

Topical Anti-Inflammatory Lignans from *Haplophyllum hispanicum*

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The present paper reports the results of the methanol extract of *Haplophyllum hispanicum* Spach on single or repeated local 12-O-tetradecanoylphorbol acetate (TPA) administration and in the oxazolone-induced contact-delayed hypersensitivity mouse ear edemas. Two topical anti-inflammatory aryl naphthalide lignans were isolated from the active fractions of the methanol extract. They were identified by spectroscopic methods, including ¹³C NMR and heteronuclear multiple bond correlation (HMBC), as diphyllin acetyl apioside and tuberculin. The former was the most active on acute TPA edema with a ID₅₀ of 0.27 μmol/ear.

Introduction

As part of our study of Spanish medicinal plants active against irritative and inflammatory dermatological processes, we investigated *Haplophyllum hispanicum* Spach, a perennial herb endemic of the mediterranean area. The alcohol extract of this plant is a reputed remedy in certain skin diseases and its usefulness is being medically ascertained. The present paper describes the effect of the methanolic extract on various models of cutaneous inflammation and reports the isolation, identification and activity of two anti-inflammatory lignans of the 1-aryl-2,3-naphthalide type.

Results and Discussion

The MeOH extract of *Haplophyllum hispanicum* was primarily tested against two experimental models of acute inflammation, TPA-induced ear and carrageenan-induced paw edemas in mice (Table I). It showed a 50% reduction of the ear edema when it was administered topically at the same dose as indomethacin (0.5 mg/ear, inhibition = 86%), whereas when given orally it did not inhibit the paw edema to a significant degree in 5 h (inhibition = 37%). In a second stage, the ex-

tract was assayed against two other inflammatory conditions, oxazolone-induced delayed hypersensitivity and the multiple-dose TPA-induced response (Table I), which share certain modes of cellular response and the same target organ (mouse ear skin), and differ in their inflammation generating mechanism. In the first case it depends on an immunological T-lymphocyte activation, whereas the second derives from a direct activation of phospholipase C through the diacylglycerol analogue TPA. The increase in ear thickness produced by oxazolone was in fact magnified (+18%) by treatment with the plant extract, indicating that some constituents may cooperate with the sensitizing agent. This is related to the phototoxicity of many rutaceous plants, for this noxious effect is a particular from of delayed hypersensitivity. On the other hand, the effect on chronic TPA-edema and epidermic proliferation was slighter because of a 39% decrease in ear thickness, and does not seem of great interest.

Following a guided bioassay of the anti-inflammatory activity on the TPA-induced acute ear edema, the active compounds were isolated by chromatographic techniques. They were identified by spectroscopic methods (¹H, ¹³C NMR and MS) as two aryl naphthalene lignans: 4-O-(5''-O-acetyl)-(β-D-apiofuranosyl)-6,7-dimethoxy-1-(3',4'-methylenedioxyphenyl)-3-hydroxymethylnaphthalene-2-carboxylic acid lactone (diphyllin acetyl apio-

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Table I. Effect of MeOH extract on the different models of inflammation in comparison with reference drugs.

Samples	% Inhibition of ear edema induced by			% Inhibition of paw edema induced by carrageenan		
	TPA (single)	TPA (repeated)	Oxazolone	1 h	3 h	5 h
MeOH extract	50.0**	33.9	-18.1	11.5	19.7	36.6
Indomethacin	85.6**	—	—	—	—	—
Dexamethasone	—	81.2**	85.3**	—	—	—
Phenylbutazone	—	—	—	20.1	63.6**	34.2

** $p < 0.01$ by Dunnet's *t*-test compared with the control group.

Single: acute edema was induced by single topical application of TPA.

Repeated: chronic inflammation was induced by multiple topical application of TPA.

side, compound **1**) and its deacetylated derivative 4-O-(β -D-apiofuranosyl)-6,7-dimethoxy-1-(3',4'-methylenedioxyphenyl)-3-hydroxymethylnaphthalene-2-carboxylic acid lactone (tuberculatin, compound **2**).

This is the first time that diphyllin acetyl apioside and tuberculatin have been identified in *H. hispanicum*. However, the former was previously described in *H. tuberculatum* (Sheriha and Abou Amer, 1984) and *H. buxbaumii* and the latter in *H. buxbaumii*.

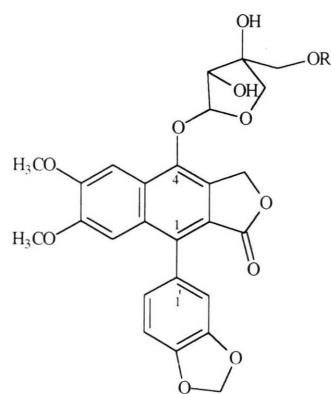
The ^1H and ^{13}C NMR spectra of compound **1** were complex because of signal spleeting, a phenomenon noted previously for other lignans of this type. Spin-spin coupling patterns between protons were obtained from a COSY-45 experiment and heteronuclear interactions by means of HC-COBI dec (direct H–C bonding) and HMBC (long-range C–H coupling). The data obtained were in close agreement with that published for other diphyllin glycosides (Al-Abed *et al.*, 1990). The ^{13}C

NMR assignments for **1** are published here for the first time.

The ^1H and ^{13}C NMR spectra of compound **2** were similar to those of **1** with the exception of the singlet (δ 2.17) assignable to the acetyl group and the resonance of the carbonyl group (δ 171.91) which allowed the identification of compound **2** as tuberculatin.

Regarding the pharmacological activity (Table II), the 50% inhibitory dose of diphyllin acetyl apioside and tuberculatin for acute TPA inflammation were 0.27 and 1.23 $\mu\text{mol/ear}$, respectively. By comparison with indomethacin, the former was a more potent inhibitor. The different anti-inflammatory activity between the two lignans could be explained on the basis of the lipophilicity of their structures since the acetyl group increases the apolar character favoring its cutaneous absorption.

The activity of lignans against inflammation induced by TPA has also been demonstrated by gomisin A, gomisin J and wuweizisu C, belonging to the dibenzocyclooctadiene type. These showed strong inhibitory effect in the mouse ear test, particularly gomisin A (Yasukawa *et al.*, 1992). As far as we can ascertain from the literature, no other



Diphyllin acetyl apioside R = Ac
Diphyllin apioside R = H

Table II. Inhibitory effect of diphyllin acetyl apioside, tuberculatin and MeOH extract of *Haplophyllum hispanicum* on TPA-induced inflammation in mice.

Sample	ID ₅₀ $\mu\text{mol/ear}$	I.R.
MeOH extract	—	50**
Diphyllin acetyl apioside	0.27	81**
Tuberculatin	1.23	67** ^a
Indomethacin	0.29	86**

ID₅₀ = 50% Inhibitory Dose. I.R. = Inhibition Ratio at 0.5 mg/ear; ^a 2 mg/ear. ** $p < 0.01$ by Dunnet's *t*-test compared with the control group.

lignans appear to have been recorded as active in this test. However, some aryl naphthalene derivatives, notably justicidin E from *Justicia procumbens* L. (Acanthaceae) have shown to possess high inhibitory activity against 5-lipoxygenase (Therrien *et al.*, 1993) and, consequently, may exhibit strong anti-inflammatory activity. In fact, the activation of 5-lipoxygenase seems to be an event directly related to the action of TPA since it has been reported that TPA increases cysteinyl leukotrienes levels in mouse ear (Fürstenberger *et al.*, 1994). Additionally, it induces in isolated epidermal cells ornithine decarboxylase (ODC) activity which is reduced by lipoxygenase inhibitors (Yamamoto *et al.*, 1987), what indirectly implies leukotrienes in proliferative response to TPA.

Material and Methods

General experimental procedures

Identification was carried out by ^1H (400 MHz), ^{13}C (75 MHz) and heteronuclear multiple bond correlation (HMBC) NMR spectra (Bax and Summers, 1986) on a Bruker AMX-400 spectrometer in CDCl_3 . High resolution FAB-MS were recorded on a VG Analytica Fisons spectrometer. Compounds were visualized by 365 nm UV light and aminoethyl ester of diphenylboric acid 1% in MeOH (Neu's reagent), ammonium ceric sulphate 2.5% in 20% nitric acid or sulphuric anisaldehyde. Silica gel 60, silica gel 60G and precoated silica gel 60G F₂₅₄ plates (Merck) were used for CC, TLC and prep. TLC, respectively. HPLC-DAD analysis was performed using a Merck-Hitachi HPLC system (L-6200 pump) equipped with an L-3000 photodiode array detector (DAD) and a prepacked analytical column (12.5×0.7 mm) of Lichrospher RP-18 (5 μm). The following conditions were used: eluents: H_2O + TFA 0.05% (A), MeOH + TFA 0.05% (B). Elution profile: 0–30 min, 30% A. Flow rate was 1 ml/min, column pressure 60–80 bar, and the UV detector was set at 255 nm.

Plant material

Aerial parts of *Hapophyllum hispanicum* (Rutaceae) were collected in June, 1993, at El Vedat de Torrent (Valencia, Spain). A voucher specimen

was deposited at the Herbarium of the Faculty of Pharmacy of Valencia.

Extraction and isolation

Fresh aerial portions of flowering *H. hispanicum* (172 g) were cut and macerated with methanol for 24 h at room temperature. This process was repeated three times and the extracts combined. Solvent was removed under reduced pressure and the MeOH extract (15 g) was subjected to gel-filtration over Sephadex LH-20, eluting with MeOH to yield seven fractions (I–VII). After bioassay on the TPA experimental model of acute inflammation, fraction IV was rechromatographed on a silica gel column with CH_2Cl_2 EtOAc and EtOAc–MeOH mixtures, and finally pure MeOH yielding twelve fractions (IV_{1-12}). Further purification of the fraction eluted with CH_2Cl_2 –EtOAc (1:1) (fr. IV_8) was performed by twice developed preparative TLC on silica gel with CH_2Cl_2 –MeOH (9:1) to yield compound **1** (50.9 mg). Fraction eluted with EtOAc (fr. IV_9) gave compound **2** (28.6 mg) after fractionating over a silica gel column eluting with CH_2Cl_2 and CH_2Cl_2 –MeOH mixtures. The purity of both compounds was confirmed by TLC and HPLC-DAD and were identified by spectroscopic methods.

Diphyllin acetyl apioside (compound **1**): $\text{C}_{28}\text{H}_{24}\text{O}_{12}$. – ^{13}C NMR (CDCl_3): δ 136.22 (C-1), 119.34 (C-2), 129.05/129.01 (C-3), 144.87 (C-4), 100.64 (C-5), 152.14 (C-6), 150.50 (C-7), 106.50 (C-8), 130.89 (C-9), 126.97 (C-10), 67.42 (C-3a), 170.34 (C-2a), 128.38 (C-1'), 110.82/110.92 (C-2'), 147.73* (C-3'), 147.71* (C-4'), 108.38 (C-5'), 123.75/123.82 (C-6'), 56.30 (6-OCH₃), 56.05 (7-OCH₃), 101.46 (–OCH₂O–), 111.22 (C-1''), 78.56 (C-2''), 77.92 (C-3''), 74.91 (C-4''), 66.69 (C-5''), 21.00 (CO–CH₃), 171.91 (CO–CH₃). – ^1H NMR (CDCl_3): δ 7.57 (1 H, s, C-5 H), 7.059/7.054 (1 H, s, C-8 H), 6.950/6.940 (1 H, d, J = 8 Hz, C-5' H), 6.810/6.770 (1 H, d, J = 2 Hz, C-2' H), 6.76 (1 H, dd, J = 8.0, 2.0 Hz, C-6' H), 6.08, 6.05 (2 H, 2×s, O–CH₂–O), 5.49 (2 H, ABq, J = 17.0 Hz, C-3a H), 5.49 (1 H, s, H-1''), 4.06 (3 H, s, C-6–OCH₃), 3.80 (3 H, s, C-7–OCH₃), 4.43 (1 H, brs, C-2'' H), 4.38 (2 H, s, C-5'' H), 4.27 (1 H, d, J =

* Interchangeable.

10.1 Hz, C-4" H), 4.05 (1 H, *d*, *J* = 10.1 Hz, C-4" H), 2.17 (3 H, *s*, -CO-CH₃). – MS: *m/z* 554 (4%) 422 (8), 380 (100). – UV (MeOH, λ_{max}): 260 nm. – HPLC-DAD: *rt* 3.58 min.

Tuberculatol (compound **2**): C₂₆H₂₂O₁₁. – ¹H NMR (CDCl₃): δ 7.54 (1 H, *s*, H-5), 7.02 (1 H, *s*, H-8), 6.919/6.911 (H, *d*, *J* = 8 Hz, 5'-H), 6.78/6.71 (2 H, *m*, 2'-H, 6'-H), 6.07, 6.03 (2×1 H, 2×*s*, O-CH₂-O), 5.50 (1 H, *s*, H-1"), 5.48, 5.41 (2 H, ABq, *J* = 14.9 Hz, H₂-3 a), 4.51 (1 H, *d*, *J* = 2.2 Hz, H-2"), 4.19, 4.03 (2 H, ABq, *J* = 10 Hz, H₂-4"), 4.03 (3 H, *s*, 6-OCH₃), 3.91, 3.85 (2 H, ABq, *J* = 11.2 Hz, H₂-5"), 3.77 (3 H, *s*, 7-OCH₃).

Biological procedures

Animals

Groups of five female Swiss mice weighing 25–30 g were used. All animals were maintained in suitable nutritional and environmental conditions throughout the experiments.

Pharmacological test

They were performed as previously reported (Recio *et al.*, 1994).

12-O-tetradecanoylphorbol acetate (TPA)-induced mouse ear edema

An edema was induced on the right ear by topical application of 2.5 μ g/ear of TPA (Sigma) in acetone. The left ear (control) received vehicle (acetone or 70% aq. EtOH). MeOH extract, all the fractions and the isolated compounds, dissolved in 70% aq. EtOH, were applied topically (0.5 mg/ear), simultaneously with TPA. The standard drug indomethacin (Sigma, St. Louis) was administered at the same dose.

Carrageenan-induced mouse paw edema

MeOH extract was dissolved in EtOH/Tween 80/H₂O (2:2:20 v/v/v) and administered orally at 100 mg/kg (0.50 ml), 1 h before carrageenan injection. A reference group was treated with phenylbutazone (100 mg/kg, *p.o.*). A control group received the vehicle only.

Mouse ear edema induced by multiple topical applications of TPA

Description of the method procedure was reported earlier (Recio *et al.*, 1995). Chronic inflammation was induced by topical application of 10 μ l of TPA (82.5 μ g/ear) to both the inner and outer surface of each ear with a micropipette on alternate days. MeOH extract was dissolved in 70% aq. EtOH and applied topically (1 mg/ear) twice daily for four days, in the morning, immediately after TPA application, and 6 h later. Dexamethasone was used as the reference drug (0.05 mg/ear). The swelling induced was assessed in terms of the increase in the thickness of the mean of five pairs of treated ears over that of the mean of five pairs of non treated ears.

Oxazolone-induced contact-delayed hypersensitivity mouse ear edema (Young and De Young, 1989)

Female mice were sensitized by topical application the shaven ventral abdomen of 50 μ l of a 2% (w/v) solution of oxazolone (Sigma) in acetone on 2 consecutive days (days 1 and 2). Challenge was performed on day 6 by application of 30 μ l of 2% oxazolone to the right ear. Left ears served as control and were treated with acetone alone. Ear edema was calculated as the difference of thickness between right and left ears. MeOH extract and dexamethasone were applied (30 μ l) to right ears (6 h after challenge, single application) and 24, 48, 72 and 96 h after challenge (repeated dosage). Ear thickness measurements of treated and control ears were made with a Micrometer Mitutoyo Serie 239 at 6, 24, 48, 72 and 96 h after challenge and just before drug application. The final measurement was performed immediately before sacrifice.

Statistics

Percentages of edema reduction are expressed by the mean with S. E. M. Dunnet's *t*-test for unpaired data was used for statistical evaluation.

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