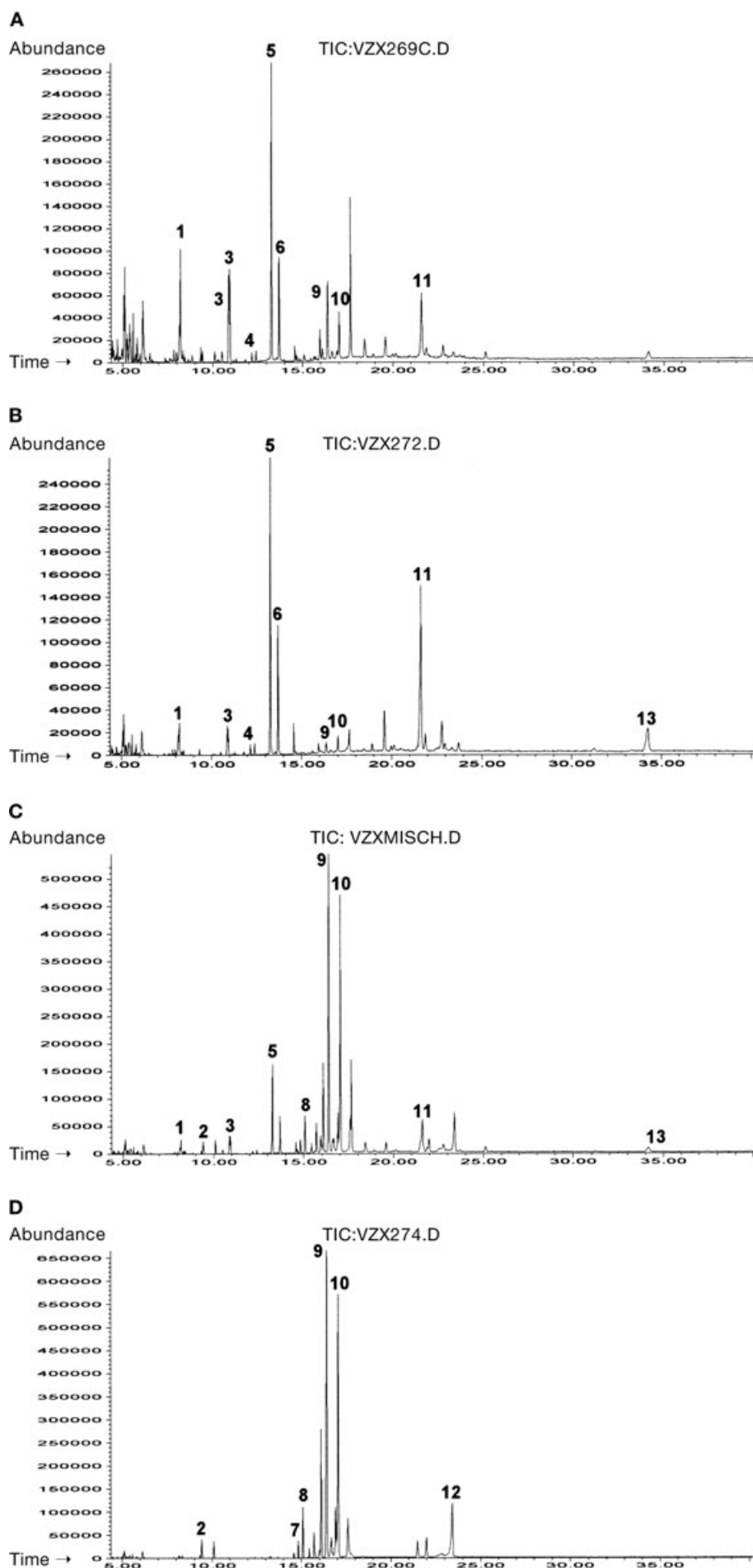


Fig. 1 GC-MS of extracts **A**, **B**, **C** and **D** as described in Materials and Methods. 1 = furanoeremophilane; 2 = 3-desoxy-neopetasol; 3 = n. i. eremophilanolide; 4 = 9-oxofuraneremophilane; 5 = 8 β H-eremophilanolide; 6 = 8 α H-eremophilanolide; 7 = isobutyryl-neopetasol; 8 = methacryloylpetasol; 9 = petasin; 10 = isopetasin; 11 = 2-angeloyl-8 β H-eremophilanolide; 12 = iso-S-petasin; 13 = 2-Mta-8 β H-eremophilanolide.

(COX-2) from sheep placental cotyledones (both: Cayman Chemical Company, Ann Arbor, MI, USA) as previously described [17], [18]. The incubation mixture contained 180 μ L 0.1 M TRIS/HCl-buffer (pH 8.0) (Roth, Karlsruhe, Germany), 5 μ M hematin (porcine, ICN, Aurora, Ohio, USA), 18 mM epinephrine hydrogen tartrate (Fluka, Buchs, Switzerland), 0.2 U of enzyme preparation and 50 μ M Na₂EDTA (only COX-2 assay, Titriplex III, Merck, Darmstadt, Germany). 10 μ L of each compound solution were added and the mixture preincubated for 5 min at room temperature. The reaction was started by adding 10 μ L of 5 μ M arachidonic acid (Cayman Chemical Company) in EtOH p.a. and incubated at 37 °C. The reaction was terminated after 20 min by addition of 10 μ L 10% formic acid.

The concentration of PGE₂, the main metabolite of arachidonic acid in this reaction, was determined by a competitive PGE₂ EIA kit (R&D Systems, Minneapolis, MN, USA), which was used as described by the manufacturer. The sensitivity of the kit was less than 36.2 pg/mL and its range was 39–5000 pg/mL. All samples were diluted in EIA buffer.

The EIA was evaluated by an ELISA reader “rainbow” (TECAN) and the PGE₂ concentration was determined as previously described [17], [18]. Inhibition of COX refers to reduction of PGE₂ formation in comparison to a blank run without inhibitor. All IC₅₀ values of the extracts and isopetasin were determined for both enzymes by measuring at least three different concentrations. Inhibition values are means of at least three experiments and each sample was tested in duplicate. IC₅₀ values were calculated with these means after graphic representation with logarithmic regression using the following logistic functions:

$$y = A + B \times \ln x$$

$$A = \frac{\sum y - B \times \sum x}{n}$$

$$B = \frac{n \times \sum xy - \sum x - \sum y}{n \times \sum x^2 - (\sum x)^2}$$

$$IC_{50} = \frac{e^{(50-A)}}{B}$$

Indomethacin (ICN), NS-398 and nimesulide (both Cayman Chemical Company) were used as COX-inhibiting positive controls.

Cultivation of primary rat microglial cells

Cortices and midbrains were dissected from newborn rat pups (Wistar) and the meninges and blood vessels were removed. Tissues were collected into 0.1 M PBS, washed 4× with cold 0.1 M PBS, homogenized mechanically without enzymes, and filtered through a 70 μ M nylon cell strainer (Falcon, Fisher Scientific, Pittsburgh, PA). PBS was replaced in 2 centrifugation steps (1000×g, for 10 min at 4 °C) with DMEM containing 10% FBS. The cells were then suspended into culture medium and plated into cell culture plates at 1×10⁵ cells/mm² and maintained in a humidified incubator at 37 °C and 5% CO₂. The medium was changed at the 2nd day *in vitro* and once a week thereafter. This procedure results in mixed glial cultures, consisting of dividing

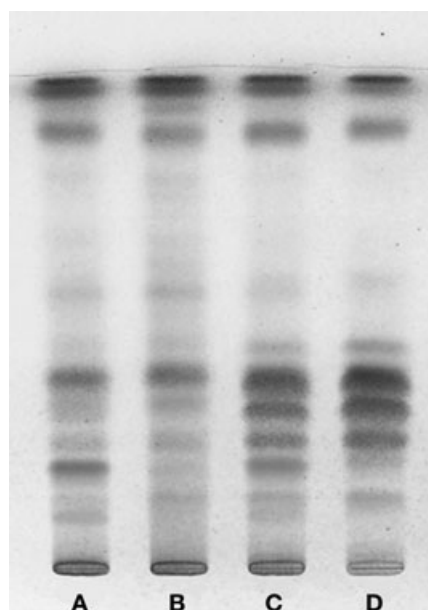


Fig. 2 Thin layer chromatogram of petasites extracts **A**, **B**, **C** and **D** as described in Materials and Methods. The yellow brown zones in the R_f range 0.2 to 0.4 are petasins.

astrocytes and microglial cells. After 2 weeks *in vitro*, microglia were harvested once a week by carefully collecting the medium until the mixed cultures were 2–3 months old. The purity of microglial cultures is about 98%.

Microglial cells were seeded in 24-well cell culture plates and pre-incubated with the extracts for 30 min in the indicated doses. Thereafter, cells were treated with LPS (100 ng/mL) for 24 h. Supernatants were harvested, centrifuged at 10,000×g for 10 min and levels of PGE₂ in the media were measured by enzyme immunoassay (EIA) (Biotrend, Köln, FRG) according to the manufacturer's instructions. Standards from 39 to 2500 pg/mL were used, sensitivity of the assay was 36.2 pg/mL.

Western blot analysis of COX synthesis, phosphorylated p42/44 and p38 MAPK, non-phosphorylated p42 and I κ B α

Microglial cells were seeded in 75 cm² flasks (Greiner, Germany) or 24-well multiwell plates (Falcon) and exposed to LPS (1 ng/mL) in the presence or absence of *Petasites* extract solutions for 24 h. Cells were then lysed in 1.3×SDS (sodium dodecyl sulfate)-containing sample buffer without DTT or bromophenol blue containing 100 μ M orthovanadate. Lysates were homogenized by repeated passage through a 26-gauge needle. Protein contents were measured using the bicinchoninic acid method (BCA protein determination kit from Pierce, distributed by KFC Chemikalien, München, Germany) according to the manufacturer's instructions (standards (BSA, Sigma) ranging from 0.2 μ g/ μ L to 4 μ g/ μ L; optical density read at 570 nm). Before electrophoresis, bromophenol blue and DTT (final concentration, 10 mM) were added to the samples. For Western blotting, 20 to 60 μ g of protein from each sample was subjected to SDS-PAGE (polyacrylamide gel electrophoresis) on a 7.5% (COX-1, COX-2) or 12% (p38 MAPK, p42/44 MAPK, I κ B α) gel under reducing conditions. Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) by semi-dry blotting. The membrane was blocked overnight at 4 °C using Rotiblock (Roth, Karlsruhe, Germany) and for another hour at room temperature before incubation with the primary antibody (goat

Table 1 Inhibition of COX-1 and COX-2 in the COX-1/-2 *in vitro* assay by *Petasites* extracts **A**, **B**, **C** and **D** (means; %) \pm SDE. The IC_{50} values of the positive controls were 0.9 μ M (COX-1) and 0.8 μ M (COX-2) for indomethacin, 50.7 μ M (COX-1) and 2.6 μ M (COX-2) for NS-398 respectively [17], [18]

	5 μ g/mL		10 μ g/mL		25 μ g/mL		50 μ g/mL	
	COX-1	COX-2	COX-1	COX-2	COX-1	COX-2	COX-1	COX-2
A	-6.3 \pm 7.7	-1.5 \pm 13.3	-2.1 \pm 7.4	16.2 \pm 11.9	0.5 \pm 12.5	47.2 \pm 12.8	-0.5 \pm 6.7	62.8 \pm 7.4
B	-31.1 \pm 6.5	1.1 \pm 4.6	-19.6 \pm 4.1	3.2 \pm 7.7	-14.5 \pm 10.4	24.1 \pm 12.3	1.5 \pm 4.6	52.1 \pm 5.0
C	-9.6 \pm 7.3	-4.6 \pm 8.3	1.1 \pm 13.5	28.1 \pm 12.3	9.3 \pm 6.6	58.3 \pm 8.4	23.1 \pm 9.6	71.2 \pm 6.9
D	-1.7 \pm 7.2	8.4 \pm 10.1	-1.0 \pm 9.9	35.6 \pm 9.8	3.0 \pm 9.8	59.3 \pm 8.0	25.0 \pm 18.5	71.2 \pm 5.1

anti-COX-2; goat anti COX-1; both from Santa Cruz, Heidelberg, Germany), 1 : 1000 dilution; rabbit anti phospho p38 MAPK (NEB/CellSignalling, Schwalbach, Germany), 1:500; rabbit anti p42 MAPK (detecting total p42 MAPK; SantaCruz, Heidelberg, Germany), 1 : 1000; rabbit anti phospho p42/44 MAPK (Promega, Mannheim, Germany), 1 : 20 000; goat anti-I κ B α (SantaCruz, Heidelberg, Germany) diluted in Tris-buffered saline (TBS) containing 0.1 % Tween 20 (Merck, Darmstadt, Germany) and 1 % bovine serum albumin (BSA, Sigma) for 2 h. After extensive washing (three times for 15 min each in TBS containing 0.1 % Tween 20), proteins were detected with peroxide-coupled donkey anti-rabbit IgG (Amersham-Pharmacia Biotech, 1 : 25,000) using chemiluminescence (ECL) reagents (Amersham-Pharmacia Biotech). All Western blots were repeated three times from three different experiments.

Table 2 IC_{50} values of petasin, isopetasin and the extracts **A**, **B**, **C** and **D** in the COX-1,2 *in vitro* assay. IC_{50} values are indicated as μ M (petasin, isopetasin) or μ g/mL (extracts), respectively. The IC_{50} values of the positive controls were 0.9 μ M (COX-1) and 0.8 μ M (COX-2) for indomethacin, 50.7 μ M (COX-1) and 2.6 μ M (COX-2) for NS-398, and 246.3 μ M (COX-1) and 36.4 μ M (COX-2) for nimesulide, respectively [17], [18]

	COX-1	COX-2
Petasin	> 400	> 400
Isopetasin	> 400	> 400
A	> 400	30.4
B	> 400	60.6
C	> 400	22.6
D	> 400	20.0

Table 3 Inhibition of COX-1 and COX-2 by isopetasin in the COX-1/-2 *in vitro* assay (means; %) \pm SDE. The IC_{50} values of the positive controls were 0.9 μ M (COX-1) and 0.8 μ M (COX-2) for indomethacin, 50.7 μ M (COX-1) and 2.6 μ M (COX-2) for NS-398, and 246.3 μ M (COX-1) and 36.4 μ M (COX-2) for nimesulide, respectively [17], [18]

	COX-1	COX-2
5 μ M	-30.5 \pm 12.8	-6.0 \pm 13.3
10 μ M	-15.4 \pm 13.4	-3.2 \pm 11.2
25 μ M	-15.3 \pm 6.8	3.7 \pm 8.7
50 μ M	-7.9 \pm 4.3	3.5 \pm 10.3

Measurement of PGE₂ in microglial cells

Supernatants of treated microglial cells were harvested, centrifuged at 10,000 \times g for 10 min and levels of PGE₂ in the media were measured by enzyme immunoassay (EIA) (Biotrend, Köln, Germany) according to the manufacturer's instructions. Standards from 39 to 2500 pg/mL were used; sensitivity of the assay was 36.2 pg/mL.

Data analysis

Statistical analysis was performed by the "Institut für Biochemische Analysen und Methodenentwicklung GbR", Freiburg (Germany). Original data were converted into % values of lipopolysaccharide control and mean \pm SE calculated. IC_{50} values were calculated by computerized non-linear regression analysis using the following logistic function:

$$1 - \frac{I_{\max} \times 10^{Lex}}{10^{(-pIC_{50})} + 10^{Lex}}$$

where I_{\max} is the maximal inhibition approached (between 0 and 1), Lex the logarithm of doses of the inhibitor and pIC_{50} the negative logarithm of IC_{50} .

Results and Discussion

Special commercial lipophilic extracts from the rhizomes of *Petasites hybridus* prepared with supercritical carbon dioxide with different content of petasin and isopetasin (**A**: 2.1% and 0.4%, **B**: 0.2% and 0.1%, **C**: 12.1% and 6.1%, **D**: 21.9% and 9.4%) were tested for inhibition of COX-1 and COX-2 isoenzymes. The extracts showed only little inhibitory activity on COX-1 (IC_{50} > 400 μ g/mL). However, they exhibited strong inhibitory effect on COX-2 (Table 1). The IC_{50} values for COX-2 were determined as 30.4 μ g/mL (**A**), 60.6 μ g/mL (**B**), 22.6 μ g/mL (**C**), and 20.0 μ g/mL (**D**), respectively (Table 2). Pure petasin and isopetasin did not inhibit COX-1 or COX-2 (IC_{50} > 400 μ M for both enzymes) (Tables 2, 3).

In order to evaluate the COX-2 inhibitory activity in a cellular model, we tested the effect of the *Petasites hybridus* extracts **A**, **C** and **D** in rat microglial cells, which have been shown to express COX-2 and to synthesize PGE₂ after treatment with lipopolysaccharides (LPS) [19]. As shown in Fig. 3A–C, all *Petasites* extracts

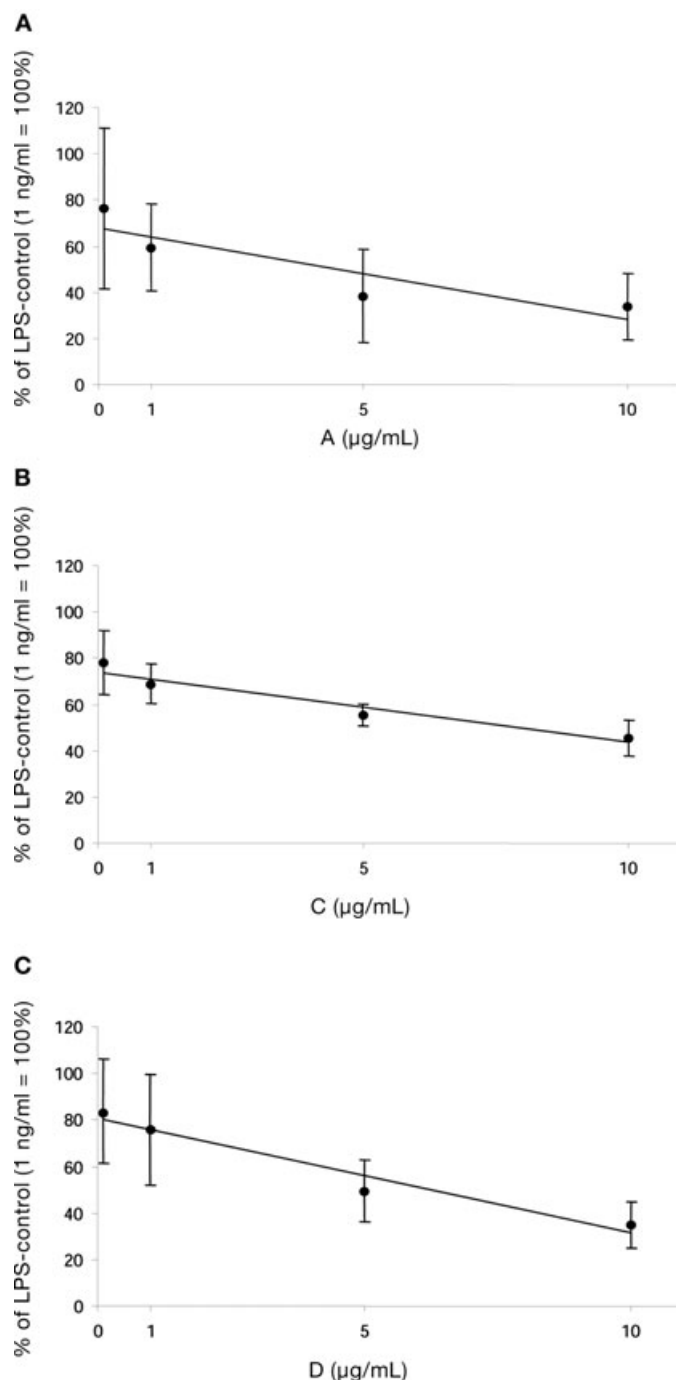


Fig. 3 Inhibitory effects of 3 different *Petasites hybridus* extracts on PGE₂ synthesis in lipopolysaccharide-stimulated rat microglial cells. Cells were preincubated with the extracts **A**, **C**, and **D** at the indicated concentrations for 30 min and subsequently treated with lipopolysaccharide (1 ng/mL) for 24 h. PGE₂ in the supernatants was measured by an enzyme immunoassay as described in Materials and Methods. Data are expressed as mean \pm SE.

dose-dependently inhibited LPS-induced and thus COX-2-mediated PGE₂ release in primary rat microglial cells, with IC₅₀ values in an equipotent range (**A**: 2.4 µg/mL [1.2 µg/mL; 4.5 µg/mL]; **C**: 5.8 µg/mL [4.2 µg/mL; 8.2 µg/mL] and **D**: 4.6 µg/mL [3.1 µg/mL; 6.7 µg/mL]). The data presented here suggest that the special lipophilic *Petasites hybridus* extracts, which are used for the treatment of migraine, are selective inhibitors of COX-2 mediated PGE₂ release.

We further investigated whether the PGE₂ inhibitory effects of the *Petasites hybridus* extracts were due to an inhibition of the expression and synthesis of COX-1 or COX-2 protein, since the inhibition of COX-2-mediated PGE₂ release in primary rat microglial cells might be due an inhibition of COX-2 expression as shown for caffeine [14] or by inhibition of COX-2 enzyme activity as shown for ascorbic acid [20]. To this end, we investigated the effects of **C** on COX-2 synthesis by Western blot experiments which revealed that the *Petasites hybridus* extract inhibited the lipopolysaccharide (LPS)-induced synthesis of COX-2 proteins in microglial cells in a dose-dependent manner. Starting with slight inhibition at 1 µg/mL, COX-2 synthesis was totally blocked at 5 µg/mL (Fig. 4A) of **C**. In contrast to COX-2, COX-1 immunoreactivity was not affected (Fig. 4B), suggesting that this extract does not inhibit COX-1 synthesis. The COX-1 Western blot experiments also provided evidence that the extract was not toxic to microglial cells in the doses tested.

Central COX-2 expression is regulated by various signal transduction pathways including p38 MAPK [21], p42/44 MAPK [22], and NF-κB [19], [21]. Therefore, we were interested in whether *Petasites hybridus* extract **C** interferes with these signal transduction pathways. Whereas LPS-induced activation of p38 MAPK and IκBα degradation was not affected by extract **C** (Figs. 5A and B), LPS-induced phosphorylation and thus activation of p42/44 MAPK was prevented by extract **C** (Fig. 6A) as well as by extract **D** (Fig. 6B) indicating that at least inhibition of the p42/44 pathway is one mechanism by which *Petasites hybridus* extract is inhibiting COX-2 synthesis in microglial cells.

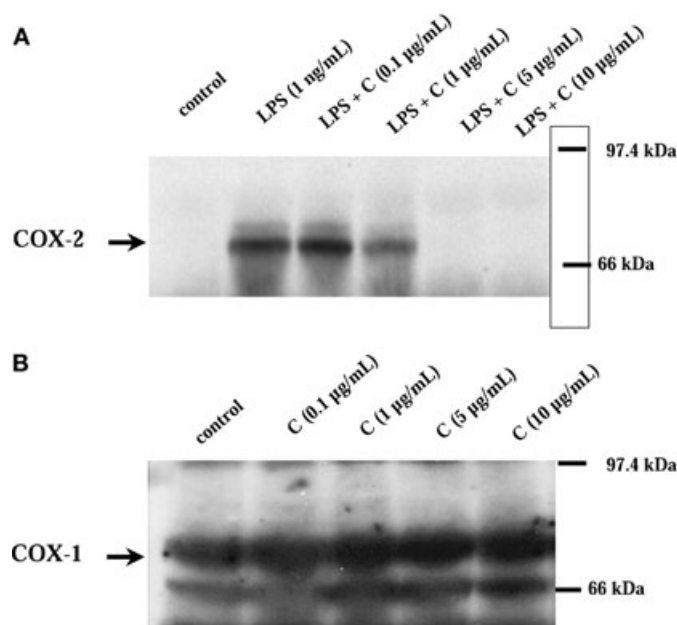


Fig. 4 *Petasites hybridus* extract **C** inhibits COX-2 (a) but not COX-1 (b) synthesis in lipopolysaccharide-stimulated microglial cells. Cells were either left untreated or were stimulated with lipopolysaccharide (1 ng/mL) for 24 h in the absence or presence of different concentrations of **C**. Western blotting with anti-COX-2 and anti COX-1 antibodies was used to detect the amount of COX-2 protein in whole cell lysates as described in Materials and Methods.

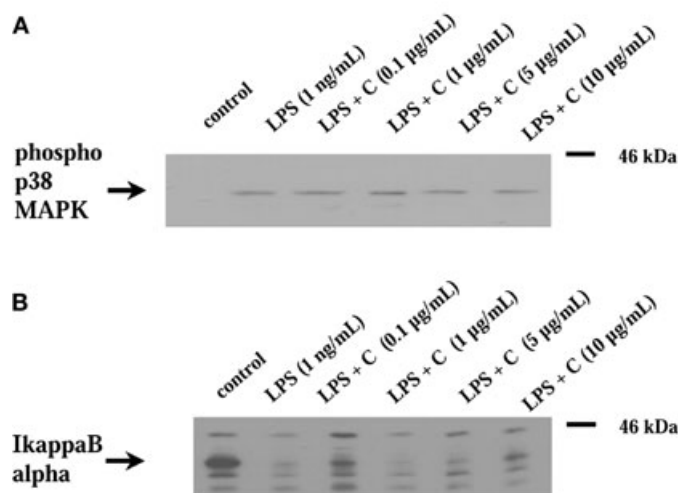


Fig. 5 LPS-induced phosphorylation of p38 MAPK (**a**) and the degradation of IkappaB alpha (**b**) is not inhibited by *Petasites hybridus* extract **C**. Total cell protein prepared from control and LPS/C treated cells was subjected to SDS-PAGE followed by Western blotting using polyclonal antibodies that recognize the phosphorylated (and thus active) form of p38 MAPK and IkappaB alpha.

These data suggest that supercritical carbon dioxide extracts from *Petasites hybridus* prevent COX-2 protein synthesis which might be another major mechanism of its PGE₂ inhibitory effects. One possible mechanism by which *Petasites hybridus* inhibited COX-2 synthesis is the inhibition of p42/44 MAP kinases (erk1/erk2) in microglia. Such an inhibition has previously been described also for parthenolide [15]. Microglia constitute part of the local CNS innate immune system. They appear in several morphological variants of resting and activated types and communicate with other immune cells via chemokines, cytokines and growth factors. In case of brain injury activated microglia concentrate themselves at damaged parts, obtain macrophage-like properties (phagocytosis), release several cytopathic secretory products and are able to present antigens. Thereby they perpetuate the pathological cycles of inflammation [23]. But also other, so far not identified, mechanisms might contribute to the inhibitory effects of *Petasites hybridus*.

Previously, Scheidegger et al. reported that the synthesis of PGE₂ in fibroblasts was stimulated by extracts of *Petasites* in a dose- and time-dependent manner [8]. This difference to our findings may be due to the different assay models or to a different composition of the extract, or, most likely, to the difference of the cell system (fibroblasts vs. primary cells of the CNS from the rat [23], [24]. Microglia are known to act differently to most peripheral cells regarding the inhibition of COX-2 [14].

Different assay models may also be the reason for the difference in the calculated IC₅₀ values (isolated enzymes in the COX-1- and COX-2-assay vs. cultivated animal cells in the microglia experiments) [24], [25], [26].

The observed effect is independent of the petasin and isopetasin contents, since the three different extracts showed equipotent inhibition regardless the content of these compounds. Our data therefore suggest that petasin and isopetasin are not the key fac-

tors responsible for the COX-2-mediated inhibition of PGE₂ in microglia. As plant extracts are complex mixtures of various ingredients, the reason for the only weak inhibition of COX-2 by **B** may be due to the difference in extract composition.

Petasin-type constituents are responsible for effects like inhibition of leukotriene formation [7] or spasmolytic activity. Wang et al. described a direct, dose-dependent Ca²⁺ antagonism of L-type voltage-dependent Ca²⁺ channel activity by S-petasin [27] and iso-S-petasin [28] in vascular smooth muscle cells which may account for the hypotensive and bradycardiac action. The negative chronotropic and inotropic properties of S-petasin also can be attributed mainly to inhibition of voltage-dependent Ca²⁺ channels but not to blockade of dihydropyridine binding sites of L-type Ca²⁺ channel or to muscarinic receptor activation [29]. Direct affection of L-type Ca²⁺ channels by S-petasin was also found in NG108-15 neuronal cells [30]. S-Isopetasin allosterically antagonizes carbachol in isolated guinea pig atria [31]. Moreover, Berger et al. found that eremophilanes of *Petasites hybridus* extracts are also effective in inhibiting dopamine-D₂ and histamine-H₁ receptors of guinea-pig cerebellum and calf striatum in a radioligand assay [32].

Since petasin and isopetasin did not show any relevant direct inhibitory effect on COX-2, other compounds in the extract must be responsible for this effect. This observation corresponds to the results of Brune et al. [6] and Bickel et al. [7] who found that isopetasin appears to be a selective inhibitor of lipoxygenase and does not affect the cyclooxygenase (COX-1) system of macrophages. Therefore, the active principle responsible for COX-2 inhibition seems to be different from the compounds inhibiting leukotriene formation. The elucidation of the chemical nature of the COX-2 inhibiting principle is in progress.

In conclusion, due to its PGE₂ and COX-2 inhibitory effects, special extracts of the rhizomes of *Petasites hybridus* can be consid-

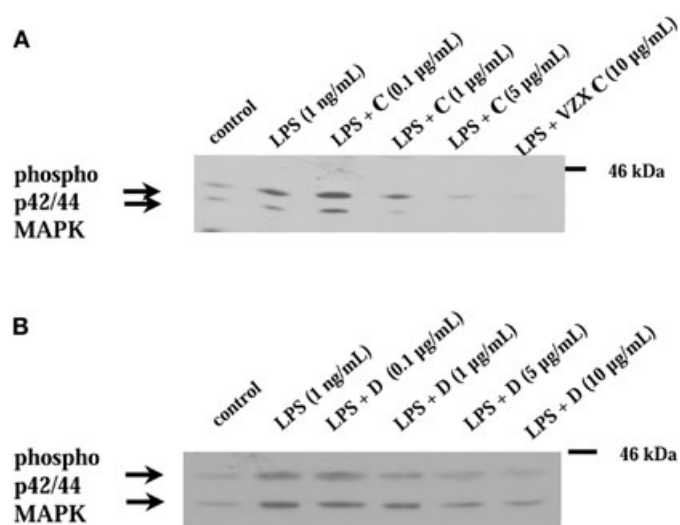


Fig. 6 LPS-induced phosphorylation of p42/44 MAPK is inhibited by *Petasites hybridus* extract **C** (**A**) and **D** (**B**). Total cell protein prepared from control and LPS/parthenolide-treated cells was subjected to SDS-PAGE followed by Western blotting using polyclonal antibodies recognizing the phosphorylated (and thus active) forms of p42/44 MAPK. The phosphorylated p42/44 MAPKs are indicated by arrows.

ered to be powerful tools against COX-2-mediated inflammation. Several constituents seem to be responsible for the therapeutic effect of *Petasites* extracts and the application of extracts, therefore, is still reasonable.

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