Isolation of Two Flavonoids from *Tanacetum microphyllum* as PMA-Induced Ear Edema Inhibitors

Jacqueline Martinez, Ana Maria Silván, Maria José Abad,* Paulina Bermejo, and Angel Villar

Department of Pharmacology, Faculty of Pharmacy, University Complutense, 28040 Madrid, Spain

Monica Söllhuber

Department of Organic Chemistry, Faculty of Pharmacy, University Complutense, 28040 Madrid, Spain

Received December 13, 1995[⊗]

The CH_2Cl_2 extract of *Tanacetum microphyllum* exhibited antiinflammatory activity on PMA-mouse ear model. Two antiinflammatory flavonoids, 5,7-dihydroxy-3,6,4′-trimethoxyflavone (santin) (**1**) and 5,7-dihydroxy-3,4′-dimethoxyflavone (ermanin) (**2**), were isolated.

In view of the well-known side effects of classical cyclooxygenase inhibitors and glucocorticoids, antiphlogistic agents having other mechanisms of action are being intensively investigated. One of the possible new ways of antiinflammatory action is the inhibition of protein kinase C (PKC). Activation of PKC leads to a number of intracellular signal transduction pathways implicated in the pathogenesis of inflammation, including phospholipase A_2-dependent arachidonic acid release, eicosanoid production, and reactive oxygen metabolite formation.[1]

Recent studies demonstrate that the topical administration of phorbol myristate acetate (PMA) to mouse ears induces an inflammatory response resulting from PKC activation.[2] The effects of topical administration of selected antiinflammatory drugs and a variety of structurally related PKC inhibitors, have been documented using this model.[3−6] Despite the fact that most research in developing antiinflammatory agents has been directed along these lines, recent trends include the search for compounds in sources that have, for various reasons, been explored considerably less, including higher plants.[7,8] Ethnomedicine provides a source of information about these plants, which is of great value in identifying possible pharmacologically active substances.

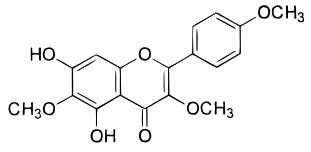
In searching for natural products as potential antiinflammatory agents, investigations have been conducted on *Tanacetum microphyllum* DC. (Compositae), an endemic species of the Iberian Peninsula, widely used in traditional medicine. Previously, pharmacological activity had been reported for extracts of this plant,[9] and the identification of three compounds with antiinflammatory activity in vivo and in vitro: hydroxyachillin, a sesquiterpene lactone of the guaianolide type,[10] and two flavonoids, centaureidin and 5,3′-dihydroxy-4′-methoxy-7-carbomethoxyflavonol.[11,12]

As part of a mechanism-based screening for novel inhibitors of PKC from a variety of natural sources, the CH_2Cl_2 extract of *T. microphyllum* was investigated in the PMA-mouse ear model and was selected for fractionation. The subsequent fractionation of the extract, with parallel pharmacological studies, led to the isolation and identification of two new active principles of *T. microphyllum*. Their characterization and biological activity are reported in this paper.

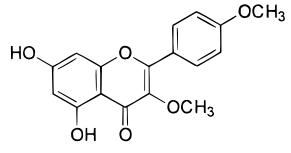
The CH_2Cl_2 extract of *T. microphyllum* has been shown to demonstrate significant antiedema activity on carrageenan-induced paw edema in rats and mice.[9,11] This extract (and all subsequent fractions) was tested using the PMA-induced ear model in mice, to evaluate its antiinflammatory properties. The effects on swelling and other inflammatory parameters are described here.

Screening of CH_2Cl_2 extract on a PMA-induced edema in mice gave positive results. The extract significantly inhibited PMA-induced mouse ear edema in a dose-dependent manner. At the highest dosage (3 mg/ear) it was more potent than the reference agent indomethacin, even 5 h after administration of PMA (Table 1). All fractions of the extract also reduced swelling more potently than indomethacin, fractions A, B, F, and G (3 mg/ear) being the major active fractions with inhibition around 80%. The vascular permeability response to PMA application was considerably reduced by the test materials (data not shown). The mean inhibition of PMA-induced permeability was 68% with CH_2Cl_2 extract at a dosage of 3 mg/ear, and around 40% with indomethacin at the same dose.

Bioassay-guided fractionation of the crude CH_2Cl_2 extract, using medium-pressure liquid chromatography (MPLC) and flash chromatography, yielded compounds **1** and **2**.



1
Santin



2
Ermanin

The spectral data identified compound **1** as 5,7-dihydroxy-3,6,4′-trimethoxyflavone (santin)[13,14] and compound **2** as 5,7-dihydroxy-3,4′-dimethoxyflavone (ermanin).[15,16]

* To whom correspondence should be addressed. Phone: (91) 394 18 71. FAX: (91) 394 17 64.

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1997.

S0163-3864(96)00163-2 CCC: $14.00

Table 1. Inhibitory Effects of *Tanacetum microphyllum* CH_2Cl_2 Extract and Flavonoids **1** and **2** on PMA-Induced Mouse Ear Edema[a]

group[b]	dosage (mg/ear)	inhibition ear edema (%) 1 h	3 h	5 h
indomethacin	3	47.3 ± 7.14[c]	38.6 ± 4.21[c]	51.2 ± 10.1[c]
CH_2Cl_2 extract	1.5	30.9 ± 4.21[c]	47.3 ± 5.16[c]	42.4 ± 5.27[c]
CH_2Cl_2 extract	3	57.3 ± 2.23[c]	71.5 ± 1.66[c]	76.0 ± 2.08[c]
compound **1**	3	80.5 ± 0.60[c]	76.3 ± 5.83[c]	81.1 ± 8.06[c]
compound **2**	3	95.1 ± 2.44[c]	71.1 ± 4.78[c]	78.3 ± 3.06[c]

[a] Female Swiss mice (six per group) were used. Data are presented as mean ± S.E.M. [b] Drug was administered 1 h prior to PMA. [c] Student's *t*-test $p \leq 0.01$.

Although within the Compositae these flavonoids are not so rare, ermanin has not been found previously in the genus *Tanacetum*. Santin has already been isolated from diverse genera such as *Achillea*,[17] *Pluchea*,[18] and *Neurolaena*,[19] while ermanin has been described in *Baccharis*[20] and *Ericameria*.[15] In the genus *Tanacetum*, santin has been reported only in *T. microphyllum*, and in *T. parthenium*.[21]

The pharmacological test was utilized in order to check whether these flavonoids were the compounds responsible for the biological activity exhibited by the crude extract. In the present paper, santin and ermanin were demonstrated to prevent significantly the development of PMA-induced ear edema in mice, being more active than indomethacin (Table 1). Maximum inhibition was observed 1 h after the administration of PMA, although the effect of both compounds continued at +5 h. These findings were supported by vascular permeability analysis, which indicated a reduction of PMA-induced permeability of 82% with santin and 92% with ermanin.

In conclusion, santin (**1**) and ermanin (**2**) were isolated as two of the biologically active principles from *T. microphyllum* using PMA-induced ear edema in mice.

Experimental Section

General Experimental Procedures. The UV spectra were determined on a UV−vis Beckmann DU-40 spectrophotometer in MeOH and usual reagents[22,23]: NaOMe, $AlCl_3$, HCl, NaOAc, and H_3BO_3. The ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker 250 AC spectrometer. Merck Si gel (70−230 mesh) and Sephadex LH-20 were used for column chromatographic separation, and analytical TLC was done 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 aerial parts of *T. microphyllum* were collected in September 1993, near Móstoles (Madrid). A voucher specimen (identified by Dr. Sanchez Mata) was deposited in the Botany Department Herbarium at the Faculty of Pharmacy, University Complutense, Madrid, Spain.

PMA-Induced Ear Edema. Ear edema was induced in female Swiss mice (8 weeks old), as previously described by Carlson et al.[24] The mice were divided into groups of six and had access to food and water *ad libitum*. Each mouse received 2 μg/ear of PMA (at concentrations of 100 μg/mL in Me_2CO) on the right ear. The phlogistic agent was applied by an automatic pipette in 10 μL volumes to both the inner and outer surfaces of the ear. The left ear (control) received Me_2CO or vehicle. Test compounds, CH_2Cl_2 extract (1.5 and 3 mg/ear), fractions (3 mg/ear), and isolated compounds (3 mg/ear) were applied topically 1 h before treatment in Me_2CO, and in some cases, 95% EtOH was used to solubilize the drug prior to dilution with Me_2CO. Indomethacin (3 mg/ear) was used as a reference drug. Ear edema was measured at various time points after PMA administration with a micrometer and was calculated by subtracting the thickness of the left ear (vehicle control) from right ear (treated ear). Data obtained are expressed as mean ± S.E.M. Unpaired Student's *t*-test was used to determine statistical significance.

In order to determine the plasma extravasation, PMA-treated mice were injected via tail vein with Evans' blue dye (1% in phosphate buffer, pH 7.2), immediately before PMA administration. Four hours later, ear punch samples were removed, weighed, and placed in formamide for 24 h at 60 °C to extract dye content. Evans' blue dye was quantified in the formide phase spectrophotometrically at 620 nm.

Extraction and Fractionation of Compounds 1 and 2. The chopped non-woody aerial parts (2 kg) of *T. microphyllum* were dried at room temperature and extracted sequentially in a Soxhlet extractor with hexane, CH_2Cl_2, EtOAc, and MeOH. The CH_2Cl_2 extract (100 g) was obtained by concentration in a vacuum and separated by MPLC (Labomatic AG MD 80/100). The extract was chromatographed on a 74-mm i.d. column (Labochrom PGC FA6) and eluted with a gradient of increasing amounts of EtOAc in CH_2Cl_2 to 100% EtOAc, which was followed by a gradient of increasing amounts of MeOH in EtOAC to 100% of MeOH (flow rate: 20 mL/min). Similar fractions, checked by TLC, were combined to create seven fractions (A−G). The active fraction B (3.5 g) was chromatographed by MPLC, on a 50-mm i.d. column (Labochrom PGC FA3). Elution with toluene−EtOAc gradient solvent system, starting with 100% toluene with increasing amounts of EtOAc to 50% (flow rate: 2 mL/min), afforded nine fractions (B_1−B_9). Fraction B_3 (52.4 mg) (in the eluate containing 5% EtOAc in toluene) was chromatographed on a 14-mm i.d. column chromatograph (Sephadex LH-20), using MeOH as eluent. Finally, the fraction was purified on a 14-mm i.d. flash column eluted with $CHCl_3$−MeOH (99:1) and afforded compounds **1** (13 mg) and **2** (5.5 mg).

Compound 1. This compound was identified as santin. The UV data agreed with the literature.[13,14]

Compound 2. This compound was identified as ermanin. The UV data agreed with the literature[15,16].

Acknowledgment. This work was supported in part by a Scientific Research Grant from the Madrid Autonomous Community. The technical assistance of Ms. Brooke-Turner is gratefully acknowledged.

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NP960163U