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Assessment of the anti-inflammatory activity and free radical scavenger activity of tiliroside

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Abstract

Three flavonoids, gnaphaliin, pinocembrin and tiliroside, isolated from *Helichrysum italicum*, were studied in vitro for their antioxidant and/or scavenger properties and in vivo in different models of inflammation. In vitro tests included lipid peroxidation in rat liver microsomes, superoxide radical generation in the xanthine/xanthine oxidase system and the reduction of the stable radical 1,1-diphenyl-2-pycryl-hydrazyl (DPPH). Acute inflammation was induced by application of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) to the mouse ear or by subcutaneous injection of phospholipase A_2 or serotonin in the mouse paw. Eczema provoked on the mouse ear by repeated administration of TPA was selected as a model of chronic inflammation. The flavonoids were assayed against sheep red blood cell-induced mouse paw oedema as a model of delayed-type hypersensitivity reaction. The most active compound, both in vitro and in vivo, was tiliroside. It significantly inhibited enzymatic and non-enzymatic lipid peroxidation (IC_{50} = 12.6 and 28 μM, respectively). It had scavenger properties (IC_{50} = 21.3 μM) and very potent antioxidant activity in the DPPH test (IC_{50} = 6 μM). In vivo, tiliroside significantly inhibited the mouse paw oedema induced by phospholipase A_2 (ED_{50} = 35.6 mg/kg) and the mouse ear inflammation induced by TPA (ED_{50} = 357 μg/ear). Pinocembrin was the only flavonoid that exhibited anti-inflammatory activity in the sheep red blood cell-induced delayed-type hypersensitivity reaction. However, only tiliroside significantly reduced the oedema and leukocyte infiltration induced by TPA. As in the case of other flavonoids, the anti-inflammatory activity of tiliroside could be based on its antioxidant properties, although other mechanisms are probably involved.

Keywords: Tiliroside; Gnaphaliin; Pinocembrin; Anti-inflammatory activity; Antioxidant activity

1. Introduction

Flavonoids are substances of low molecular weight and are present in all vascular plants. The basic flavonoid structure consists of 15 carbon atoms (C_6–C_3–C_6), arranged in three rings labelled A, B and C. They are subdivided according to the presence of an oxo group at position 4, a double bond between carbons 2 and 3, or a hydroxyl group in position 3 of the C ring. Compounds within a class differ in the substitution of A and B rings (Fig. 1).

Apart from their physiological role in plants, flavonoids are known to possess anti-inflammatory, antioxidant, anti-allergic, hepatoprotective, anti-thrombotic, antiviral and anti-carcinogenic activities. The remarkable array of biochemical and pharmacological actions of these substances is illustrated by their effects on the function of various mammalian cellular systems, the immune system and inflammatory cells. These actions can be explained by the antioxidant activity exhibited by many flavonoids. Their antioxidant mechanisms include the inhibition of enzymes involved in the formation of reactive oxygen species (xanthine oxidase, protein kinase C, lipoxygenase, cyclooxygenase, NADH oxidase, etc.) or the chelation of trace elements (free iron or copper) which are potential enhancers of free radical generation. Besides scavenging, flavonoids may stabilise free radicals involved in oxidative processes by complexing with them (see reviews of Middleton et al., 2000; Pietta, 2000). The pharmacological capacities of flavonoids have been closely studied and important structure–activity relationships have been established (Pietta, 2000; Heijnen et al., 2002). The most relevant features of active flavonoids are the presence of a catechol

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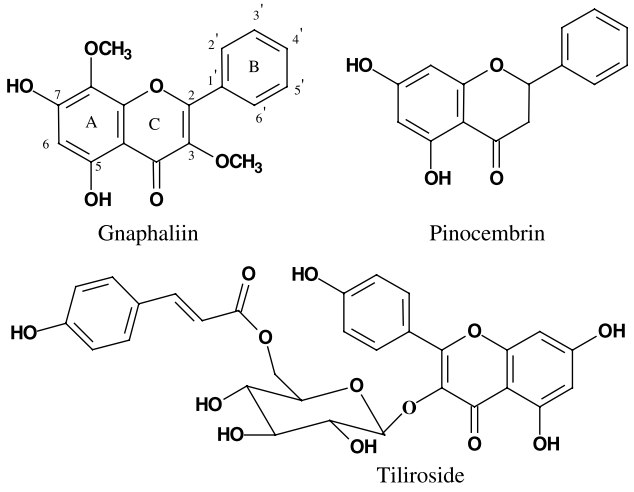

Fig. 1. Structures of flavonoids used in this study.

moiety or *O*-3′,4′-dihydroxy structure in ring B, the double bond between C-2 and C-3 and a free 3-hydroxy group in the C ring. In addition, characteristics such as the spatial conformation or the lipophilicity of the molecule have an important influence on its pharmacological activity (Heijnen et al., 2002).

In a previous paper (Sala et al., 2002), we established the anti-inflammatory and antioxidant properties, in vivo and in vitro, respectively, of extracts obtained from the Mediterranean medicinal plant *Helichrysum italicum* (Asteraceae). Such activities can be attributed to the presence of phenolic substances such as flavonoids. Three different flavonoids—identified as gnaphaliin (a methoxyflavone), pinocembrin (a flavanone) and tiliroside (a flavonol acyl-glucoside)—were isolated from the active extracts (Sala et al., 2001).

Continuing our investigation of new drugs obtained from anti-inflammatory medicinal plants, we present the effects of these naturally occurring compounds in different in vivo and in vitro models of inflammation so as to determine if these substances are responsible for the antioxidant and anti-inflammatory activities noted in the methanolic extract.

2. Materials and methods

2.1. Reagents

The isolation from anti-inflammatory extracts of *H. italicum* and the identification of 5,7-dihydroxy-3,8-dimethoxyflavone or gnaphaliin, 5,7-dihydroxyflavanone or pinocembrin and kaempferol-3-*O*-(6″-*p*-coumaroyl)-β-D-glucoside or tiliroside have been reported previously (Sala et al., 2001). Serotonin creatinine sulphate and Tween 80 were from Fluka Chemika-Biochemika (Buchs, Switzerland); leukotriene B_4 enzyme immunoassay kit was from Cayman Chemicals (Ann Arbor, MI, USA); acetone and methanol of analytical grade were from Baker (Deventer, Holland); ethanol 96% and methanol analytical grade, were from Panreac (Barcelona, Spain). The rest of the reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals

Female Wistar rats weighing 180–200 g and groups of six Swiss female mice weighing 25–30 g were used. All animals were fed on a standard diet ad libitum. Housing conditions and all in vivo experiments were approved by the institutional Ethics Committee of the Faculty of Pharmacy, University of Valencia (Spain), according to the guidelines established by the European Union on Animal Care (CEE Council 86/609).

2.3. Microsomal lipid peroxidation

Lipid peroxidation inhibiting activity was determined as described previously (Sala et al., 2002). Reaction mixtures contained rat liver microsomes and test products (100 and 50 μM).

Non-enzymatic peroxidation was induced by $FeSO_4$ and ascorbate. At room temperature, the amount of lipid peroxidation was determined by measuring absorbance at 535 nm, using the 2-thiobarbituric acid method. Butylated hydroxytoluene was used as a positive control. Enzymatic lipid peroxidation was started by addition of CCl_4 to the reaction mixture containing microsomes and an NADPH-generating system. After a 15-min incubation at 37 °C, thiobarbituric acid-reactive substances were determined as above.

2.4. Superoxide radical scavenging activity

Superoxide radical was generated by enzymatic oxidation of hypoxanthine with xanthine oxidase grade I (0.006 U) and was detected by the reduction of nitroblue tetrazolium, followed spectrophotometrically at 560 nm. Assay details are described elsewhere (Sala et al., 2002). Pyrogallol was used as a positive control. The influence on enzyme activity of flavonoids was evaluated by measuring the uric acid formation from xanthine (2–25 μM). After a 15-min incubation, absorbance was measured at 295 nm. Allopurinol was used as reference compound.

2.5. Superoxide radical generation by human leukocytes

Cells were obtained from human buffy coats. After centrifugation, the polymorphonuclear leukocyte-rich pellets were resuspended in Hanks' balanced salt solution. For the superoxide generation assay, the protocol described by Montesinos et al. (1995) was used. Superoxide release was induced by addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA; 1 μM). After a 10-min incubation at 37 °C, the precipitate was dissolved in dimethyl sulfoxide–HCl (95:5, v/v) and measured using a Labsystem Multiscan MCC/340 at 560 nm.

2.6. DPPH scavenging activity

Reduction of the stable free radical was determined according to Cavin et al. (1998), with some modifications. 1,1-Diphenyl-2-pycryl-hydrazyl (DPPH) in methanol (1.5 ml; 20 mg/l) was added to 0.75 ml of a solution of test compounds in methanol (100 μM). Absorbance at 517 nm was determined spectrophotometrically after 5 min and the scavenging activity was calculated as a percentage of radical reduction. Butylated hydroxytoluene and quercetin-3-*O*-glucoside were used as reference compounds.

2.7. Cytotoxicity assay

Cytotoxicity of the flavonoids was measured by a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983). Rat peritoneal neutrophils were exposed to 5 μl of the products (100 μM, final concentration) for 30 min at 37 °C, and then incubated with MTT. After 3–4 h at 37 °C, the formazan product was solubilised in dimethyl sulfoxide. Absorbance was measured at 490 nm using a Labsystems Multiskan MCC/340 plate reader. A decrease in absorbance indicated a decrease in cell viability.

2.8. Inhibition of leukotriene B_4 production from rat polymorphonuclear leukocytes

Rat peritoneal leukocytes (95% viability, trypan blue exclusion test) were prepared according to Safayhi et al. (1995). For 5-lipoxygenase product formation from endogenous arachidonic acid, leukocytes were stimulated at 37 °C for 5 min with the calcium ionophore A23187 (1.9 μM). The cells were incubated in the presence of the test compounds at a final concentration of 100 μM. The reaction was stopped with methanol/1 N HCl (97:3). The production of leukotriene B_4, after purification on a solid phase extraction system, was determined by a leukotriene B_4 enzyme immunoassay kit, according to the manufacturer's instructions.

2.9. Phospholipase A_2-induced paw oedema in mouse

Details of the method were described previously (Giner-Larza et al., 2001). Phospholipase A_2 from *Naja mossambica* (2 U in 25 μl sterile saline) was injected s.c. into the right hind paw. The test compounds (80 mg/kg) were injected i.p. 30 min before phospholipase A_2, and the reference drug cyproheptadine (10 mg/kg) was administered p.o. 60 min prior to inflammation induction. Products and reference drug were dissolved in Tween 80–ethanol–saline (1:1:10). Oedema was measured with a plethysmometer (Ugo Basile) 30, 60 and 90 min after challenge and is expressed as the difference between the volume of the right and left paws. The control group was treated only with phospholipase A_2. Inhibition is expressed as a percentage of control.

2.10. Serotonin-induced paw oedema in mouse

Oedema was induced in the right hind paw by subplantar injection of serotonin (3% w/v in saline, 25 μl). Gnaphaliin and pinocembrin were dissolved in olive oil, while tiliroside was dissolved in ethanol–saline (1:19). They were administered s.c. at a dose of 50 mg/kg (0.1 ml), 3 h before serotonin injection. A group received the reference drug dexamethasone (0.5 mg/kg) dissolved in ethanol–saline (1:19). The right and left paw volumes were measured with a plethysmometer (Ugo Basile) 12 min after inflammation induction. Oedema is expressed as above. Details of the method have been reported earlier (Hernández et al., 2001).

2.11. Sheep red blood cell-induced delayed-type hypersensitivity

The experiment was performed according to the protocol described previously (Góngora et al., 2000). Red blood cells (2×10^7) in phosphate buffer solution were injected s.c. into the shaved back of mice. The mice were challenged 5 days later by injecting 1×10^8 red blood cells into the right hind paw. The flavonoids (15 mg/kg) and dexamethasone (10 mg/kg) were administered i.p. immediately before and 16 h after challenge. The paw volumes were measured with a plethysmometer (Ugo Basile) 18, 24 and 48 h after challenge. The oedema is expressed as above.

2.12. TPA-induced mouse ear oedema

Oedema was induced by topical application of 2.5 μg of TPA per ear. Test compounds and the standard drug indomethacin were applied topically (0.5 mg/ear), simultaneously with TPA (Giner et al., 2000). Flavonoids were dissolved in ethanol–water (8:2), while indomethacin was dissolved in acetone. The 50% inhibitory dose (ID_{50}) was determined by applying the flavonoids at four different doses, ranging from 500 to 62 μg/ear for gnaphaliin, 500 to 15 μg/ear for pinocembrin and 500 to 250 μg/ear for tiliroside. Ear thickness was measured before TPA application and 4 h after, and oedema is expressed as the increase in thickness due to inflammation.

2.13. Mouse ear inflammation induced by multiple topical applications of TPA

Inflammation was induced by topical application on alternate days (five applications) of 2 μg of TPA to each ear (Stanley et al., 1991). Compounds (0.5 mg/ear) and dexamethasone (0.05 mg/ear) were applied topically twice daily for 4 days. On the last day, the compounds were applied only in the morning. The mice were killed by cervical dislocation, and two ear punches from each animal ($n = 5$) were taken. Eight ear samples were frozen in hexadecyltrimethylammonium bromide for the myeloperoxidase assay and two were placed in 4% formaldehyde for

histology. Details of the method have been described earlier (Giner et al., 2000; Giner-Larza et al., 2001).

2.14. Myeloperoxidase assay

In accordance with the method described by De Young et al. (1989), each ear sample, placed in an Eppendorf tube with hexadecyltrimethylammonium bromide, was thawed, homogenised and centrifuged. Enzyme activity was determined in the supernatant, using a colorimetric method and a Labsystems Multiskan MCC/340 plate reader set to measure absorbance at 620 nm. Details of the method have been described earlier (Giner-Larza et al., 2001).

2.15. Histology

Ear samples were fixed in 4% neutral-buffered formalin. Each sample was cut longitudinally into equal halves. Half of each was embedded in paraffin, cut into 3–4 μm sections and stained with haematoxylin–eosin. Epithelium thickness was evaluated using an objective × 100 and expressed as the mean ± S.D. of the number of epidermal layers from the basal to the granulous stratum, inclusive (Giner et al., 2000).

2.16. Statistical analysis

Data are expressed as means ± S.E.M. Statistical evaluation was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. Statistical significance is expressed as $*P<0.05$ or $**P<0.01$. The inhibitory concentrations or inhibitory doses 50% (IC_{50} or ID_{50}, respectively) were calculated from the concentration or dose/effect regression lines.

3. Results

3.1. Effect on microsomal lipid peroxidation

Tiliroside was the only flavonoid that suppressed the lipid peroxidation initiated in rat liver microsomes by Fe^{2+}/ascorbate (Fig. 2A). Inhibitory concentration 50 (IC_{50}) values for tiliroside and butylated hydroxytoluene were 28 and 6.2 μM, respectively. At the concentration of 100 μM, all the compounds inhibited enzymatic lipid peroxidation to an extent similar to that of butylated hydroxytoluene (Fig. 2B). IC_{50} values were determined for tiliroside and butylated hydroxytoluene (12.6 and 5.5 μM, respectively).

3.2. Superoxide radical scavenging activity

The scavenging effect of flavonoids on radical superoxide was determined in the hypoxanthine/xanthine oxidase system. Only tiliroside showed activity in this test (Fig. 3).

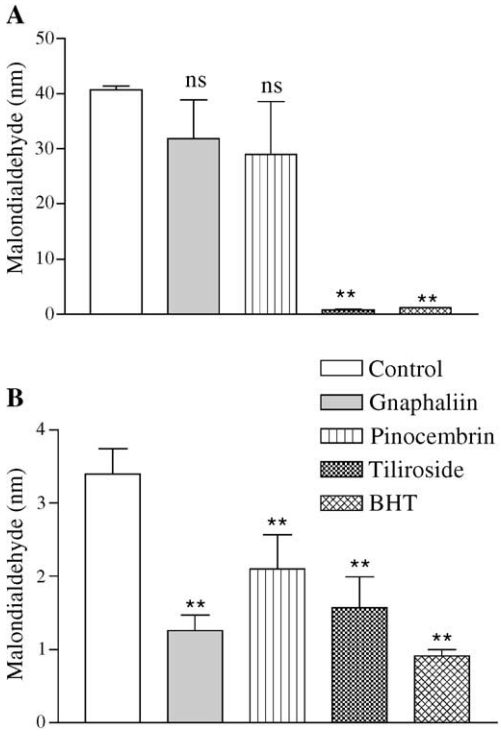

Fig. 2. Effects of flavonoids and the reference drug butylated hydroxytoluene (BHT) at the final concentration of 100 μM on lipid peroxidation in rat liver microsomes stimulated with (A) Fe^{2+}/ascorbate and (B) CCl_4/NADPH. Each bar value is the mean of triplicate measurements. Statistical significance of difference from the control: $**P<0.01$, ns = not significant by Dunnett's multiple comparison test.

The IC_{50} (21.3 μM) was in the range of that of pyrogallol (17.7 μM). The pronounced inhibition of superoxide-induced reduction of nitro blue tetrazolium could partially depend on a moderate direct inhibition of xanthine oxidase (30% inhibition at 50 μM), but superoxide scavenging activity was also involved. This scavenging property was also observed against the superoxide radical generated by human leukocytes stimulated by TPA (data not shown), the IC_{50} being twice as high as that in the hypoxanthine/xanthine oxidase system (42 μM).

3.3. DPPH scavenging activity

Of all the compounds tested, tiliroside showed the highest scavenging/reducing properties at 100 μM (Fig. 4). The IC_{50} was about 6 μM, in the same range as that of the flavonol quercetin-3-*O*-glucoside.

3.4. Effect on leukotriene B_4 generation by rat peritoneal leukocytes

Previously, cell viability in the presence of the flavonoids was assayed in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide test. At 100 μM, no substance showed toxicity against rat peritoneal leukocytes (viability >95%).

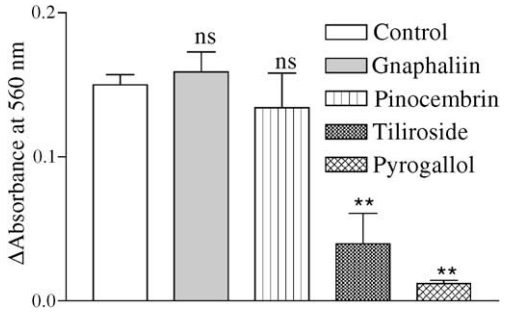

Fig. 3. Activity of flavonoids and the reference drug pyrogallol as scavengers of superoxide anion. Results of nitroblue tetrazolium reduction are expressed as the increase (Δ) in absorbance per minute. The final concentration of compounds was 100 μM, except for tiliroside, which was 50 μM. Each value is the mean of triplicate measurements ± S.E.M. Statistical significance of difference from the control: $^{**}P<0.01$, ns = not significant by Dunnett's multiple comparison test.

The effect of the flavonoids at 100 μM on the production of leukotriene B_4 in vitro was estimated using an enzyme immunoassay kit. The results for tiliroside suggest that this substance significantly stimulates arachidonic acid metabolism, expressed in terms of leukotriene B_4 generation (pg/ml). However, ghaphaliin and pinocembrin suppressed leukotriene B_4 formation (Table 1).

Table 1
Effect of flavonoids on LTB_4 production by rat peritoneal leukocytes

	Leukotriene B_4	
	(pg/ml ± S.E.M.)[a]	Percent inhibition
Control[b]	336 ± 97	
Gnaphaliin[c]	20 ± 5[d]	94
Pinocembrin[c]	15 ± 4[d]	96
Tiliroside[c]	>500	–

[a] Values are reported as the mean ± S.E.M. of three different experiments.

[b] Rat peritoneal polymorphonuclear leukocytes stimulated by calcium ionophore A23187.

[c] Polymorphonuclear leukocytes were incubated with the flavonoids at 100 μM for 5 min at 37 °C.

[d] $P<0.01$, statistical significance of difference from the control by Dunnett's multiple comparison test.

3.5. Effects on phospholipase A_2-induced mouse paw oedema

Fig. 5 shows the inhibition of phospholipase A_2-induced paw oedema at the various time points analysed (30, 60 and 90 min). All the flavonoids (80 mg/kg, i.p.) reduced paw oedema after 30 min. Tiliroside and pinocembrin showed the strongest effect at 60 min (68% and 63% inhibition, respectively), while gnaphaliin reduced the inflammatory lesion by about 50% at 60 min. After 90 min, the anti-inflammatory activity of flavonoids and cyproheptadine (10 mg/kg, p.o.) subsided. The dose/response relationship of tiliroside and pinocembrin yielded for tiliroside an ID_{50} of 35.6 mg/kg ($r^2=0.9746$, $P=0.0254$ considered significant) and for pinocembrine an ID_{50} of 58.9 mg/kg ($r^2=0.9529$, $P=0.0470$ considered significant) 1 h after irritant injection.

3.6. Effects on serotonin-induced mouse paw oedema

The vascular leakage induced by subcutaneous injection of serotonin in the mouse paw triggers oedema, which reached a maximum 12 min after application. Flavonoids at 50 mg/kg, administered s.c. 3 h before serotonin, slightly reduced oedema formation (<40%). Gnaphaliin was the most active compound in this test (Fig. 6).

3.7. Effect on sheep red blood cell-induced delayed-type hypersensitivity

In this model of inflammation, the reaction was maximal at 18 h and considerably reduced 48 h following challenge. Flavonoids (15 mg/kg, i.p) were administered immediately prior to and 16 h after challenge (Fig. 7). They produced a significant reduction in the elicitation phase only at 18 h.

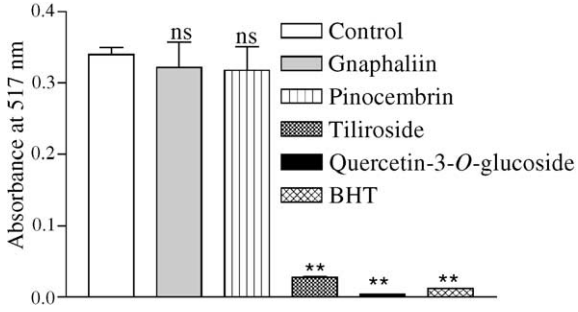

Fig. 4. Scavenging effects of flavonoids and the reference drugs butylated hydroxytoluene (BHT) and quercetin-3-*O*-glucoside on the DPPH radical. Compounds were tested at the final concentration of 100 μM. Antioxidant activity is shown as the mean ± S.E.M. Statistical significance of difference from the control: $^{**}P<0.01$, ns = not significant by Dunnett's multiple comparison test.

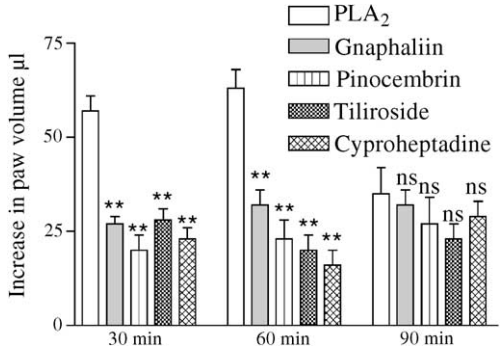

Fig. 5. Effects of flavonoids (80 mg/kg i.p.) and cyproheptadine (10 mg/kg p.o.) on *N. mossambica* phospholipase A_2 (PLA_2)-induced mouse paw oedema. $n=6$ animals. Times on abscissa indicate the interval between phospholipase A_2 injection and measurement of oedema. Increase in paw volume is given as the mean ± S.E.M. Statistical significance of difference from the control: $^{**}P<0.01$, ns = not significant by Dunnett's multiple comparison test.

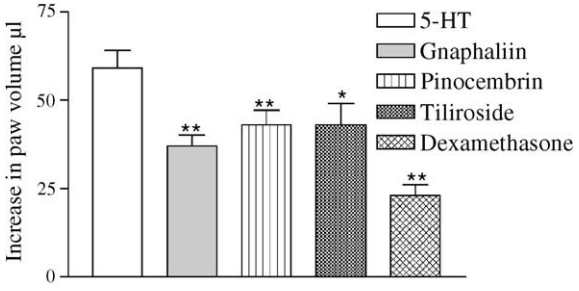

Fig. 6. Effects of flavonoids on serotonin (5-HT)-induced mouse paw oedema. Flavonoids (50 mg/kg) and dexamethasone (0.5 mg/kg) were administred subcutaneously. Footpad oedema was induced 3 h later by subplantar injection of serotonin. Footpad volume was measured 12 min after treatment with the irritant. $n=6$. Increase in paw volume expressed as the mean ± S.E.M. Statistical significance of difference from the control: $^{**}P<0.01$, $^{*}P<0.05$ by Dunnett's multiple comparison test.

Pinocembrin was the most active (48% inhibition), while only gnaphaliin still had a slight anti-oedema effect after 48 h. Dexamethasone (10 mg/kg) had an anti-inflammatory effect throughout the experimental period.

3.8. Effects on mouse ear oedema induced by TPA

The ear oedema induced by TPA was reduced significantly by all three flavonoids, which produced an effect similar to that of indomethacin when administered topically. All the flavonoids produced a dose-dependent decrease in oedema. The most potent compound was pinocembrin (61 vs. 125 µg/ear for indomethacin) (Table 2).

3.9. Effects on mouse ear inflammation induced by multiple applications of TPA

Only tiliroside (0.5 mg/ear × 7 applications) significantly reduced oedema formation at the dose assayed, by nearly 50%. Dexamethasone (0.05 mg/ear) inhibited oedema formation by 83% (Fig. 8A). However, all the compounds reduced neutrophil infiltration, measured as myeloperoxidase activity. As shown in Fig. 8B, tiliroside was, once again, the most active flavonoid, with a percentage of inhibition similar to that of dexamethasone (88% and 96%, respectively).

Table 2
Anti-inflammatory effect of the flavonoids on acute TPA-induced ear oedema

Product	ID_{50} (mg/ear)[a]	ΔEar thickness[b] (µm ± S.E.M.)	I.R.[c]
Control	–	187 ± 16	–
Gnaphaliin	0.210[d]	52 ± 16[e]	72
Pinocembrin	0.061[f]	35 ± 19[e]	81
Tiliroside	0.357[g]	37 ± 7[e]	80
Indomethacin	0.125[h]	39 ± 9[e]	79

[a] 50% inhibitory dose.
[b] Ear thickness expressed as the mean difference between thickness before and after challenge ± S.E.M. $n=6$.
[c] Inhibition ratio percentage at 0.5 mg/ear with respect to the control treated only with TPA.
[d] Coefficient of determination (r^2) for the linear regression = 0.9890, $P=0.0111$ (ANOVA test, significant).
[e] $P<0.01$ with respect to the control group (Dunnett's t-test).
[f] $r^2=0.9950$, $P=0.0008$ (ANOVA test, significant).
[g] $r^2=0.9747$, $P=0.0444$ (ANOVA test, significant).
[h] $r^2=0.9995$, $P=0.0231$ (ANOVA test, significant).

With respect to the histological study, the control group of ear samples treated with TPA showed inflammatory lesions characterised by the presence of intraepithelium

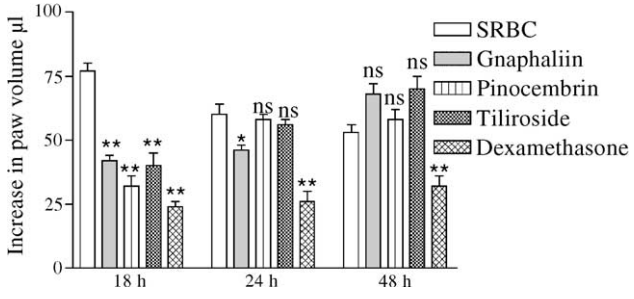

Fig. 7. Effects of flavonoids on sheep red blood cell-induced delayed hypersensitivity. Flavonoids (15 mg/kg) and dexamethasone (10 mg/kg) were administered intraperitoneally immediately before and 16 h after challenge. Footpad volume was measured 18, 24 and 48 h after challenge. $n=6$. Increase in paw volume expressed as the mean ± S.E.M. Statistical significance of difference from the control: $^{**}P<0.01$, $^{*}P<0.05$, ns = not significant by Dunnett's multiple comparison test.

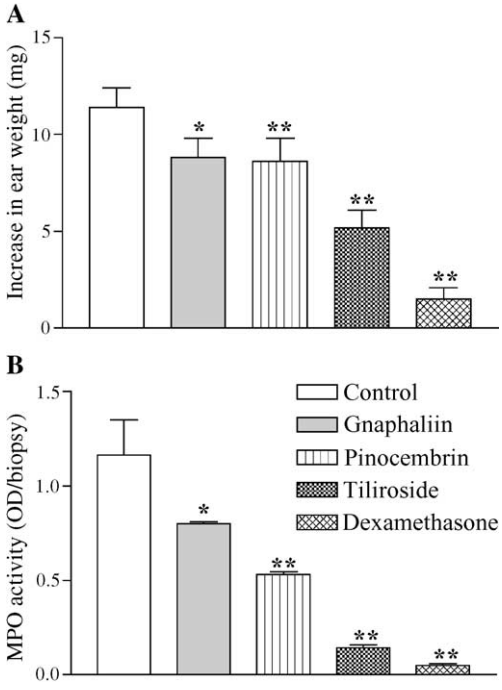

Fig. 8. Effects of flavonoids (repeatedly 0.5 mg/ear) and dexamethasone (repeatedly 0.05 mg/ear) on chronic inflammation induced by TPA in mice (2 µg/ear). $n=5$ animals. (A) Increase in ear weight in mg (mean ± S.E.M.). (B) Neutrophil accumulation assessed as myeloperoxidase activity. Each column with a vertical bar represents the mean for eight ear samples with S.E.M. Statistical significance of difference from the control: $^{**}P<0.01$, $^{*}P<0.05$, ns = not significant by Dunnett's multiple comparison test.

microabscesses, infiltration of polymorphonuclear leukocytes and macrophages in conjuctive tissue, and epidermal hyperplasia, hypertrophy and hyperkeratosis (Fig. 9A). Epithelium thickness was increased by 5.7 ± 0.3 cells and the main infiltrating cells in the skin were neutrophils. The dexamethasone-treated tissues showed no evidence of lesions and epithelium thickness was 4.4 ± 0.2 cells (Fig. 9B). The tiliroside-treated samples (Fig. 9C) showed no, or at best, mild dermal inflammation and lymphocytes were the predominant inflammatory cells. This flavonoid was not capable of reducing the epidermal hyperplasia (7.0 ± 0.2 cells).

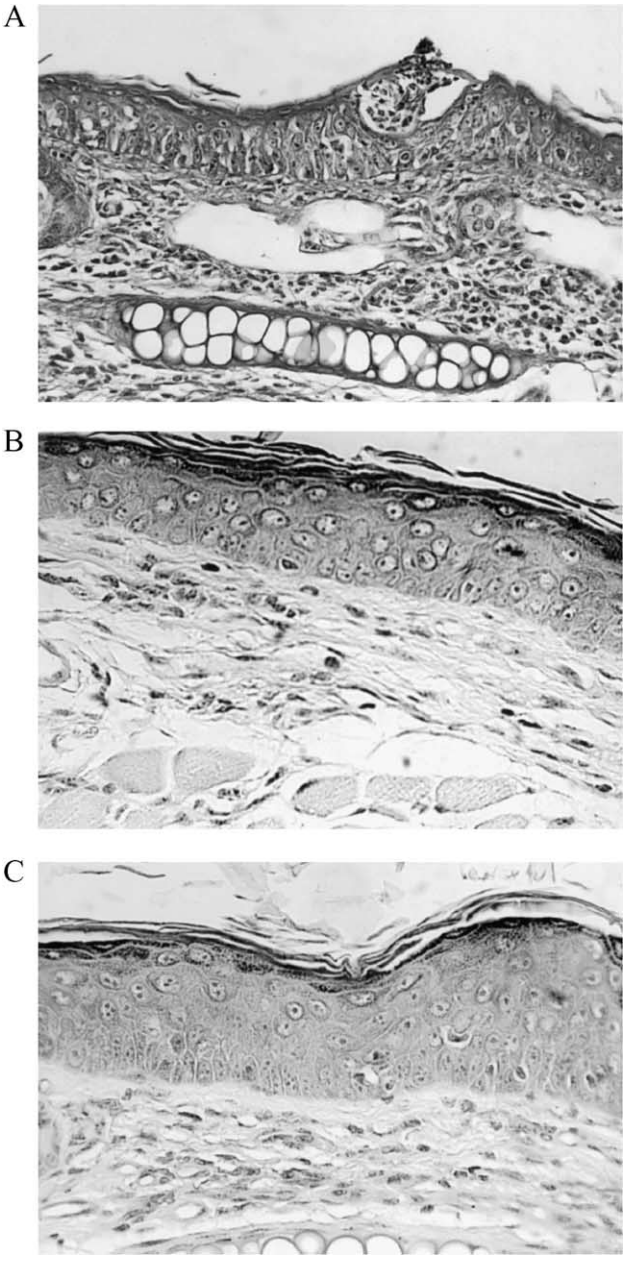

Fig. 9. Ear tissues after repeated application of TPA. (A) Control × 25. (B) Ear treated with dexamethasone, × 25. (C) Ear treated with tiliroside, × 25.

4. Discussion

The flavonoids gnaphaliin, pinocembrin and tiliroside, isolated from the aerial parts of *H. italicum* (Sala et al., 2001), were assayed in vitro and in vivo in order to determine if they are responsible for the antioxidant and the anti-inflammatory activities noted with the active extracts of this medicinal plant.

The aglycones gnaphaliin and pinocembrin do not contain a hydroxyl group in the B ring and show differences at the 3-position in the C-ring. Pinocembrin lacks a 3-hydroxy group, whereas gnaphaliin possesses a methoxyl group in this position. Tiliroside is an acylated glucoside of the antioxidant flavonol kaempferol. Both, tiliroside and kaempferol contains a 4′-hydroxy group in B-ring, while the former has the 3-hydroxy in C-ring blocked by a glucose, which in turn is esterified with a *p*-coumaroyl group.

Gnaphaliin and pinocembrin inhibited enzymatic lipid peroxidation, possibly by inhibiting the enzymes involved in the initiation of lipid peroxidation, whereas it appears that tiliroside also acted by scavenging free radicals. According to previous reports, it seems that the most important requirement for antioxidant activity is the presence of a catechol group in ring B. In fact, compounds possessing this group are the most effective inhibitors of lipid peroxidation ($IC_{50} < 10$ μM) (Middleton et al., 2000). Despite the lack of the catechol moiety in ring B, tiliroside inhibited microsomal lipid peroxidation induced by Fe^{2+}/ascorbate. This was the only compound from *H. italicum* that was active against superoxide radical generation and reduction of the DPPH radical. The pharmacological activity noted with these substances was in accordance with the data reported in the literature for compounds of similar structure (Middleton et al., 2000; Pietta, 2000; Heijnen et al, 2002). Flavanones, such as pinocembrin, are weak antioxidants due to the lack of conjugation of the 2,3 double bond with the 4-oxo group. The presence of the 3-hydroxy group in the heterocyclic ring enhances radical scavenging activity, while the presence of additional hydroxyl or methoxyl groups at positions 3,5 and 7 of rings A and C seems to be less important (Pietta, 2000). Tiliroside, despite being a glycoside, retains the antioxidant capacity of its aglycone, kaempferol and, in fact, has an IC_{50} in the same range (28 and 23.8 μM, respectively) (Mora et al., 1990). This behaviour could be based on additional factors such as the electron-donating effect of other positions (5 and 7, mainly) or the molecular conformation of tiliroside. In this way, tiliroside is not a planar molecule, because of the presence of the acylated glucose, whose conformation is a chair pyran ring. This permits the approximation of the oxo-group from the *p*-coumaroyl residue to the 4′ -hydroxy in ring B, and this could influence the active sites of tiliroside or even constitute an active site, similar to that formed by the oxo-group at C-4 and the near hydroxyl at C-3, in kaempferol (Pannala et al., 2001).

Leukotrienes are molecules involved in inflammation, asthma and allergies, as well as other physiological and pathological processes. The effect of flavonoids on arachidonic acid metabolism via lipoxygenase has been examined by various authors, who have indicated that the antioxidant properties of flavonoids are important for lipoxygenase inhibition (Middleton et al., 2000). When we examined the inhibitory effect of pinocembrin and gnaphaliin on arachidonate metabolism in rat peritoneal leukocytes stimulated with cation ionophore A23187, we observed that both strongly reduced the production of leukotriene B_4. However, neither exhibited antioxidant activity, suggesting that said inhibition was dependent on the interaction of flavonoids with enzymes such as 5-lipoxygenase or phospholipase A_2. Similar behaviour has been reported for methylated flavonoid aglycones such as cirsiliol and sideritoflavone (Alcaraz and Ferrándiz, 1987; Laughton et al., 1991). In contrast, tiliroside stimulated leukotriene B_4 production, while, as explained above, exerting anti-peroxidative activity. To explain this finding, it is suggested that the inhibition of fatty acid peroxidation may lead to higher levels of arachidonic acid, which would be catalytically transformed in leukotrienes, instead of being randomly degraded by spontaneous peroxidation.

The anti-inflammatory effects of flavonoids have been widely demonstrated (see reviews of Manthey, 2000; Middleton et al., 2000), but this is the first report on the activity in vivo of tiliroside and pinocembrin.

The oedema induced by subcutaneous injection of phospholipase A_2 is produced by several types of inflammatory mediators, with mast cell degranulation apparently playing a major role (Cirino et al., 1989; Hartman et al., 1991). The three flavonoids may diminish the oedema induced by this enzyme, principally by preventing histamine or serotonin release. This hypothesis is supported by the fact that most flavonoids inhibit histamine release from human mast cells (Middleton et al., 2000). Tiliroside, the most potent substance in this test, has previously been described as a very potent anti-complement agent in the classical complement pathway (Jung et al., 1998). The activated complement fragments, mainly C3a and C5a, induce the release of mediators from mast cells, causing reactions that resemble those of immediate hypersensitivity. In this way, both mechanisms could be implicated in the anti-inflammatory effect of tiliroside.

The acute inflammation induced by a single dose of TPA is characterised by erythema, oedema and polymorphonuclear leukocyte infiltration, while repeated doses of TPA cause further inflammatory cell infiltration and epidermal hyperplasia. We found that tiliroside was effective in both the acute and chronic TPA models. It inhibited dose-dependently the acute inflammation, although its potency was lower than that of pinocembrin. The chronic inflammation induced by repeated application of TPA is inhibited principally by corticosteroids such as dexamethasone (Stanley et al., 1991). Gnaphaliin, pinocembrin and tiliroside moderately reduced the oedema and markedly decreased the leukocyte infiltration. In our histological study of ear samples treated with tiliroside, the reduction in neutrophil recruitment at the site of inflammation was confirmed. In this way, particular flavonoids had a pronounced inhibitory effect on enzyme systems in activated cells such as neutrophils, T and B cells, etc. (Middleton, 1998).

Reactions to sheep red blood cell-induced delayed-type hypersensitivity were maximal at 18 h and disappeared 48 h after challenge. In this kind of delayed-type hypersensitivity reaction, the allergen enters directly in the dermis and, of the cells present, the T type CD4+ are those that are mainly responsible for the reaction (Grabbe and Schwarz, 1998). Yamada and Sugasawa (1996) suggested that mast cells are involved in the late phase of the delayed-type hypersensitivity induced by sheep red blood cells, and that histamine and leukotrienes could have an important role in this kind of reaction. Among the three compounds, the flavanone, pinocembrin, was the most active. The methoxyflavone, gnaphaliin, and the flavonol glycoside, tiliroside, exerted a weak anti-oedema effect. It has previously been reported that the flavanone glucoside, plantagoside (Yamada et al., 1989), inhibits the in vitro immune response of mouse spleen cells to sheep red blood cells. However, in mice treated with the flavonol glycosides, mauritianin and myricitrin, there is no decrease in the delayed-type hypersensitivity reaction to sheep red blood cells (Takeuchi et al., 1986; Yasukawa et al., 1990).

In conclusion, regarding flavonoid aglycones, the effect of the flavanone pinocembrin should be highlighted because of its relative potency in the acute TPA test and its activity in cell-mediated allergy and leukotriene production. The compound was active even though it lacks some of the chemical features generally considered necessary for the effect of flavonoids. According to the results found for tiliroside, it is probable that the anti-inflammatory effect observed in certain models, principally those in which phospholipase A_2 is implicated, could be related to its antioxidant activity. However, the paradoxical pro-oxidant effect, noted as leukotriene B_4 production in stimulated neutrophils, which is dependent on the activation of 5-lipoxygenase, suggests that other mechanisms may be involved.

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