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Letter to the Editor

In vivo anti-inflammatory activity and flavonoid identification of medicinal *Eupatorium* species

In the last years, it is becoming clear that inflammation plays a key role in the development and progression of various chronic diseases, including cancer, type-2 diabetes, Alzheimer's disease, cardiovascular and renal diseases.[1] The limitation of current anti-inflammatory therapies is acknowledged, and continuous efforts are made to improve the available treatments and to find new drugs with less secondary effects. In this search, plants that have long been used in traditional medicine for inflammation, pain and wound healing, represent a rich source of new anti-inflammatory compounds.

One of the most popular and extensively used species from the genus *Eupatorium*, *Eupatorium perfoliatum*, has been widely used by native Indians in North America and nowadays it is used there and in Europe for inflammation-associated diseases like arthritis and rheumatism.[2]

In a previous work we have found four flavonoids with significant anti-inflammatory activity in the TPA-induced ear edema in mice and in the NF-κB expression assays, isolated through bioassay-guided fractionation of an active extract of *Eupatorium arnottianum*.[3] These results led us to evaluate the anti-inflammatory activity of other five native medicinal species of *Eupatorium*, with traditional uses for inflammation and pain by indigenous and rural cultures, and to investigate the presence of some of those anti-inflammatory flavonoids in the active extracts of the selected species.

Eupatorium subhastatum, *Eupatorium laevigatum*, *Eupatorium macrocephalum*, *Eupatorium hecatanthum* and *Eupatorium candolleanum* are described as used in folk medicine for the treatment of different inflammation-related ailments. These herbs are consumed either as teas, gargarisms, by chewing different plant parts or used topically in different forms. Stems, leaves and fresh flowers of *E. hecatanthum* are chewed against teeth pain and stomachaches[4,5]; decoctions or infusions of its aerial parts are used in washes for gangrene and ulcerations.[6] *E. laevigatum* leaves are used for wound healing and as analgesic.[7,8] *E. macrocephalum* flowers are chewed for sore throat.[4] *E. subhastatum* is used to cure pimples and insect bites, in gargarisms for sores, and as descongestive.[8] Although these species have been widely used in folk medicine, no pharmacological data related to inflammation has been reported previously.

Dichloromethane and aqueous extracts of *E. subhastatum*, *E. laevigatum*, *E. macrocephalum*, *E. hecatanthum* and *E. candolleanum* were assayed in the topical and systemic models of inflammation: TPA-induced ear edema and carrageenan-induced edema assays. Furthermore, HPLC profiling and flavonoid identification of the active dichloromethane (DCM) extracts of the five species was carried out. Acute toxicity of the aqueous extracts was also evaluated.

Plants were collected in the surroundings of Paraná, Entre Ríos province (Argentina) in February 2008. *E. subhastatum* was collected in Castelar, Buenos Aires province (Argentina) in December 2008. Specimens were identified by Ing. Juan de Dios Muñoz, and Ing. Gustavo Giberti. Voucher specimens were deposited at Instituto de Tecnología Agropecuaria de Entre Ríos Herbarium and at the Herbarium of the Museo de Farmacobotánica Juan A. Domínguez, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires.

Aqueous extracts (5%) were prepared according to Argentine Pharmacopoeia VI edn.[9] Briefly, 200 ml of boiling distilled water were added to 10 g of air-dried aerial parts in a stopper flask and left to settle for 20 min. The aqueous extracts were filtered and lyophilized. Dichloromethane extracts (DCM) were prepared by maceration of 25 g of the dried and ground aerial parts in a stopper flask in contact with 250 ml of dichloromethane during 24 h. Extracts were then filtered and re-extracted twice. The filtered extracts were mixed and dried under vacuo. The yields of all dried extracts were calculated referring them to 100 g of dried plant material.

The *in vivo* assays were performed according to international guiding principles and local regulations concerning the care and use of laboratory animals for biomedical research.[10] Animals were housed in standard environmental conditions (22 ± 1 °C, with a 12 h light/dark cycle) with free access to a standard commercial diet and water *ad libitum*.

The ear edema assay was carried out according to Carlson et al.[11] Groups of 10 animals (male Swiss mice, 25–30 g) were used. The right ear of each mouse received a topical application of 2.5 μg of 12-O-tetradecanoylphorbol-13 acetate (TPA) as 0.125 μg/μl acetone solution (10 μl to each side of the ear). Extracts (0.5, 1 and 2 mg/ear/20 μl, dissolved in acetone), were applied topically immediately after TPA. The left ear, used as control, received the vehicle only. Indomethacin was used as reference drug (0.5 mg/ear/20 μl). After 4 h, the animals were sacrificed. Disks of 6 mm diameter were removed from each ear and their weight was determined. The swelling was measured as the difference in weight between the punches obtained from right and left ears.

Carrageenan-induced edema assay was performed as described by Winter et al.[12] Groups of 5 female Sprague–Dawley rats (150–200 g) were used. Paw swelling was elicited with 0.1 ml 1% carrageenan in 0.9% (w/v) saline injected in the right hind foot. Extracts (100 and 200 mg/kg) and the reference drug indomethacin (10 mg/kg) dissolved in EtOH–Tween 80–water 5:5:90 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

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plethysmometer (Ugo Basile) at 0 and 1, 3, and 5 h after the 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 control animals.

For acute toxicity assays, groups of 10 CF-1 mice (6 weeks old), 5 male and 5 female, were used. The control group received only vehicle (water) and the remaining groups received increasing doses up to 2 g/kg (0.5 ml/25 g body weight) of the different aqueous extracts of *Eupatorium* spp., orally, by means of a gastric catheter. The number of deaths, weight loss, abdominal constrictions, palpebral ptosis, movement, lethargy, stereotypy, ataxia, tremors, convulsions, diarrhea and presence of secretions were recorded. Animals were observed twice a day, for up to 15 consecutive days.

Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Dunnett's test and Bonferroni test. A p value <0.05 was considered significant. Results were expressed as mean ± SEM.

DAD–HPLC analysis of the active DCM extracts was performed in a Waters 600/DAD Waters 2996 device. Empower Pro data processor, UV range: 200–400 nm, was used. 0.5 g of each dried DCM extract was resuspended in 10 ml of a mixture of hexane–diethyl ether 1:1 and 30 ml 70% methanol were added. After shaking, the organic phase was discarded. The methanolic–aqueous phase was taken to dryness. The dried methanol residues were dissolved in 2 ml methanol HPLC-grade and filtered by 0.45 μm filters. 20 μl of this solution were injected. Jaceosidin, nepetin, hispidulin and eupatilin (reference compounds previously isolated from *E. arnottianum*) were used. These compounds were dissolved in methanol at the concentration of 0.5 mg/ml and 20 μl of each solution were injected. Extracts and reference compounds were run in two chromatographic systems as follows:

System 1. Column: Phenomenex Luna C18, 5 μm, 250 × 4.6 mm. Solvent A: water–formic acid 97:2.5. Solvent B: methanol–formic acid 97:2.5. Gradient 0–5 min: 50% B; 30 min: 100% B. Flow: 1 ml/min. System 2. Column: Agilent Zorbax Eclipse XDB-C8, 5 μm, 150 × 4.6 mm. Solvent A: water–formic acid 97:2.5. Solvent B: methanol–formic acid 97:2.5. Gradient 0–10 min: 45% B. 15 min: 60% B. 20–25 min: 100% B. Flow: 1 ml/min. Peak purity was determined by comparison of the UV spectra at upslope and downslope inflexion points. Identification of the peaks in the active extracts was done by comparing the retention times (Rt) and UV spectral data with those of the reference compounds.

The yields of DCM extracts (w/w) were: *E. subhastatum* 15.1%, *E. laevigatum* 20.0%, *E. macrocephalum* 9.0%, *E. hecatanthum* 7.3%, *E. candolleanum* 6.2%. Aqueous extracts yields (w/w) were: *E. subhastatum* 15.0%, *E. laevigatum* 19.0%, *E. macrocephalum* 14.0%, *E. hecatanthum* 19.6% and *E. candolleanum* 15.8%.

The five tested species were effective in reducing the ear thickness induced by TPA (Table 1). *E. subhastatum*, *E. laevigatum*, and *E. hecatanthum* DCM extracts were the most active ones, with maximum inhibition of 86.4, 89.7, 94.4% and ED_{50} of 0.35, 0.51, 0.92 mg/ear, respectively. *E. candolleanum* and *E. macrocephalum* also reduced the ear edema (ED_{50} 1.0 and 0.79 mg/ear), but produced a lower maximum inhibition. The aqueous extracts could not be tested due to their low solubility either in acetone or absolute ethanol, solvents used to dissolve the dried extracts in the topical TPA assay.

Only DCM and aqueous extracts of *E. subhastatum*, at a dose of 200 mg/kg, were active in the carrageenan-induced paw edema assay, both producing a moderate edema inhibition (37% in the 5th hour). None of the other species presented activity in this assay (100 and 200 mg/kg).

Table 1
Topical anti-inflammatory activity of dichloromethane extracts of *E. subhastatum*, *E. laevigatum*, *E. hecatanthum*, *E. macrocephalum* and *E. candolleanum* in the TPA-induced mouse ear oedema.

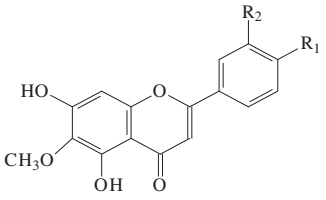
Treatment	Weight (mg)	Oedema inhibition (%)
Control	21.4 ± 0.9	0
Indomethacin	5.1 ± 0.8*	76.2
E. subhastatum DCM		
0.5 mg	9.2 ± 1.4*	57.0
1.0 mg	3.6 ± 0.5**	83.2
2.0 mg	2.9 ± 0.8**	**86.4**
E. laevigatum DCM		
0.5 mg	13.0 ± 1.3*	39.2
1.0 mg	2.2 ± 0.2**	**89.7**
2.0 mg	3.3 ± 0.8**	84.6
E. hecatanthum DCM		
0.5 mg	16.4 ± 0.4	23.4
1.0 mg	10.7 ± 0.8*	50.0
2.0 mg	1.2 ± 0.1**	**94.4**
E. macrocephalum DCM		
0.5 mg	13.5 ± 2*	36.9
1.0 mg	9.1 ± 0.8**	57.5
2.0 mg	8.9 ± 1.0**	58.4
E. candolleanum DCM		
0.5 mg	13.6 ± 0.6*	36.4
1.0 mg	9.3 ± 0.9**	56.5
2.0 mg	10.7 ± 1.7*	50.0

DCM: dichloromethane extract. Results were obtained by topical administration of different doses (0.5, 1 and 2 mg/ear) of each extract and 0.5 mg/ear of indomethacin. Statistical differences compared to controls ($n = 10$) were determined by Dunnett's test $^{*}p < 0.05$, $^{**}p < 0.01$. Bold numbers mean higher maximum inhibitions obtained.

The administration of aqueous extracts of the tested species was not lethal even up to an oral dose of 2000 mg/kg p.o. Therefore, their oral LD_{50} was higher than 2 g/kg in mice. No significant difference in body weight gain was observed between the control and any of the treated groups at any period time. Besides, the extracts did not produce any sign of toxicity during the observation period and at necropsy no macroscopic changes in organs could be detected in the treated groups, demonstrating that these extracts did not produce acute toxicity.

Jaceosidin, nepetin, eupatilin and hispidulin (Fig. 1) were identified by HPLC–DAD in the active dichloromethane extracts of the investigated species.

Jaceosidin was identified in all the tested species with the exception of *E. laevigatum*. This compound proved to be the major one in *E. subhastatum*, *E. macrocephalum* and *E. candolleanum*.



	R_1	R_2
Nepetin	**OH**	**OH**
Jaceosidin	**OH**	**OCH_3**
Hispidulin	**OH**	**H**
Eupatilin	**OCH_3**	**OCH_3**

Fig. 1. Structure of the flavonoids identified in *Eupatorium* DCM extracts.

Nepetin was identified in *E. macrocephalum* and *E. hecatanthum*, while eupatilin was present in *E. macrocephalum* and *E. candolleanum*. Hispidulin was only identified in *E. laevigatum*.

Jaceosidin, nepetin and hispidulin have demonstrated anti-inflammatory effect in the same experimental model used in this investigation[3,13]; besides, eupatilin inhibited croton oil induced edema.[14]

Other flavonoid compounds were detected in the DAD–HPLC profiles of the extracts, so further investigations should involve isolation and identification of these compounds and the determination of their participation in the anti-inflammatory activity of *Eupatorium* extracts included in this study.

Since there are no previous references about the toxicity of these species, the safety of their aqueous extracts was evaluated. The oral LD_{50} of the aqueous extracts were greater than 2 g/kg in mice. The lack of acute toxic effects of the aqueous extracts of these plants via the oral route in mice could support their safety as traditional medicines.

In conclusion, this is the first report on the anti-inflammatory activity and acute toxicity of these *Eupatorium* species. The topical anti-inflammatory effect found for these species could support their medicinal uses for localized inflammations. The presence of the active compounds eupatilin, jaceosidin, nepetin and/or hispidulin in these DCM extracts is reported here for the first time and could justify, at least in part, the observed anti-inflammatory effects of these extracts in the TPA assay.

The finding of anti-inflammatory effect and absence of acute toxicity of the studied species, together with the chromato graphic flavonoid profiling and the identification of active principles in their extracts, can contribute to the quality control and serve as a support for the efficacy and security of these traditional medicines.

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