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Artigo

Anti-inflammatory activity of *Heterotheca subaxillaris* var. *latifolia* (Buckley) Gandhi & R.D. Thomas, Asteraceae

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RESUMO: "Atividade antiinflamatória de *Heterotheca subaxillaris* var. latifolia (Buckley) Gandhi & R.D. Thomas (Asteraceae)". A atividade antiinflamatória de extratos éter petróleo, diclorometânico e metanólico de *Heterotheca subaxillaris* var. latifolia, foi testada pelo método de edema induzido pelo 12-*O*-tetradecanoil phorbol acetato (TPA) na orelha do camundongos e pelo método do edema de pata induzido por carragenina em ratos. O extrato diclorometânico mostrou atividade antiinflamatória significativa (91% inibição) no edema de orelha induzido por TPA (administração tópica de 1 mg/orelha). Não houve efeitos significativos no teste do edema induzido pela carragenina. O fracionamento bioguiado das frações ativas levou ao isolamento dos flavonoides majoritários santina, pectolinaringenina, 3,6-dimetoxi-5, 7,4 '-trihidroxiflavona e hispidulina.

Unitermos: *Heterotheca subaxillaris* var. *latifolia,* Asteraceae, atividade antiinflamatória, flavonoides.

ABSTRACT: The anti-inflammatory activity of the petroleum ether, dichloromethane and methanol extracts from *Heterotheca subaxillaris* var. *latifolia* were assayed using 12-*O*-tetradecanoyl phorbol acetate (TPA) induced ear edema test in mice and carrageenan-induced paw edema test in rats. The dichloromethane extract showed a significant anti-inflammatory activity (91% of inhibition) in TPA-induced ear edema test (topical administration at 1 mg/ear). No effects were seen on carragenan-induced edema. Bio-guided fractionation deal to the isolation of the major flavonoids santin, pectolinaringenin, 3,6-dimethoxy-5,7,4'-trihydroxyflavone and hispidulin present in the active fractions.

Keywords: *Heterotheca subaxillaris* var. *latifolia*, Asteraceae, anti-inflammatory activity, flavonoids.

INTRODUCTION

Heterotheca subaxillaris var. *latifolia* (Buckley) Gandhi & R.D. Thomas, Asteraceae, is a weed widely distributed in the north western and central regions of Argentina. It is locally known as "alcanfor" because of its aromatic odour. It is also known in Mexico, where the infusion of the entire plant is used orally by the Kickapoo Indians to ease menstrual pains (Latorre & Latorre, 1977).

Previous phytochemical screening has described the isolation of terpene derivatives from *H. latifolia* (Bohlmann et al., 1980; Bohlmann et al., 1985). Recently, we described the isolation and identification of five flavonoids from this species (Rojo et al., 2004). The composition of the essential oil from the leaves and flowers has also been evaluated (Lincoln & Lawrence, 1984; Bandoni et al., 1986; Duschatzky et al., 1998). Essential oil obtained from *H. latifolia* of San Luis Province, Argentina, was found to inhibit Junin virus (García et al., 2003). However, there are no data in the literature concerning the possible pharmacological effects and the active constituents of this plant.

In this study, different extracts from the aerial parts of *H. subaxillaris* var. *latifolia* and the fractions obtained from dichloromethane extract were tested for their anti-

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inflammatory activity. Isolation of santin, pectolinaringenin, 3,6-dimethoxy-5,7,4'-trihydroxyflavone and hispidulin from the active fractions are reported.

MATERIAL AND METHODS

Plant material

Aerial parts of *Heterotheca subaxillaris* var. *latifolia* (Buckley) Gandhi & R.D. Thomas were collected from San Luis Province (Argentine). A voucher specimen was deposited at the Museum of Botany and Pharmacognosy "Carlos Spegazzini", Faculty of Exact Sciences, National University of La Plata, (LPE 927).

Preparation of extracts

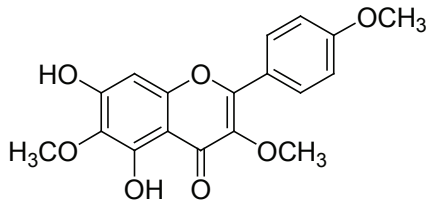
Air-dried and powered plant (500 g) was successively macerated with petroleum ether (60-80 °), CH_2Cl_2 and MeOH for 48 h at room temperature. By removing the solvents *in vacuo*, crude extracts of increasing polarity were obtained (3.1, 7.8 and 60.9 g respectively).

Fractionation of active extract

The active CH_2Cl_2 extract (1.9 g) was submitted to chromatografic columnn on Sephadex® LH20 (2.5 x 60 cm) eluted with $CHCl_3$ (500 mL), $CHCl_3$:MeOH (1:1) (500 mL) and MeOH (500 mL). Five fractions: CI (650 mg, 25-250 mL), CII (415 mg, 250-500 mL), CMI (364 mg, 500-750 mL), CMII (297 mg, 750-1000 mL) and MI (96 mg, 1000-1500 mL) were obtained. Fraction MI was obtained in small quantity and its activity could not be assessed.

Isolation and identification

Active fractions CMI and CMII were chromatographed on preparative Silicagel 60 plate using toluene:EtOAc (7:3) as mobile phase to give 3,6,4′-trimethoxy-5,7-dihydroxyflavone (santin) (**1**) and 6,4′-dimethoxy-5,7-dihydroxyflavone (pectolinaringenin) (**2**) from fraction CMI and 3,6-dimethoxy-5,7,4′-trihydroxyflavone (**3**) and 6-dimethoxy-5,7,4′-trihydroxyflavone (hispidulin) (**4**) from fraction CMII. The structures of the isolated flavonoids (**1-4**) were determined on the basis of spectral data (UV, MS, ^1H and ^{13}C NMR) and in comparison with authentical samples (Rojo et al., 2004).



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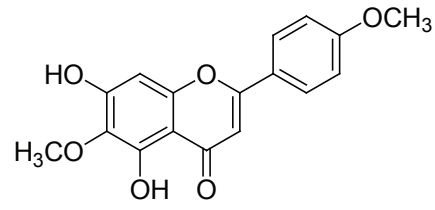

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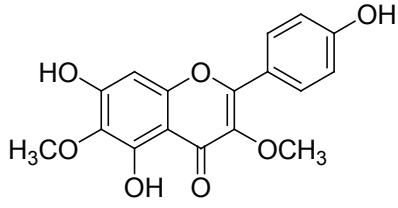

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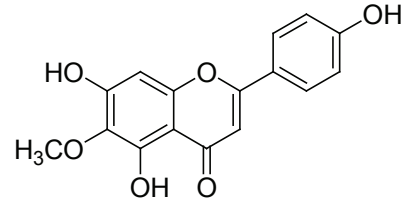

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Animals

Male Wistar rats (150-200 g) and male Swiss mice (25-30 g) were used taking into account international principles and local regulations concerning the care and use of laboratory animals (Olfert et al., 1993). The animals were housed in standard environmental conditions (25±1 °C, with a 12 h light/dark cycle) with free access to a standard commercial diet and water *ad libitum*.

Anti-inflamatory evaluation

TPA-induced ear edema in mice

Ear edema was induced according to Carlsson et al. (1989). Groups of ten animals each were used. The right ear of each mouse received a topical application of 2.5 µg of 12-*O*-tetradecanoylphorbol-13 acetate (TPA) in 20 µL acetone (10 µL to each side of the ear).

Petroleum ether, MeOH, CH_2Cl_2 extracts and fractions CI, CII, CMI and CMII (dissolved in EtOH 80%),

were applied topically immediately after TPA at the dose of 1 mg/ear. Left ear, used as control, received the vehicle. Indomethacin (0.5 mg/ear) was used as reference drug. After 4 h, animals were sacrificed by cervical dislocation. Disks of 6 mm diameter were removed from each ear and the weight was determined. The swelling was measured as the difference in weight between the punches from right and left ears, and expressed as an increase in ear thickness.

Carrageenan-induced edema in rats

Groups of five animals each were used. Paw swelling was elicited with 0.1mL 2% carrageenan in 0.9% saline (w/v) injected in the right hind paw under the plantar aponeurosis (Winter et al., 1962). Petroleum ether, MeOH and CH_2Cl_2 extracts and fractions CI, CII, CMI CMII (200 mg/kg) dissolved in EtOH-Tween 80-water 5:5:90 and the reference drug indomethacin (10 mg/kg) were administered orally 1 h before carrageenan injection. A control group received the vehicle only (5 mL/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer (Ugo Basile) at time 0 and 1, 3, and 5 h after carrageenan injection. The difference between the left and the right paw volumes (indicating the degree of inflammation) was determined and the percent inhibition of edema was calculated in comparison to the control animals.

Statistical analysis

Results are expressed as mean±SEM. Differences between the control and treated groups were tested for significance using a one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test. P values < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Petroleum ether, dichloromethane and methanol extracts obtained from the aerial parts of *Heterotheca subaxillaris* var. *latifolia* (Buckley) Gandhi & R.D. Thomas, where tested in two models of inflammation, TPA-induced ear edema in mice (topical administration at 1 mg/ear) and carrageenan induced paw edema in rats (oral administration of 200 mg/kg) giving different results. This could be due to the mechanism of the phlogistic agents and/or the administration way used.

The dichloromethane extract, when applied at the dose of 1 mg/ear, exerted a 91% inhibition of the TPA-ear edema, whereas petroleum ether, and methanol extracts did not show any significant anti-inflammatory activity (Table 1).

Chromatographic fractionation of the CH_2Cl_2 extract yielded five fractions and four of them (CI, CII, CMI, CMII) were tested under the same experimental conditions.

All dichloromethane fractions assayed were capable to inhibit significantly the development of TPA-induce ear edema, being the major active fractions CMI and CMII with inhibition of 77.33 and 90.42% respectively. These values were greater than those produced by 0.5 mg/kg of indomethacin (Table 1). Four flavonoides, santin (**1**), pectolinaringenin (**2**) from fraction CMI and 3,6-dimethoxy-5,7,4'-trihydroxyflavone (**3**) and hispidulin (**4**) from CMII were isolated and identified as the major constituents of the active fractions.

The results obtained in the carragenan-induced paw edema are shown in Table 2. It should be noted that petroleum ether, methanolic and dichloromethane extracts (200 mg/kg *p.o.*) were inactive. Only fraction CII induced a moderate but significant inhibition of paw-swelling of 50 and 40% at 3 and 5 h respectively, while indomethacin (10 mg/kg *p.o.*) showed a marked inhibitory effect of 70 and 62% at the same times.

Topical administration of TPA provides a skin inflammation model suitable for the evaluation of topical and systemic anti-inflammatory agents, besides providing some information of the mechanism involved. TPA binds and activates the protein kinase C (PKC) by interaction at the diacylglycerol site, inducing a pronounced inflammatory response. PKC is a Ca^{++} and phospholipid-dependent protein kinase which is present in one of the earliest events in the cascade of signal transduction pathways leading to a variety of cellular responses such as secretion, gene expression, cellular proliferation, cellular differentiation and muscle contraction. This enzyme is related to the pathogenesis of inflammation with phospholipase A2-dependent arachidonic acid release and eicosanoid production (Castagna et al., 1982; Nishizuka, 1984, 1988) and many natural flavonoids have been reported to inhibit PKC (Ferriola et al., 1989; Argullo et al., 1997). Therefore, the antiinflammatory activity showed by dichloromethane extract and fractions on the TPA-mouse ear model could be mediated by the inhibition of this enzyme. On the other hand, flavonoids may inhibit the cyclo-oxygenase and/or the 5-lipoxygenase pathways of arachidonate metabolism.

Williams et al. (1995) described that the flavonoid tanetin, which structure was revised to santin (Wiliams et al., 1999), inhibited both enzymes with similar potency when using rat leukocytes activated by the calcium ionophore A. 3,6-dimethoxy-5,7,4'-trihydroxyflavone, gave a similar enzyme profile to santin, shown to be active against acute inflammation in mice induced by TPA (12-*O*-tetradecanoylphorbol acetate) (Recio et al., 1995). Therefore, the isolation and identification of compounds with anti-inflammatory effects as santin, 3,6-dimethoxy-5,7,4'-trihydroxyflavone and hispidulin (Gil et al., 1994) as well as other related flavonoids as pectolinaringenin from fraction CMI and CMII could justify the observed anti-inflammatory activity.

Table 1. Topical anti-inflammatory activity of the extracts and CH_2Cl_2 fractions of Heterotheca subaxillaris var. latifolia in 12-*O*-tetradecanoylphorbol acetate-induced ear edema in mice.

Treatment	Dose (mg/ear)	Edema (mg)[a]	Inhibition (%)
Control	------	11.69±0.43	----
Petroleum ether	1	9.33±0.36	20.19
CH_2OH	1	8.56±0.54	26.77
CH_2Cl_2	1	1.21±0.25*	91.11
Fraction CI	1	5.16±0.70*	55.86
Fraction CII	1	5.20±0.76*	55.52
Fraction CMI	1	2.65±0.62*	77.33
Fraction CMII	1	1.12±0.33*	90.42
Indomethacin	0.5	3.48±0.59*	70.23

[a]Values are mean ± SEM, n = 10; *P < 0.01 vs. control, Dunnett´s test.

Table 2. Oral anti-inflammatory activity of *Heterotheca subaxillaris* var. *latifolia* extracts and CH_2Cl_2 fractions on carrageenan induced paw edema in rats.

Treatment	Dose mg/kg *p.o.*	Edema volume (mL)		
		1 h	3 h	5 h
Control	---	0.23±0.07	1.73±0.25	2.25±0.23
Petroleum ether	200	0.22±0.06	1.68±0.31	2.12±0.21
CH_2OH	200	0.25±0.04	1.71±0.22	2.09±0.26
CH_2Cl_2	200	0.26±0.06	1.62±0.23	2.05±0.22
Fraction C I	200	0.22±0.05	1.86±0.26	2.28±0.17
Fraction C II	200	0.16±0.06	0.86±0.32*	1.35±0.24*
Fraction CM I	200	0.21±0.07	1.50±0.14	1.97±0.14
Fraction CM II	200	0.23±0.06	2.14±0.17	2.37±0.16
Indomethacin	10	0.16±0.03	0.52±0.22*	1.09±0.27*

Values are mean ± SEM, n = 5; *P < 0.05 vs. control, Dunnett´s test.

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