Anti-Inflammatory Properties of a Lipid Fraction Obtained from *Sideritis javalambrensis*

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A lipid fraction obtained by activity-guided fractionation from the hexane extract of *Sideritis javalambrensis* was evaluated for anti-inflammatory activity. This fraction significantly inhibited paw oedema induced by carrageenan as well as ear oedema induced by 12-O-tetradecanoylphorbol 13-acetate (TPA) in mice after oral or topical administration, respectively. Quantitation of the specific marker myeloperoxidase (MPO) demonstrated that its topical anti-inflammatory activity was associated with reduction in cell infiltration into inflamed tissues. The lipid fraction significantly decreased leukocyte granular enzyme release (β-glucuronidase), but failed to inhibit superoxide generation. Histamine release from mast cells was also suppressed in a concentration-dependent manner. In addition, non-toxic concentrations of this fraction reduced nitric oxide (NO) generation in lipopolysaccharide (LPS)-treated J774 macrophages. Taken together, these results suggest that the lipid fraction exerts in vivo anti-inflammatory activity with the partial contribution of inhibitory actions on some inflammatory responses.

Key words * S. javalambrensis; inflammation; leukocyte functions; histamine; nitric oxide

Inflammation is a complex pathophysiological process mediated by a variety of signalling molecules produced by leukocytes, macrophages and mast cells as well as by the activation of complement factors which bring about oedema formation as a result of extravasation of fluid and proteins and accumulation of leukocytes at the inflammatory site. A large number of mediators produced by these cells play a key role as arachidonic acid metabolites like prostaglandins and leukotrienes, reactive oxygen species, hydrolytic enzymes, histamine, nitric oxide. The genus *Sideritis* (Lamiaceae) embraces a great number of species that are traditionally used in Spanish folk medicine for their anti-inflammatory and gastroprotective properties to treat certain disorders that are accompanied by inflammation. Several anti-inflammatory compounds have been obtained from plants of this genus, mainly flavonoids and terpenoids.

We have been investigating the phytochemistry of *Sideritis javalambrensis* and have shown that its hexane and methanol extracts are markedly anti-inflammatory in the rat adjuvant arthritis model. According to the revised literature, glycerolipids are found much less commonly as anti-inflammatory agents and relatively little literature data are available on the pharmacological activities of this type of compounds. This prompted us to study the anti-inflammatory profile of a lipid fraction obtained from this plant against different experimental models of acute inflammation in mice. In addition, interaction of this fraction with functional properties of leukocytes as well as its ability to modify some responses related to the inflammatory process were analyzed in order to investigate the mechanisms underlying its anti-inflammatory activity.

MATERIALS AND METHODS

*Chemicals* Cell culture reagents were obtained from Life Technologies (Barcelona, Spain). The remaining reagents were purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.).

*Plant Material* Aerial parts of *Sideritis javalambrensis* were collected in Sierra Javalambre, Teruel province (Spain). A voucher specimen has been deposited in the Department of Botany, Faculty of Pharmacy, Complutense University, Madrid (Spain).

*Extraction and Fractionation* Aerial parts of *S. javalambrensis* (1.1 kg) were dried, powdered and extracted with n-hexane (yield 41.5 g). The extract (27.5 g) was fractionated by flash column chromatography with silicagel (70–230 mesh) and eluted with CH₃C₂H₄OAc mixtures. Fractions were collected and pooled according to results from TLC. Ten fractions were obtained and checked for anti-inflammatory activity in the mouse paw oedema model. The most active fraction (weight 6.0 g) was further chromatographed on a silica gel column and eluted with n-hexane/EtOAc (93 : 7), yielded the test fraction (4.4 g), which was homogenous on TLC with different solvent mixtures. H-NMR spectra signals exhibited at δ 0.86 (CH₃), 1.23 [CH₂]₃n and 2.29 (CH₂–CH⁻CH–COO⁻) suggested the presence of long chain fatty acids. H-NMR and H-COSY spectra indicated that the signals at δ 4.1, 4.3 and 5.3 belong to 62.1 (t) and 68.9 (d) ppm C-NMR signals. A DEPT experiment also indicated that the signals corresponded to methylene or more precisely oxymethylenes as shown by their chemical shifts. Therefore, a short chain polyol part-like that of glycerol must be present. As no 1-H-NMR signal appeared between 5.5 and 8.0 ppm (aromatic protons), C-NMR signals between 125 and 135 ppm can be attributable to isolated double bonds, probably in the supposed fatty acid residue correlating with the presence of the multiplet centred at 5.29 ppm. A huge multiplet near 1.30 ppm corresponded to aliphatic methylene protons. C-NMR signal 171–172.4 ppm corresponds to the carbonic of ester groups.

The IR spectra demonstrated the presence of OH (3100–3700 cm⁻¹), CH and CH₂ (2866–3020 cm⁻¹), C=O (ester 1740 cm⁻¹) and C–O–C (ester 1150 cm⁻¹). UV spectrum showed an UVmax at 229 nm, indicating the presence of an
unsaturated methyl ester.

The results obtained from the spectral data are in accord with a glycerolipid structure, as was evidenced by alkaline hydrolysis and GC-MS analysis of the methyl esters of the fatty acids, being linoleic acid (C18:2) (51.4%), oleic acid (C18:1) (37.7%) and palmitic acid (C16:0) (5.0%) the main fatty acids.

**In Vivo Assays. Carrageenan-Induced Mouse Paw Oedema** Swelling was induced by a modification of the technique of Sugishita et al. Female Swiss mice (25—30 g) divided into groups of eight were used. Lipid fraction (50 and 100 mg/kg dissolved in ethanol/Tween 80 : H2O 1 : 1 : 18 v/v/v) were administered p.o. 1 h before inflammation was induced on the left paw by the subplantar injection carrageenan (0.05 ml; 3% w/v in saline). A reference group was treated with indomethacin (10 mg/kg p.o.). Paw volumes were measured before carrageenan injection and 3, 5, and 7 h after using a plethysmometer.

**12-O-Tetradecanoylphorbol-13-acetate (TPA)-Induced Mouse Ear Oedema** TPA (2.5 µg/ear), dissolved in 20 µl of acetone, was applied topically to the right ear of female Swiss mice (25—30 g). Lipid fraction dissolved in 20 µl of acetone, was applied topically simultaneously with TPA administration. The left ear (control) received only acetone. The reference drug, indomethacin, was administered at the same doses. After 4 h, the animals were sacrificed 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.13 Ear sections were homogenized in 750 µl of saline, and after centrifugation at 10000 g for 15 min at 4°C, myeloperoxidase activity was measured in supernatants, as described before.14

**In Vitro Assays. Preparation of Rat Peritoneal Leukocytes** Peritoneal leukocytes were elicited from male Wistar rats (250—300 g) as described before15 and resuspended in complete Hanks Balanced Salt Solution (HBSS) at 2.5 x 10^6 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 lipid fraction. 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 nicotinamide adenine dinucleotide (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-100.16

**β-Glucuronidase Release** 10 µl of leukocytes supernatants were added to 5 mm 4-methylumbelliferyl-β-D-glucuronic acid 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 measured fluorimetrically with excitation set 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** Cell aliquots (1 ml) were preincubated with test compounds (superoxide dismutase and lipid fraction) or vehicle (DMSO) for 10 min at 37°C. PMA (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.17

**Histamine-Release from Rat Peritoneal Mast Cells** Rat peritoneal mast cells were prepared as previously described.18 Aliquots of cell suspension (0.5 ml, 106 cells/ml) were preincubated at 37°C with vehicle (0.1% DMSO), lipid fraction or reference compound (sodium cromolyn) for 10 min and then, the release reaction was triggered by the addition of stimulants (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 1000 g. Histamine contents in the supernatants were assayed fluorimetrically after condensation with o-phthalaldehyde19 and expressed as a percentage of the total cellular histamine after correction for spontaneous release (rarely exceeding 6%). The total content of histamine was measured after treatment of the cell suspension with 5% perchloric acid.

**Nitric Oxide (NO) Generation** The mouse macrophage cell line J774 obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, U.K.) was cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2 mm L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml) and 10% foetal calf serum until they reached confluence. To induce iNOS (inducible isoform of nitric oxide synthase) fresh culture medium containing E. coli lipopolysaccharide (LPS, 1 µg/ml) was added. Nitrite accumulation in the medium was measured after 24 h. Where appropriate, lipid fraction (5—250 µg/ml), dexamethasone (1 µM) were added to the medium 0.5 h before or 20 h after induction with LPS. Stock solutions were made in DMSO and further diluted in culture medium (DMSO final concentration 0.01%). This concentration of DMSO did not affect nitrite production or respiration. NO formation was measured as the stable product nitrite in culture supernatant with the Griess reagent.20

**Cell Viability** Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide] to formazan.21 Cells in 96-well plates were exposed to the lipid fraction for 24 h and incubated with MTT (0.2 mg/ml) for 60 min. Culture medium was removed and the cells solubilized in DMSO (100 µl). The extent of reduction of MTT to formazan within cells was quantitated by measurement of OD540 with a microplate reader. Viability was set as 100% in untreated cells.

**Statistical Analysis** Data values are given as mean ± S.E.M. For differences between controls and treated groups, Student's t-test for unpaired samples was used. p values <0.05 were considered to be significant.

**RESULTS AND DISCUSSION**

In this study, the anti-inflammatory properties of a lipid fraction obtained from the hexane extract of S. javalambrensis have been evaluated. The hexane extract reduced the inflammatory response of adjuvant arthritis in rats, as described previously.10

Figure 1 summarizes the effect of the lipid fraction on mouse paw oedema induced by carrageenan. Oral pretreatment (1 h before carrageenan) with 50 and 100 mg/kg signifi-
Fig. 1. Effects of the Lipid Fraction and Indomethacin on Carrageenan-Induced Mouse Paw Oedema

Values represent the means±S.E.M. (n=8). Indomethacin (10 mg/kg) and Lipid fraction (LF 50 and 100 mg/kg) were administered p.o. 1 h before carrageenan injection. Significance relative to control values *= p<0.05, ** p<0.01.

scently reduced the edematous response. This inhibitory effect was observed at the three time points considered 3, 5, and 7 h after oedema induction at both doses administered. Indomethacin significantly inhibited this response.

Evaluation of the topical anti-inflammatory activity of the lipid fraction was performed in the TPA-induced mouse ear oedema. The phorbol ester (TPA) provides a skin inflammation model suitable for evaluation of both topical and systemic anti-inflammatory agents and it has been extensively applied in studies of anti-inflammatory products. As shown in Fig. 2A topical application of the lipid fraction significantly suppressed the extent of swelling by 35, 41 and 53% at the doses of 0.25, 0.5 and 1 mg/ear, respectively.

Myeloperoxidase (MPO), a haemoprotein located in azurophil granules of neutrophils has been used as a biochemical marker for neutrophil infiltration into tissues. A high level of this enzyme activity was noted in TPA-treated ears 4 h after induction of inflammation. The observed topical anti-inflammatory activity was confirmed by quantifying the levels of MPO, which was extracted from ear biopsy. These studies revealed that this lipid fraction significantly inhibited this parameter by 43, 51 and 66% at the doses tested (Fig. 2B). Indomethacin was more effective on myeloperoxidase than on oedema, which was also attenuated.

In order to investigate the mechanisms underlying the anti-inflammatory activity of this lipid fraction, we have studied its ability to modify some responses related to the inflammatory process.

During inflammation, neutrophils stimulated by various agents release reactive oxygen species and stored proteolytic enzymes, which have been assigned prominent roles in causing inflammatory tissue reactions. Therefore, inhibition of cell mediated injury responses could be considered an additional mechanism for attenuating inflammation. Addition of calcium ionophore A23187 to leukocyte suspensions causes release of the azurophil granular enzyme β-glucuronidase. This response was significantly inhibited in a concentration-dependent manner by this fraction (Fig. 3). At the concentra-

Fig. 2. Effect of the Lipid Fraction and Indomethacin on TPA-Induced Mouse Ear Oedema (A) and Myeloperoxidase Activity (Absorbance at 630 nm) in Supernatants of Homogenates from TPA-Treated Ears (B)

Lipid fraction and indomethacin were administered topically at the time of TPA application (2.5 μg/ear). Values are expressed as means±S.E.M., from nine mice. ** p<0.01.

Fig. 3. Effect of the Lipid Fraction and Indomethacin on Lactate Dehydrogenase (LDH) and β-Glucuronidase Release from Calcium Ionophore A23187-Treated Rat Leukocytes

Values are expressed as means±S.E.M. from two separate experiments. * p<0.05, ** p<0.01.
tions tested in our study (5—100 µg/ml), it did not exert any cytotoxic effects, as no increase in LDH release was observed compared to control group. However, the fraction failed to affect TPA-stimulated oxidative burst as no inhibition on superoxide generation was observed (data not shown).

Mast cell degranulation followed by the release of histamine plays a pivotal role in the inflammatory response. As shown in Fig. 4, this fraction exerted a significant concentration-dependent inhibition on histamine release from mast cells stimulated by compound 48/80 or calcium ionophore A23187. At the highest concentration tested (100 µg/ml), percentages of inhibition achieved were 47.7% with compound 48/80 and 33.9% with calcium ionophore A23187.

Inflammatory macrophages play a central role in the inflammatory process by secreting large amounts of mediators that control the initiating process of inflammation. The synthesis of NO, which depends on nitric oxide synthase (NOS), is an important pharmacological target since overproduction of NO and its metabolites, seems to be associated primarily with inflammatory disorders. Activation of J774 macrophages with bacterial lipopolysaccharide LPS (1 µg/ml) is known to cause the induction of iNOS enzyme and it has been reported as a suitable cell model for testing new anti-inflammatory agents.

Finally, we examined the inhibitory effects of the lipid fraction on NO production. The fraction (250 µg/ml) in the absence of LPS did not alter the basal nitrite concentration. Pre-treatment of macrophages for 30 min with various concentrations of the lipid fraction (5—250 µg/ml) caused a concentration-dependent reduction of NO generation in the culture medium at 24 h, as shown in Fig. 5. The DMSO concentration used as vehicle had no effect on nitrite production. Dexamethasone (an inhibitor of iNOS induction) at 1 µM inhibited nitrite accumulation by 50.3%. Experiments performed by adding this fraction 20 h after LPS stimulation showed that the inhibitory response decreased. Based on MTT assays, viability was not reduced significantly after 24 h of incubation with fraction concentrations up to 250 µg/ml, nor decreased cellular respiration (Fig. 6). Therefore, it does not induce cytotoxicity and/or nonspecific depression of cellular respiration in J774 macrophages. Moreover, since LPS
induces a loss of cell viability within 24 h of culture, it was of interest to evaluate whether the tested fraction might protect against LPS-induced damage of these cells. As shown in Fig. 6, lipid fraction (250 µg/ml) added 30 min before LPS, exerted a moderate but significant protective effect against LPS-induced cytotoxicity.

The observation that the maximal effects were obtained when the fraction was added prior to macrophage challenge suggests an inhibitory action on the transcriptional mechanism leading to iNOS expression as it was unable to inhibit NOS activity once expressed in the activated cells. Linoleic and oleic acids, the most abundant fatty acids present in this fraction have also been reported as inhibitors of NOS expression.20

Recently, a potentially important role of NO generated by iNOS has also been suggested in experimental models of acute inflammation induced by carrageenan.29,30 The present findings provide a mechanism by which the anti-inflammatory properties of this fraction could be mediated.

In summary, we can conclude that this lipid fraction effect- ivally controls acute inflammation in experimental models after either oral or topical administration with a partial contribution of inhibitory actions on some cellular inflammatory responses. Isolation of the active constituents of this fraction and evaluation of their anti-inflammatory activity are in progress.

REFERENCES