Topical Anti-inflammatory Activity of Flavonoids and a New Xanthone from *Santolina insularis*

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Bioactivity-guided fractionation of the methanol extract from the leaves of *Santolina insularis* led to the isolation of one new xanthone, (*E*)-3-{6-[(*E*)-3-hydroxy-3-oxo-1-propenyl]-9-oxo-9*H*-xanthen-2-yl}-2-propenoic acid, together with six known flavonoids: hispidulin, nepetin, cirsimaritin, rhamnocitrin, luteolin and luteolin 7-*O*-*β*-D-glucopyranoside. The structures were elucidated by means of 1D-, 2D-NMR spectroscopy and mass spectrometry. The topical anti-inflammatory activity of all isolated compounds and extracts was investigated employing the croton oil-induced dermatitis in mouse ear. The most active compound, luteolin, showed an ID_{50} of 0.3 μmol/cm^2 and prevented ear oedema more effectively than an equimolar dose of indomethacin within 24 h.

Key words: Santolina insularis, Anti-inflammatory Activity, Flavonoids

Introduction

Santolina species (Asteraceae, tribe Anthemideae) are widely used in traditional medicine for their anti-inflammatory properties. Various germacrane derivatives and coumarins with antiphlogistic activities have previously been isolated from plants of this genus (Silván *et al.*, 1996; Sala *et al.*, 2000). *Santolina insularis* (Genn. ex Fiori) Arrigoni is an endemic shrub growing in the mountainous area of Sardinia. The plant has been used in folk medicine as an intestinal vermifuge against horse strongyloidiasis and as a parasite repellent (Ballero and Fresu, 1991). The anti HSV-1 and anti HSV-2 activity of the essential oil and the chemical composition of the acetone extract from the aerial parts of S. *insularis* have previously been studied (Poli *et al.*, 1997; De Logu *et al.*, 2000; Valenti *et al.*, 2001; Fattorusso *et al.*, 2004) but no biological investigations on non-volatile constituents have been carried out so far. In this study we investigated the composition of the methanolic extract which showed anti-inflammatory activity by inhibiting croton oil-induced ear oedema in mice (Tubaro *et al.*, 1986) by 75%.

Material and Methods

Plant material

S. insularis was collected in Ussassai (NU), Sardinia, Italy, in May 2002. The plant material was identified by Prof. Bruno De Martis (Università di Cagliari, Dipartimento di Scienze Botaniche) and a voucher specimen (No. 0310) was deposited in the Herbarium of the Dipartimento Farmaco Chimico Tecnologico, Università di Cagliari.

Chemicals

Croton oil and indomethacin were purchased from Sigma products (Milano, Italy). Ketamine hydrochloride was purchased from Virbac S.r.l. (Milano, Italy).

Animals

Male CD-1 mice (28–32 g weight) were supplied by Harlan Italy (Udine, Italy).

General experimental procedures

UV spectra were recorded on a Cintra 5 spectrophotometer (GBC Scientific Equipment, Victoria, Australia). EI-MS spectra were taken on a

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QMD 1000 instrument at 70 eV using a direct inlet system, and CI-MS were measured on a Shimadzu LC-MS-2010. Melting points were determined on a Köfler apparatus and are uncorrected. NMR spectra were recorded at 25 °C on a Varian UNITY INOVA 400 MHz spectrometer, operating at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. Compounds were measured in DMSO-d_6 or CD_3OD. As internal standard the resonances of the residues of the undeuterated solvents were used. Column chromatography was carried out under TLC monitoring using Kiesel gel 60 (400 ÷ 440 mesh; Merck) and Sephadex LH-20 (25–100 μm; Pharmacia). For vacuum liquid chromatography (VLC) LiChroprep C-18 (Merck) was used. TLC was performed on silica gel 60 F_{254} or RP-18F_{254} (Merck).

Extraction and isolation

Air-dried and powdered leaves of *S. insularis* (1200 g) were ground and extracted with petroleum ether (7 l) by percolation at room temperature; that resulted in 56.7 g dried extract. The remaining plant material was then extracted with CH_2Cl_2 (5 l), giving 102.3 g dried extract, and MeOH (3.5 l), yielding further 200.8 g of crude extract.

A portion of the MeOH extract (12 g) was prefractionated by VLC over RP-18, using a step gradient of H_2O/MeOH/CH_3CN [H_2O, H_2O with increasing amounts of MeOH (25% each step (total volume: 500 ml)), MeOH, MeOH with increasing amounts of CH_3CN (25% each step), CH_3CN]. The collected fractions were evaporated *in vacuo* and examined by TLC. Homogeneous fractions, showing similar spots on TLC plates, were pooled to give 4 major fractions (F1–F4). The anti-inflammatory active fraction F1 (0.58 g) was fractionated on an open column of Sephadex LH-20, eluted with MeOH and resulted in luteolin 7-*O*-*β*-D-glucopyranoside (**3**) (6.9 mg), and (*E*)-3-{6-[(*E*)-3-hydroxy-3-oxo-1-propenyl]-9-oxo-9*H*-xanthen-2-yl}-2-propenoic acid (**1**) (16 mg). The bioactive fraction F4 (280 mg) was purified by open column chromatography over Sephadex LH-20 using CH_2Cl_2/CH_3OH (3:1) as eluent. Six fractions (F4.1–F4.6) were obtained, of which one was pure luteolin (**2**) (10 mg) and another pure hispidulin (**4**) (9.6 mg). Subfraction F4.3 (35 mg) was subjected to open column chromatography on silica gel using a mixture of *n*-hexane and EtOAc (1:2) as eluent to give pure cirsimaritin (**7**) (6.9 mg). Subfractions F4.6 (25 mg) and F4.5 (25 mg) were purified by open column chromatography on silica gel using a mixture of *n*-hexane and EtOAc (1:1) as eluent and yielded nepetin (**5**) (8.5 mg) and rhamnocitrin (**6**) (5.8 mg).

(E)-3-{6-[(E)-3-hydroxy-3-oxo-1-propenyl]-9-oxo-9H-xanthen-2-yl}-2-propenoic acid (**1**): Yellow amorphous solid. – M.p. 200–201 °C. – UV (MeOH): λ_{max} = 238, 260, 312, 380 nm. – ^{1}H and ^{13}C NMR: see Table I. – CI-MS: *m/z* = 337 $[M+H]^+$ (40), 317 (100), 286 (70), 268 (50).

Anti-inflammatory activity

The mice were anaesthetised by intraperitoneal injection of 145 mg/kg ketamine [2-(2-chlorophenyl)-2-(methylamino)cyclohexanone] hydrochloride. Inflammation was induced on the right ear (surface: about 1 cm^2) by the application of 80 μg of croton oil. The tested substances were dissolved together with the croton oil in 42% aqueous ethanol (methanolic extract and its fractions) or acetone/ethanol (1:1 v/v; pure compounds). After 6 h, the mice were sacrificed and a punch (6 mm ∅) was excised from both the ears. Inflammation was measured as oedema formation and was quantified by the weight difference between treated and untreated (opposite) ear samples. The anti-inflammatory activity was expressed as the percentage of inhibition of the oedema in the mice treated with the substances under study, in comparison with control mice, treated with the irritant alone. The pharmacological data were analysed by the Student's *t*-test, considering a probability level lower than 0.05 as indicative of significance. All animal experiments complied with the Italian D. L. n. 116 of January 27, 1992 and associated guidelines of the European Communities Council Directive of November 24, 1986 (86/609 ECC).

Results and Discussion

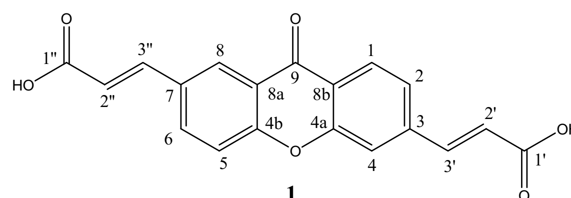
The methanolic extract of the dried leaves of *S. insularis* was fractionated by VLC, followed by purification with open column chromatography over Sephadex LH-20 or silica gel, to give one new compound, **1**, together with six known flavonoids, **2**–**7**.

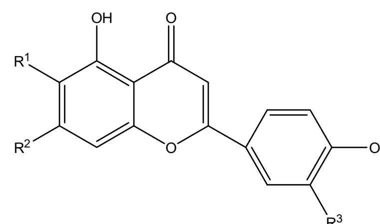
The CI-MS of compound **1** showed an ion peak at *m/z* 337 ($[M+H]^+$) corresponding to the molecular formula $C_{19}H_{12}O_6$. The UV spectrum of **1** exhibited characteristic absorption bands of a xan-

Table I. ^{1}H and ^{13}C NMR data for compound **1** (in CD_3OD)[a].

Position	δ_H[b]	δ_C[c]
1	6.87 d (8)	116.8
2	7.04 dd (2, 8)	123.2
3		128.2
4	7.14 d (2)	115.5
4a		149.8
4b		149.6
5	6.89 d (8)	116.9
6	7.02 dd (2, 8)	123.3
7		127.9
8	7.17 d (2)	115.6
8a		128.4
8b		129.0
9		181.8
1′		169.3
2′	6.39 d (15.6)	115.9
3′	7.68 d (15.6)	147.0
1″	6.50 d (15.6)	169.7
2″	7.70 d (15.6)	116.4
3″		147.1

[a] *J* values (in Hz) in parentheses.
[b] Measured at 400 MHz.
[c] Measured at 100 MHz.





	R¹	R²	R³
2	H	OH	OH
3	H	*O*-*β*-D-Glc	OH
4	OCH_3	OH	H
5	OCH_3	OH	OH
6	H	OCH_3	H
7	OCH_3	OCH_3	H

Fig. 1. Structures of the isolated compounds: (*E*)-3-{6-[(*E*)-3-hydroxy-3-oxo-1-propenyl]-9-oxo-9*H*-xanthen-2-yl}-2-propenoic acid (**1**), luteolin (**2**), luteolin 7-*O*-*β*-D-glucopyranoside (**3**), hispidulin (**4**), nepetin (**5**), rhamnocitrin (**6**), and cirsimaritin (**7**).

thone (λ_{max} 238, 260, 312, 380 nm). Besides the 13 carbon signals of the xanthon nucleus, the ^{13}C NMR spectrum (Table I) of **1** exhibited six signals indicating the presence of two propenoic acid moieties (δ 169.7, 169.3, 147.1, 147.0, 115.9, 116.4). In the ^{1}H NMR spectrum (Table I) the large coupling constant of the olefinic protons of the carboxylic chains at δ 6.39 (1H, d, J = 15.6 Hz), 6.50 (1H, d, J = 15.6 Hz), 7.68 (1H, d, J = 15.6 Hz), 7.70 (1H, d, J = 15.6 Hz), indicated a *trans* geometry. In addition, the ^{1}H NMR spectrum of compound **1** revealed two groups of each three aromatic protons in an ABX spin system. The carboxylic chains were attached to C-3 and C-7 of the xanthon nucleus judged from the correlation signals observed in the HMBC spectrum between H-3′ at δ 7.68 with C-2 (δ 123.2), C-3 (δ 128.2) and C-4 (δ 115.5), and between H-3′′ at δ 7.71 with C-6 (δ 123.3), C-7 (δ 127.9) and C-8 (δ 115.6). The structure of compound **1** was confirmed by extensive 1D and 2D experiments as (*E*)-3-{6-[(*E*)-3-hydroxy-3-oxo-1-propenyl]-9-oxo-9*H*-xanthen-2-yl}-2-propenoic acid (Fig. 1).

Table II. Effect of the tested compounds on the global oedematous response.

Compound	Dose [μmol/cm²]	N[a]	Oedema [mg][d] mean ± S. E.	Reduction (%)
Controls	–	10	6.8 ± 0.2	–
MeOH extract	1000[b]	10	1.7 ± 0.1[c]	75
1	0.3	10	2.7 ± 0.3[c]	20
2	0.3	10	2.7 ± 0.2[c]	62
3	0.3	10	4.7 ± 0.3[c]	31
4	0.3	10	3.5 ± 0.4[c]	49
5	0.3	10	3.6 ± 0.3[c]	47
6	0.3	10	4.9 ± 0.3[c]	31
7	0.3	10	4.0 ± 0.2[c]	44
Indomethacin	0.3	10	2.9 ± 0.3[c]	59

[a] Number of animals.
[b] Dose expressed in μg/cm².
[c] $p < 0.05$ at the Student's *t*-test.
[d] Weight difference between treated and untreated ear sample.

In addition to the new xanthone described above, six known flavonoids, luteolin (**2**), luteolin 7-*O*-*β*-D-glucopyranoside (**3**), hispidulin (**4**), nepetin (**5**), rhamnocitrin (**6**), and cirsimaritin (**7**), were isolated from the same extract. They were iden-

tified by spectral and chemical data and by comparison with literature data (Agrawal, 1989).

All the isolated compounds inhibited the croton oil-induced ear oedema in mice (Table II). Luteolin (**2**) (0.3 μmol/cm^2) was the most active compound and led to 62% oedema reduction after topical application while indomethacin (0.3 μmol/cm^2), which was used as reference compound, led to 59% oedema reduction. The methoxylated flavonoids hispidulin (**4**), nepetin (**5**), cirsimaritin (**7**) and rhamnocitrin (**6**) showed reduced anti-inflammatory activity compared to luteolin. Hence, methylation of one of the free hydroxyl groups results in a clear decrease of activity. Compound **1** was found to be only moderately active showing 20% oedema reduction after topical application.

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