Anti-Inflammatory and Immunomodulating Properties of a Sterol Fraction from *Sideritis foetens* Clem.

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A sterol fraction composed of campesterol (7.6%), stigmasterol (28.4%) and $\beta$-sitosterol (61.1%) was obtained by activity-guided fractionation of the acetone extract of *Sideritis foetens* Clem. This sterol fraction showed anti-inflammatory activity in *in vivo* murine models of inflammation. It decreased carrageenan paw oedema in mice after oral administration of 30 and 60 mg/kg and inhibited mouse ear oedema induced by 12-$\alpha$-tetradecanoylphorbol acetate (TPA) after topical application. Quantitation of the neutrophil specific marker myeloperoxidase (MPO) demonstrated that its topical anti-inflammatory activity was associated with reduction in neutrophil infiltration into inflamed tissues. Non-cytotoxic concentrations of the sterol fraction inhibited leukocyte granular enzyme release ($\beta$-glucuronidase) and superoxide generation. However, it did not show any significant inhibitory effect on histamine release from mast cells. *In vitro* modulatory activity towards the classical pathway of the complement system shown by this fraction would correlate with the anti-inflammatory profile shown *in vivo*.

Key words sterols; *Sideritis foetens*; inflammation; polymorphonuclear leukocyte

Interest in the phytochemical study of species of the *Sideritis* genus (Lamiaceae) has been stimulated by their pharmacological activity and their wide range of medicinal folk uses in Spain such as vulnerary, anti-infectious, anti-inflammatory, anti-rheumatic and anti-ulerous applications. Various flavonoids and terpenoids have been isolated from this genus.1,2) We have recently reported the anti-inflammatory properties of a lipid fraction obtained from *Sideritis javalambrensis* and those of the diterpenoid (ent-13(16),14-labdadiene-6$\alpha$,8$\alpha$,18-triol-andalusol-) isolated from the acetone extract of *Sideritis foetens*, an endemic plant of Southern Spain.3,4,5) Inflammation is normally a localized protective response which serves to destroy, dilute, or wall-off both the injurious agent and the injured tissue. The inflammatory response is coordinated by cells such as macrophages, lymphocytes, leukocytes and mast cells. A large number of mediators produced by these cells play a key role, for example, arachidonic acid metabolites such as prostaglandins and leukotrienes, reactive oxygen species, hydrolytic enzymes, histamine, nitric oxide, cytokines, etc.5–8) Acute inflammation is associated with a rapid, early infiltration of polymorphonuclear leukocytes and increased vascular permeability at the site of injury.

In this paper, we have evaluated the *in vivo* anti-inflammatory properties of a sterol fraction obtained from *S. foetens* on different inflammatory responses in mice. In addition, the interaction of this fraction with the functional properties of leukocytes and mast cells was analyzed *in vitro*. Immunomodulating activity through interaction with complement activation was also investigated.

MATERIALS AND METHODS

**Plant Material** The aerial parts of *Sideritis foetens* were collected in Sierra de Gádor, Almería province (Spain). A voucher specimen has been deposited in the Department of Botany, Faculty of Pharmacy, Complutense University, Madrid (Spain).

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mice (25—30 g). Sterol fraction, dissolved in 20 μl of acetone, was applied topically (at doses of 0.25, 0.5 and 1 mg/ear) simultaneously with TPA administration. The left ear (control) received only acetone. The reference drug, indomethacin, was administered at the same doses, and β-sitosterol at 0.5 mg/ear. After 4 h, the animals were killed by cervical dislocation and a 6 mm biopsy was obtained from both ears and immediately weighed. The increase in the weight of the right ear punch over that of the left indicated the oedema. Ear sections were homogenized in 750 μl of saline, and after centrifugation at 10000 g (final concentration 1 m), myeloperoxidase activity was measured in supernatants, as described before.11)

In Vitro Assays. Preparation of Rat Peritoneal Leukocytes Peritoneal polymorphonuclear leukocytes were elicited from male Wistar rats (250—300 g) as described before12) and resuspended in complete Hanks Balanced Salt Solution (HBSS) at 2.5×10⁶ cells/ml. Aliquots (0.5 ml) of leukocytes were stimulated with calcium ionophore A23187 (final concentration 1 μM) at 37 °C for 10 min with or without prior addition of the sterol fraction (0.5—50 μg/ml), β-sitosterol (41.4 μg/ml) or vehicle (ethanol). Supernatants were used for assay of lactate dehydrogenase (LDH) and β-glucuronidase release.

LDH Release: LDH was determined by measuring the rate of oxidation of NADH at 340 nm using sodium pyruvate as substrate. The total cellular content of LDH was measured in cells treated with 0.05% Triton X-100.13)

β-Glucuronidase Release: 10 μl of leukocyte supernatant was added to 5 ml 4-methylumbelliferyl-β-d-glucuronide and incubated for 25 min at 37 °C. The reaction was terminated by adding a solution containing 0.1 M NaHCO₃ and 0.25 M Na₂CO₃. The amount of released 4-methylumbelliferone was fluorimetrically measured with excitation at 356 nm and emission at 500 nm. The total cellular β-glucuronidase content was measured by lysing a portion of cells with 0.05% Triton X-100. Results for enzyme release are expressed as a percentage of this amount.

Superoxide Generation Aliquots of 1 ml rat peritoneal leukocytes were preincubated with test compounds (superoxide dismutase, sterol fraction and β-sitosterol) or vehicle (ethanol) for 10 min at 37 °C. TPA (1 μM) was added to induce superoxide generation which was estimated as the reduction of ferricytochrome c measured as the change in absorbance at 550 nm.14) These values were converted to nmol O₂⁻/2.5×10⁶ cells/10 min using an extinction coefficient of 2.1×10⁵ M⁻¹ cm⁻¹. Superoxide dismutase (SOD) was used as positive control.

Histamine-Release from Rat Peritoneal Mast Cells Rat peritoneal mast cells were prepared as previously described.15) Aliquots of cell suspension (0.5 ml, 10⁶ cells/ml) were preincubated at 37 °C with vehicle (ethanol) or test compounds (sodium cromolyn and sterol fraction) for 10 min and then the release reaction was triggered by addition of stimulus (compound 48/80 10 μg/ml or calcium ionophore A23187 1 μM). The reaction was terminated 15 min later by adding ice-cold Tyrode solution and the mix was centrifuged 10 min at 10000 g. Histamine in the supernatant was assayed fluorimetrically after condensation with o-phthalaldehyde and expressed as a percentage of the total cellular histamine after correction for spontaneous release (rarely exceeding 6%). The total histamine content was measured after treatment of the cell suspension with 5% perchloric acid.

Anti-Complementary Activity The inhibition of complement activity was determined by a modified version of the microassay described by Klerx.16) Briefly, the sterol fraction (8.8—140 μg/ml), dissolved in DMSO was diluted in the appropriate buffer (DMSO conc. <1%). Logarithmic dilutions were made with the classical pathway (CP) buffer, VSB⁺⁺ pH 7.35 (0.142 M sodium chloride, 4.95 mM sodium barbital supplemented with 0.5 mM Mg²⁺ and 0.15 mM Ca²⁺) or with the alternative pathway (AP) buffer, EGTA-VB pH 7.35 (0.142 mM sodium chloride, 4.95 mM sodium barbital supplemented with 5 mM Mg²⁺ and 8 mM EGTA). Thereafter, 50 μl of a dilution (1.25%) of HPS in VSB⁺⁺ (CP) or 25 μl of a dilution (50%) of HPS in EGTA-VB (AP) were added to the wells. After a standard preincubation at 37 °C for 30 min, 50 μl of a suspension of sensitized sheep erythrocytes (CP) or 25 μl of a suspension of uncoated rabbit erythrocytes (AP) were added. Separate experiments were performed reducing the preincubation time (15, 0 min).

Plates were then incubated at 37 °C for 60 min (CP) or 30 min (AP) and centrifuged. To quantify haemolysis, 50 μl of the supernatant were mixed with 200 μl of water and absorbances at 405 nm measured. The concentration of the sample giving 50% inhibition in the test system (IC₅₀) was calculated.

Statistical Analysis Data values are given as mean± S.E.M. [IC₅₀ values were calculated from at least four significant concentrations (n=6) by regression analysis]. For differences between controls and treated groups Student's t-test for unpaired data was used.

RESULTS AND DISCUSSION

The anti-inflammatory and immunomodulating properties of a sterol fraction obtained from the acetone extract of Sideritis foetens and composed of campesterol (7.6%), stigmasterol (28.4%) and β-sitosterol (61.1%) have been evaluated. In vivo anti-inflammatory activity was assayed by testing this fraction on different acute inflammatory models. The acetone extract of S. foetens (350 mg/kg) demonstrated a significant anti-inflammatory activity on carrageenan-induced mouse paw oedema. Activity-guided fractionation of this extract led to subfracton B and subsequently to a sterol fraction. The sterol fraction at p.o. doses of 30 or 60 mg/kg suppressed hind paw swelling between 3 and 7 h after carrageenan administration, achieving the highest inhibitory effect 3 h after carrageenan injection at both doses administered (30.1 and 37.4% inhibition, respectively). Indomethacin exerted a higher effect and decreased oedema formation with inhibition% of 18.7 (1 h), 41.4 (3 h), 51.0 (5 h) and 35.6 (7 h) at the dose of 10 mg/kg p.o. (Table 1). The anti-inflammatory activity of β-sitosterol, the main component of this sterol fraction, in this murine model was previously reported.17)

Physiological activation of protein kinase C (PKC) by diacylglycerol can be mimicked by tumor-promoting phorbol esters such as TPA. The induction of PKC-dependent signal transduction pathways by TPA results in a number of cellular responses that are believed to contribute to the pathogenesis of many inflammatory diseases.18) In experimental animal models TPA induces significant inflammatory responses
characterized by oedema, neutrophil infiltration, prostaglandin production and increases in vascular permeability. Topical application of the sterol fraction profoundly affected ear oedema induced by TPA in mice, compared with control animals, inhibiting this inflammatory response by 41%, 47.2 and 73.6% at the doses tested (Fig. 1B).

A high level of myeloperoxidase activity, an indicator of neutrophil accumulation, was noted on TPA-treated ears 4 h after induction of inflammation. The observed topical anti-inflammatory activity was confirmed by quantitating the levels of MPO, which was extracted by ear biopsy. The sterol fraction significantly inhibited myeloperoxidase activity by 19.8%, 25.0% and 29.3% at the doses tested (Fig. 1B). Therefore, inhibition of cell-mediated responses could be an additional mechanism for attenuating inflammation. Activation of rat leukocytes with calcium ionophore A23187 caused a calcium-dependent secretion of the contents of the lysosomal granules. This can be measured after pelleting the cells by centrifugation assaying the amounts of the enzyme B-glucuronidase in the supernatant. As shown in Table 2, leukocytes preincubated with the sterol fraction (0.5—50 μM) secreted lower levels of B-glucuronidase compared with control group (30.3% at the highest concentration tested, 50 μM). This effect could be explained by a stabilizing effect on membranes, as it has been reported that sterols decrease the membrane sensitivity to calcium, neutralizing the inflammatory activity of the enzyme B-glucuronidase.

In order to investigate the mechanisms underlying the anti-inflammatory activity of this fraction we have investigated its ability to modify certain responses related to the inflammatory process. Production of free oxygen radicals by leukocytes and secretion of granular enzymes are mechanisms supporting inflammation and tissue destruction. Therefore, inhibition of cell-mediated responses could be an additional mechanism for attenuating inflammation. Activation of rat leukocytes with calcium ionophore A23187 caused a calcium-dependent secretion of the contents of the lysosomal granules. This can be measured after pelleting the cells by centrifugation assaying the amounts of the enzyme B-glucuronidase in the supernatant. As shown in Table 2, leukocytes preincubated with the sterol fraction (0.5—50 μM) secreted lower levels of B-glucuronidase compared with control group (30.3% at the highest concentration tested, 50 μM). This effect could be explained by a stabilizing effect on membranes, as it has been reported that sterols decrease the membrane sensitivity to calcium, neutralizing the inflammatory activity of the enzyme B-glucuronidase.

Table 1. Oral Anti-Inflammatory Activity of the Acetone Extract, Sterol Fraction and Indomethacin on Carrageenan-Induced Mouse Paw Oedema

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>% Increase in foot volume after carrageenan injection (% inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (vehicle)</td>
<td>55.0±1.9</td>
<td>55.8±1.9</td>
</tr>
<tr>
<td>Acetone extract</td>
<td>350</td>
<td>38.9±3.3  (29.3)</td>
</tr>
<tr>
<td>Subfraction B</td>
<td>100</td>
<td>40.2±2.4  (26.9)</td>
</tr>
<tr>
<td>Sterol fraction</td>
<td>30</td>
<td>43.1±6.2  (21.6)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10</td>
<td>45.2±9.5  (17.8)</td>
</tr>
</tbody>
</table>

Values represent the mean±S.E.M. (n=8). Compounds were administered orally 1 h before carrageenan injection. *p<0.05, **p<0.01 with respect to the control group. (Student’s t-test)

Table 2. Interaction of the Sterol Fraction with Leukocyte Functions in Vitro

<table>
<thead>
<tr>
<th></th>
<th>LDH release (%)</th>
<th>B-Glucuronidase release (%)</th>
<th>Superoxide generation (nmol/2.5×10⁶ cell/10min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.7±0.9</td>
<td>20.1±1.3</td>
<td>39.5±1.3</td>
</tr>
<tr>
<td>Sterol fraction 0.5 μM/ml</td>
<td>7.5±2.6</td>
<td>19.8±2.0</td>
<td>41.3±0.7</td>
</tr>
<tr>
<td>5 μM/ml</td>
<td>8.7±1.5</td>
<td>16.6±2.5</td>
<td>36.4±2.2</td>
</tr>
<tr>
<td>10 μM/ml</td>
<td>8.9±1.2</td>
<td>15.4±2.1</td>
<td>35.1±1.2</td>
</tr>
<tr>
<td>25 μM/ml</td>
<td>9.2±1.8</td>
<td>14.8±1.8**</td>
<td>31.6±1.2</td>
</tr>
<tr>
<td>50 μM/ml</td>
<td>9.9±1.5</td>
<td>14.0±2.2**</td>
<td>29.5±0.5**</td>
</tr>
<tr>
<td>β-Sitosterol 41.1 μM/ml</td>
<td>8.9±1.6</td>
<td>14.9±1.6*</td>
<td>28.9±0.6*</td>
</tr>
<tr>
<td>SOD (37.5 U/ml)</td>
<td></td>
<td></td>
<td>0.0±0.0**</td>
</tr>
</tbody>
</table>

Values are expressed as means±S.E.M. from 3 tests. *p<0.05, **p<0.01 with respect to control group (Student’s t-test). SOD, superoxide dismutase.
increase in LDH release was observed.

In addition, this fraction suppressed TPA-stimulated oxidative burst as only weak inhibition on superoxide generation was observed at the concentrations tested (0.5—50 μg/ml). β-Sitosterol (41.4 μg/ml) also reduced this response (Table 2).

Mast cell degranulation followed by histamine release plays a pivotal role in the inflammatory response. Incubation of mast cells with increasing concentrations of the sterol fraction did not affect histamine secretion, independently of the stimulus used (calcium ionophore A23187 or compound 48/80) (data not shown).

Finally, this fraction was examined for immunomodulating properties through inhibition of classical and alternative complement pathways. The complement system is recognized as an important pathway of host defence against infection, as well as mediating immunological and inflammatory reactions. As shown in Fig. 2, the sterol fraction (8.8—140 μg/ml) inhibited the haemolytic activity of the classical complement pathway (IC50: 35.5 μg/ml, standard conditions). Modification of preincubation conditions at 37 °C (15, 0 min) results in a decrease of the inhibitory activity indicating complement consumption during preincubation. However, this activity was not decreased when preincubation was performed at 4 °C suggesting that the interference does not occur at the enzymatic level. Each sterol was reported to occur at the enzymatic level. Each sterol was reported to

Although the in vivo anti-inflammatory activity of phytosterols was previously investigated, in the present work we have reported new insights into the mechanism of action of sterols on cellular systems as the sterol fraction interacted with leukocyte functions and exerted a modulatory activity towards the classical pathway of the complement system.

From these data and previous investigations on Sideritis plant species, we can suggest that different kind of lipids (glycerolipids, sterols) contribute to the anti-inflammatory profile of this genus, although the spectra of action of these compounds is different.

REFERENCES