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ANTI-INFLAMMATORY COMPOUNDS FROM *SIDERITIS JAVALAMBRENSIS* *N*-HEXANE EXTRACT

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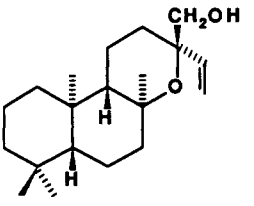
ABSTRACT.—The anti-inflammatory activities of the *n*-hexane extract of *Sideritis javalambrensis* and several purified fractions were investigated using the carrageenan mouse paw edema test. Progressive fractionation led to the isolation of the active principles *ent*-16-hydroxy-13-*epi*-manoyl oxide [**1**] and esters of tyrosol with palmitic, stearic, behenic, and lignoceric acids.

The aerial parts of plants belonging to the genus *Sideritis* (Lamiaceae) have been used in Spain for a long time to treat inflammatory conditions. To date the active principles identified include the diterpenoid borjatriol from *Sideritis mugronensis* Borja (1) and the flavonoids hypolaetin-8-*O*-glucoside from the same species (2) and sideritoflavone from *Sideritis leucantha* Cav. (3). These compounds, unlike the classical nonsteroidal anti-inflammatory agents, appear to have mechanisms of action independent of cyclo-oxygenase inhibition as they fail to decrease this enzyme activity in several in vitro tests while hypolaetin-8-*O*-glucoside and sideritoflavone inhibit lipoxygenases from different sources (4–7). In previous work the anti-inflammatory activity of the hexane extract of *Sideritis javalambrensis* Pau was demonstrated in rats using the rat paw carrageenan edema and the adjuvant-carrageenan-induced inflammation tests (8). In continuation of our studies in this field, the present work was intended to carry out the separation of this extract searching for the anti-inflammatory activity of resulting fractions using the carrageenan mouse paw edema test (9), a method where only small amounts of sample are required.

RESULTS AND DISCUSSION

The separation of the *S. javalambrensis* *n*-hexane extract by cc yielded five fractions with the anti-inflammatory activity primarily seen in fractions II and III (200 mg/kg ip) but at percentages of inhibition slightly less than 80 mg/kg ip of phenylbutazone (50.6% and 52.7% for fractions II and III, respectively, against 60.1% for the anti-inflammatory standard, at the +3 h determination). As fractions II and III showed a similar behavior and tlc revealed the presence of common components, both fractions were combined and chromatographed on a Si gel column, giving fractions a–d, which were



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also studied using the carrageenan mouse paw edema. The inhibition exerted by such fractions at 100 mg/kg ip was significant only for fractions a and b. The active principles present in fractions a and b were isolated by cc and preparative tlc and designated isolate 1 and 2, respectively. In the inflammation test used, isolate 2 (75 mg/kg ip) appeared to exert a consistent anti-inflammatory effect throughout the experiment while the inhibition of edema shown by isolate 1 at the same dose tended to increase 3 to 7 h after carrageenan administration (Figure 1). Inhibitory effects were consistently higher for phenylbutazone (80 mg/kg ip) before the +3 h determination, when the effect of isolate 1 (60.1% of inhibition) approximated that of the standard anti-inflammatory agent (63.5% of inhibition).

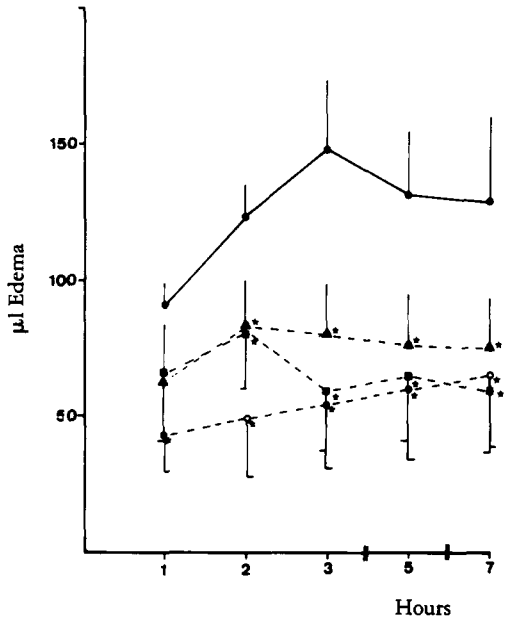

FIGURE 1. Effects of **1**, isolate 2, and phenylbutazone on carrageenan edema. Results are expressed as mean ± SD ($n = 6$). Compound **1** and isolate 2 were administered at a dose of 75 mg/kg ip, and phenylbutazone was administered at 80 mg/kg ip. $^{*}P < 0.05$. Control (●), **1** (■), isolate 2 (▲), phenylbutazone (○).

Isolate 1 was identified by its spectral data as *ent*-16-hydroxy-13-*epi*-manoyl oxide [**1**], a new *ent*-13-*epi*-manoyl oxide derivative, recently isolated by us from the same species (10). The ^{1}H-nmr spectrum of isolate 2 showed an A_2B_2 system centered at 6.87 ppm indicating the presence of four aromatic protons, two at 6.7 ppm and the others downfield at 7.0 ppm, suggesting the presence of a hydroxyl group at the para position. The resonance at 4.17 ppm referred to the benzylic methylene. The signals at 2.80 and 2.18 ppm, related to the protons of an oxygen-bearing methylene group, are most likely part of a primary ester group and the α-methylene of a fatty acid, respectively. A broad singlet at 1.26 ppm was assigned to the protons of methylene groups, and the resonance at 0.8 ppm resulted from the terminal methyl group. The ^{13}C-nmr spectrum indicated the presence of one carbonyl group (173.9 ppm), aromatic carbons (154.2–115.4 ppm), one of them resonating downfield assigned to a carbon bearing a hydroxyl function, and one oxymethylene carbon (64.9 ppm). Finally, the group of signals between 34.3 and 22.7 ppm were assigned to methylene carbons and the resonance

at 14.1 ppm to methyl groups. The ms of isolate 2 displayed a base peak at *m/z* 120 which corresponds to the alcoholic moiety of the ester. Peaks at high *m/z* values did not seem to correspond to molecular ions. Isolate 2 was subjected to saponification and derivatization to form the methyl esters. Gc-ms examination showed the presence of four fatty acids: palmitic ($[M]^+$ 270), stearic ($[M]^+$ 298), behenic ($[M]^+$ 354), and lignoceric ($[M]^+$ 382). Thus, isolate 2 is formed by esters of *p*-hydroxyphenylethanol (tyrosol) with palmitic, stearic, behenic, and lignoceric acids. Its spectral data are in good agreement with those reported for an isolate of *Sideritis ochroleuca* De Noe formed by esters of the same alcohol with palmitic, stearic, arachidic, behenic, and lignoceric acids (11), although in our gc-ms study the methyl ester of arachidic acid was not detected.

Realizing that ip administration of test materials can lead to counterirritation and false positive results for anti-inflammatory activity, a second extraction of plant material was performed following the above procedures in order to get enough of the active principles to confirm their activity using oral administration. As can be seen in Figure 2, the effect of these drugs at 125 mg/kg po was not significant 1–2 h after injection of carrageenan, but they exerted a significant inhibitory effect from 3 to 7 h after inducing edema. The inhibitory effects increased dose-dependently and at 250 mg/kg po the degree of action of both drugs was comparable to that of phenylbutazone (100 mg/kg po), with inhibitory rates at 3 h of 55.8, 50.8, and 47.7% for isolates 1 and 2 and phenylbutazone, respectively. Thus, both compounds are active by oral administration although higher doses are needed. While the inhibitory effects of these agents were weak 1–2 h after inducing carrageenan edema, they became remarkable in the second stage of this test when nonsteroid anti-inflammatory drugs are known to act strongly. Therefore, isolates 1 and 2, obtained from a *Sideritis* species endemic to Sierra Javalambre, Spain, can be considered as new natural anti-inflammatory agents.

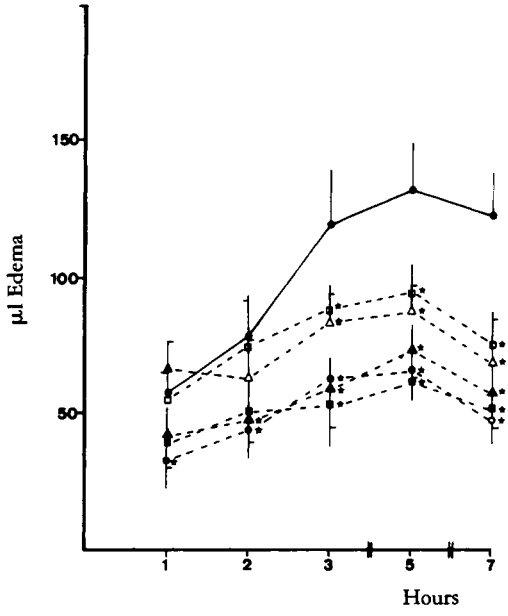

FIGURE 2. Effects of **1**, isolate 2, and phenylbutazone administered po on carrageenan edema. Results are expressed as mean ± SD ($n = 12$). $^*P < 0.05$. Control (●), **1** (125 mg/kg) (□), **1** (250 mg/kg) (■), isolate 2 (125 mg/kg) (Δ), isolate 2 (250 mg/kg) (▲), phenylbutazone (100 mg/kg) (○).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Spectra were recorded on the following apparatus: ms, VG Mass >Lab 12-250 at 75 eV. ^{1}H-nmr spectra, Bruker WP-200 (200 MHz) and ^{13}C-nmr spectra, Bruker WP-200 (50 MHz). Chemical shifts given in ppm relative to TMS ($\delta = 0$). Gc-ms analysis was carried out on a Konik-2000 chromatograph equipped with a 0.2 mm id × 20 m SE30 capillary column programmed from 100° to 250° at 5°/min, coupled to a VG12-250 (75 eV) spectrometer.

PLANT MATERIAL.—Aerial parts of *S. javalambrensis* were collected at Sierra Javalambre, Teruel, Spain, in July 1984, for the first isolation process and in July 1986, for the second one. Voucher specimens were deposited in the Herbarium of the Department of Botany, University of Valencia, Spain.

EXTRACTION AND ISOLATION.—Dried, ground plants (3.61 kg) were extracted with *n*-hexane. The extract (155 g) was chromatographed (Si gel 60 Merck, elution with CH_2Cl_2 and CH_2Cl_2/MeOH mixtures). Isolates 1 and 2 were purified by cc (Si gel 60 Merck, MeCl) followed by preparative tlc [Si gel 60 Merck, CH_2Cl_2-EtOAc (95:5)]. Isolate **1** was identified by its spectral data as *ent*-16-hydroxy-13-*epi*-manoyl oxide [**1**] (10).

ISOLATE 2.—Isolate 2 (78 mg): eims *m/z* (rel. int.) 455 (3), 425 (11), 407 (7), 398 (5), 377 (4), 368 (4), 352 (3), 254 (3), 242 (3), 120 (100); ^{1}H nmr ($CDCl_3$) 6.87 (4H, q, 8.6 Hz), 4.17 (2H, t, 7.05 Hz), 2.80 (2H, t, 6.98 Hz), 2.18 (2H, t, 7.30 Hz), 1.26 (bs), 0.8 (3H, t); ^{13}C nmr ($CDCl_3$) 173.9 (s), 154.2 (s), 130.6 (s), 115.4 (s), 64.9 (s), 34.2 (d), 29.7 (s), 29.4 (q). After saponification with alcoholic KOH, the acidic fraction was treated with CH_2N_2 and the resulting esters analyzed by gc-ms. The methyl esters of palmitic ([M]$^+$ 270), stearic ([M]$^+$ 298), behenic ([M]$^+$ 354), and lignoceric ([M]$^+$ 382) acids were identified.

MOUSE PAW EDEMA ASSAY.—Following the procedures of Sugishita *et al.* (9), groups of six male albino mice (20–25 g) received ip or po 0.5 ml of test substance or phenylbutazone (Geigy) dissolved in EtOH-Tween 80-H_2O (1:1:18), while the control group received only the vehicle. One hour after drug administration, 0.05 ml of a 3% solution/suspension of carrageenan (λ-carrageenan, Ricorvi) in saline was injected into the subplantar area of the right hind paw. Paw volumes were measured using a plethysmometer (Ugo Basile) 1, 2, 3, 5, and 7 h after carrageenan injection, and the volume of edema was expressed for each animal as the difference between the carrageenan-injected and contralateral paws. The percent inhibition of edema was calculated for each group with respect to its vehicle-treated control group. Statistical analysis of data was performed using the two-tailed Student's *t*-test for unpaired samples with the Bonferroni correction for multiple comparisons (12).

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