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Anti-inflammatory Effects of South American *Tanacetum vulgare*

GUILLERMO R. SCHINELLA*, ROSA-MARÍA GINER, MARÍA DEL CARMEN RECIO, PERLA MORDUJOVICH DE BUSCHIAZZO*, JOSÉ-LUIS RÍOS AND SALVADOR MÁÑEZ

*Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Spain and *Cátedra de Farmacología, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, CIC Provincia de Buenos Aires, Argentina*

Abstract

In recent years the role of phenolic compounds and sesquiterpene lactones, particularly parthenolide, in the anti-migraine and anti-inflammatory effects of *Tanacetum parthenium* (Asteraceae) has attracted much attention. However, the closely-related cosmopolitan species *T. vulgare* has remained outside the mainstream of research in this field.

After treating the aerial parts of *T. vulgare* with dichloromethane and methanol, and applying conventional column and thin-layer chromatographic techniques, it was possible to isolate from the moderately lipophilic fractions the principles responsible for the anti-inflammatory activity of this plant against the mouse-ear oedema induced by 12-*O*-tetradecanoylphorbol 13-acetate. These were identified by ultraviolet and nuclear magnetic resonance spectroscopy as parthenolide (93% oedema inhibition at 0·5 mg/ear, ID50 (dose of drug inhibiting the oedema by 50%) = 0·18 μmol/ear) and the methoxyflavones jaceosidin (80% oedema inhibition at 0·5 mg/ear, ID50 = 0·50 μmol/ear), eupatorin, chrysoeriol and diosmetin.

Because in molar terms the potency of parthenolide was nearly three times greater than that of the most active of the flavones and because it is obtained from the plant in considerably larger amounts, the flavonoids must only be partially responsible, and to a minor extent, for the observed in-vivo anti-inflammatory local effect.

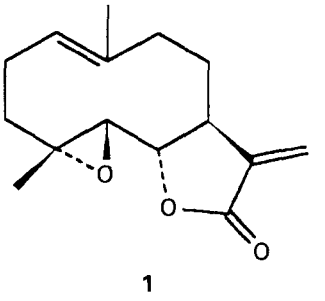
Although many species of the genus *Tanacetum* (Asteraceae), such as *T. annuum*, *T. balsamita*, *T. indicum*, *T. nubigenum*, *T. santolinoides*, *T. parthenium* and *T. vulgare*, are used therapeutically around the world (Brown et al 1996), the last two are undoubtedly the best characterized. *T. vulgare* L., tansy (*Chrysanthemum vulgare* (L.) Bernh.), is a cosmopolitan plant for which a huge number of varieties and chemotypes have been described. It furnishes the crude toxic drug Tanaceti flos, included for years in some western pharmacopoeias because of its vermifuge and emenagogue properties (Evans 1996) and its anti-inflammatory activity in acute and chronic tests (Mordujovich-Buschiazzo et al 1996). *T. parthenium* (L.) Schultz Bip., feverfew, has attracted increasing interest during recent years because of its efficacy in treating arthritis, fever, migraine and skin diseases. Its main anti-inflammatory activity has been attributed to the sesquiterpene lactone parthenolide, because of its ability to impair platelet activation (Heptinstall et al 1992), the induction of cyclooxygenase-2 expression in macrophages (Hwang et al 1996) and the activation of the nuclear transcription factor κB (Bork et al 1997). However, not only parthenolide but also other undetermined compounds present in the plant were able to interfere with the production of inflammatory mediators such as leukotriene B_4 and thromboxane B_2 from leukocytes both in rodents and in man (Sumner et al 1992). A new flavonoid called tanetin (6-hydroxykaempferol-3,7,4′-trimethyl ether), also isolated from this species, strongly reduces cyclooxygenase- and 5-lipoxygenase-mediated catabolism of arachidonic acid (Williams et al 1995).

Additional research has recently been reported for a third taxon, *T. microphyllum* DC., a species from Central Spain which contains the anti-inflammatory principles 7-hydroxyachillin, a sesquiterpene lactone, and the flavonoids ermanin,

Correspondence: S. Máñez, Av. V. A. Estellés s/n, 46100 Burjassot, Valencia, Spain.

santin, centaureidin and a 7-carboxymethyl analogue of tamarixetin (Abad et al 1993, 1995; Martínez et al 1997).

The aim of the current work was to identify the active constituents of *T. vulgare* from South America (Mordujovich-Buschiazzo et al 1996). For this purpose the plant was collected, extracted with solvent, and the most active fractions were separated.



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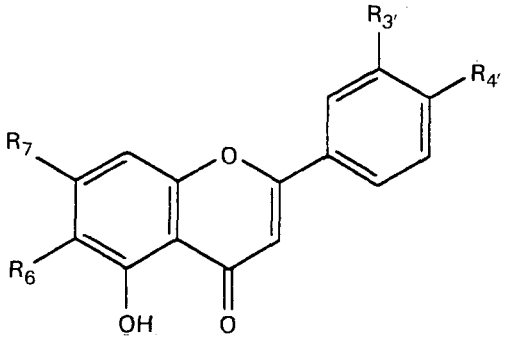

	R_6	R_7	$R_{3'}$	$R_{4'}$
2	H	OH	OCH_3	OH
3	OCH_3	OH	OCH_3	OH
4	H	OH	OH	OCH_3
5	OCH_3	OCH_3	OH	OCH_3

Figure 1. Structures of compounds **1**–**5**.

Material and Methods

Plant material

Aerial parts of *T. vulgare* cultivated as an ornamental plant in Buenos Aires province, Argentina, were collected while in flower and authenticated by Dr M. Nájera, Departmento de Botánica, Universidad Nacional de la Plata. A voucher sample (LPE no. 968) was deposited in the Museo de Botánica y Farmacognosia Carlos Spegazzini in La Plata.

Chemicals

Methanol, aluminium chloride, sodium acetate and sodium methoxide for UV spectroscopy were purchased from Merck (Darmstadt, Germany). Solvents for NMR spectroscopy, carrageenan λ, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and indomethacin were purchased from Sigma (St Louis, MO).

Isolation and identification procedures

Air-dried and powdered material was extracted with dichloromethane in a Soxhlet apparatus and then macerated with methanol. After concentration, the dichloromethane extract (7% of plant dry weight) was dissolved in warm ethanol, treated with 4% aqueous lead acetate, left standing for one night and filtered. From the filtrate, a chloroform extract (1·9% of plant dry weight) was obtained and subjected to gel-filtration on a 40cm × 3cm Sephadex LH-20 (Pharmacia) column (column A) with methanol to yield 57 × 10-mL fractions. Fractions 25–30 were combined and separated on a 40cm × 2·5cm silica gel column (column B) by eluting with 100mL $CHCl_3$ and 300mL $CHCl_3$–C_2H_5OAc (9 : 1) to give thirty 10-mL fractions. Those numbered 13–20 contained parthenolide (**1**) (21% w/w of chloroform extract). By preparative TLC on silica gel 60 F_{254} (Merck) with double-development with $CHCl_3$–CH_3OH (15 : 1) we obtained four flavonoid aglycones from fractions 47–57 from column A: chrysoeriol (**2**), jaceosidin (**3**), diosmetin (**4**) and eupatorin (**5**). Their w/w percentages of the chloroform extract were 0·3, 0·9, 0·5 and 1·3, respectively. The structures of the compounds are given in Figure 1.

Spectral data of isolated compounds

^1H and ^{13}C NMR spectra were recorded by means of a Varian 400MHz spectrometer and UV-vis spectra by means of a Perkin-Elmer Lambda 15 spectrophotometer.

Parthenolide (4,5-epoxygermacra-1(10),11(13)-dien-6β,7αH-12,8-olide; **1**). ^1H NMR (400MHz, $CDCl_3$, δ ppm, TMS = 0) 6·33 d (J = 3Hz), H-13a; 5·62 d (J = 3Hz), H-13b; 5·20 br d (J = 7·7Hz), H-1; 3·85 t (J = 4·9Hz), H-6; 2·78 d (J = 4·9Hz), H-5 ; 2·78 m, H-7; 2·40 m, H-2 and H-9′; 2·15 m, H-2′, H-3′, H-8′ and H-9; 1·73 m, H-7; 1·70 s (3 H), CH_3-14; 1·29 s (3H), CH_3-15; 1·24 m , H-3. ^{13}C NMR (100MHz, $CDCl_3$, (δppm, TMS = 0) 169·23 (C-12), 139·19 (C-11), 134·56 (C-10), 125·23 (C-1), 121·22 (C-13), 82·42 (C-6), 66·36 (C-5), 61·50 (C-4), 47·64 (C-7), 41·18 (C-9), 36·32 (C-3), 30·62 (C-8), 24·12 (C-2), 17·26 (C-15), 16·93 (C-14).

Chrysoeriol (5,7,4′-trihydroxy-3′-methoxyflavone; **2**). UV (λ_{max} nm) CH_3OH: (252) 268 (277) 343. +$AlCl_3$: 260 (275) 297, 359 (386). +$AlCl_3$-HCl: 260 (275) 294, 356 (388). +$NaOCH_3$: 270, 323, 396 (↑). +NaOAc:

273, 309, 374. The symbols (↑) and (↓) indicate respectively increases and decreases in absorbance relative to the same band recorded in methanol.

Jaceosidin (5,7,4′-trihydroxy-3′,6-dimethoxyflavone;3). UV (λ_{max} nm) CH_3OH: 274 (290) 341. +$AlCl_3$: 259 (280) 297, 311, 368 (393). +$AlCl_3$-HCl: (257) (282) 295 (311) 360 (388). +$NaOCH_3$: (254) 274, 326, 404 (↑). +NaOAc: 274, 320, 379. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm, TMS = 0) 7·48 dd (J = 8·2 and 2·2 Hz), H-6′; 7·46 d (J = 2·2 Hz), H-2′; 6·91 d (J = 8·2 Hz), H-5′; 6·56 s , H-8; 6·47 s, H-3; 3·95 s (3H), OCH_3-6; 3·85 s (3H), OCH_3-3′. ^{13}C NMR (100 MHz, $CDCl_3$, δ ppm, TMS = 0) 183·79 (C-4), 165·89 (C-2), 162·74 (C-7), 155·14 (C-5), 153·87 (C-9), 152·25 (C-3′), 149·56 (C-4′), 133·92 (C-6), 123·74 (C-1′), 121·63 (C-6′), 116·12 (C-5′), 110·46 (C-2′), 104·50 (C-10), 103·41 (C-3), 96·29 (C-8), 60·74 (OCH_3 at C-6), 56·60 (OCH_3 at C-3′).

Diosmetin (5,7,3′-trihydroxy-4′-methoxyflavone; 4). UV (λ_{max} nm) CH_3OH: 269, 339. +$AlCl_3$: 258, 275, 293, 359 (383). +$AlCl_3$-HCl: (258) 278, 293, 356 (385). +$NaOCH_3$: (265) 275, 308, 370 (↓). +NaOAc: (265) 274, 313, 367.

Eupatorin (5,4′-dihydroxy-6,7,3′-dimethoxyflavone; 5). UV (λ_{max} nm) CH_3OH: (267) 274, 342. +$AlCl_3$: 262, 282 (296) 364 (398). +$AlCl_3$-HCl: (257) 283 (298) 359 (391). +$NaOCH_3$: 274, 310, 370 (↓). +NaOAc: (251) 273, 313, 369. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm, TMS = 0) 7·56 dd (J = 8·4 and 2·5 Hz), H-6′; 7·45 d (J = 2·5 Hz), H-2′; 7·08 d (J = 8·4 Hz), H-5′; 6·51 s , H-8; 6·32 s, H-3; 3·92 s (3H), 3·90 s (3H), 3·80 s, (3H), OCH_3.

Pharmacological assays (Cuéllar et al 1997)

Animals. Experiments were performed on groups of six female Swiss albino mice, 25–30 g, housed in a temperature-controlled room with a 12 h light–dark schedule. A standard diet was freely available to all animals.

12-O-tetradecanoylphorbol 13-acetate-induced mouse-ear oedema. Oedema was induced on the right ear by topical application of 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 2·5 μg) in acetone (20 μL). The left ear received only acetone or, when necessary, 70% aqueous C_2H_5OH. Extracts, isolated compounds and indomethacin were applied at a dose of 0·5 mg/ear, simultaneously with TPA. Oedema was expressed as the mean difference between the thicknesses of the right and left ears, measured with a Mitutoyo Series 293 microcaliper. ID50 values (dose of drug inhibiting the oedema by 50%) were calculated by linear regression of the dose–response relationship for four doses of drug between 0·05 and 0·5 mg/ear.

Carrageenan-induced mouse paw oedema. A solution of carrageenan in saline (3%, 0·05 mL) was injected into the right hind foot. The volumes of the injected and contralateral paws were measured, by means of a plethysmometer (Ugo Basile), 1, 3 and 5 h after induction of swelling. The oedema was expressed as the mean difference between the volumes of the right and left feet.

Statistics. Oedema reductions (mean ± s.e.m.) for each group were compared with those for the control group by use of Dunnett's *t*-test for unpaired data.

Results

Identification of the compounds isolated

The ^{1}H NMR spectrum of compound **1** revealed the presence of two coupled gem-olefinic protons (δ 6·33 and 5·62, d, J = 3 Hz), an olefinic proton at δ 5·20, two aliphatic protons at δ 3·85 and 2·78, six methyl protons at δ 1·70 and 1·29, and two broad multiplets (δ 2·15–2·40) that account for eight aliphatic methylenes. The ^{13}C NMR spectrum contained fifteen signals, five were characteristic of a α-methylene-γ-lactone (δ 169·23, –CO–O–; 139·13, > C=CH_2; 121·22, = CH_2; 82·42, > CH–O–CO–; 47·64, > CH–C(–R)=CH_2), two attributable to a pair of olefinic carbons (δ 134·56 and 125·23), two others to carbinolic carbons (δ 66·36 and 61·50), and two representative of two methyl groups (δ 16·93 and 17·96). On the basis of these data, **1** was characterized as a sesquiterpene lactone and then identified as parthenolide by comparison with the previously published NMR data for this compound (Castañeda-Acosta et al 1993) and by direct comparison of the gas-chromatographic retention time with that of an authentic sample (data not shown).

Initial determination of the structure of the flavonoids was performed on the basis of UV spectral data obtained from solutions of the pure compounds and in the presence of standard shift reagents (Mabry et al 1970; Voirin 1983). Simple monomethyl derivatives of luteolin (compounds **2** and **4**) were identified in this way. Compounds **3** and **5** were investigated by ^{1}H NMR spectroscopy. In both cases, two singlets in the 6·0–6·5 ppm range indicated the existence of an A ring-trisubstituted flavone, in which the proton at the lower frequency corresponds to H-3 and that at the higher frequency to H-8. Ring B gave three signals characteristic of

an *o*-di-*O*-substituted aromatic structure—as also did compounds **3** and **5**. Two methoxy proton signals were present in the ^{1}H NMR spectrum of **3** and three such signals in that of **5**. The increased absorbance of the band I in the UV spectrum upon addition of $NaOCH_3$ indicated the presence of a free 4′-OH in compound **5**, while the opposite behaviour in the same test made it possible to attribute a 3′-OH, 4′-OCH_3 structure to compound **4**. 6-OCH_3 substitution was revealed for both flavones by the bathochromic shifts observed in the presence of $AlCl_3$-HCl (17–19nm); these are indicative of 5-OH, 6-OCH_3 flavones (Sakakibara & Mabry 1977).

Anti-inflammatory activity

Data obtained during tests to determine the activity of extracts and pure compounds in the TPA-induced ear oedema test are shown in Table 1. The slight efficacy of methanolic extract compared with that of more lipophilic extracts reveals that the bulk of the anti-inflammatory principles of the plant are confined to the low-polarity fraction. Compound **1** (93% inhibition, Table 2) and its mother extract had almost the same effect, almost complete suppression of oedema. Of the flavones, only **3** strongly counteracted the inflammation elicited by TPA (80% inhibition). Both **1** and **3** therefore qualified as the most probable active principles of the plant and so for these compounds the ID50 was calculated from the results of the TPA test, and their anti-inflammatory activity after oral administration was tested against carrageenan oedema. The results are summarized in Table 3. In this model, inhibition of the inflammatory process was much more modest—only 24% oedema reduction was obtained for parthenolide after 1 h.

Discussion

Chemotypes within *Tanacetum vulgare* have been differentiated mainly on the basis of their essential oil constituents, a practice which has led to apellations such as thujone type, camphor type, chrysanthenyl acetate type, etc., arising from the name of the main volatile compound (Nano et al 1979). When germacranolides (parthenolide among them) were described in *T. vulgare* samples collected in the Piedmont region of Italy, sesquiterpenoids acquired importance as systematic markers in the species (Nano et al 1980). Later, parthenolide was also identified in *T. vulgare* growing in the Netherlands. In this instance, it should be noted that the percentage of parthenolide measured by HPLC in 14 samples varied greatly, from total absence to 1·33% in flower heads and to 0·46% in leaves (Hendriks & Bos 1990). In the current study we isolated a representative amount of 0·40% from complete aerial parts, implying that the actual content could be slightly higher in the plant. It can therefore be considered a good source of parthenolide.

Table 1. Effect of *T. vulgare* extracts on mouse-ear oedema induced by 12-*O*-tetradecanoylphorbol 13-acetate.

Treatment	Increase in thickness (mm × 10^{-3} ± s.e.m.)	Inhibition (%)
Control	182·1 ± 23·2	–
Dichloromethane extract	71·0 ± 14·1**	61
Methanol extract	114·0 ± 35·3	37
Chloroform extract	15·0 ± 11·0**	92
Indomethacin	47·6 ± 11·1**	74

Extracts and standard drug were applied at 0·5 mg/ear. **$P < 0.01$, significantly different from control (Dunnett's *t*-test, n = 6).

Eupatorin and jaceosidin were, in this order, the most abundant flavones isolated. They present the same hydroxyl substitution pattern, characterized by the superimposition of a 6-OCH_3 group over the 5,7,3′,4′ tetrasubstitution, which is also shared by the other commonest flavones chrysoeriol and diosmetin. In previous papers the presence of these flavones, except eupatorin, had been reported in *T. vulgare* together with 3,6-dimethyl-6-hydroxyflavonols not observed in the current work, e.g. jaceidin or axillarin (Adikhodzhaeva & Bankovska 1977; Ognyanov & Todorova 1983). That our sample lacked these flavonols is remarkable not only because they have previously been found in the same species, but also because their absence seems to indicate separation from *T. parthenium* and *T. microphyllum*, for which many 6-oxygenated flavonols, for example methyl derivatives of quercetagetin and 6-hydroxykaempferol, have been reported (Abad et al 1993, 1995; Williams et al 1995; Martínez et al 1997). Consequently, methylflavonoids or, even better, methylflavonols, could be used for chemosystematic purposes, even more so when parthenolide does not seem to be a valid tool for differentiating the major taxa within *Tanacetum*.

Evaluation of the pharmacological results supports the idea that parthenolide is the main in-vivo anti-inflammatory principle of *T. vulgare*, because it had the maximum effect in TPA ear oedema, as did indomethacin, and its potency was about four times greater than that of jaceosidin. Furthermore, orally administered parthenolide induced a significant decrease in carrageenan paw oedema that was, nevertheless, below the effectiveness of an intraperitoneally administered chloroform extract (Mordujovich-Buschiazzo et al 1996).

Table 2. Effect of the isolated compounds on mouse-ear oedema induced by 12-*O*-tetradecanoylphorbol 13-acetate.

Treatment	Increase in thickness ($mm \times 10^{-3}$) ± s.e.m.	Inhibition (%)	ID50 (μmol/ear)
Control	139·5 ± 22·5	–	–
Parthenolide	9·2 ± 7·0**	93	0·18 (0·12–0·23)
Chrysoeriol	92·8 ± 17·6	33	NT
Jaceosidin	28·0 ± 6·5**	80	0·50 (0·41–0·59)
Diosmetin	74·5 ± 13·1*	47	NT
Eupatorin	74·4 ± 22·2	47	NT
Indomethacin	20·8 ± 4·7**	85	0·35 (0·15–0·43)

*$P < 0·05$, **$P < 0·01$, significantly different from control (Dunnett's *t*-test, n = 6). ID50 is the dose resulting in 50% inhibition; the values in parentheses are the 95% confidence limits. NT = not tested.

Table 3. Effect of the main active principles on carrageenan-induced paw oedema.

Treatment	Increase in paw volume ($mL \times 10^{-2}$)			Inhibition (%)		
	1h	3h	5h	1h	3h	5h
Control	9·3 ± 0·3	11·3 ± 0·5	10·3 ± 0·6	–	–	–
Parthenolide	7·0 ± 1·2*	10·2 ± 0·2	10·2 ± 1·0	25	10	0
Jaceosidin	8·6 ± 0·4	8·8 ± 0·8	10·3 ± 0·6	8	22	0
Phenylbutazone	7·2 ± 0·2*	4·2 ± 0·4**	7·1 ± 0·6*	23	63	31

Compounds and standard drug were administered at 100 mg kg^{-1}. *$P < 0·05$, **$P < 0·01$, significantly different from control (Dunnett's *t*-test, n = 6).

It should be admitted that the eventual importance of the methylflavones as anti-inflammatory agents is likely to be quite minor, for both their individual effect and concentration in raw material and extracts are lower than for parthenolide. Nevertheless, jaceosidin is more potent than the flavonoids isolated from *Tanacetum microphyllum*, santin and ermanin, which had similar efficacy when applied at a six-fold higher dose (Martínez et al 1997). Of the flavones reported here the highest activity was found for jaceosidin the only one with two methyl groups. This indicates that a moderate amount of methylation provides an appropriate equilibrium between two opposite effects arising from hydroxyl methylation—an increase in lipophilicity, and therefore of skin penetration, and a blocking of bioreactive sites, such as free hydroxyl groups in phenolic compounds. Moreover, it can be observed that a 6-*O*-methyl-5,6,7-trioxygenated A ring is more favourable for topical activity than is a 5,7-dihydroxyl structure. The influence of a 4′-*O*-hydroxyl-3′-*O*-methoxylated B ring or of its reciprocal 3′-*O*-hydroxyl-4′-*O*-methoxy form is unclear and requires further research.

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