Immunomodulatory and Anti-Inflammatory Activity of Selected Osthole Derivatives

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From osthole [7-methoxy-8-(3-methyl-but-2-enyl)-chromen-2-one] (I), obtained by selective extraction of \textit{Peucedanum ostruthium} (L.) W. Koch roots, ostholic acid (II) was synthesized 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 \textit{in vitro}, and 4 was the most suppressive. The effects of 1 and 3 on concanavalin A- and pokeweeds mitogen-induced mouse splenocyte proliferation were inhibitory and those of 4 stimulatory. The compounds were also tested for their activity on tumour necrosis factor \(\alpha\) 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 \(\alpha\) (rat cells), whereas compound 2 stimulated the production of both cytokines. Compounds 1, 2 and 3 were also strongly inhibitory on tumour necrosis factor \(\alpha\) production in human blood cells (73, 78 and 80% inhibition at 10 \(\mu\)g/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: \textit{Peucedanum ostruthium} (L.) W. Koch, Osthole Derivatives, Humoral Immune Response, Carrageenan

Introduction

\textit{Peucedanum ostruthium} (L.) W. Koch (syn. \textit{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 XIX\textsuperscript{th} century, \textit{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 antipyretic 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 \textit{et al.}, 2003; Borges \textit{et al.}, 2005). In an \textit{in vitro} study coumarins from \textit{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 \textit{et al.}, 2005). Recently, bioactivity-guided fractionation led to a successful isolation of antiosteoporotic components, with osthole as the major compound (Zhang \textit{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 \textit{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.](image-url)
(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% NH₄Cl solution to lyse 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 · 10⁸/100 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 · 10⁶ 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 10⁶ 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 centri-
fuged, 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 \mu g/ml of LPS. The compounds were present at concentrations of 10 and 100 \mu g/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 ºC until cytokine determinations by bioassays using WEHI 164.13 and 7TD1 indicator cell lines sensitive to the actions of tumour necrosis factor alpha (TNF-\alpha) 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 \mu g/ml of LPS. The compounds were added at doses of 10 and 100 \mu g/ml and the solvents in the appropriate dilutions. The supernatants were harvested and the levels of TNF-\alpha 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-\mu g 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 \mu g/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 \mu g/ml. On the other hand, 4 was stimulatory at 1 \mu g/ml and amide 2 suppressive at 1 \mu g/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 \mu g/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).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Humoral immune response (AFC · 10⁶)</th>
<th>Proliferation to concanavalin A (OD 550/630 nm)</th>
<th>Proliferation to pokeweed mitogen (OD 550/630 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>92.25 (4.52)</td>
<td>0.1287 (0.0044)</td>
<td>0.2489 (0.0035)</td>
</tr>
<tr>
<td>DMSO</td>
<td>1003.50 (83.03)</td>
<td>0.4862 (0.0196)</td>
<td>0.4515 (0.0039)</td>
</tr>
<tr>
<td>DMF</td>
<td>1187.50 (59.41)</td>
<td>0.5772 (0.0104)</td>
<td>0.4090 (0.0086)</td>
</tr>
<tr>
<td>LF</td>
<td>260.00 (11.55)</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>1</td>
<td>640.00 (45.46)</td>
<td>0.3870 (0.0059)</td>
<td>0.3577 (0.0047)</td>
</tr>
<tr>
<td>2</td>
<td>745.75 (43.79)</td>
<td>0.5965 (0.0131)</td>
<td>0.4652 (0.0031)</td>
</tr>
<tr>
<td>3</td>
<td>775.00 (4.24)</td>
<td>0.3707 (0.0063)</td>
<td>0.3520 (0.0021)</td>
</tr>
<tr>
<td>4</td>
<td>529.25 (17.24)</td>
<td>0.5262 (0.0054)</td>
<td>0.3695 (0.0045)</td>
</tr>
</tbody>
</table>

- The compounds were used at 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.

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. 2, 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 pretreatment 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.


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