Antiinflammatory Activity of Coumarins from *Santolina oblongifolia*

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Four coumarins were isolated from the EtOAc extract of the flower-tops of *Santolina oblongifolia* Boiss. (Compositae). They were identified as 7-methoxycoumarin (herniarin) (**1**), 6,7-dihydroxycoumarin (aesculetin) (**2**), 6-methoxy-7-glucosidylcoumarin (scopolin) (**3**), and 6-hydroxy-7-methoxycoumarin (scopoletin) (**4**). This is the first report of the isolation of aesculetin and scopolin from the genus *Santolina*. The isolated coumarins showed marked activity as inhibitors of eicosanoid-release from ionophore-stimulated mouse peritoneal macrophages.

Members of the *Santolina* genus have been of interest due to their excellent medicinal value. Different classes of natural products have been isolated from these species, including flavonoids,[1,2] terpenoids,[3,4] coumarins,[5,6] and polyacetylenes.[7,8]

In Spanish traditional medicine, *Santolina oblongifolia* Boiss. (referred to as "manzanilla de Gredos") is used as a folk remedy against a variety of diseases such as inflammatory complaints and to achieve beneficial effects on the digestive tract. We have recently examined the antiinflammatory activity of its organic extracts against adjuvant-carrageenan-induced inflammation (ACII) in rats (unpublished data).

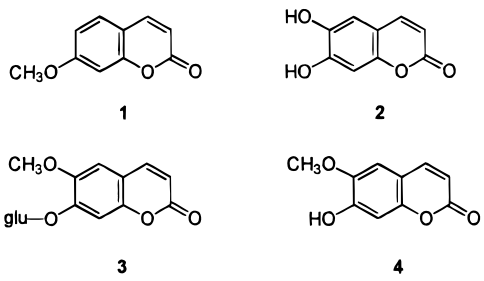
As part of a mechanism-based screening, an EtOAc extract of *S. oblongifolia* showed significant activity in ACII, and this was selected for fractionation. Bioassay-guided fractionation of the extract led to the isolation and identification of four coumarins. To investigate whether the inhibition of eicosanoid release by coumarins contributes to the antiinflammatory activity of this plant drug, we have studied their effects on PGE_2- and LTC_4-release from ionophore-stimulated mouse peritoneal macrophages.

This paper describes the isolation, structure determination, and biological activity of these compounds.

As noted above, the EtOAc extract of *S. oblongifolia* demonstrated significant antiinflammatory activity in ACII. In the acute phase of this model, the extract was capable of 30% inhibition of the edema induced by carrageenan. In the prolonged phase, the extract reduced the inflammation produced by the phlogistic agent, with an inhibition percentage higher than that of the reference drug indomethacin. At 150 mg/kg, the extract caused a 67% reduction of edema, compared to 36% reduction at 1 mg/kg in the indomethacin reference. Additionally, the mean body weight was found to be lower in the control group than in the EtOAc extract and indomethacin groups.

Chromatographic purification of the extract yielded four coumarins (compounds **1**−**4**).

The spectral characteristics of compound **1** identified this isolate as 7-methoxycoumarin (herniarin).[9] Within the Compositae, herniarin has already been isolated in such diverse genera as *Anthemis*,[10] *Artemisia*,[11] and *Santolina*, specifically in *S. oblongifolia*.[5]



The spectral characteristics of compounds **2** and **3**, when compared with the literature, were consistent with those of 6,7-dihydroxycoumarin (aesculetin),[12] and 6-methoxy-7-glucosidylcoumarin (scopolin),[13] respectively. Although within the Compositae these coumarins are not so rare, aesculetin and scopolin has never been found previously in the genus *Santolina*. Aesculetin has already been isolated from such diverse genera as *Artemisia*[14] and *Bidens*,[15] while scopolin has been described in *Artemisia*[14] and *Anthemis*.[16] The spectral data of compound **4** revealed its identity as 6-hydroxy-7-methoxycoumarin (scopoletin).[12] Within the Compositae, scopoletin has already been isolated from *Anthemis*,[10] *Artemisia*,[17] and *Baccharis*.[18] In the genus *Santolina*, scopoletin has been reported only in *S. oblongifolia* and *S. pinnata*.[2]

Pharmacological tests were undertaken in order to determine whether these coumarins were the compounds responsible for the antiinflammatory activity exhibited by the crude extract of *S. oblongifolia*.

Prostaglandins (PGs) and leukotrienes (LTs) are two classes of arachidonic-acid-derived mediators, which are involved in the initiation and maintenance of a variety of inflammatory diseases. From the cyclooxygenase products, PGE_2 is a powerful vasodilating agent and mediates pain and edema production in inflamed areas.[19] From the family of LT-type mediators, of which the 5-lipoxygenase is the key enzyme, LTC_4 is an important mediator of bronchial asthma and acute inflammation.[20]

The coumarins obtained from *S. oblongifolia* showed considerable activity as inhibitors of eicosanoid release from ionophore-stimulated mouse peritoneal macrophages. Herniarin (**1**), scopoletin (**4**), scopolin (**3**), and aesculetin (**2**) presented a dose-related response to PGE_2 release, with IC_{50} values of 84, 5, 77, and 11 μmol,

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Table 1. Inhibition of PGE_2-Release from Mouse Peritoneal Macrophages Stimulated with Calcium-Ionophore A23187 (10^{-6} mol) by Coumarins Isolated from *Santolina oblongifolia*

compounds	PGE_2 (ng/mL)[a]	% inhibition
control	11.5 ± 7.0	0.0
indomethacin (100 μM)	2.40 ± 0.4[b]	78.13
herniarin (100 μM) (**1**)	3.95 ± 0.5[c]	65.65
scopoletin (100 μM) (**4**)	2.60 ± 0.2[b]	77.39
scopolin (100 μM) (**3**)	2.80 ± 0.1[b]	75.65
aesculetin (100 μM) (**2**)	3.25 ± 0.3[b]	71.73

[a] Values determined by Student's *t*-test. [b] $p = <0.01$. [c] $p = <0.05$.

Table 2. Inhibition of LTC_4-Release from Mouse Peritoneal Macrophages Stimulated with Calcium-Ionophore A23187 (10^{-6} mol) by Coumarins Isolated from *Santolina oblongifolia*

compounds	LTC_4 (ng/mL)[a]	% inhibition
control	10.20 ± 4.2	0.0
NDGA (25 μM)	2.17 ± 0.3[b]	78.68
herniarin (100 μM) (**1**)	9.90 ± 2.3	2.94
scopoletin (100 μM) (**4**)	9.30 ± 3.6	8.80
scopolin (100 μM) (**3**)	4.59 ± 0.2[c]	55.00
aesculetin (100 μM) (**2**)	2.94 ± 1.3[b]	71.20

[a] Values determined by Student's *t*-test. [b] $p < 0.01$. [c] $p = <0.05$.

respectively. The inhibition at the highest dosage was around 70% (Table 1), as effective as the reference drug indomethacin (IC_{50} = 22 μmol). In the LTC_4-release assay, only aesculetin (**2**) showed a significant effect (IC_{50} = 18 μmol), with an inhibition percentage similar to the reference drug, nordihydroguaiaretic acid (NDGA) (IC_{50} = 6 μmol) (Table 2). Scopolin (**3**), at the highest dosage, showed an inhibition rate of 55%, while herniarin (**1**) and scopoletin (**4**) had no significant effect on LTC_4 release.

In conclusion, our results indicate that the EtOAc extract of *S. oblongifolia* contained at least four active principles able to inhibit eicosanoid release from mouse peritoneal macrophages at relatively low concentrations, contributing to the antiinflammatory activity of this plant drug. However, the presence of other putative active compounds in this plant cannot be excluded.

Experimental Section

General Experimental Procedures. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker 250 AC spectrometer (operating at 250 MHz and 62.5 MHz, respectively). Merck Si gel (70−230 mesh) and Sephadex LH-20 were used for column chromatography separation, and analytical TLC was performed on Merck Si gel 60 F_{254} plates; TLC spots/bands were located by a UV lamp and/or by spraying with "oleum," followed by heating. All the chemicals used were purchased from Sigma Chemical Co.

Plant Material. The flower-tops of *S. oblongifolia* were collected in July 1992, in Puerto del Pico, Avila, Spain. A voucher specimen (identified by Dr. Sanchez Mata) was deposited in the Botany Department Herbarium, Faculty of Pharmacy, University Complutense, Madrid, Spain.

Extraction and Fractionation of Compounds 1−4. The air-dried plant material (2.5 kg) was extracted sequentially in a Soxhlet extractor with hexane, CH_2Cl_2, EtOAc, and MeOH. The EtOAc extract (20 g) was obtained by concentration *in vacuo*, and chromatographed on a Si gel flash column (6.5 × 30 cm). Elution initially with CH_2Cl_2−EtOAc−toluene (8:1:0.5), with a gradient solvent of CH_2Cl_2−EtOAc (8:3 → 2:8), and finally with MeOH afforded six fractions (A−F). Fraction B (780 mg, in the eluate containing 1% EtOAc and 0.5% toluene in CH_2Cl_2), fraction E (2.62 g, in 50% EtOAc in CH_2Cl_2), and fraction F (3.6 g, in 75% EtOAc in CH_2Cl_2), were purified on a Sephadex LH-20 column (3 × 20 cm) using MeOH as eluent. Similar fractions, checked by TLC, were combined to create 10 fractions (B_1−B_3; E_1−E_3; F_1−F_4). Fractions B_3, E_3, and F_3 were found to contain a single compound, yielding herniarin (**1**), aesculetin (**2**), and scopolin (**3**). Fraction C (1.55 g, in the eluate containing 1% EtOAc and 0.5% toluene in CH_2Cl_2) was separated by MPLC (Labomatic AC MD 80/100). The fraction was chromatographed on a Si gel column (5.2 × 60 cm) (Labochrom PGC FA3), and eluted with dioxane−hexane (45:55) (flow rate: 2 mL/min), yielding three fractions (C_1−C_3). Fraction C_2 allowed the spontaneous crystallization of compound **4**.

Compound 1: identified as herniarin; ^{1}H-NMR and ^{13}C-NMR data agreed with the literature.[9]

Compound 2: identified as aesculetin; ^{1}H-NMR and ^{13}C-NMR data agreed with the literature.[12]

Compound 3: identified as scopolin; ^{1}H-NMR and ^{13}C-NMR data agreed with the literature.[13]

Compound 4: identified as scopoletin; ^{1}H-NMR and ^{13}C-NMR data agreed with the literature.[12]

Adjuvant-Carrageenan-Induced Inflammation (ACII). ACII was induced in Wistar rats, according to the method described by Mizushima *et al.*[21] The EtOAc extract was dissolved in Tween 80−carboxymethylcellulose−H_2O (5.7:1:94.3, v/w/v), and administered orally at 150 and 300 mg/kg, 1 h before carrageenan injection and every day throughout the experiment. A reference group was treated with indomethacin (1 mg/kg). A control group received the vehicle only.

In Vitro Eicosanoid-Release Assay. Preparation of Test Samples. Each isolated compound was dissolved in DMSO, and assayed at concentrations ranging from 25 to 100 μg/mL. Indomethacin (50−100 μg/mL) and NDGA (12.5−25 μg/mL) were used as reference compounds (PGE_2 and LTC_4, respectively).

Cultivation of Macrophages. Macrophages from NMRI male mice were collected by peritoneal lavage with Dulbecco's phosphate buffer saline. The cells (2 × 10^6) were incubated overnight in 35-mm Petri dishes in 2 mL of Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2. Then, nonadherent cells were washed off and 2 mL of fresh DMEM (without FCS), containing test compounds, were added. Controls contained DMSO (basic level of released eicosanoid) or reference compounds. This addition of fresh DMEM was followed 1 h later by the addition of either 10 μL DMSO or DMSO containing the calcium-ionophore A23187 (10^{-6} mol).[22] Two hours later, the contents of cell dishes were collected in glass vials, and cell layers were checked for viability.

Enzyme Immunoassay (EIA). The EIA procedure for measurement of eicosanoids (PGE_2 and LTC_4) was performed essentially as described by Pradelles *et al.*[23] The plates were coated with goat anti-mouse polyclonal antibodies in 20 mL of potassium phosphate buffer 5 × 10^{-2} mol (200 μL/well). The plates were incubated overnight at 4 °C, followed by exhaustive washing to remove unbound monoclonal antibodies. Monoclonal

antibody, eicosanoid conjugated with acetylcholinesterase (eicosanoid tracer), and test samples were incubated in the plate for 18 h at 4 °C. The plate was washed to remove any unbound reagents, and Ellman's reagent (which contains the substrate to acetylcholinesterase) was added. The product of this enzymatic reaction had a distinct yellow color, which was determined spectrophotometrically at 412 nm. The results of the macrophage assays comprised mean ± SD for groups of five culture dishes. Significance values were calculated using Student's *t*-test.

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