Immunomodulatory and Anti-Inflammatory Activity of Selected Osthole Derivatives

Michał Zimecki[a], Jolanta Artym[a], Wojciech Cisowski[b], Irena Mażol[b], Maciej Włodarczyk[b], and Michał Gleńsk[b,*]

[a] Department of Experimental Therapy, Institute of Immunology and Experimental Therapy, R. Weigla str. 12, 53–114, Wroclaw, Poland

[b] Department of Pharmacognosy, Wroclaw Medical University, Nankiera sq. 1, 50–140 Wroclaw, Poland. E-mail: michalg@farmgn.am.wroc.pl

* Author for correspondence and reprint requests

Z. Naturforsch. **64 c**, 361–368 (2009); received October 17/December 3, 2008

From osthole [7-methoxy-8-(3-methyl-but-2-enyl)-chromen-2-one] (**I**), obtained by selective extraction of *Peucedanum ostruthium* (L.) W. Koch roots, ostholic acid (**II**) was synthetized as a result of its oxidation with chromium trioxide. From ostholic acid, through its chloride, four amides were obtained: the morpholide **1**, the *p*-chloro-benzylamide **2**, the piperidine **3** and the *N*-methyl-piperazide **4**. Except for **1**, other compounds have not been described before. The amides **1**–**4** and their precursor osthole (**I**) were tested for their potential activities in selected immunological assays. The compounds showed moderate inhibitory activity in the humoral immune response to sheep erythrocytes in mice *in vitro*, and **4** was the most suppressive. The effects of **1** and **3** on concanavalin A- and pokeweed mitogen-induced mouse splenocyte proliferation were inhibitory and those of **4** stimulatory. The compounds were also tested for their activity on tumour necrosis factor α and interleukin 6 production, induced by lipopolysaccharide, in cultures of rat peritoneal cells and human peripheral blood mononuclear cells. Compounds **1**, **3** and **4** inhibited tumour necrosis factor α (rat cells), whereas compound **2** stimulated the production of both cytokines. Compounds **1**, **2** and **3** were also strongly inhibitory on tumour necrosis factor α production in human blood cells (73, 78 and 80% inhibition at 10 μg/ml, respectively). On the other hand, **2** and **4** stimulated the interleukin 6 production (2- to 3-fold stimulation). In addition, **2** and **4** suppressed the carrageenan-induced inflammation in mice (56.5% and 68.3% inhibition, respectively). In summary, the compounds predominantly displayed suppressive and anti-inflammatory activities in the investigated models.

Key words: *Peucedanum ostruthium* (L.) W. Koch, Osthole Derivatives, Humoral Immune Response, Carrageenan

Introduction

Peucedanum ostruthium (L.) W. Koch (syn. *Imperatoria ostruthium* L.) belongs to the Apiaceae family and is a perennial herb growing in Central Europe (Geiger, 1840; Hegi, 1975). The roots have been used in folk medicine as panaceum and, until the end of the XIXth century, *P. ostruthium* was included in European pharmacopoeias (Codex der Pharmacopöen, 1844–1845; Pharmacopoea Germanica, 1882). In traditional medicine this plant material was used as antiphlogistic and antibacterial agent for the treatment of rheumatism and fever as well as against digestive tract disorders (Madaus, 1938; Muszyński, 1957; Teuscher and Lindequist, 1994).

Some studies have proved that coumarins present in the roots possess antibacterial, antiphlogistic and antipyretic activities (Hiermann and Schantl, 1998; Schinkovitz *et al.*, 2003; Borges *et al.*, 2005). In an *in vitro* study coumarins from *P. ostruthium* were found to act as inhibitors of acetylcholinesterase that may play an important role in the treatment of Alzheimer's disease symptoms (Urbain *et al.*, 2005). Recently, bioactivity-guided fractionation led to a successful isolation of antiosteoporotic components, with osthole as the major compound (Zhang *et al.*, 2007).

The literature provides also information regarding multiple biological activities of simple coumarins such as: anticoagulant, anti-inflammatory and enzyme inhibition properties (reviewed in Borges *et al.*, 2005).

The aim of the present study was to evaluate potential activities of new coumarin derivatives in selected immunological tests.

Material and Methods

Plant material and extraction

The roots of *Peucedanum ostruthium* (L.) W. Koch were collected at the beginning of September 2007 near Karpacz (Sudety Mountains, Poland). A voucher specimen has been deposited at the Department of Pharmacognosy, Wroclaw Medical University, Wroclaw, Poland. The raw plant material was dried at ambient temperature and finally ground to powder. Powdered roots (920 g) were exhaustively extracted using a Soxhlet apparatus with petroleum ether for 120 h. After extraction and cooling procedures osthole was crystallized. Subsequently, precipitated osthole was drained off and the solvent removed by distillation. The remaining product (40 g of a greasy substance) was dissolved in boiling methanol and left for a final osthole precipitation. From the applied amount of the plant material, after extraction and crystallization procedures, about 10 g of pure osthole (**I**) was isolated.

Osthole oxidation

Osthole (6 g) was dissolved in 150 ml of glacial acetic acid, and 3 g of chromium(VI) oxide were added. As a result 1.1 g of ostholic acid was obtained. This method was applied by us previously (Cisowski and Rządkowska-Bodalska, 1979).

Ostholic acid chloride and amides preparation

After dissolving 0.8 g of ostholic acid in anhydrous toluene, the mixture was treated with thionyl chloride at boiling temperature for 2 h. Toluene and excess of thionyl chloride were removed by distillation. The remaining product was dissolved in 10 ml of anhydrous toluene and the solution divided into four equal parts (2.5 ml each). To 2.5 ml of the ostholic acid chloride solution, 0.3 ml of morpholine was added. First, the mixture was kept for 12 h at room temperature and then heated for 2 h at 60 °C. Afterwards, the flask's content was evaporated to dryness and the remaining product was mixed with aqueous hydrochloric acid. The creamy precipitate was formed and collected on a Schott glass filter (G3). Finally, the precipitate was recrystallized from methanol, providing 0.063 g of the morpholide **1**. In a similar way, from the acid chloride, the corresponding amides **2**, **3**, and **4** were obtained (Fig. 1).

7-Methoxy-8-(3-methyl-but-2-enyl)-chromen-2-one (osthole, **I**): Colourless crystals (crystallized from methanol); m.p. 82–83 °C. – Yield: 10.0 g. – NMR and MS data are in good agreement with data given in the literature.

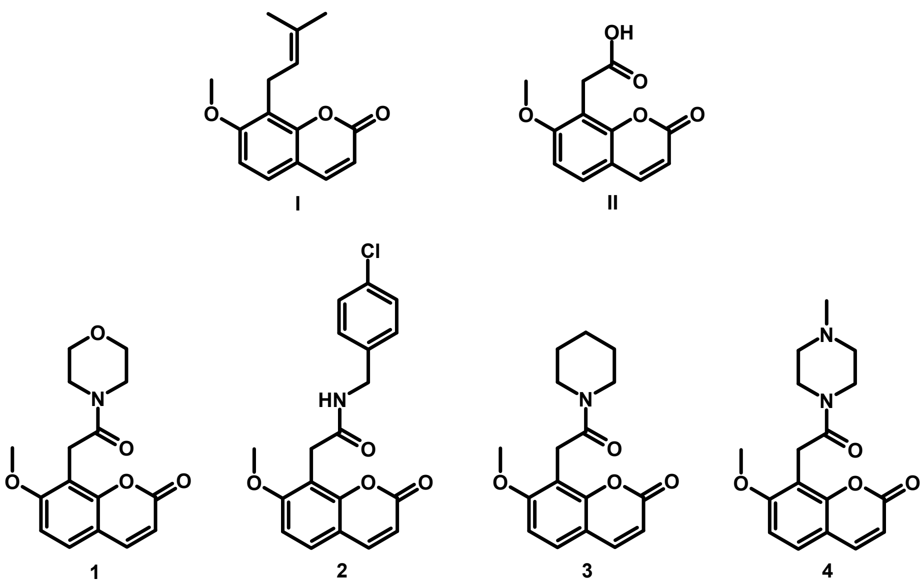

Fig. 1. Chemical structures of compounds **I**, **II**, **1**–**4**.

(7-Methoxy-2-oxo-2H-chromen-8-yl) acetic acid (ostholic acid, **II**): Colourless crystals [crystallized from a mixture of methanol and acetic acid (10:1)]; m.p. 255–257 °C. – Yield: 1.1 g.

7-Methoxy-8-(2-morpholin-4-yl-2-oxo-ethyl)-chromen-2-one (**1**): Cream-coloured crystals; m.p. 170–172 °C. – Yield 0.063 g.

N-(4-Chloro-benzyl)-2-(7-methoxy-2-oxo-2H-chromen-8-yl)-acetamide (**2**): White crystals (crystallized from methanol); m.p. 242–243 °C. – Yield: 0.126 g.

7-Methoxy-8-(2-oxo-2-piperidin-1-yl-ethyl)-chromen-2-one (**3**): Cream-coloured crystals (crystallized from methanol); m.p. 165–166 °C. – Yield: 0.064 g.

7-Methoxy-8-[2-(4-methyl-piperazin-1-yl)-2-oxo-ethyl]-chromen-2-one (**4**): Yellow crystals (crystallized from methanol); m.p. 191–194 °C. – Yield: 0.026 g.

Details concerning NMR and MS data of all compounds can be obtained by direct contact with the lead author.

Animals

12-week-old CBA mice were delivered from Ilkowice near Kraków, Poland, and 3-month-old Wistar rats from the Institute of Laboratory Medicine, Łódź, Poland. The animals were fed a commercial, pelleted food and filtered water *ad libitum*. The local ethics committee approved the study.

Reagents and antigens

Concanavalin A (ConA), pokeweed mitogen (PWM), dimethylsulfate (DMSO), dimethylformamide (DMF), lipopolysaccharide (LPS) from *E. coli* (serotype O111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and carrageenan were from Sigma. Sheep red blood cells (SRBC) were delivered by Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland.

Preparation of the compounds for the studies

Compounds **1**, **3** and **4** were initially dissolved in DMSO and compound **2** in DMF (1 mg in 0.1 ml of the solvents). Further dilutions of the compounds were performed in RPMI 1640 medium for the *in vitro* tests and in 0.9% NaCl for the *in vivo* use. The cell cultures contained appropriate dilutions of the solvents for respective compound concentrations (1, 10 and 100 μg/ml).

Proliferative response of splenocytes to mitogens

The mice were sacrificed and the spleens were isolated aseptically. The organs were pressed against a plastic screen into 0.83% NH_4Cl solution to lyze the erythrocytes (5 min incubation at room temperature). The cells were washed twice with Hanks' medium (Hanks, 1976), passed through a glass wool column to remove debris, and re-suspended in a culture medium, referred to as the culture medium, consisting of RPMI 1640 medium supplemented with 10% fetal calf serum, sodium pyruvate, 2-mercaptoethanol and antibiotics. Then the cells were distributed into 96-well flat-bottom tissue culture plates at a density of $2 \cdot 10^5$/100 μl/well. 2.5 μg/ml of ConA or PWM were added to induce cell proliferation. The compounds were added to the cultures at doses of 1, 10 and 100 μg/ml. After a 3-d incubation, the cell proliferation was determined using a colorimetric MTT assay (Hansen *et al.*, 1989). The results are presented as the mean optical density (OD) at 550 nm ± standard error (SE) from quadruplicate determinations.

Secondary humoral immune response to sheep erythrocytes in vitro

Mice were primed with 0.2 ml of sheep erythrocyte (SRBC) suspension, administered intraperitoneally. After 4 d, the splenocytes were isolated and a single cell suspension was prepared in the culture medium. The cells were incubated in 24-well culture plates at a density of $5 \cdot 10^6$ cells/ml with the addition of 50 μl of 0.005% SRBC. The compounds were added to the cultures at the beginning of a 4-d incubation period in a cell culture incubator, in doses of 10 and 100 μg/ml. After 4 d, the number of antibody-forming cells (AFC) against SRBC was determined according to Mishell and Dutton (1967). The results are shown as the mean number of AFC from 4 wells ± SE, calculated per 10^6 viable spleen cells.

Induction of cytokines in rat peritoneal cells

The peritoneal cavities of rats were washed with 10 ml of Hanks' medium, the cells were centrifuged, washed twice with Hanks' medium, and re-suspended in the culture medium at a density of $5 \cdot 10^6$ cells/ml. The cells were stimulated by addition of 5 μg/ml of LPS. The compounds were present at concentrations of 10 and 100 μg/ml. The control cultures contained appropriate concentrations of the solvents (DMSO or DMF). After 24 h of incubation the supernatants were harvested and kept frozen at -20 °C until cytokine determinations by bioassays using WEHI 164.13 and 7TD1 indicator cell lines sensitive to the actions of tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), respectively (Espevik and Nissen-Meyer, 1986; Van Snick *et al.*, 1986). Concentrations of the cytokines were presented in pg/ml.

Induction of cytokines in human peripheral blood mononuclear cells (PBMC)

The venous blood from a single donor was separated on a ficoll-uropoline gradient (density of 1.077 g/ml). The mononuclear cells of the interphase were washed twice with Hanks' medium and re-suspended in the culture medium at a density of $5 \cdot 10^6$/ml. The cells in 24-well culture plates ($5 \cdot 10^6$/ml/well) were stimulated overnight with 5 μg/ml of LPS. The compounds were added at doses of 10 and 100 μg/ml and the solvents in the appropriate dilutions. The supernatants were harvested and the levels of TNF-α and IL-6 determined by the bioassays (Espevik and Nissen-Meyer, 1986; Van Snick *et al.*, 1986).

Carrageenan inflammation in mice

Carrageenan was dissolved in 0.9% saline to obtain a 2% solution. The mice were given 0.05 ml of the carrageenan solution, intradermally into the hind foot pads, and after 3 h the foot pad edema was measured using a caliper. The compounds were injected intraperitoneally, at 200-μg doses, 48 and 24 h before the carrageenan injection. The results are presented as mean values of the net increase of the foot pad thickness (in millimeters) ± SE.

Statistics

The results are presented as mean values ± SE. Levene's test and Brown-Forsyth's test were used to determine the homogeneity of variance between groups. The variance was homogenous and analysis of variance (one way ANOVA) was applied, followed by post hoc comparison with Tukey's test to estimate the significance of the difference between groups. Significance was determined at $p \leq 0.05$. The statistical analysis was performed using Statistica 6.1 for Windows.

Results

Effects of the compounds on the secondary humoral immune response of mouse splenocytes to sheep erythrocytes in vitro

The compounds, used at concentrations of 10 and 100 μg/ml, were tested for their ability to affect the secondary humoral immune response of mouse splenocytes *in vitro*, expressed as the number of AFC to sheep erythrocytes. Leflunomide (LF) was used as a suppressive reference drug. The results (Table I) showed that the compounds exhibited moderate, but statistically significant inhibitory actions which were, however, not so distinctly dose-dependent as in the case of LF. The strongest inhibitory action was demonstrated by **4** (47.25% inhibition). For comparison, the respective dose of LF inhibited the response by 74.1%. The suppressive effects of the compounds were compared with the appropriate control solvents (DMSO and DMF) as described in Materials and Methods.

Effects of the compounds on concanavalin A- and pokeweed mitogen-induced proliferation of mouse splenocytes

The compounds were also assayed for their effects on the proliferative response of mouse splenocytes to T-cell (ConA) and B-cell (PWM) mitogens (Ferguson *et al.*, 1976). The results shown in Table I revealed small but statistically significant suppression of the proliferative response of cells to ConA by **1** and **3** at 100 μg/ml. On the other hand, **4** was stimulatory at 1 μg/ml and amide **2** suppressive at 1 μg/ml (data not shown).

Compounds **1** and **3** were also suppressive in the model of the proliferative response of cells to PWM (Table I). Interestingly, amide **2** stimulated the proliferative response of cells to PWM. Compound **4** weakly stimulated the proliferation; a significant stimulation was observed only at 10 μg/ml (not shown). In general, the alterations in the proliferative response of splenocytes to mitogens by the studied compounds were differential.

Table I. Effects of the compounds on the secondary humoral immune response to sheep erythrocytes and proliferative response of mouse splenocytes to mitogens. The results are presented as mean values from quadruplicate determinations ± standard error (SE).

Compound[a]	Experimental model		
	Humoral immune response (AFC · 10^6)	Proliferation to concanavalin A (OD 550/630 nm)	Proliferation to pokeweed mitogen (OD 550/630 nm)
None	92.25 (4.52)	0.1287 (0.0044)	0.2489 (0.0035)
DMSO[b]	1003.50 (83.03)	0.4862 (0.0196)	0.4515 (0.0039)
DMF[c]	1187.50 (59.41)	0.5772 (0.0104)	0.4090 (0.0086)
LF	260.00 (11.55)	Not done	Not done
1	640.00 (45.46)	0.3870 (0.0059)	0.3577 (0.0047)
2	745.75 (43.79)	0.5965 (0.0131)	0.4652 (0.0031)
3	775.00 (4.24)	0.3707 (0.0063)	0.3520 (0.0021)
4	529.25 (17.24)	0.5262 (0.0054)	0.3695 (0.0045)

[a] The compounds were used at 100 μg/ml.
[b] DMSO was used at respective dilution and was the solvent for **1**, **3** and **4**.
[c] DMF was used at respective dilution and was the solvent for **2**.
Statistics: Humoral immune response: LF *vs.* DMSO, $p = 0.0001$; DMSO *vs.* **1**, $p = 0.0004$; DMSO *vs.* **3**, NS; DMSO *vs.* **4**, $p = 0.0001$; DMF *vs.* **2**, $p = 0.0001$ (ANOVA). Proliferation to ConA: DMSO *vs.* **1**, $p = 0.0002$; DMSO *vs.* **3**, $p = 0.0002$; DMSO *vs.* **4**, NS; DMF *vs.* **2**, NS (ANOVA). Proliferation to PWM: DMSO *vs.* **1**, $p = 0.0002$; DMSO *vs.* **3**, $p = 0.0002$; DMSO *vs.* **4**, $p = 0.0002$; DMF *vs.* **2**, $p = 0.0002$ (ANOVA).

Effects of the compounds on TNFα and IL-6 production by cultures of rat peritoneal exudate cells stimulated with lipopolysaccharide

Fig. 2A and B demonstrate the effects of the preparations on LPS-induced TNF-α and IL-6 production by pooled rat peritoneal cell cultures. The actions of the compounds on cytokine production were differential. Marked stimulatory effects on both TNF-α and IL-6 production were found by application of **2** at 100 μg/ml. Compounds **1**, **3** and **4** were inhibitory with regard to TNF-α production, in particular at 100 μg/ml. Whereas **3** and **4** did not enhance the IL-6 production, **1** caused about a 2-fold increase of the IL-6 level.

Effects of the compounds on lipopolysaccharide-induced TNF-α and IL-6 production in the cultures of human PBMC

Effects of the compounds on cytokine production were also tested on PBMC (Fig. 3A and B). It appeared that **1**, **3** and **4** were strongly inhibitory with respect to TNF-α production by these cells at 10 μg/ml (73.0, 78.2 and 80% inhibition, respectively). Strong inhibition of the TNF-α production by DMSO at 100 μg/ml did not allow to evaluate the activity of the compounds at this dose. Likewise, the other solvent (for **2**) was also strongly inhibitory for the TNF-α production making the evaluation of the activity of **2** not possible. Both solvents, however, did not alter the IL-6 production by PBMC, and we found an exceptionally high stimulatory action of **2** (2- and 3-fold stimulation for 10 and 100 μg/ml concentrations) and a 3-fold stimulation of the IL-6 production by **4** at 100 μg/ml.

Effects of the compounds on the carrageenan-induced inflammation in mice

The mice were given the amides **2** and **4** and the reference compound osthole intraperitoneally, 48 and 24 h before the injection of carrageenan. The results (Fig. 4) showed that **2** and **4** significantly reduced the foot pad edema (by 56.5 and 68.3%, respectively). However, administration of osthole led to even stronger (80.7%) inhibition.

Discussion

In this study we demonstrated differential immunomodulatory activities of the new coumarin derivatives in several conventional immunological tests. In the model of secondary humoral immune response *in vitro* we found that the compounds were moderately inhibitory with regard to the number of AFC. That action could have an association with the ability of some compounds (in particular compound **4**) to inhibit the TNF-α production in LPS-induced peritoneal

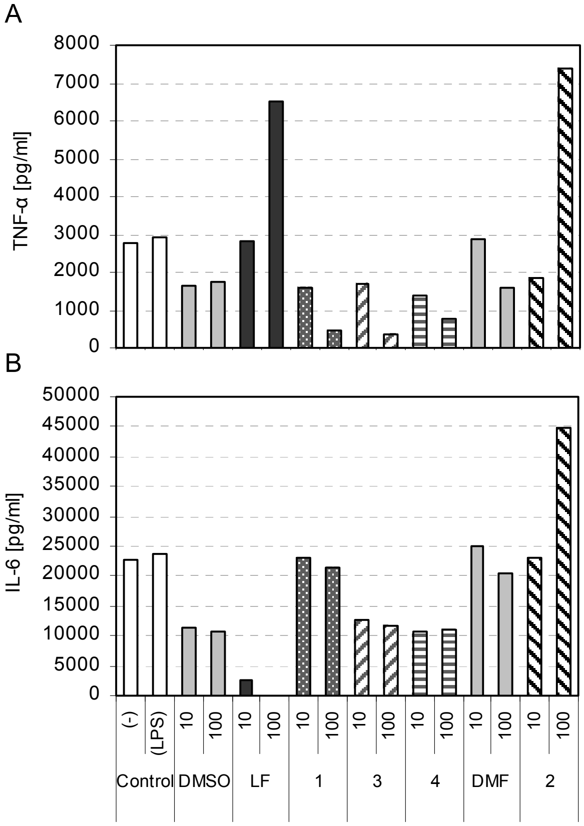

Fig. 2. Effects of the compounds on (A) TNF-α and (B) IL-6 production by rat peritoneal exudates cells. The compounds were used at concentrations of 10 and 100 μg/ml. DMSO was used at respective dilution and was the solvent for **1**, **3** and **4**. DMF was used at respective dilution and was the solvent for **2**. The results are presented as cytokine concentrations expressed in pg/ml.

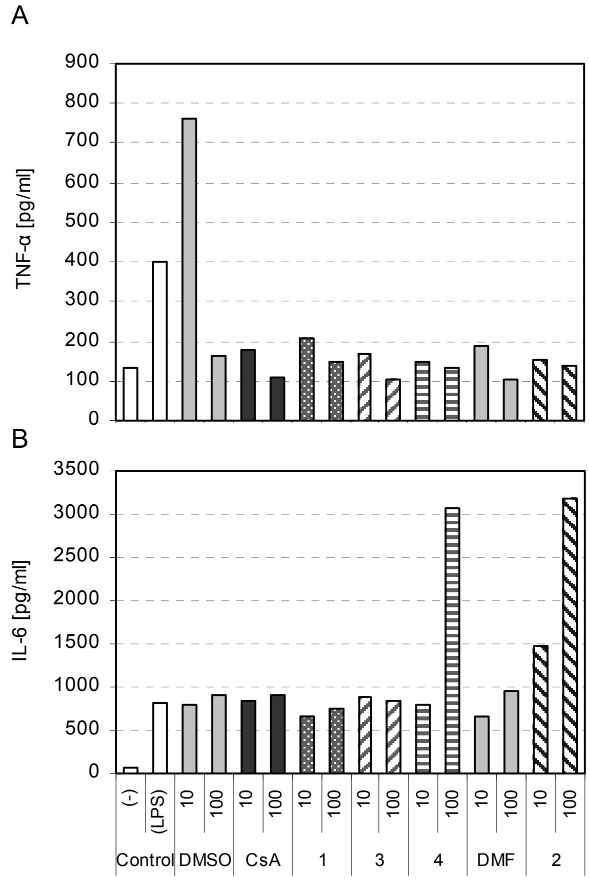

Fig. 3. Effects of the compounds on (A) TNF-α and (B) IL-6 production by human peripheral blood mononuclear cells. The compounds were used at concentrations of 10 and 100 μg/ml. DMSO was used at respective dilution and was the solvent for **1**, **3** and **4**. DMF was used at respective dilution and was the solvent for **2**.

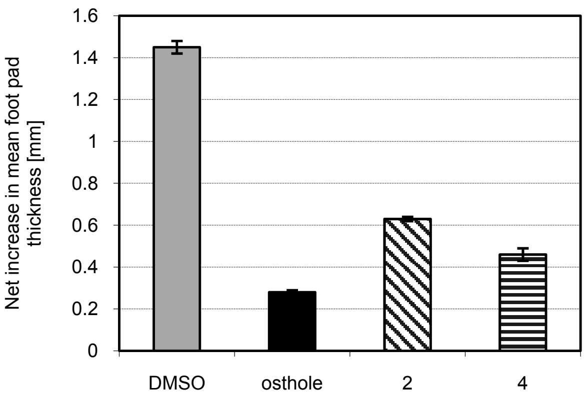

Fig. 4. Effects of the compounds on carrageenan-induced inflammation in mice. Statistics: DMSO *vs.* osthole, p = 0.0001; DMSO *vs.* **2**, p = 0.0001; DMSO *vs.* **4**, p = 0.0001; osthole *vs.* **2**, p = 0.0001; osthole *vs.* **4**, p = 0.0001 (ANOVA). The compounds were injected intraperitoneally, at 200-μg doses, 48 and 24 h before the carrageenan injection. The results are presented as mean values of the net increase of the foot pad thickness (in millimeters) ± SE.

exudate rat cell cultures and the PBMC cultures since TNF-α is essential for initiation of the immune response (Wellborn *et al.*, 1996). Reports on effects of coumarin derivatives on the humoral immune response are practically absent. In an available report, the authors showed that pretreatement of mice with coumarin derivatives enhanced the humoral immune response to *E. coli* lyzates, soluble worm antigen preparations and cancer bladder tissue homogenates (Maghraby and Bahgat, 2004). That phenomenon was also associated with an increase of CD4+ T cell levels.

The effects of the compounds on other parameters of the immune response, such as the proliferative response of splenocytes to T- and B-cell mitogens, were differential, *i.e.* suppressive or stimulatory. These findings are in agreement with other *in vivo* (Lelung *et al.*, 2005) and *in vitro* (Barreiro *et al.*, 2006) data. Esculetin (Lelung *et al.*, 2005) was shown to increase the mitogenic response of mouse splenocytes to ConA and LPS and, in addition, to induce the LAK (lymphokine-activated killer cell) activity in splenic lymphocytes. A suppressive activity for the mitogen-induced proliferation was regulated, on the other hand, by scopoletin (Barreiro *et al.*, 2006) which was not associated with induction of cell toxicity by the compound.

The compounds used in the present investigation were strongly inhibitory with regard to TNF-α production in human and rat cell cultures. That is also a feature of other coumarin derivatives tested in various experimental models (Corsini *et al.*, 2001; Bucolo *et al.*, 2003; Cheng *et al.*, 2004; Kim *et al.*, 2006; Yoshikawa *et al.*, 2006; Zhao *et al.*, 2007). The studies indicated that the suppressive action of coumarins occurs at the pretranscriptional level (Corsini *et al.*, 2001; Kim *et al.*, 2006).

The inhibitory actions of the coumarin derivatives are consistent with other studies (Kontogiorgis and Hadjipavlou-Litina, 2005). That action may be associated with the inhibitory effect on histamine release from mast cells (Tsuruga *et al.*, 1991), and these cells play a crucial role in the early phase of the carrageenan-induced inflammatory reaction (Vinegar *et al.*, 1987). The ability of the compounds to stimulate the IL-6 production found in the PBMC model could also have a significance since IL-6 counteracts the action of TNF-α (Tilg *et al.*, 1994). Nevertheless, the inhibitory actions of the compounds in the humoral immune response and in the carrageenan-induced inflammation cannot be solely explained by the effects of the compounds on the TNF-α and IL-6 production which were not always consistent in the applied models. Therefore, other mechanisms of inhibitory actions of the compounds are plausible and await further research.

Barreiro A. M. L., Cremaschi G., Werner S., Coussio J., Ferraro G., and Anesini C. (2006), *Tilia cordata* Mill. extracts and scopoletin (isolated compound): differential growth effects on lymphocytes. Phytother. Res. **20**, 34–40.

Borges F., Roleira F., Milhazes N., Santana L., and Uriarte E. (2005), Simple coumarins and analogues in medicinal chemistry: occurrence, synthesis and biological activity. Curr. Med. Chem. **12**, 887–916.

Bucolo C., Cuzzocrea S., Mazzon E., and Caputi A. P. (2003), Effects of cloricromene, a coumarin derivative, on endotoxin-induced uveitis in Lewis rats. Invest. Ophthalmol. Vis. Sci. **44**, 1178–1184.

Cheng J. F., Chen M., Wallace D., Tith S., Arrhenius T., Kashiwagi H., Ono Y., Ishikawa A., Sato H., Kozono T., Sato H., and Nadzan A. M. (2004), Discovery and structure-activity relationship of coumarin derivatives as TNF-alpha inhibitors. Bioorg. Med. Chem. Lett. **14**, 2411–2415.

Cisowski W. and Rządkowska-Bodalska H. (1979), Synthesis of some derivatives of osthol. Part I. Polish J. Chem. **53**, 1527–1531.

Codex der Pharmacopöen (1844–1845), Leopold Boß-Verlag, Leipzig.

Corsini E., Lucchi L., Binaglia M., Viviani B., Bevilacqua C., Monastra G., Marionovich M., and Galli C. L. (2001), Cloricromene, a semi-synthetic coumarin derivative, inhibits TNF-alpha production at a pretranscriptional level. Eur. J. Pharmacol. **418**, 231–237.

Espevik T. and Nissen-Meyer J. (1986), A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumour necrosis factor from human monocytes. J. Immunol. Methods **95**, 99–105.

Ferguson R. M., Schmidtke J. R., and Simmons R. L. (1976), Inhibition of mitogen-induced lymphocyte transformation by local anesthetics. J. Immunol. **116**, 627–634.

Geiger P. L. (1840), Handbuch der Pharmacie. C. F. Winter, Heidelberg.

Hanks J. (1976), Hanks' balanced salt solution and pH control. Tissue Culture Association Manual **3**, 3.

Hansen M. B., Nielsen S. E., and Berg K. (1989), Reexamination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods **12**, 203–210.

Hegi G. (1975), Illustrierte Flora von Mitteleuropa. Parey Verlag, Berlin, Hamburg.

Hiermann A. and Schantl D. (1998), Antiphlogistic and antipyretic activity of *Peucedanum ostruthium*. Planta Med. **64**, 400–403.

Kim J. H., Jeong J. H., Jeon S. T., Kim H., Ock J., Suk K., Kim S. I., Song K. S., and Lee W. H. (2006), Decursin inhibits induction of inflammatory mediators by blocking nuclear factor-kappaB activation in macrophages. Mol. Pharmacol. **69**, 1783–1790.

Kontogiorgis C. A. and Hadjipavlou-Litina D. J. (2005), Synthesis and anti-inflammatory activity of coumarin derivatives. J. Med. Chem. **48**, 6400–6408.

Lelung K. N., Lelung P. Y., Kong L. P., and Lelung P. K. (2005), Immunomodulatory effects of esculetin (6,7-dihydroxycoumarin) on murine lymphocytes and peritoneal macrophages. Cell. Mol. Immunol. **2**, 181–188.

Madaus G. (1938), Lehrbuch der biologischen Heilmittel. Georg Thieme Verlag, Leipzig.

Maghraby A. and Bahgat M. (2004), Immunostimulatory effect of coumarin derivatives before and after infection of mice with the parasite *Schistosoma mansoni*. Arzneim. Forsch. **54**, 545–550.

Mishell R. I. and Dutton R. W. (1967), Immunization of dissociated spleen cell cultures from normal mice. J. Exp. Med. **126**, 423–442.

Muszyński J. (1957), Farmakognozja. PZWL, Warszawa.

Pharmacopoea Germanica (1882), 2nd ed. R. von Decker's Verlag, Berlin.

Schinkovitz A., Gibbons S., Stavri M., Cocksedge M. J., and Bucar F. (2003), Ostruthin: an antimycobacterial coumarin from the roots of *Peucedanum ostruthium*. Planta Med. **69**, 369–371.

Teuscher E. and Lindequist U. (1994), Biogene Gifte: Biologie, Chemie, Pharmakologie, 2nd ed. Gustav-Fischer-Verlag, Stuttgart.

Tilg H., Trehu E., Atkins M. B., Dinarello C. A., and Mier J. W. (1994), Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumour necrosis factor receptor p55. Blood **83**, 113–118.

Tsuruga T., Ebizuka Y., Nakajima J., Chun Y. T., Noguchi H., Iitaka Y., and Sankawa U. (1991), Biologically active constituents of *Magnolia salicifolia*: inhibitors of induced histamine release from rat mast cells. Chem. Pharm. Bull. **39**, 3265–3271.

Urbain A., Marston A., and Hostettmann K. (2005), Coumarins from *Peucedanum ostruthium* as inhibitors of acetylcholinesterase. Pharm. Biol. **43**, 647–650.

Van Snick J., Vink A., Uyttenhove C., Coulie P. G., Rubira M. R., and Simpson R. J. (1986), Purification and NH_2-terminal amino acid sequence of a T-cell derived lymphokine with growth factor activity for B-cell hybridomas. Proc. Natl. Acad. Sci. USA **83**, 9679–9683.

Vinegar R., Truax J. F., Selph J. L., Johnston P. R., Venable A. L., and McKenzie K. K. (1987), Pathway to carrageenan-induced inflammation in the hind limb of the rat. Fed. Proc. **46**, 118–126.

Wellborn M. B. 3rd, Van Zee K., Edwards P. D., Pruitt J. F., Kaibara A., Vauthey J. N., Rogy M., Castleman W. L., Lowry S. F., Kenney J. S., Stueber D., Ettlin U., Wipf B., Loetscher H., Copeland E. M. 3rd, Lesslauer W., and Moldawer L. L. (1996), A human tumour necrosis factor p75 receptor agonist stimulates *in vitro* T cell proliferation but does not produce inflammation or shock in the baboon. J. Exp. Med. **184**, 165–171.

Yoshikawa M., Nishida N., Ninomiya K., Ohgushi T., Kubo M., Morikawa T., and Matsuda H. (2006), Inhibitory effects of coumarin and acetylene constituents from the roots of *Angelica furcijuga* on D-galactosamine/lipopolysaccharide-induced liver injury in mice and on nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages. Bioorg. Med. Chem. **14**, 456–463.

Zhang Q., Qin L., He W., Van Puyvelde L., Maes D., Adams A., Zheng H., and De Kimpe N. (2007), Coumarins from *Cnidium monnieri* and their antiosteoporotic activity. Planta Med. **73**, 13–19.

Zhao L., Tao J. Y., Zhang S. L., Pang R., Jin F., Dong J. H., and Gou Y. J. (2007), Inner anti-inflammatory mechanism of petroleum ether extract from *Melilotus suaveolens* Ledeb. Inflammation **30**, 213–223.