Study of the Topical Anti-Inflammatory Activity of *Achillea ageratum* on Chronic and Acute Inflammation Models

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We have produced a chloroform extract from *Achillea* which includes stigmasterol and sitosterol. By comparing it with the pure compounds an anti-inflammatory effect (with mouse ears) is assumed.

The topical anti-inflammatory effect of the chloroform extract from *Achillea ageratum* (Asteraceae)and of stigmasterol and β-sitosterol, isolated of this extract has been evaluated, against to 12–0-tetradecanoylphorbol acetate (TPA)-induced mouse ear edema, using simple (acute model) and multiple applications (chronic model) of the phlogistic agent. Myeloperoxydase activity also was studied in the inflamed ears.

In the acute model the extract exerted a dose-dependent effect. All the doses assayed (1, 3 and 5 mg/ear) significantly reduced the edema (50%, 66% and 82%, respectively). The isolated sterols stigmasterol and β-sitosterol (with doses of 0.5 mg/ear) had similar effect as the extract with doses of 1 and 3 mg (59% and 65% respectively).

In the chronic model the anti-inflammatory effect generally was a more moderate one. The highest dose of the extract decreased the edema reduction to 26% with the highest dose of the extract applied. With the compounds the effect decreased to 36% with stigmasterol, and 40.6% with β-sitosterol.

Myeloperoxydase activity (MPO) was reduced by the extract and the compounds in the acute model, however, in the chronic edema, the enzyme inhibition was very weak with all treatments even with the standard substance.

These results indicate that the chloroform extract of *Achillea ageratum* and some of the its components stigmasterol and β-sitosterol are more effective as topical anti-inflammatory agents in acute than in the chronic process and their action is markedly influenced by the inhibition of neutrophil migration into inflamed tissue.

Introduction

Different species of *Achillea* genus (Asteraceae), has been widely used in traditional medicine mainly for its tonic, stimulant or anti-inflammatory activity in digestive diseases (Della Logia, 1992; Goldberg, 1969; Al Hindawi, 1989; Font Quer, 1990).

The species *Achillea ageratum* is a plant of the mediterranean region, known by the popular names of "agerato", "hierba luisa", "artemisia real" or "altarreina" (Font Quer, 1990). We have previously shown the composition of the essential oils, and it the spasmolytic (Puerta and Herrera, 1995) and antimicrobial properties (Puerta *et al.*, 1996) had been studied. Besides the analgesic, antipyretic and anti-inflammatory activities of different extracts of this plant also have been recently investigated (García *et al.*, 1997).

In the present work and following with the phytochemical and pharmacological study of this species, we had isolated two terpenic compounds: β-sitosterol and stigmasterol from a chloroform extract. Different types of terpenic compounds had been reported having anti-inflammatory properties (Recio *et al.*, 1995; Akhisia *et al.*, 1996). Especially some phytosterols have been shown by to exert inhibition on edema induced by 12–0-tetradecanoylphorbol acetate (TPA) (Della Loggia, 1994). For this reason we had evaluated the topical anti-inflammatory effect of the chloroform extract and of the compounds stigmasterol and β-sitosterol on TPA-induced auricular edema in mouse. Simple and repeated applications of the phlogistic

agent were used. To determine the possible influence of the extract and of the compounds on leukocyte migration, myeoloperoxydase activity was also measured.

Material and Methods

Plant material

The aerial parts of *Achillea ageratum* were collected in the flowering period in "Prado del Rey" (Cádiz, Spain) and authenticated in the laboratory of Vegetal Biology of the Faculty of Pharmacy (Sevilla, Spain). A voucher specimen was deposited in the herbarium of this laboratory (SEVF).

Extraction and isolation

The dried plant material (500 g) was succesively extracted with chloroform in a Sohxlet apparatus. The extract obtained was concentrated under reduced pressure using a rotatory evaporator to eliminate the organic solvent, yielding a dry residue (6.3%). 2.5 g of this residue was chromatographed on a silicagel column (60 g, 0.063–0.200 mm and 0.25–0.5 mm, Merck, Darmstadt) and eluted with solvent portions of increasing polarity of a mixture of n-hexane, diethyl ether, ethyl acetate and methanol, yielding 175 fractions (15 ml). Fractions 78–115, corresponding to the n-hexane/diethyl ether (30:70 v/v) eluate, yielded a crystalline fraction (1.4 mg). (TLC silicagel developed with n-hexane/diethyl ether (50:50 v/v) gave a grey-green spot with oleum reagent, (R_F 0.24). Fractions 116–129, corresponding to the diethyl ether/ethyl acetate (80:20 v/v) eluate, yielding another crystalline fraction (1.1 mg). (TLC silicagel developed with diethyl ether/ethyl acetate (50:50 v/v) gave a grey spot with oleum reagent, R_F 0.62).

The two compounds isolated were analysed by gas liquid chromatography (GLC), gas chromatography-mass spectrometry (GC-MS), and NMR spectrum (Bruker AMX-500) and identified as stigmasterol and β-sitosterol. Analytical data are as follows:

Stigmasterol: M. E. *m/z* (%): 412 (M^+, 30), 394 (17), 300 (21), 255 (38), 133 (26), 97 (29), 83 (72), 69 (58), 55 (100), 43 (33).

^{13}C-NMR (CDCl$_3$ 500 Hz): δ 140.8 (C-5), 138.4 (C-22), 129.3 (C-23), 121.7 (C-6), 70.8 (C-3), 42.3

(C-13), 42.0 (C-4), 36.8 (C-1), 36.6 (C-10), 31.8 (C-2).

^{1}H-NMR (CDCl$_3$, 300 Hz): δ 0.687 (s, 18-H$_3$), 0.842–0.746 (d, 26-H$_3$, 27-H$_3$, 29-H$_3$), 1.001 (s, 19-H$_3$), 1.017 (d, 21-H$_3$), 4.95–5.20 (m, 22-H , 23-H), 5.35 (d, *J* = 5.18, 6-H).

β-sitosterol: M. E. *m/z* (%): 414 (M+, 15), 396 (17), 318 (20), 329 (22), 303 (41), 213 (45), 107 (85), 81 (100), 67 (86).

^{13}C-NMR (CDCl$_3$, 500 MHz): δ 140.7 (C-5), 121.7 (C-6), 71.7 (C-3), 36.1 (C-4), 33.9 (C-1), 33.7 (C-2), 31.6–24.3 (C-7, C-11, C-12, C-15, C-16, C-22, C-23, C-28).

^{1}H-NMR (CD CI$_3$, 300 MHz): δ 0.676 (s, 18-H$_3$), 0.829 (d, *J* 6.8 Hz, 26-H$_3$), 0.912 (d, *J* = 6.5 Hz; 21-H$_3$), 1.013 (s, 19-H$_3$), 5.38 (m, 6-H).

The GC was recorder on Chrompack-CD 9000 using helium as carrier gas.

GC-MS was performed on a Carlo Erba gas chromatograph linked to a Kratos MS 80 mass spectrometer equipped with a NBSLIB 2 data system, using cross-linked 5% phenylmethyl silicone (OV-5, 25m × 0.25 mm × 0.23 μm). The compounds were identified by spectral data and comparison with literature data on the composition of different vegetable oils (Itoh *et al.*, 1981; Kornfeldt, 1981; Lercker *et al.*, 1981).

Animals

Groups of six female Swiss mice weighing 25–30 g each (from Animal Service of Seville University) were used in the acute and chronic inflammation tests. All animals were maintained in suitable nutritional and environmental conditions throughout the experiments.

12–0-Tetradecanoylphorbol acetate (TPA)-induced mouse ear edema

On the right ear was induced an edema by topical application of 2.5 μg/ear of TPA in acetone. The compounds stigmasterol and β-sitosterol (0.5 mg/ear) and the chloroform extract (1, 3 and 5 mg/ear), dissolved in the vehicle were applied topically, simultaneously with TPA. Indomethacin (SIGMA, St. Louis) (0.5 mg/ear) was used as standard drug (De Young *et al.*, 1989).

Mouse ear edema induced by multiple topical application of TPA (chronic model)

The method of Stanley *et al.* (1991) was followed. 10 µl of TPA (2.5 µg/ear) were applied topically to both surfaces of each ear on alternate days (0, 2, 4, 7 and 9 at 10 hours). The extract (5 mg) and stigmasterol and β-sitosterol (0.5 mg/ear) dissolved in acetone were applied topically twice daily for four days immediately after TPA application and once at 10.00 h of day 10. Dexamethasone (0.05 mg/ear) was used as reference drug.

Myeloperoxydase assay

Myeloperoxydase activity was determined using a modified method of Suzuki *et al.* (1983). The tissue (ear punch) was homogenized for 45 s at 0 °C, by means of a Polytron PT 1200, in sodium phosphate buffer (pH 5.4, 80 mM; 0.75 ml) containing hexadecyltrimethylammonium bromide (0.5%). The homogenate was centrifuged at $1200 \times g$ and 4 °C for 15 min. For the assays supernatant (200 µl) was mixed with sodium phosphate buffer (pH 5.4, 80 mM) hexa decyltrimethylammonium bromide (0.5% w/v) and tetramethylbenzidine (1.6 mM added as 18.6 mM stock solution dissolved in N-N'-dimethylformamide). The mixture was then heated to 37 °C and the reaction started by addition of H_2O_2 (0.026%). Each tube containing the complete reaction mixture was incubated for exactly 3 min at 37 °C an the reaction was then terminated by addition of 1.46 M sodium acetate (pH 3.0) and placed on ice. The absorbance of each tube was determined at 620 nm and corrected by subtracting the blank value. We define 1 unit of activity as the difference of absorbance min^{-1}, between the untreated control group and the treated groups, in a final reaction volume of 3 ml. The absorbance for the control-untreated group (which received only TPA) was the 100% activity. Percentage inhibition was calculated by comparison of results from drug-treated and untreated control groups.

Statistical analysis

Values are given as arithmetic means ± S. E. M. The significance of differences between means was evaluated by Student's t-test for unpaired data ($n = 6$). Each experiment was tested in triplicate.

Results and Discussion

Sterols stigmasterol (1.4 mg) and β-sitosterol (1.1 mg) were isolated and identified from the chloroform extract of the aerial parts of *Achillea ageratum,* by chromatographic procedure, spectral and bibliography data as described the Materials and Methods section.

The extract and the compounds were active against acute inflammation in mice induced by TPA, producing significant reductions in edema (see Table I). The highest doses of the extract exerted an edema inhibition similar to the reference drug, indomethacin. This result indicates that the anti-inflammatory property of this extract could be attributed, to some extent, to the isolated sterols, but probably they are not the only active principles responsible and other bioactive components may be involved, or perhaps both compounds may operate in a synergistic manner.

With respect to the pharmacological modulation of inflammation induced by repeated applications of TPA (Table I) the extract had little effect on ear weight (26% of reduction) while the compounds

Table I. Effect of *Achillea ageratum* on TPA-induced edema in the acute and chronic models.

Groups	Dose (mg/ear)	Acute edema weight (mg)	Acute edema inhibition (%)	Chronic edema weight (mg)	Chronic edema inhibition (%)
Control	0	17.60±0.56	0	22.50±1.95	0
Chloroform Ext.	1	8.70±0.18**	50.6	n.t.	n.t.
Chloroform Ext.	3	5.91±0.56***	66.4	n.t.	n.t.
Chloroform Ext.	5	3.08±0.57***	82.5	16.58±0.98***	26.3
Stigmasterol	0.5	7.22±0.97***	59.0	13.37±0.90***	36.4
β-Sitosterol	0.5	6.18±0.37***	64.9	14.30±0.66***	40.6
Indomethacin	0.5	3.12±0.48***	82.3	n.t.	n.t.
Dexamethasone	0.05	n.t.	n.t.	9.75±0.86***	56.7

*** $p < 0.001$ ** $p < 0.01$ n.t. non tested

Table II. Myeloperoxydase activity (MPO) of the extract and of the compounds isolated from *A. ageratum* in the inflamed ears with simple and multiple applications of TPA (acute and chronic models).

Groups	Dose (mg/ear)	Acute model: Absorbance value	Acute model: MPO (units)	Acute model: MPO (Inhibition %)	Chronic model: Absorbance value	Chronic model: MPO(munit)	Chronic model: MPO (Inhibition%)
Control	0	0.869±0.06	96	0	0.750±0.1	83	0
Chloroform Ext.	1	0.268±0.04***	29	63.3	n.t.	n.t.	n.t.
Chloroform Ext.	3	0.224±0.02***	24	69.3	n.t.	n.t.	n.t.
Chloroform Ext.	5	0.108±0.02***	12	85.2	0.595±0.18	66	20.7
Stigmasterol	0.5	0.228±0.01***	25	73.8	0.520±0.09	57	30.7
β-Sitosterol	0.5	0.136±0.03***	15	83.3	0.480±0.03*	53	34.9
Indomethacin	0.5	0.032±0.01***	35	99.4	n.t.	n.t.	n.t.
Dexamethasone	0.05	n.t.	n.t.	n.t.	0.463±0.11**	51	38.3

*** p<0.001   * p<0.1 n.t. non tested.

had an acceptable anti-inflammatory effect (36% stigmasterol and 40.6% β-sitosterol) although less than in the first model.

The MPO activity was strongly reduced by the extract and the compounds in the acute inflammation model (Table II). This result shows that they have strong effect on cellular migration, mainly due to polymorphonuclear leukocytes. However in the chronic model the enzyme inhibition was more slight with all the treatments assayed (Table II).

Such differences between acute and chronic anti-inflamatory properties must be the result of differences in the ability of the extract and of the compounds to counteract the diverse cellular and biochemical events that characterize the inflammatory process. The extract and the compounds, stigmasterol and β-sitosterol seem to be active against the primary vascular response mediated by amines and prostaglandins. They are less active in antagonizing the chronic phase in which the secretion of hydrolytic enzymes and the activation of mononuclear phagocytic cells lead tissue degeneration. In this phase TPA increases ornithine decarboxylase and 8-lipoxygenase activities and induces the transcription of the IL -1β gene (Alcaraz and Ríos, 1991).

In summary, these results show that *A. ageratum* and it components stigmasterol and β-sitosterol are an effective topic anti-inflammatory agents mainly in acute inflammations; their effect on leukocyte migration to the inflamed site might be an important aspect of the mechanism of their action.

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