Andalusol, a Diterpenoid with anti-Inflammatory Activity from *Siderits foetens* Clemen

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The anti-inflammatory activity of andalusol (*ent*-13(16),14-labdadiene-6α,8α,18-triol), a diterpenoid obtained from the acetone extract of *Sideritis foetens* Clemen. has been investigated. This compound was able to inhibit acute inflammatory processes induced by carrageenan or 12-*O*-tetradecanoylphorbol acetate (TPA) after oral or topical administration. Quantitation of the neutrophil specific marker, myeloperoxidase (MPO), demonstrated that its topical anti-inflammatory effect was associated with reduction in neutrophil infiltration into inflamed tissues. Interaction of andalusol with leukocyte functions and histamine release from mast cells was analyzed *in vitro*. At a concentration of 100 μM andalusol decreased β-glucuronidase release from calcium ionophore A23187-stimulated rat peritoneal leukocytes. However, it failed to affect superoxide generation on TPA-stimulated leukocytes and it was non toxic to leukocytes up to 100 μM (assayed in terms of lactate dehydrogenase release). Andalusol produced a dose-dependent inhibition on histamine release from rat peritoneal mast cells stimulated by compound 48/80 or calcium ionophore A23187. These results suggest that andalusol possesses an anti-inflammatory profile, and it is in part responsible for the anti-inflammatory activity attributed to this plant.

Introduction

The genus *Sideritis* (Lamiaceae) embraces a great number of species that are traditionally used for their anti-inflammatory and gastroprotective properties. Several anti-inflammatory substances have been identified in extracts of plants from this genus, mainly flavonoids and terpenoids (Villar *et al.,* 1984a; Villar *et al.,* 1984b; Barberán *et al.,* 1987; de las Heras and Hoult, 1995).

In the present study, we report the *in vivo* anti-inflammatory activity of the diterpenoid andalusol (*ent*-13(16),14-labdadiene-6α,8α,18-triol) obtained from the acetone extract of *Sideritis foetens* Clemen. In addition, the interaction of this compound with functional properties of leukocytes (superoxide generation and azurophil granular enzyme secretion) and histamine release from mast cells was analyzed *in vitro* as part of the investigations into its mechanism of action.

Nothing has been previously reported on the pharmacological effects of andalusol. We have, therefore, attempted to determine if andalusol was responsible for the anti-inflammatory activity exhibited by the acetone extract of *Sideritis foetens*.

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Material and Methods

Plant material

The aerial parts of *Sideritis foetens* were collected in May 1994 in Sierra de Gádor (Berja), Almería province (Spain). A voucher specimen has been deposited in the Department of Botany, Faculty of Pharmacy, Complutense University, Madrid (Spain).

Isolation of andalusol

The carrageenan-induced mouse paw oedema model was used for a guided-bioassay of the anti-inflammatory activity.

Dried aerial parts of *Sideritis foetens* (3 kg) were macerated with 99.5% acetone (12 l) at room temperature. The extract was evaporated *in vacuo* to yield 84.8 g of residue. The acetone extract demonstrated *in vivo* anti-inflammatory activity. This extract (11.8 g) was fractionated by flash column chromatography over a silica gel column (6 x 80

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cm, 230–400 mesh, 452 g). Elution with cyclohexane: acetone 70/30 v/v yielded andalusol (105 mg) as white needles. It was identified by comparing its ^{1}H-RMN spectral data with literature values (López *et al.,* 1977). Purity was checked by a reverse phase-HPLC method, using as mobile phase: water-methanol (30/70 v/v) in isocratic elution; column: Hypersil ODS (15 cm, 4.6 mm; 5 μm) (Shandon Science, Astmoon, U. K.); flow-rate: 1 ml/min. (Gómez-Serranillos *et al.,* 1997).

In vivo assays

Carrageenan-induced mouse paw oedema

Inflammation was induced by a modification of the method of Sugishita (Sugishita *et al.,* 1981). Female Swiss mice weighing 25–30 g divided into groups of eight were used. The acetone extract (350 mg/kg), andalusol (10, 30 and 60 mg/kg), reference drug (indomethacin 10 mg/kg) or vehicle (ethanol:tween 80:water 1:1:18 v/v/v) were administered orally 1 hour before inflammation was induced on the left paw by the subplantar injection of 0.05 ml of 3% λ-carrageenan in 0.9% saline (w/v). The paw volumes were measured before carrageenan injection and 1, 3, 5 and 7 hours after using a plethysmometer (LETICA LI 7500, Cibertec, Madrid, Spain).

12-*O*-tetradecanoylphorbol acetate (TPA) -induced mouse ear oedema

TPA (2.5 μg/ear), dissolved in acetone, was applied topically to both surfaces of the right ear of female Swiss mice (25–30 g) as described previously (Recio *et al.,* 1994). Andalusol, dissolved in 80% aqueous ethanol, was applied topically (at the doses of 0.5 and 1 mg/ear) simultaneously with TPA. The left ear (control) received acetone or vehicle. The reference drug, indomethacin, was administered at the same doses. After 4 hours, animals were sacrificed by cervical dislocation and a 6 mm biopsy was obtained from the center of the each ear and immediately weighed. The difference in the weight between the right treated ear and left ear was used as an index of oedema. The samples were used for myeloperoxidase (MPO) measurements as described below.

Myeloperoxidase (MPO) assay

Ear samples were homogenized in a tissue homogenizer system in 750 μl saline and centrifuged (10,000 × *g*, 15 min at 4ªC). Myeloperoxidase activity was measured in supernatants (Gil *et al.,* 1995). The reaction mixtures contained 50 μl supernatant, 150 μl phosphate-buffered saline (PBS), 15 μl 0.22 M NaH_2PO_4 (pH 5.4), 20 μl 0.034% H_2O_2 and 20 μl 18 mM tetramethylbencidine in 8% dimethylformamide. After 3 min incubation at 37 °C, 30 μl of 1.46 M sodium acetate buffer (pH 3.0) was added and absorbances at 630 nm were read using a microtiter plate reader.

In vitro assays

Lactate dehydrogenase (LDH) and β-glucuronidase release from rat peritoneal leukocytes

Peritoneal leukocytes were elicited from male Wistar rats (250–300 g) as described before (Charalambous *et al.,* 1994) 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 (Sigma, final concentration 1 μM) at 37 °C for 10 min with or without prior addition of andalusol (1–100 μM) or vehicle (ethanol). The supernatants were used for assay of LDH and β-glucuronidase release.

LDH release assay: The possible cytotoxicity of the compound was determined by measuring spectrophotometrically the amount of LDH released by cells into the supernatant. Enzyme activity was determined as the rate of oxidation of 40 mM NADH at 340 nm using 0.63 M sodium pyruvate as substrate. The total cellular was measured in cells treated with 0.05% Triton X-100 (de las Heras and Hoult., 1995).

β-glucuronidase release assay: Samples of leukocytes supernatants were added to 5 mM 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 $NaHCO_3$ and 0.25 M Na_2CO_3. 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 ex-

pressed as a percentage of this amount (de las Heras and Hoult., 1995).

Superoxide generation by rat peritoneal leukocytes

Aliquots of 1 ml rat peritoneal leukocytes, obtained as above, were preincubated with test compounds or vehicle for 10 min at 37 °C. They were stimulated with TPA (final concentration 1 μM) at 37 °C. After 10 min the reaction was terminated by centrifuging the tubes (400 × *g*, 10 min 4 °C). Superoxide was estimated as the reduction of ferricytochrome *c* measured as the change in absorbance at 550 nm. These values were converted to nmol $O_2^{\cdot-}$/2.5 x 10^6 cells x 10 min using an extinction coefficient of 2.1 x 10^4 M^{-1} cm^{-1}. Superoxide dismutase (SOD) was used as reference compound (de las Heras and Hoult, 1995).

Histamine-release from rat peritoneal mast cells

Rat peritoneal mast cells were prepared as previously described (Wang *et al.*, 1995). Briefly, heparinized Tyrode solution was injected into the peritoneal cavity of exsanguinated female Wistar rats (250–300 g). After abdominal massage, the cells in the peritoneal fluid were harvested and separated in 38% bovine serum albumin in glucose-free Tyrode solution. The cell pellet was washed and suspended in Tyrode solution to a final concentration of 10^6 cells/ml.

Aliquots of cell suspension (0.5 ml) were preincubated at 37 °C with vehicle (ethanol) or andalusol 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 centrifuged 10 min at 1000 × *g*. The contents of histamine in the supernatants were assayed fluorimetrically after condensation with *o*-phthalaldehyde (Shore *et al.*, 1959) and expressed as 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.

Statistics

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.

Results

HPLC analysis of andalusol

To check peak purity, the eluate was monitored with a photodiode array detector (λ=190–390 nm). The peak was considered pure when the three spectra corresponding to the up-slope, apex and down-slope was computer-normalized, superimposed, and was exact coincidence between the three spectra (match factor ≥ 99.5) (Fig. 1).

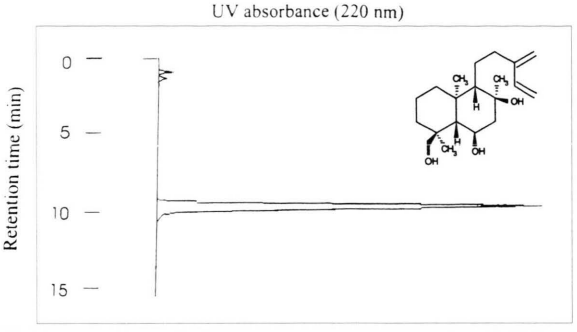

Fig. 1. Reverse phase-HPLC profile of andalusol and chemical structure.

Carrageenan-induced mouse paw oedema

The acetone extract of *Sideritis foetens* demonstrated significant anti-inflammatory activity at an oral dose of 350 mg/kg in this model, showing percentages of inhibition of 29.4, 50.0, 37.0 and 37.5% at 1, 3, 5 and 7 hours after carrageenan injection. Oral administration of andalusol also inhibited the formation of the carrageenan oedema. The highest inhibitory effect (44.8%) was observed 5 hours after carrageenan injection at the dose of 60 mg/kg. (Table I).

Mouse ear oedema assay

As can be seen in Table II, topical application of andalusol (0.5 and 1 mg/ear) significantly attenuated the TPA-induced oedema. This topical anti-inflammatory activity was confirmed by quantiting the levels of the neutrophil-specific marker (MPO), which was extracted from ears biopsy.

Table I. Effects of andalusol on carrageenan-induced mouse paw oedema.

Treatment	Dose (mg/kg)	% Increase in foot volume after carrageenan injection (% inhibition)			
		1 h	3 h	5 h	7 h
Control (vehicle)		57.6 ± 3.7	62.3 ± 2.6	53.1 ± 2.8	52.7 ± 3.2
Andalusol	10	45.7 ± 4.8 (20.7)	50.4 ± 5.2 (19.1)	43.5 ± 3.6 (18.1)	43.6 ± 2.9 (17.3)
	30	45.7 ± 2.4* (20.7)	50.2 ± 4.0 (19.4)	43.7 ± 3.7 (17.7)	43.0 ± 4.0 (18.4)
	60	32.7 ± 3.0** (43.2)	35.9 ± 1.6** (42.4)	29.3 ± 3.5** (44.8)	32.3 ± 4.3** (38.7)
Indomethacin	10	39.7 ± 2.5** (31.1)	39.1 ± 2.6** (37.2)	26.9 ± 3.0** (49.3)	28.6 ± 1.8** (45.7)

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

Table II. Effects of andalusol on TPA-induced mouse ear oedema.

Treatment	Dose (mg/ear)	Oedema (mg) (mean ± S. E. M.)	Oedema Inhibition (%)
Control (TPA)		15.7 ± 0.5	
Andalusol	0.5	11.6 ± 0.5*	26.1
	1.0	10.8 ± 1.2**	31.2
Indomethacin	0.5	2.4 ± 0.1**	84.7
	1.0	1.5 ± 0.3**	90.4

*$p<0.05$, **$p<0.01$ by Student-t test with respect to the control group (n=8).

These studies revealed that andalusol significantly inhibited MPO activity by 51.0% and 56.6% at the doses used (0.5 and 1 mg/ear) (Fig. 2). Indomethacin effectively inhibited both parameters, as expected.

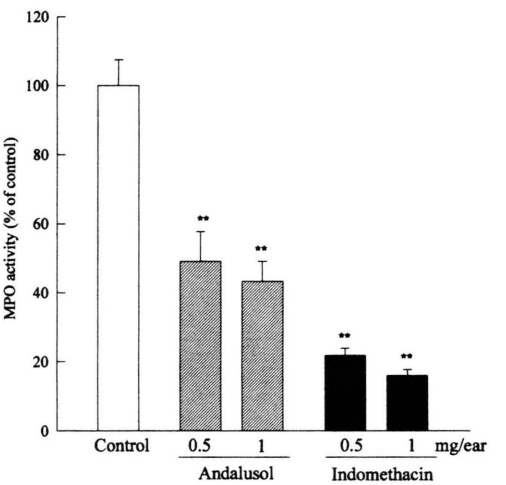

Fig. 2. Effects of andalusol and indomethacin on myeloperoxidase activity in supernatants of homogenates from TPA-treated ears. The effects of drugs were determined 4 hours after TPA application. Control (100%) mean ± S. E. M. value for MPO activity was 0.249 ± 0.019.O.D$_{630}$. (n=8). **$p<0.01$ vs control (Student's t-test).

Effects of andalusol on leukocyte functions

Andalusol was tested as potential inhibitor of various functional responses of activated leukocytes (Table III). This compound inhibited the release of β-glucuronidase to the medium at 100 μM (40.0% inhibition). Up to the higher concentration tested (100 μM), it did not cause any significant increase of LDH release, so a toxic effect on cells should be discarded. However, andalusol at 1000 μM caused increased LDH release suggesting toxicity at this top dose.

On the other hand, andalusol did not affect superoxide generation by activated leukocytes.

Effects of andalusol on histamine release from rat peritoneal mast cells

The effects of andalusol on histamine release from mast cells induced by compound 48/80 or calcium ionophore A23187 are illustrated in Figure 3. At the highest concentration tested (100 μM), andalusol significantly inhibited histamine release induced by both stimulants (24.7% of inhibition with compound 48/80 and 30.7% with

Table III. Interaction of andalusol with leukocytes functions *in vitro*.

	LDH release (% of control)	β-glucuronidase release (% of control)	Superoxide generation nmol/2.5 x10^6 cells x10 min
Control	100.0 ± 7.6	100.0 ± 9.5	24.2 ± 0.4
Andalusol 1 μM	n.t.	109.3 ± 13.1	25.1 ± 0.4
Andalusol 10 μM	92.4 ± 8.6	105.8 ± 14.4	24.9 ± 0.6
Andalusol 100 μM	96.7 ± 9.8	60.0 ± 9.3*	24.8 ± 0.8
SOD (37.5 U/ml)			0.0 ± 0.0**

Values are expressed as means ± S. E. M. for 3 tests. *p<0.05, **p<0.01 with respect to the control group (Student's *t*-test). n.t., not tested. SOD (superoxide dismutase).

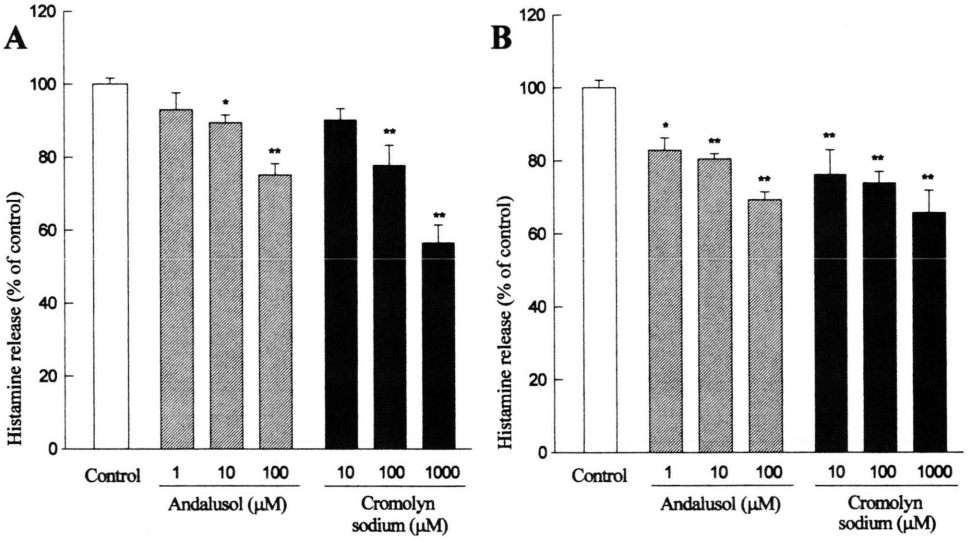

Fig. 3. Effects of andalusol and cromolyn sodium on histamine release in rat peritoneal mast cells stimulated by compound 48/80 (panel A) and calcium ionophore A23187 (panel B). Controls (100%) for histamine release were 68.7 ± 1.1% for compound 48/80 and 69.8 ± 1.5% for calcium ionophore A23187. *p<0.05, **p<0.01 vs control (Student's *t*-test).

calcium ionophore A23187). This inhibition was dose-dependent and was observed also at 1 and 10 μM. The reference drug, cromolyn sodium, inhibited dose-dependently histamine release.

Discussion

The present study has demonstrated that andalusol, a diterpenoid obtained from the acetone extract of *Sideritis foetens*, was able to inhibit experimental acute inflammation after oral or topical administration. Andalusol had already been isolated before from this plant (García-Alvarez and Rodríguez, 1980), but to our knowledge this is the first report of the pharmacological activity of this compound.

Andalusol showed significant anti-inflammatory activity on carrageenan-induced mouse paw oedema at the dose of 60 mg/kg p.o. comparable to indomethacin (10 mg/kg p.o.), used as reference drug. When andalusol was administered topically, it also decreased the TPA-induced ear mouse oedema, although its activity was lower than indomethacin at the same doses.

It is known that the acute inflammatory response is characterized by the accumulation of leukocytes at extravascular sites. Inflammatory tissue destruction is at least partially due to activated leukocytes which produce oxygen radicals and release lysosomal enzymes, such as β-glucuronidase. Therefore, we wanted to determine if the anti-in-

flammatory effect of andalusol was associated with reduction in neutrophil infiltration into the TPA-treated ears, assayed biochemically by accumulation of the neutrophil specific marker MPO. Results from these experiments proved that andalusol significantly inhibited leukocyte migration into inflamed ears at the doses tested.

On the other hand, interaction of andalusol with functional properties of leukocytes was assayed. Treatment of leukocytes with andalusol did not affect the release of superoxide anion induced by TPA. Nevertheless, andalusol decreased the secretion of lysosomal β-glucuronidase. This effect may contribute to the anti-inflammatory profile of andalusol. The inhibition of β-glucuronidase release was not due to drug induced cytotoxicity, as andalusol was non-toxic to leukocytes at the concentrations tested.

Mast cell degranulation followed by the release of histamine is the first even in the carrageenan-induced oedema (Di Rosa *et al.*, 1971), so we decided to investigate if andalusol, such as other diterpenoids (Yosikawa *et al.*, 1996) was able to inhibit histamine release from mast cells. In our assays, andalusol showed inhibition of this response when mast cells were stimulated by calcium ionophore A23187 or compound 48/80.

We can conclude that the observed anti-inflammatory activity of the acetone extract of *Sideritis foetens* might attributed in part to andalusol, although other active principles present in the extract could also be involved. Studies on interaction of andalusol with other inflammatory mediators are in progress.

Ackowledgements

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