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ARTICLE

Topical Anti-inflammatory Activity of Eupatilin, A Lipophilic Flavonoid from Mountain Wormwood (*Artemisia umbelliformis* Lam.)

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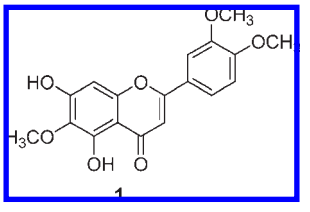
Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone) is the major lipophilic flavonoid from *Artemisia umbelliformis* Lam. and *Artemisia genipi* Weber, two mountain wormwoods used for the production of the celebrated alpine liqueur genepy. The topical anti-inflammatory activity of eupatilin was investigated using the inhibition of the Croton-oil-induced dermatitis in the mouse ear as the end point. The oedematous response and the leukocyte infiltration were evaluated up to 48 h after the induction of phlogosis, comparing eupatilin with hydrocortisone and indomethacin as representatives of steroid and non-steroid anti-inflammatory drugs, respectively. At maximum development, eupatilin significantly reduced edema in a dose-dependent manner (ID_{50} = 0.28 μmol/cm^2), showing an anti-inflammatory potency comparable to that of indomethacin (ID_{50} = 0.26 μmol/cm^2) and only 1 order of magnitude lower than that of hydrocortisone (ID_{50} = 0.03 μmol/cm^2). Within 48 h, eupatilin (0.30 μmol/cm^2) caused a global inhibition of the oedematous response (42%) higher than that of an equimolar dose of indomethacin (18%) and fully comparable to that of 0.03 μmol/cm^2 of hydrocortisone (55%). Moreover, the effect of eupatilin on the granulocytes infiltrate (32% inhibition) was similar to that of indomethacin (35% inhibition) and comparable to that of hydrocortisone (42% reduction), as confirmed by histological analysis. When our results are taken together, they show that eupatilin is endowed with potent *in vivo* topical anti-inflammatory activity, qualitatively similar to that of hydrocortisone and intermediate in terms of potency between those of steroid and non-steroid drugs.

KEYWORDS: Eupatilin; *Artemisia umbelliformis*; *Artemisia genipy*; flavonoids; polymethoxylated flavones; anti-inflammatory activity; Croton oil

INTRODUCTION

Eupatilin (5,7-dihydroxy-3′,4′,6-trimethoxyflavone, **1**) is a polymethoxhylated lipophilic flavone first isolated from *Eupatorium semiserratum* DC (Asteraceae) (*1*) and next found also in other asteraceous plants, including a series of bitter aromatic wormwoods (*2*−*7*). Eupatilin is the active ingredient of Stillen, a herbal drug from the Asian wormwood *Artemisia asiatica*, developed in South Korea for the treatment of gastritis and peptic ulcer (*6*), and is also the major lipophilic flavonoid from *Artemisia umbelliformis* Lam. and *Artemisia genipi* Weber, two mountain wormwoods used for the production of the celebrated alpine liqueur genepy (*7*−*9*).



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Eupatilin has been shown to exert cytoprotective and anti-apoptotic effects in gastric and esophageal epithelial primary cells (*6*, *10*, *11*) and is endowed with antispasmodic (*3*) and antimutagenic properties (*4*), while apoptotic and anti-proliferative activities have been demonstrated on cancer cells (*12*−*14*). Eupatilin has also been evaluated, with promising results, in

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several assays of relevance for inflammation and allergy. Thus, this flavonoid inhibits *in vitro* mast cell degranulation and histamine release, shows *in vivo* anti-allergic properties (*5*, *15*), is an antioxidant (*6*), inhibits 5-lipoxygenase and the leukotrienes synthesis (*2*, *15*−*17*), decreases prostaglandin E_2 production (*18*), and inhibits the activation of nuclear transcription factor NF-κB and the expression of cyclooxygenase-2 and different pro-inflammatory cytokines, such as interleukins (IL-4, IL-6, and IL-8) and tumor necrosis factor-α (TNF-α) (*5*, *18*). When these cellular and molecular assays are taken together, they suggest that eupatilin is a promising anti-inflammatory agent. This and the medicinal use of genepy to treat inflammatory conditions (*19*) have provided a rationale to assess the activity of eupatilin also in *in vivo* assays. To this purpose, we have used the Croton-oil-induced mouse ear dermatitis, a model of skin acute inflammation (*20*), as the end point, evaluating the effect of eupatilin on the oedematous response and leukocyte infiltration up to 48 h after phlogosis induction and comparing at an histological level its anti-inflammatory profile with that of hydrocortisone and indomethacin as representatives of steroid and non-steroid anti-inflammatory agents.

MATERIALS AND METHODS

Materials and Animals. Eupatilin has been isolated from the Swiss chemotype of *A. umbelliformis* Lam. (Asteraceae) as reported (*7*). Croton oil, indomethacin, hydrocortisone, tetramethylbenzidine (TMB), sodium azide, hexadecyltrimehylammonium bromide (HTAB), and 96-wells microtitle plates were purchased from Sigma-Aldrich (Milan, Italy). Ketamine hydrochloride (Inoketam 100) was purchased from Virbac srl (Milan, Italy). The other chemicals of analytical grade were purchased from Carlo Erba (Milan, Italy). Male CD-1 mice weighing 28−32 g were supplied by Harlan Italy (San Pietro al Natisone, Italy).

Croton-Oil-Induced Dermatitis. Topical inflammation was induced on the right ear (surface of about 1 cm^2) of anaesthetized mice (145 mg/kg ketamine hydrochloride, intraperitoneally) by application of 80 μg of Croton oil dissolved in 15 μL of acetone. Control animals received only the irritant solution, whereas other animals received both the irritant and the substances under test (*20*). At different times after the induction of dermatitis, animals were sacrificed and a punch (6 mm Ø) was taken from both the treated and untreated ears to evaluate the oedematous response and the leukocyte infiltrate. A total of 10 animals were used for each group of treatments. All animal experiments complied with the Italian D.L. no. 116 of Jan 27, 1992 and associated guidelines in the European Communities Council Directive of Nov 24, 1986 (86/609 ECC).

Evaluation of the Oedematous Response. Edema was quantified by the difference in weight between the punches taken from the treated and untreated ears. The anti-edema activity was expressed as percent inhibition of the oedematous response in animals treated with the test substances in comparison to edema of animals treated with the irritant alone (*20*).

The global effect of the tested substances on the edema development up to 48 h was quantified by calculating the areas under the curves (AUCs) representing the oedematous response up to 48 h and, subsequently, by the ratio between AUCs of these animals and AUCs of controls.

Evaluation of the Granulocytes Infiltrate. The cellular infiltrate was quantified in the treated ears measuring the myeloperoxidase activity, as index of the presence of neutrophilic granulocytes, in the same ear plug used to calculate the edema inhibition (*20*). Myeloperoxidase was extracted by HTAB, according to Bradley et al. (*21*), and the enzyme activity was measured by a colorimetric assay using TMB as chromogen (*22*). Each ear plug, suspended in 1 mL buffered saline (0.1 M sodium acetate buffer at pH 4.2), containing 0.1% HTAB (w/v), was homogenized by Ultra-Turrax (Ika-Werk, Staufen, Germany) for 5 s at 20 000 rpm. The homogenate was centrifuged at 15000*g* for 5 min, and the supernatant was used for the colorimetric assay, because preliminary experiments revealed that the pellet contained less than 5% of total myeloperoxidase activity. In each well of a 96-well microplate, 25 μL of the supernatant was mixed with 50 μL of the chromogen solution [2.83 mM TMB dissolved in 0.1 M sodium acetate buffer at pH 4.2, containing 0.1% (w/v) HTAB]. The enzyme reaction was started adding 75 μL of 0.7 mM hydrogen peroxide. After 5 min of incubation at 25 °C, the reaction was blocked by 50 μL of 4 M acetic acid, containing 10 nM sodium azide. The absorbance was read at 620 nm using an automated microplate reader (Bio-Tek Instruments, Winooski, VT). Myeloperoxidase activity was expressed as enzyme units in 1 mL of supernatant. One unit of peroxidase activity was defined as the amount of enzyme oxidizing 1 nmol of TMB/min. The enzyme activity of each sample was determined in duplicate.

The global effect of the tested substances on the whole cellular infiltrate up to 48 h was quantified by calculating the AUCs representing the time course of myeloperoxidase activity up to 48 h and, subsequently, the ratio between AUCs of these animals and AUCs of controls.

Histological Analysis. Ear biopsies, fixed in 10% formalin, were dehydrated in ascending grades of alcohol (ethanol), cleared in xilene, and embedded in paraffin wax. Sections (20 μm) were stained with hematoxylin-eosin or Giemsa and observed at a light microscope (Zeiss Axiophot, with Photometrics Cool Snaps Camera and the RS-image program).

Statistical Analysis. Pharmacological data were analyzed by one-way analysis of variance followed by the Dunnett's test for multiple comparisons of unpaired data, and a probability level lower than 0.05 was considered as significant. The dose giving 50% inhibition of the oedematous response (ID_{50}) was calculated by graphic interpolation of the logarithmic dose−effect curves.

Table 1. Anti-edema Activity of the Eupatilin, Indomethacin, and Hydrocortisone after 6 h

substance	dose (μmol/cm^2)	edema (mg) (m ± SE)	percent reduction	ID_{50} (μmol/cm^2)
controls		7 ± 0.3		
eupatilin	0.10	5.5 ± 0.6[a]	21	0.28
	0.30	3.2 ± 0.3[a]	54	
	1.00	1.1 ± 0.2[a]	84	
indomethacin	0.03	6.1 ± 0.4	14	0.26
	0.10	5.1 ± 0.2[a]	28	
	0.30	3.5 ± 0.1[a]	50	
	1.00	1.5 ± 0.1[a]	78	
hydrocortisone	0.003	6.1 ± 0.2	13	0.03
	0.010	4.9 ± 0.3[a]	30	
	0.030	2.9 ± 0.2[a]	58	
	0.100	1.7 ± 0.2[a]	76	
	0.300	0.8 ± 0.1[a]	88	

[a] $p < 0.05$ at the analysis of variance, as compared to controls.

RESULTS AND DISCUSSION

Screening of the Anti-edema Activity after 6 h. The topical anti-inflammatory effect of eupatilin, indomethacin, and hydrocortisone was screened and compared as anti-edema activity 6 h after the Croton-oil-induced ear dermatitis, at the maximum of edema formation in control mice (*20*). Eupatilin significantly reduced the oedematous response in a dose-dependent manner, causing a 21% edema reduction at the lower administered dose (0.10 μmol/cm^2) and reaching an 84% reduction at the highest dose (1 μmol/cm^2). At the same doses, the non-steroidal anti-inflammatory drug (NSAID) indomethacin induced edema reduction ranging from 28 to 78% (0.10 and 1 μmol/cm^2, respectively). As expected, the steroidal drug hydrocortisone was more active, reducing the oedematous response from 30% (0.01 μmol/cm^2) to 76% (0.10 μmol/cm^2) (**Table 1**).

The anti-edema potency of each compound was evaluated from the dose−response curve, assessing the ID_{50} value for each compound. The ID_{50} for eupatilin was 0.28 μmol/cm^2, close to that of indomethacin (ID_{50} = 0.26 μmol/cm^2), whereas hydrocortisone was more potent (ID_{50} = 0.03 μmol/cm^2; **Table 1**). Therefore, 6 h after the dermatitis induction, eupatilin showed an anti-inflammatory activity similar to that of indomethacin and only 10-fold lower than that of hydrocortisone.

Effect on the Time Course of the Croton-Oil-Induced Ear Dermatitis. The anti-inflammatory activity of eupatilin (0.30 μmol/cm^2),

indomethacin (0.30 μmol/cm^2), and hydrocortisone (0.03 μmol/cm^2) was further evaluated by both the edema and the leukocyte infiltration development up to 48 h after the dermatitis induction.

Effect on the Oedematous Response on the Whole Development of the Croton Oil Dermatitis. Control animals developed an oedematous response, which reached its maximum 6 h after the Croton oil application, and next, progressively decreased while still remaining measurable after 48 h (**Figure 1**). Eupatilin (0.30 μmol/cm^2) exerted a significant anti-edema activity at each observation time, ranging from 35% (at 24 and 48 h) to 59% (at 6 h). The effect of an equimolar dose of indomethacin was significant only at 3 and 6 h after the induction of the dermatitis, when it reduced the oedematous response by 53 and 64%, respectively. Indomethacin then substantially lost its anti-edema effect, as previously observed (*20*). Conversely, hydrocortisone (0.03 μmol/cm^2) induced a significant anti-edema reduction at all of the observation times, ranging from 69% (48 h) to 25% (6 h), and with an overall activity profile similar to that of eupatilin.

The activity profile of the three compounds on the whole oedematous response up to 48 h was quantified by calculating the AUCs shown in **Figure 1** and then the ratio between the AUCs for mice treated with each tested compound and the AUCs of control animals. Eupatilin reduced the global oedematous response by 42%, showing an effect comparable to that of hydrocortisone (55% reduction) and more than 2-fold higher than that of indomethacin (18% reduction) (**Table 2**).

Effect on the Cellular Infiltration on the Whole Development of the Croton Oil Dermatitis. The recruitment of leukocytes in the inflamed ear tissue of control animals was measured as myeloperoxidase activity and was already detectable 3 h after the induction of dermatitis. It then increased up to 24 h and next slightly decreased while also being sustained after 48 h (**Figure 2**). Eupatilin (0.30 μmol/cm^2) caused a significant reduction of the leukocyte infiltration, fully measurable from 6 h after the induction of the phlogosis (33% reduction). Indomethacin (0.30 μmol/cm^2) and hydrocortisone (0.03 μmol/cm^2) exerted a similar effect, although the activity of the steroidal drug (15% reduction) was already significant 3 h after the phlogosis induction (**Figure 2**).

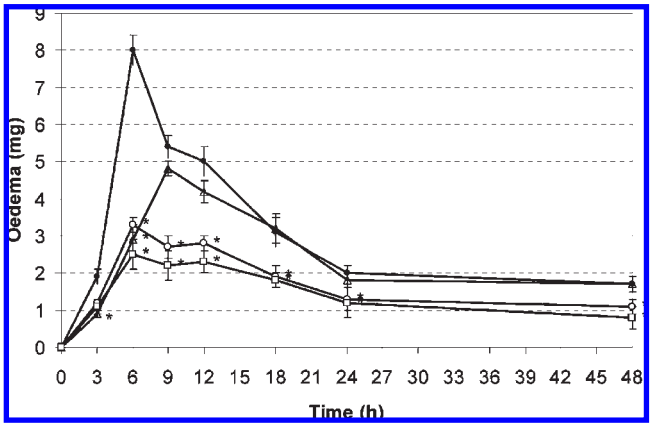

Figure 1. Effects of the different substances on the time course of the oedematous response up to 48 h (●, controls; ○, 0.3 μmol/cm^2 eupatilin; △, 0.3 μmol/cm^2 indomethacin; □, 0.03 μmol/cm^2 hydrocortisone). (*) $p < 0.05$ at the analysis of variance, as compared to controls. Each point represents the mean of the results from 10 mice.

The net effect of eupatilin on the granulocytes infiltrate (32% reduction), calculated from the AUCs of the curves in **Figure 2**, was similar to that of indomethacin (35% reduction) and comparable to that of hydrocortisone (42% reduction) (**Table 2**).

Histological Analysis. The anti-inflammatory properties of eupatilin, indomethacin, and hydrocortisone were evaluated also by histological analysis of the ear tissues. After the application of Croton oil, ear tissues of control animals showed a massive degranulation of mast cells, evident already 3 h after the dermatitis induction (**Figure 3A**). Moreover, because of edema formation, dilated blood vessels and dermal distension were observed already 3 h after the induction of the phlogosis, becoming more evident after 6 h (**Figure 4A**) and progressively attenuating after 9 h. The inflamed ear tissues of control animals were characterized also by an infiltration of neutrophilic granulocytes, already visible after 6 h but increasing progressively up to 24 h (**Figure 5A**) and still pronounced after 48 h. Ears treated with eupatilin (0.3 μmol/cm^2) showed a general reduction of all signs of inflammation, both at vascular and cellular levels, including the presence of mast cells preserved from degranulation (**Figures 3B−5B**). Ear tissues from mice treated with indomethacin (0.03 μmol/cm^2) showed a reduction in mast cell degranulation and leukocyte infiltration at the different times of observation, but starting from 9 h after phlogosis induction, the dermal tissue became distended because of the lack of its anti-edema effect (**Figure 3C−5C**). Similar to the animals treated with eupatilin (0.30 μmol/cm^2), also those treated with hydrocortisone (0.03 μmol/cm^2) showed ear tissues with attenuated vascular and cellular signs of inflammation (**Figures 3D−5D**).

Eupatilin is the major lipophilic flavonoid contained in a series of mountain wormwoods [*A. genipi* Weber, *A. umbelliformis* Lam., and *A. petrosa* (Baumg.) Jan] used for the production of genepy, a celebrated alpine liqueur traditionally associated with health claims to promote digestion and relief of airway inflammation. The presence of eupatilin is the major chemical trait shared by these plants, which are otherwise different in pattern of sesquiterpene lactones (*7*). Eupatilin has demonstrated *in vivo* clinical efficacy for the treatment of gastric ulcer and shows an interesting profile of activity in *in vitro* assays of anti-inflammatory activity. Because inflammation of the airways is a folk-medicine indication for the alpine liqueur genepy and mountain wormwoods have been used in ethnopharmacology for the topical treatment of wounds and bruises (*19*), we wondered if the promising anti-inflammatory profile of eupatilin in *in vitro* assays could be translated also in *in vivo* experiments. Our findings clearly demonstrate that eupatilin is a potent topical anti-inflammatory agent, capable of significantly inhibiting the Croton-oil-induced dermatitis in the mouse ear, acting on both the edema formation and the leukocyte infiltrate that are the hallmark of this model of inflammation.

Table 2. Effect of Eupatilin, Indomethacin, and Hydrocortisone on the Global Oedematous Response and Leukocyte Infiltrate

		edema		cell infiltrate	
substance	dose (μmol/cm^2)	AUC (mg × h) (m ± SE)[a]	percent reduction	AUC (EU × h) (m ± SE)[a]	percent reduction
controls		136.8 ± 4.6		1900.13 ± 85.66	
eupatilin	0.30	79.48 ± 4.7[b,c]	42	1291.58 ± 64.98[b]	32
indomethacin	0.30	111.93 ± 3.3[b]	18	1237.43 ± 80.23[b]	35
hydrocortisone	0.03	60.90 ± 9.3[b]	55	1098.75 ± 100.27[b]	42

[a] EU = enzyme units. AUC = area under the curve. [b] $p < 0.05$ at the analysis of variance, as compared to controls. [c] $p < 0.05$ at the analysis of variance, as compared to indomethacin.

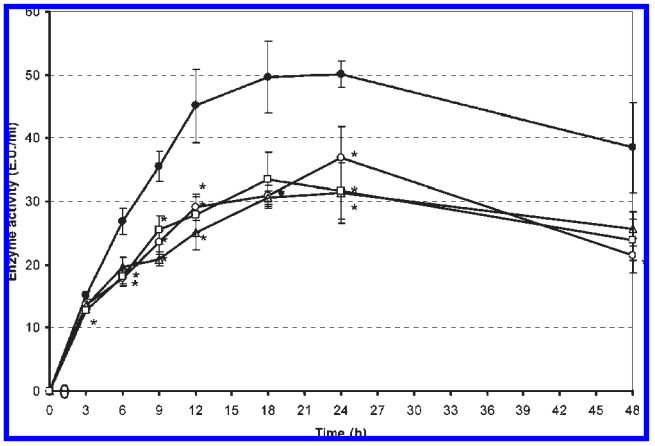

Figure 2. Effects of the different substances on the time course of cellular infiltrate up to 48 h (●, controls; ○, 0.3 μmol/cm^2 eupatilin; △, 0.3 μmol/cm^2 indomethacin; □, 0.03 μmol/cm^2 hydrocortisone). (∗) $p < 0.05$ at the analysis of variance, as compared to controls. Each point represents the mean of the results from 10 mice.

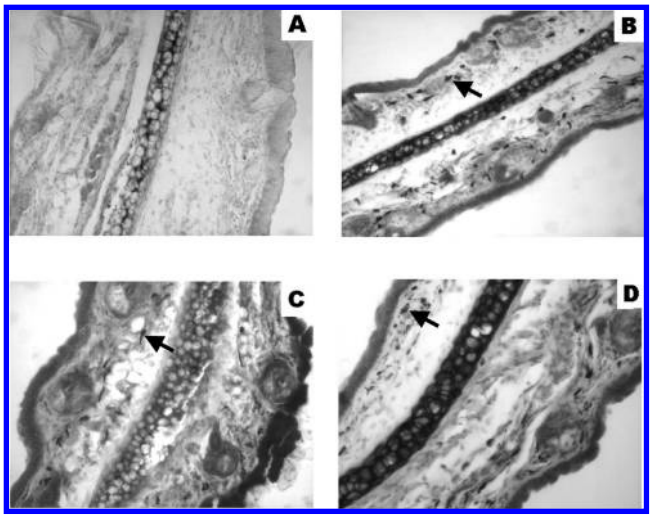

Figure 3. Section of mouse ear 3 h after the induction of the Croton oil dermatitis (A, control; B, 0.30 μmol/cm^2 eupatilin; C, 0.30 μmol/cm^2 indomethacin; D, 0.03 μmol/cm^2 hydrocortisone). Giemsa staining, 25×. In the control panel, a massive degranulation of mast cells (see arrows) is well evident already 3 h after dermatitis induction.

In particular, 6 h after the induction of the dermatitis, at the time of maximum edema response, eupatilin showed potency (ID_{50} = 0.28 μmol/cm^2) similar to that of the NSAID indomethacin (ID_{50} = 0.28 and 0.26 μmol/cm^2, respectively) and only 10-fold lower than that of the reference steroidal drug hydrocortisone (ID_{50} = 0.03 μmol/cm^2). However, by evaluating the effect on the whole edema development (up to 48 h), the antiphlogistic effect of eupatilin (0.30 μmol/cm^2) is significantly higher than that of indomethacin (0.30 μmol/cm^2), with overall reduction of the oedematous response by 42 and 18%, respectively. Thus, while indomethacin was active only 3 and 6 h after the phlogosis reduction, eupatilin exerted a significant anti-edema effect at all observation times, with an overall profile of activity similar to that of hydrocortisone (0.03 μmol/cm^2), that caused a global edema reduction of 55%. Eupatilin significantly reduced also the cellular infiltrate from 6 up to 48 h from the phlogosis induction, with an overall reduction of 32%. A similar effect was observed for indomethacin and hydrocortisone, which induced an overall reduction of the cellular infiltrate of 35 and 42%, respectively.

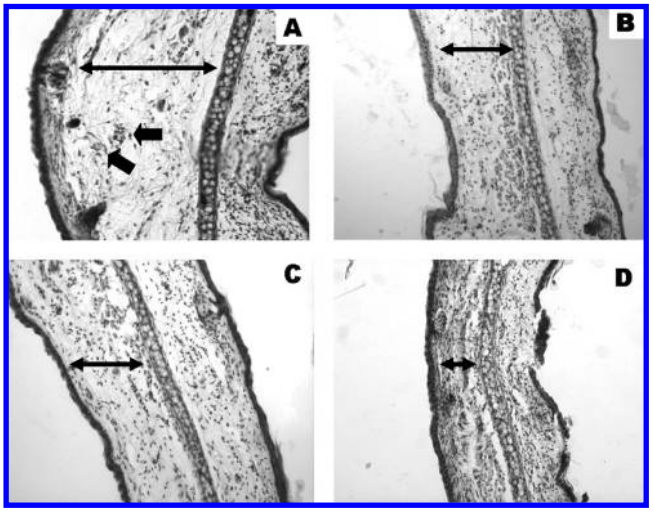

Figure 4. Section of mouse ear 6 h after the induction of the Croton oil dermatitis (A, control; B, 0.30 μmol/cm^2 eupatilin; C, 0.30 μmol/cm^2 indomethacin; D, 0.03 μmol/cm^2 hydrocortisone). Hematoxylin and eosin staining, 12.5×. Double arrows indicate the dermal distension at the inflamed side of the ear. Small arrows indicate the dilated blood vessels.

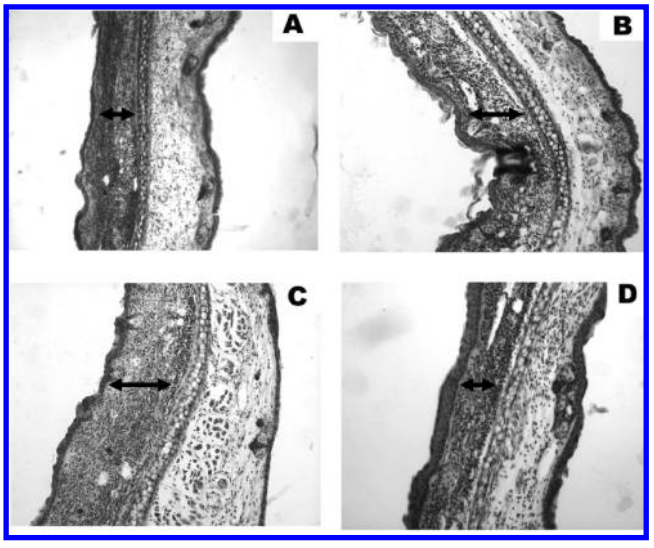

Figure 5. Section of mouse auricle 24 h after the induction of the Croton oil dermatitis (A, control; B, 0.30 μmol/cm^2 eupatilin; C, 0.30 μmol/cm^2 indomethacin; D, 0.03 μmol/cm^2 hydrocortisone). Hematoxylin and eosin staining, 12.5×. Double arrows indicate the inflamed side of the ear, where a neutrophilic granulocytes infiltrate (gray cellular aggregates) is evident.

These findings were confirmed by the histological analysis of the ear tissues at the different observation times. Thus, eupatilin attenuated the disorganization of the dermal and hypodermal connective tissues caused by the Croton-oil-induced edema formation and considerably reduced the leukocyte infiltrate. Moreover, eupatilin inhibited mast cell degranulation, in accordance with previous findings on its effects on the release of histamine *in vitro* (*15*). The mast cell degranulation was also preserved in the mice ear tissues treated with the two reference compounds, but indomethacin showed a reduced dermal distension only up to 6 h, according to its anti-edema effect.

When these observations are taken together, they suggest that eupatilin exerts anti-inflammatory activity with a mechanism substantially different from that of indomethacin. Thus, while indomethacin showed anti-edematous effects only in the first phase of the Croton oil dermatitis, eupatilin preserved its activity

also in the later phase of inflammation. The anti-inflammatory activity of indomethacin mainly depends upon the non-selective inhibition of cyclooxygenases (COXs), and COX-1 inhibition is also related to gastrointestinal depletion of cytoprotective prostaglandins (*23*, *24*). Conversely, eupatilin *in vitro* inhibits the expression of COX-2 (*18*) and shows only a marginal effect on the cyclooxygenase enzyme activity (*2*). Eupatilin has also been shown to possess antioxidant properties and to inhibit the production of the reactive oxygen species (*6*) as well as several other events and mediators involved in the inflammatory process, such as 5-lipooxygenase, the formation of the vasoactive or chemotactic leukotrienes LTD_4 and LTB_4 (*2*, *16*, *17*), and the NF-κB-dependent expression of the pro-inflammatory cytokines TNF-α, IL-4, IL-6, and IL-8 (*5*, *18*). Eupatilin is therefore a pleiotropic anti-inflammatory agent, acting on multiple molecular targets involved in inflammation, a feature that might substantially overcome the adverse effects induced by the inhibition of a single mechanism, such as the inhibition of the cyclooxygenase pathway by a NSAID (*23*).

In conclusion, we have demonstrated that the remarkable *in vitro* anti-inflammatory properties of the lipophilic flavonoid eupatilin can be translated in animal models of inflammation, supporting the traditional use of eupatilin-containing wormwoods and their preparations to treat inflammatory conditions. The overall anti-inflammatory profile of eupatilin is characterized by the interaction with several targets and is more similar to that of steroidal drugs than to that of NSAIDs. Methoxylated lipophilic flavonoids show an improved oral bioavailability with respect to their polyphenolic analogues (*25*), and also given the chemical stability of eupatilin and its clinical efficacy in the treatment of gastric ulcer (*6*), it does not seem unreasonable to assume that this rare flavonoid might be involved in the healthy gastrointestinal and anti-inflammatory properties traditionally associated with mountain wormwoods and their preparations, including genepy.

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