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Anti-inflammatory glycoterpenoids from *Scrophularia auriculata*

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Abstract

The activity of the four glycoterpenoids: two saponins, verbascosaponin A and verbascosaponin, and two iridoids, scropolioside A and scrovalentinoside, isolated from *Scrophularia auriculata* ssp. *pseudoauriculata*, were studied in different models of acute and chronic inflammation. Both saponins significantly inhibited the mouse paw edema induced by carrageenan and ear edema induced by single and multiple doses of 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Verbascosaponin A showed a potency twice as high as that of indomethacin in the acute TPA model. Verbascosaponin A and scropolioside A were active after a long latency period against ethyl phenylpropiolate edema, as are glucocorticoids. When the putative corticoid-like mechanism of the two compounds was studied, verbascosaponin A activity was notably reduced by the mRNA synthesis inhibitor, actinomycin D, while the effect of scropolioside A was partially interfered with by the anti-glucocorticoid drugs used. Both iridoids were active on the delayed type hypersensitivity reaction. They significantly reduced the inflammatory lesion and suppressed the cellular infiltration.

Keywords: Anti-inflammatory activity; Verbascosaponin A; Verbascosaponin; Scropolioside A; Scrovalentinoside; (*Scrophularia auriculata* ssp. *pseudoauriculata*)

1. Introduction

Diverse saponin-rich crude drugs have been used extensively for their anti-inflammatory properties. The most important of these drugs is probably liquorice root, which contains glycyrrhizin and its aglycone, glycyrrhetinic acid, that have been shown to have corticosteroid-like effects (Edwards et al., 1996). Among the most recent studies in this field is that on the horse chestnut, which decreases the formation of edemas induced by different phlogistic agents and its component, aescine, a mixture of oleanane saponins that prevents neutrophil recruitment, adherence and activation (Bougelet et al., 1998). Another saponin, esculentoside A from *Phytolacca esculenta*, inhibits antibody production by B lymphocytes, phagocytosis, production of anti-inflammatory mediators and cytokines by macrophages (Ju et al., 1994, 1998). As part of our screening program designated to discover anti-inflammatory agents from plant sources, we isolated two saikosaponins from *Heteromorpha trifoliata* that possess anti-inflammatory effects in acute experimental models (Recio et al., 1995b); the zanhasaponins A and B from *Zanha africana*, active as inhibitors of phospholipase A_2 activity (Cuéllar et al., 1997); and three lupane saponins from *Bupleurum fruticescens*, called fruticesaponins A, B and C, which are effective against carrageenan, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and ethyl phenylpropiolate acute edemas (Just et al., 1998).

Certain iridoid-producing plants have often been used as herbal anti-inflammatory remedies. Moreover, iridoid preparations, such as picroliv, and pure iridoid glycosides exhibit anti-inflammatory activity in a wide number of test models. It has been demonstrated that these agents are unstable in presence of acid, β-glycosidases or acylases, and that, in presence of ammonium ions, can be transformed into pyridine monoterpene alkaloids, which have been shown to have anti-inflammatory effects. This conversion can thus determine the activity of some iridoids (Ghisalberti, 1998). Lanhers et al. (1992) demonstrated the efficacy of the roots of *Harpagophytum procumbens* against carrageenan-induced edema, but its major iridoid, harpagoside, was inactive. However, Baghdikian et al.

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(1997) reported on anti-inflammatory activity of this compound when administered i.p. Recio et al. (1994) had previously demonstrated the weak effect of different iridoids against carrageenan edema; loganic acid was the most active, whereas aucubin, loganin and verbenalin, as well as acylated catalpol derivatives, were more effective against TPA ear edema. Other derivatives such as loniceroside A (Lee et al., 1995) and some iridoids from *Fraxinus* species (Ivanovskova et al., 1996) are also reported to be effective against carrageenan and phospholipase A_2 edemas.

Scrophularia auriculata is a medicinal plant used in traditional medicine against inflammatory skin diseases. Its aqueous alcohol extract showed a marked effect on both acute and chronic models of inflammation (Cuéllar et al., 1998). We have reported on the isolation, structural elucidation (Ríos et al., 1991) and topical antiedematous effect of two acylated catalpol derivatives (Giner et al., 1991). Recently, two more rhamnopyranosyl catalpol derivatives, namely scrovalentinoside and scropolioside A, and two saikosaponins possessing oleanane–triterpene skeleton, verbascosaponin A and verbascosaponin, have been described (Giner et al., 1998).

The present study continues our investigation on *S. auriculata*. We examined the effect of scrovalentinoside, scropolioside A, verbascosaponin A and verbascosaponin on different models to rationally determine their value as anti-inflammatory agents in vivo. For this purpose, we have chosen acute and chronic experimental methods that try to reproduce pathological conditions similar to the inflammatory disorders for which the plant is mostly used.

2. Materials and methods

2.1. Animals

Groups of six female Swiss mice weighing 25–30 g were used. All animals were fed a standard diet ad libitum and housed in a temperature controlled room with a 12-h light/dark schedule throughout the experiments, in accordance with the European Union regulations (CEC council 86/609).

2.2. Chemicals

Verbascosaponin A, verbascosaponin, scropolioside A and scrovalentinoside were isolated from the aerial parts of *S. auriculata* L. ssp. *pseudoauriculata* (Senn.) Bolòs et Vigo (*S. valentina* Rouy) as previously reported (Giner et al., 1998). Carrageenan, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), arachidonic acid, ethyl phenylpropiolate, oxazolone, serotonin, actinomycin D, cycloheximide, hydrogen peroxide, *N*,*N*-dimethylformamide, tetramethylbenzidine, hexadecyltrimethyl ammonium bromide, and the reference drugs, indomethacin, nordihydroguaiaretic acid and dexamethasone, were purchased from Sigma, St. Louis and sodium acetate from Panreac, Barcelona.

2.3. Carrageenan-induced mouse paw edema (Sugishita et al., 1981)

Edema was induced on the right hind paw by subplantar injection of carrageenan (3% w/v in saline, 0.05 ml). The glycoterpenoids, dissolved in Ethanol/Tween 80/H_2O (2:2:20, v/v), were administered orally at a dose of 100 mg/kg (0.5 ml), 1 h before carrageenan injection. A control group received vehicle only and a reference group was treated with indomethacin (7 mg/kg, p.o.). The volume of the injected and of the contralateral paws was measured 1, 3 and 5 h after induction of inflammation, using a plethysmometer (Ugo Basile). Edema was expressed as the increase in paw volume due to carrageenan injection, and edema inhibition was expressed as the reduction in volume with respect to the control group.

2.4. Tetradecanoylphorbol acetate (TPA)-induced mouse ear edema (Young and De Young, 1989)

Edema was induced in the ear by topical application of 10 μl of TPA in acetone (2.5 μg/ear) to both the inner and outer surface of one ear of each mouse with a micropipette. The glycoterpenoids, dissolved in 70% aqueous ethanol, were applied topically (0.5 mg/ear), immediately after TPA. The standard drug, indomethacin, was administered at the same dose. The thickness of each ear was measured before treatment and 4 h after induction of inflammation, using a micrometer (Mitutoyo Series 293). Edema was expressed as the increase in ear thickness due to TPA application and edema inhibition was expressed as the reduction in thickness with respect to the control group. The 50% inhibitory dose (ID_{50}) was determined by applying the saponins at four different doses ranging from 0.075 to 0.600 mg/ear.

2.5. Arachidonic acid-induced mouse ear edema (Young and De Young, 1989)

Arachidonic acid was dissolved in acetone at a concentration of 100 mg/ml. Edema was induced in the ear by topical application of 10 μl of arachidonic acid in acetone (2 mg/ear) to both the inner and outer surface of one ear of each mouse with a micropipette. The glycoterpenoids, dissolved in 70% aqueous ethanol, were applied topically (0.5 mg/ear), 30 min before the application of arachidonic acid. A reference group was treated with nordihydroguaiaretic acid (2 mg/ear). The thickness of the ears was measured before treatment and 1 h after induction of inflammation, using a micrometer. Edema reduction was measured and expressed in the same way as for the TPA test.

2.6. Ethyl phenylpropiolate-induced mouse ear edema (Bratssand et al., 1982)

Ethyl phenylpropiolate was dissolved in acetone at a concentration of 50 mg/ml. Edema was induced by topical application of 10 μl of ethyl phenylpropiolate in acetone (1 mg/ear) to both the inner and outer surface of one ear of each mouse with a micropipette. The glycoterpenoids, dissolved in 70% aqueous ethanol (0.5 mg/ear), were applied topically 16 h before and simultaneously with the induction of the ear edema. Dexamethasone, dissolved in acetone (0.05 mg/ear), was applied 16, 8 and 2 h before ethyl phenylpropiolate. The thickness of the ears was measured before treatment and 1 h after induction of inflammation using a micrometer. Edema reduction was measured and expressed in the same way as for the TPA test.

2.7. Block by the anti-glucocorticoid, progesterone, and mRNA or protein synthesis inhibitors of vascular permeability caused by serotonin in mice (Sugishita et al., 1983)

Progesterone (100 mg/kg, 0.1 ml) dissolved in olive oil was administered s.c. into the dorsal area 1 h before the treatment with drugs. Dexamethasone (0.5 mg/kg) dissolved in ethanol/saline (1:19, v/v) and glycoterpenoids (50 mg/kg) dissolved in olive oil were injected s.c. (0.1 ml) into the dorsal area but not near the progesterone site. Actinomycin D (2 mg/kg) or cycloheximide (6 mg/kg), dissolved in physiological saline, was given s.c. simultaneously with the test compounds and again 1.5 h later in an attempt to inhibit mRNA or protein synthesis for 3 h after application of the test compounds. Serotonin (2% w/v in saline, 0.05 ml) was injected into the right hind paw 3 h after administration of the test compounds and the edema was measured 12 min after its induction using a plethysmometer. The left paw received 0.05 ml of saline. Edema reduction was measured and expressed in the same way as for the carrageenan test.

2.8. Mouse ear edema induced by multiple topical applications of TPA (Stanley et al., 1991)

Chronic inflammation was induced by topical application of 10 μl of TPA (2 μg/ear) to both the inner and outer surface of both ears of each mouse with a micropipette on alternate days. The glycoterpenoids were dissolved in 70% aqueous ethanol and applied topically (0.5 mg/ear) twice daily for 4 days, in the morning immediately after TPA application and 6 h later. On the last day, the compounds were applied only in the morning. Dexamethasone was used as the reference drug (0.05 mg/ear). The mice were killed by cervical dislocation 6 h after the last TPA application and two ear punches were taken from each animal. Swelling was assessed in terms of mean weight increase of each ear, and swelling inhibition was expressed as weight reduction referred to the control group. Eight samples placed in hexadecyltrimethylammonium bromide were frozen for the myeloperoxidase assay, and the other four were placed in 4% formalin for preparation of histological sections.

2.9. Oxazolone-induced contact-delayed hypersensitivity mouse ear edema (Young and De Young, 1989)

Female mice were sensitized by topical application to the shaved abdomen of 50 μl of a 2% (w/v) solution of oxazolone in acetone on two consecutive days (days 1 and 2). Challenge was performed on day 6 by application of 30 μl of 2% oxazolone to both the inner and outer surface of both ears of each mouse. Glycoterpenoids (0.5 mg/ear) and dexamethasone (0.05 mg/ear) were applied topically (30 μl) to both ears 6 h after challenge (single application) and 24, 48, 72 and 96 h after challenge (repeated dosage). Ear thickness of treated and control groups was measured with a micrometer 24, 48, 72, 96 and 102 h after challenge and every day just before drug application. The final measurement was performed immediately before the animals were killed. The thickness of each ear was measured as described for the TPA test and activity was expressed as inhibition percentage referred to the control group.

2.10. Myeloperoxidase assay (De Young et al., 1989)

Each ear sample, placed in an Eppendorf tube with 0.75 ml of 80 mM sodium phosphate buffer (pH = 5.4) containing 0.5% hexadecyltrimethylammonium bromide, was homogenized (45 s at 0°C) and decanted into a microfuge tube. After addition of a second 0.75 ml aliquot of hexadecyltrimethylammonium bromide in phosphate buffer saline, the sample was centrifuged at 11 200 g at 4°C for 20 min. The supernatant (30 μl × triplicate) was added to 200 μl of a mixture containing 100 μl of 80 mM phosphate buffer saline, 85 μl of 0.22 M phosphate buffer saline (pH = 5.4) and 15 μl of H_2O_2 0.017% in a 96-well microtiter plate. The reaction was started by adding 20 μl of 18.4 mM tetramethylbenzidine in 8% aqueous dimethylformamide. The mixture was incubated for 3 min at 37°C and then placed on ice. The reaction was stopped by addition of 30 μl 1.46 M NaOAc (pH = 3.0). Enzyme activity was determined colorimetrically using a Labsystems Multiskan MCC/340 plate reader set to measure absorbance at 620 nm.

2.11. 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 to 4 μm sections and stained with hematoxylin–eosin, trichrome stain, periodic acid Schiff and toluidine blue. Epithelium thickness was evaluated using a lens ×100 and expressed

as the mean ± S.E.M. of the number of epidermal layers from the basal to the granulous stratum, both included. A representative area of the inflammatory cellular response was then selected for semiquantitative cell counting with a lens 40×, in 20 fields. The inflammatory cells, lymphocytes, macrophages and neutrophils, were counted in the papillary and in the reticular dermis/subcutis layers and conventionally expressed as 1-2-3 units, according to their relative abundance. Mastocytes were evaluated with a semiquantitative method and expressed as the mean ± S.E.M. of the number of total cells in an area of 1000 μm^2, on a toluidine blue-stained slice.

2.12. Statistics

The data are expressed as means ± S.E.M. Inhibition percentages are calculated from the differences between treated and non-treated tissues, and are referred to the control treated only with the inflammatory agent. One-way analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons of unpaired data was used for statistical evaluation.

3. Results

3.1. Carrageenan-induced mouse paw edema

The oral administration of either saponins or iridoids reduced the carrageenan paw edema. The saponins inhibited the edema by more than 50% at 1 h, and their effect decreased slightly at 3 and 5 h (Fig. 1). Treatment with verbascosaponin A gave a significant swelling inhibition (52% at 1 h), higher than that of indomethacin (34%) and comparable to it in the later phase (3 h, 39% vs. 44%; 5 h, 32% vs. 35%). Of the iridoids, scropolioside A was more active than scrovalentinoside, with 33% and 13% edema inhibition respectively at 3 h, but they produced similar values at 1 h (28% and 26%) and at 5 h (23% and 25%, respectively) (Fig. 1).

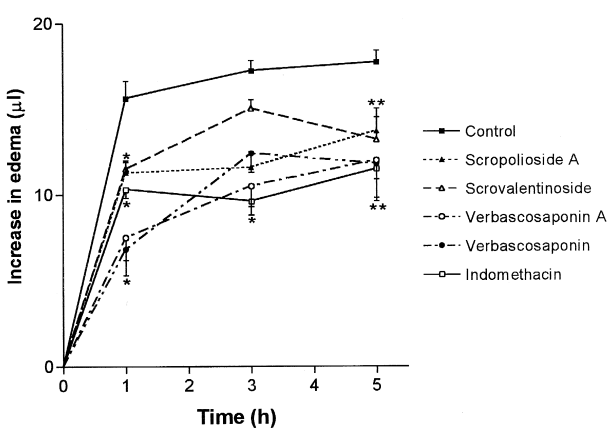

Fig. 1. Effects of glycoterpenoids on carrageenan-induced mouse paw edema. Glycoterpenoids (100 mg/kg) and indomethacin (7 mg/kg) were orally administered. Footpad edema was induced 1 h later by subplantar injection of carrageenan (3% w/v in saline). Footpad volume was measured 1, 3 and 5 h after treatment with the irritant. Each point represents the mean of six increases in footpad volume with S.E.M. Statistically significant difference from the control is expressed by $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$ by Dunnett's multiple comparison test.

Table 1
Anti-inflammatory effect of the glycoterpenoids on acute 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ear edema
TPA (2.5 μg/ear) was topically applied on ear surface simultaneously with test compounds (0.5 mg/ear). Ear thickness was measured before and 4 h after treatment with the irritant.

Product	ID_{50}^{a} (μmol/ear)	ΔEar thickness[b] (μm ± S.E.M.)	I.R.[c]
Control	–	201 ± 15	–
Scropolioside A	–	97 ± 20[d]	52
Scrovalentinoside	–	107 ± 13[d]	47
Verbascosaponin A	0.32[e] (0.42–0.25)	54 ± 6[d]	73
Verbascosaponin	0.18[f] (0.28–0.16)	56 ± 3[d]	72
Indomethacin	0.35[g] (0.43–0.15)	34 ± 11[d]	83

[a] 50% inhibitory dose.
[b] Ear thickness expressed as the mean of the difference between thickness before and after challenge ± S.E.M.
[c] Inhibition ratio percentage with respect to the control treated only with TPA.
[d] $P < 0.01$ with respect to the control group (Dunnett's *t*-test).
[e] r (correlation coefficient) = 0.9995, $P = 0.0208$ (ANOVA test, significant).
[f] $r = 0.9958$, $P = 0.0042$ (ANOVA test, significant).
[g] $r = 0.9993$, $P = 0.0231$ (ANOVA test, significant).

3.2. TPA-induced mouse ear edema

When topically administered, the four glycoterpenoids efficiently suppressed the TPA ear edema (Table 1). Both saponins produced a dose-dependent edema reduction. Their ID_{50} were determined to be 0.32 μmol/ear for verbascosaponin A and 0.18 μmol/ear for verbascosaponin. Both values indicate greater potency than that of indomethacin (0.35 μmol/ear) (Table 1). Both iridoids also inhibited the acute TPA ear edema although to a lesser extent than did the saponins. Scrovalentinoside and scropolioside A reduced it by 52% and 47%, respectively (Table 1).

3.3. Arachidonic acid-induced mouse ear edema

Neither iridoids nor saponins had any appreciable effect on arachidonic acid-induced edema. Scrovalentinoside, scropolioside A and verbascosaponin only produced 6%, 2% and 4% inhibition of the edema, respectively, while verbascosaponin A even increased it. The reference drug, nordihydroguaiaretic acid, reduced the edema by 54%.

3.4. Ethyl phenylpropiolate-induced mouse ear edema

The effect of topically administered glycoterpenoids on ethyl phenylpropiolate ear edema is summarized in Table

2. When saponins were applied 16 h before the irritant, ethyl phenylpropiolate, only verbascosaponin A was active and reduced the edema by 67% (Table 2). The behavior of the two iridoids also differed. Scrovalentinoside produced 56% inhibition, while scropolioside A only gave 37% reduction (Table 2). None of the glycoterpenoids gave significant inhibition of edema when administered simultaneously with ethyl phenylpropiolate. Both iridoids produced 33% edema reduction whereas the saponins were even somewhat inflammatory. The reference drug dexamethasone gave 82% inhibition when applied 16 h before the irritant, 70% inhibition 8 h before, but only reduced the edema by 20% with 2 h pretreatment.

3.5. Serotonin-induced mouse paw edema and block by the anti-glucocorticoid, progesterone, and mRNA or protein synthesis inhibitors of vascular permeability caused by serotonin in mice

The results from the ethyl phenylpropiolate model show that scrovalentinoside and verbascosaponin A may act after a latency period as do glucocorticoids. Therefore, a model of glucocorticoid receptor and genomic effect block was applied to them to determine whether they indeed have a glucocorticoid-like behavior. We had already reported that neither progesterone, actinomycin D nor cycloheximide given alone produced any significant effect on the serotonin-induced paw edema (Recio et al., 1995a). As seen in Table 3, scrovalentinoside and verbascosaponin A reduced the edema induced by serotonin in mouse paw by 73% and 64%, respectively. The anti-inflammatory action of verbascosaponin A against serotonin paw edema was affected by the block of the glucocorticoid receptor with progesterone, was greatly decreased by the mRNA synthesis inhibitor, actinomycin D, and slightly reduced by the protein synthesis inhibitor, cycloheximide. The activity of scrovalentinoside on serotonin-induced edema was also affected in presence of progesterone, actinomicyn D and cycloheximide (Table 3).

Table 2
Anti-inflammatory effect of the glycoterpenoids on ethyl phenylpropiolate-induced ear edema
Test compounds (0.5 mg/ear) were topically applied on ear surface 16 h before ethyl phenylpropiolate (1 mg/ear). Ear thickness was measured before and 1 h after treatment with the irritant.

Product	Δ Ear thickness[a] (μm ± S.E.M.)	I.R.[b]
Control	124 ± 18	–
Scropolioside A	78 ± 12[c]	37
Scrovalentinoside	55 ± 10[d]	56
Verbascosaponin A	41 ± 7[d]	67
Verbascosaponin	122 ± 2[e]	2
Dexamethasone	22 ± 9[d]	82

[a] Ear thickness expressed as the mean of the difference between thickness before and after challenge ± S.E.M.
[b] Inhibition ratio percentage with respect to the control treated only with ethyl phenylpropiolate.
[c] $P < 0.05$ with respect to the control group (Dunnett's *t*-test)
[d] $P < 0.01$ with respect to the control group (Dunnett's *t*-test).
[e] Not significant.

Table 3
Influence of progesterone, actinomycin D and cycloheximide on the anti-inflammatory effects of glycoterpenoids
Glycoterpenoids (50 mg/kg) and dexamethasone (0.5 mg/kg) were s.c. administered. Footpad edema was induced 3 h later by subplantar injection with serotonin (2% w/v in saline) and footpad volume was measured 12 min later. Progesterone (100 mg/kg) was s.c. administered 1 h before treatment with test compounds. Actinomycin D (2 mg/kg) and cycloheximide (6 mg/kg) were given s.c. simultaneously with test compounds, and again 1.5 h later.

Product	Δ Paw volume[a] (μl ± S.E.M.)	I.R.[b]
Control (serotonin)	56 ± 9	–
Serotonin + scrovalentinoside	15 ± 5[c]	73
Serotonin + scrovalentinoside + progesterone	39 ± 4[d]	30
Serotonin + scrovalentinoside + actinomycin D	28 ± 4[e]	50
Serotonin + scrovalentinoside + cycloheximide	34 ± 7[c]	39
Serotonin + verbascosaponin A	20 ± 4[c]	64
Serotonin + verbascosaponin A + progesterone	35 ± 6[e]	37
Serotonin + verbascosaponin A + actinomycin D	47 ± 4[f]	16
Serotonin + verbascosaponin A + cycloheximide	30 ± 4[e]	46
Serotonin + dexamethasone	14 ± 5[c]	75
Serotonin + dexamethasone + progesterone	38 ± 7[f]	32
Serotonin + dexamethasone + actinomycin D	40 ± 2[f]	28
Serotonin + dexamethasone + cycloheximide	43 ± 6[f]	23

[a] Paw volume expressed as the mean of the difference between right and left paw volume ± S.E.M.
[b] Inhibition ratio percentage with respect to the control treated only with serotonin.
[c] $P < 0.01$ with respect to the control group (Dunnett's *t*-test).
[d] $P < 0.05$ with respect to the group treated with the same product in the absence of the inhibitor (Student's *t*-test)..
[e] Not significant
[f] $P < 0.01$ with respect to the group treated with the same product in the absence of the inhibitor (Student's *t*-test).

3.6. Mouse ear edema induced by multiple topical applications of TPA

Both saponins were also active on this model of chronic inflammation. Verbascosaponin A reduced the edema by 67% and verbascosaponin did so by 58% (Fig. 2a). The latter compound caused a parallel decrease of 65% in neutrophil infiltration, detected from the myeloperoxidase activity, while verbascosaponin A only produced a 32% reduction (Fig. 2b). Both iridoids, scropolioside and

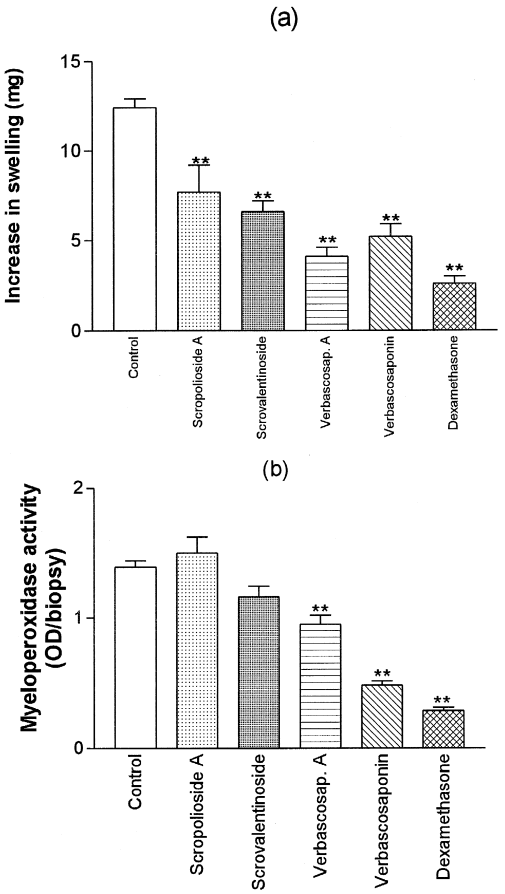

Fig. 2. Effects of glycoterpenoids on chronic inflammation induced by repeated doses of TPA. TPA (2 μg/ear) was topically applied on ear surface on alternate days. Glycoterpenoids (0.5 mg/ear) and dexamethasone (0.05 mg/ear) were topically applied on ear surface for the last 4 days, immediately after TPA application and 6 h later. (a) Ear swelling expressed as the mean weight increase of each ear. (b) Neutrophil accumulation assessed from myeloperoxidase activity. Each column with a vertical bar represents the mean for six animals (a) and eight ear samples (b) with S.E.M. Statistically significant difference from the control is expressed by $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$ by Dunnett's multiple comparison test.

scrovalentinoside A, also inhibited the swelling and reduced it by 38% and 47%, respectively (Fig. 2a), but they did not reduce myeloperoxidase activity (Fig. 2b).

3.7. Oxazolone-induced contact-delayed hypersensitivity mouse ear edema

In this test, in which only corticosteroid drugs are significantly active, verbascosaponin A and verbascosaponin showed a moderate effect (20% and 32% swelling reduction at 24 h, respectively; Fig. 3a). Verbascosaponin A gave a 37% inhibition of myeloperoxidase activity, while verbascosaponin was inactive (Fig. 3b). However, the iridoids were slightly more active than the saponins in both the initial phase and throughout the process. Although the iridoids are isomers, scrovalentinoside was more active than scropolioside A both on sensitization (37% vs. 24% at 24 h) and inflammation (36% vs. 16% at 102 h) (Fig. 3a), while on neutrophil infiltration, scropolioside A was more active (31% inhibition of myeloperoxidase activity) (Fig. 3b).

3.8. Histology

Histological study of non-treated ear samples showed no detectable morphological lesions. They had an epithe-

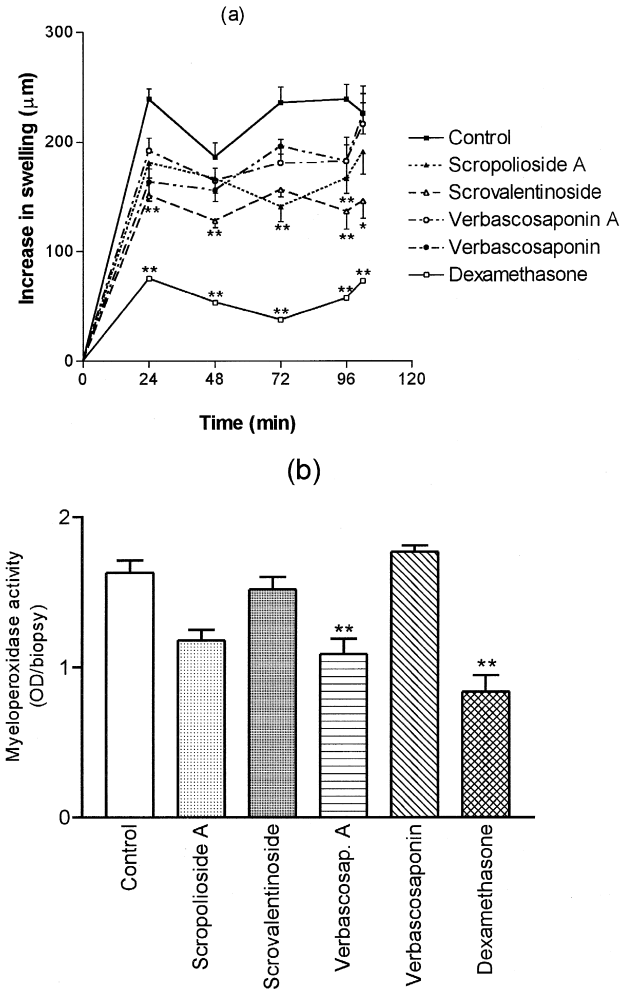

Fig. 3. Effects of glycoterpenoids on delayed-type hypersensitivity induced by oxazolone. Sensitization of dorsal skin with oxazolone (50 μl, 2% w/v in acetone) for 2 consecutive days and subsequent challenge of ears 5 days later with oxazolone (30 μl, 2%). The process resulted in an ear swelling reaction measured at 24, 48, 72, 96 and 102 h after challenge and just before drug application. Glycoterpenoids (0.5 mg/ear) and dexamethasone (0.05 mg/ear) were topically applied on ear surface 6, 24, 48, 72 and 96 h after challenge. (a) Ear swelling expressed as the mean thickness increase of each ear. (b) Neutrophil accumulation assessed from myeloperoxidase activity. Each column with a vertical bar represents the mean for six animals (a) and eight samples (b) with S.E.M. Statistically significant difference from the control is expressed by $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$ by Dunnett's multiple comparison test. (a) Edema formation, (b) neutrophil accumulation. The bars represent the means ± S.E.M. for six animals (a) and eight samples (b). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group (by Dunnett's multiple comparison test).

lium thickness of 2.6 ± 0.1 cells, and the number of mastocytes found in the dermis was 12.1 ± 1.0 cells (Fig. 4).

The control group of ear samples obtained after the multidose TPA test showed inflammatory lesions characterized by the presence of intraepithelium microabscesses, infiltration of polymorphonuclear leukocytes and macrophages in conjunctive, epidermal hyperplasia, hypertrophia and hyperkeratosis. Epithelium thickness was increased (7.5 ± 0.6 cells) as well as the number of mastocytes (11.7 ± 0.1), and the cellular inflammatory ratio was neutrophil–macrophage–lymphocyte (3:1:1) (Fig. 5a). The dexamethasone-treated tissues presented no lesion, epithelium thickness was 5.7 ± 0.3 cells and the number of mastocytes 15.5 ± 0.8 (Fig. 5b). The verbascosaponin-treated samples showed no inflammation or only a mild one, dermal fibrosis, areas with collagen and an increase in fibroblast cells. The epithelium thickness was 5.9 ± 0.3, the number of mastocytes 15.5 ± 0.1 (Fig. 5c). The verbascosaponin A-treated ears presented fibrosis with an increase in collagen and fibroblasts, granulation/scar tissue and angiogenesis. The epithelium thickness was 5.6 ± 0.2 cells, the number of mastocytes 15.3 ± 0.9, and the cellular inflammatory ratio was neutrophil–macrophage–lymphocyte (1:3:1).

In the case of ear samples with delayed-type hypersensitivity induced with oxazolone, the control group biopsies showed a severe inflammatory lesion, affecting both the perichondrium and muscle, and necrotic ulcers extended

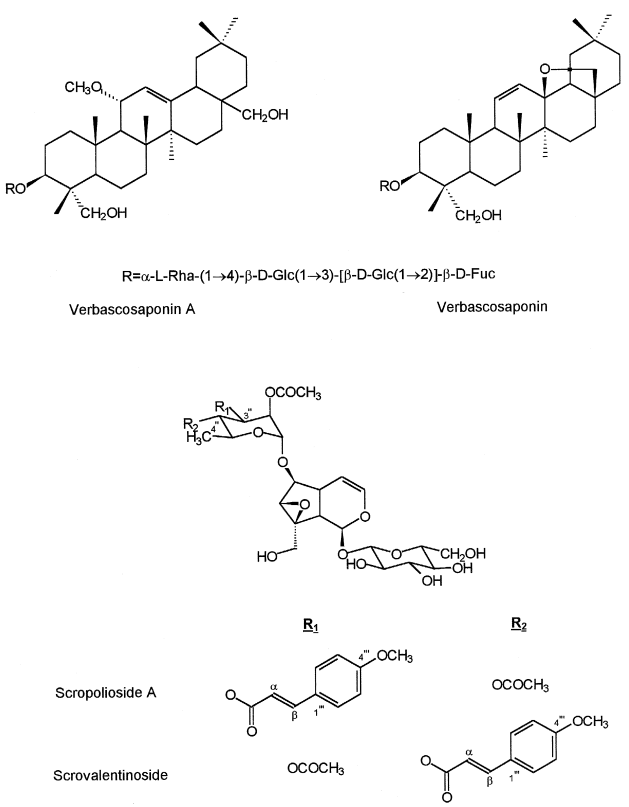

Fig. 4. Chemical structures of the test compounds.

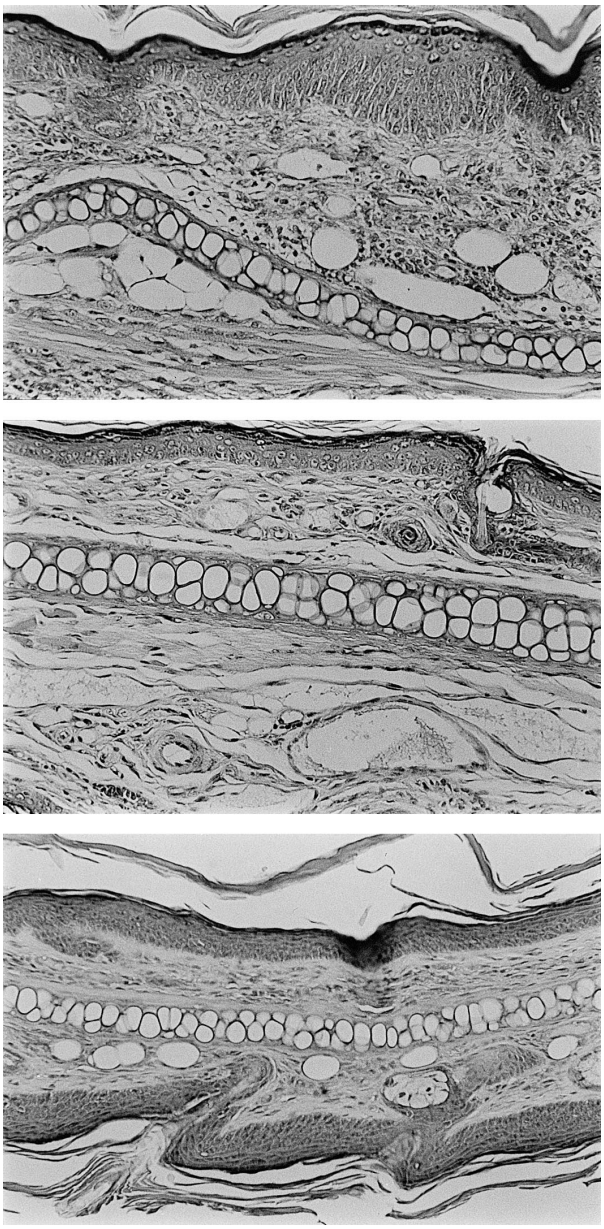

Fig. 5. HE-stained sections of mouse ears (×16) after repeated topical application of TPA. (a) Control: + inflammatory lesion with intraepithelial microabscesses. Diffuse inflammation of the conjunctive tissue by polymorphonuclear leukocytes and macrophagues. Epithelial reaction with hyperplasia and hypertrophia of the dermis. Hyperkeratosis areas. (b) Ears treated with dexamethasone: diffuse lesion in the dermis with increase in fibroblasts and lymphocyte infiltration and few polymorphonuclear leukocytes. (c) Ears treated with verbascosaponin: no inflammation. Dermal fibrosis with increase in fibroblasts.

along the whole surface of the ear. The epithelium thickness was 4.5 ± 1.0 cells, the number of mastocytes 4.9 ± 0.3, and the cellular inflammatory ratio was neutrophil–macrophage–lymphocyte (3:2:1) (Fig. 6a). The dexamethasone-treated tissues presented a mild inflammatory lesion, discrete fibrosis and hyperkeratosis. The epithelium thickness was similar to that of non-treated ears (2.4 ± 0.1

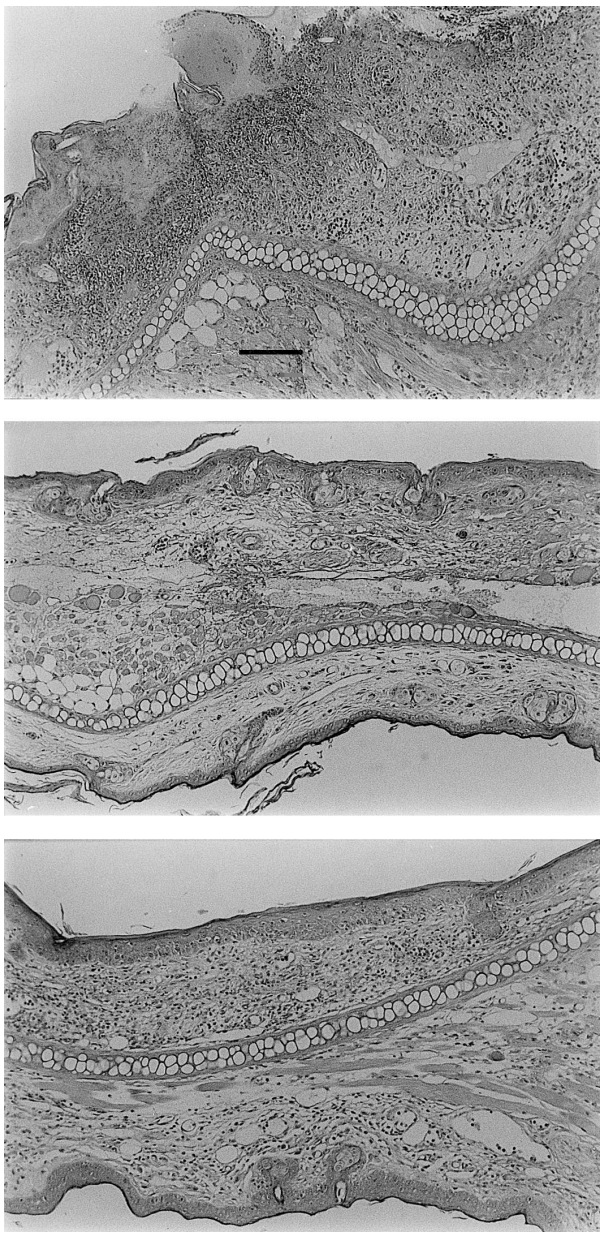

Fig. 6. HE-stained sections of mouse ears (×10) after topically applied oxazolone. (a) Control: ears with severe inflammatory lesion affecting the cartilage (perichondritis) and the muscle (miositis). Necrotic ulcers with inflammatory displasias. (b) Ears treated with dexamethasone: no inflammation. Fibrosis. Isolated ulcers at the root. (c) Ears treated with scrovalentinoside: focal inflammatory lesion. Fibrosis and hyperplasia of epithelium. Ulcers were not observed.

cells) and cellular infiltration was markedly reduced in number and extension. Only a few neutrophils were observed (2/field) (Fig. 6b). The scrovalentinoside-treated biopsies presented histological changes similar to those obtained with dexamethasone treatment, a mild inflammatory lesion, discrete fibrosis and hyperkeratosis. The epithelium thickness was 3.2 ± 0.4 cells and the number of mastocytes 4.9 ± 0.3 (Fig. 6c). The scropolioside A-treated ear samples presented a moderate inflammatory lesion, mixed inflammatory infiltration, hyperkeratosis and discrete fibrosis of the conjunctive layer. The epithelium thickness was slightly greater (3.7 ± 0.3 cells), the number of mastocytes 3.6 ± 1.2, and the cellular inflammatory ratio was neutrophil–macrophage–lymphocyte (2:1:2).

4. Discussion

The four glycoterpenoids, verbascosaponin A, verbascosaponin, scrovalentinoside and scropolioside A assayed on various acute and chronic experimental models showed anti-inflammatory activity against all the inducers with the exception of arachidonic acid. This is the first time that the effects of these saikosaponins and iridoids on diverse inflammatory conditions have been determined, although the activity of some related saponins such as buddleiasaponin I (Bermejo et al., 1998) and the so-called saponins 1 and 2 from *H. trifoliata* (Recio et al., 1995b) have previously been reported.

The intraplantar injection of carrageenan in rats leads to paw edema, the first phase of which results from the concomitant release of histamine, serotonin and kinins and the second phase correlates with the elevated production of prostaglandins, oxygen-derived free radicals, production of inducible cyclooxygenase and local neutrophil infiltration and activation. Verbascosaponin and verbascosaponin A suppressed the edematous response 1h after carrageenan injection, and this effect decreased slightly with time. However, the saikosaponins isolated from *H. trifoliata*, namely saponin 1 (a 3β,16β,23-trihydroxy-13,28-epoxy-olean-11-en derivative) and saponin 2 (a 3β,23,28-trihydroxy-11α-methoxyolean-12-en derivative), had no significant activity per os (Recio et al., 1995b). It seems that our saponins do not undergo gastrointestinal inactivating metabolism or at least, not to the same extent as has been described for other saikosaponins (Shimizu et al., 1985). Only a slight edema reduction was observed when the iridoids were assayed in this model.

The skin inflammation produced by a single application of the protein kinase C activator, TPA, is characterized by erythema, edema and polymorphonuclear leukocyte infiltration, while repeated doses of TPA cause edema, inflammatory cell infiltration and epidermal hyperplasia. The acute inflammation was significantly suppressed by cyclooxygenase, lipoxygenase and phospholipase A_2 inhibitors and corticosteroids, while the chronic process did not respond to the usual cyclooxygenase inhibitors. Verbascosaponin and verbascosaponin A were effective in both the acute and chronic TPA models. They inhibited dose dependently the acute inflammation, and the potency of verbascosaponin was similar to that of indomethacin, while verbascosaponin A was twice as potent (Table 2). Histologically, both saponins reduced the inflammatory lesion and cellular infiltration, but verbascosaponin was more effec-

tive against tissue damage and cellular infiltration. This was further confirmed by the decrease in myeloperoxidase activity. The mechanism of action, however, has not yet been established. It has recently been reported that other closely related saponins such as buddleiasaponin I, isolated from *Scrophularia scorodonia*, and the so-called saikosaponins 1 and 2, from *Bupleurum rigidum* (Bermejo et al., 1998), significantly inhibited the generation of both prostaglandin E_2 and leukotriene C_4 by calcium ionophore-stimulated mouse peritoneal macrophages and thromboxane B_2 release induced by calcium ionophore in human platelets. This led the authors to consider these compounds dual cyclooxygenase/lipoxygenase inhibitors with preponderant anti-lipoxygenase activity (Bermejo et al., 1998).

The application of ethyl phenylpropiolate produces an increase in the vascular permeability with leukocyte ear infiltration into the ears. Ethyl phenylpropiolate ear edema was inhibited by glucocorticoids applied topically 16 h before this irritant because they act after a latency period during which protein synthesis is regulated. The response to ethyl phenylpropiolate was notably reduced by verbascosaponin A, while verbascosaponin was inactive. It must be emphasized that as with dexamethasone, the active products loose activity on their simultaneously application with ethyl phenylpropiolate. Assaying the block of the anti-inflammatory effects of verbascosaponin A on serotonin-induced paw edema was done in order to investigate if the mechanism was possibly related to that of glucocorticoids. Verbascosaponin A was active against serotonin paw edema, but this effect was substantially reduced by the mRNA synthesis inhibitor, actinomycin D. The aforementioned *Heteromorpha* saponins 1 and 2 were also active against ethyl phenylpropiolate ear edema. However, in contrast with the present results, we had found earlier that only one of these compounds, saponin 1, inhibited serotonin paw edema, and this anti-inflammatory action was markedly reduced by mRNA and protein synthesis inhibitors (Recio et al., 1995b). Therefore, the presence of the epoxy bridge and the methoxyl group seems to have no influence on this mode of action. When we used scrovalentinoside with this model, its strong serotonin edema inhibition was partially interfered with by the drugs used. These experimental data suggest that the mechanism of action of verbascosaponin A and scropolioside A may be in part related to that of the glucocorticoids.

The T cells are the main kind of leukocytes involved in delayed hypersensitivity, a process for which oxazolone-induced inflammation is a key model. This test is useful in the search for drugs that could inhibit the inflammation and tissue destruction caused by the type IV allergic reaction. In contrast with the results obtained in the other models assayed, the ear swelling due to oxazolone-induced delayed-type hypersensitivity was reduced by the pair of iridoid isomers, scrovalentinoside and scropolioside A, and the saikosaponins were not active. Both iridoids, but especially scrovalentinoside, were active both in the early stage and throughout the inflammatory process and decreased the edema. Scrovalentinoside produced histological changes similar to those with dexamethasone, reduced the inflammatory lesion and suppressed the cellular infiltration (macrophages and neutrophils). This demonstrates the efficacy of these iridoids on the delayed-type hypersensitivity reaction. However, it is strange that other catalpol–iridoid derivatives such as scropolioside A, koelzioside and 6-*O*-(3″-*O*-*p*-methoxy-cinnamoyl)-α-L-rhamnopyranosyl catalpol, have been described as immunostimulants, because they increased the macrophage migration index, a parameter which has been correlated with macrophage activation and delayed-type hypersensitivity response (Garg et al., 1994). Moreover, harpagoside, a 8β-oxy-8α-methyl derivative, also exhibited induction of an immune response (Garg et al., 1994). Nevertheless, it should be noted that other iridoids such as hydramacrosides A and B behaved as antiallergic principles that inhibited the histamine release induced from mast cells by an antigen–antibody reaction (Yosikawa et al., 1994), and arbortristosides A and C were active on passive cutaneous anaphylaxis and presented mast cell stabilizing activity (Gupta et al., 1995).

In conclusion, the four glycoterpenoids, verbascosaponin A, verbascosaponin, scrovalentinoside and scropolioside A are the main anti-inflammatory compounds from *S. auriculata* ssp. *pseudoauriculata*. The saponins are more effective in the acute models of inflammation while the iridoids are more effective with the delayed-type hypersensitivity reaction.

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