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Effectiveness of *Vernonia scorpioides* ethanolic extract against skin inflammatory processes

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ABSTRACT

Ethnopharmacological relevance: Vernonia scorpioides (Asteraceae) is a native Brazilian medicinal plant that is commonly used to treat skin disorders. Considering the traditional use of *Vernonia scorpioides* and the lack of information about its pharmacological properties, we investigated the topical anti-inflammatory effect of the ethanolic extract of *Vernonia scorpioides* (EEVS) on acute and chronic cutaneous inflammation models in mouse.

Materials and methods: The topical anti-inflammatory effect of EEVS was evaluated against acute models (12-O-tetradecanoylphorbol acetate (TPA)- and arachidonic acid (AA)-induced mouse ear oedema) and chronic models (multiple applications of croton oil).

Results: The EEVS caused a dose-related inhibition of oedema in both the TPA- and AA-induced acute models (DI_{50} = 0.24 and 0.68 mg/ear with an inhibition of 80 ± 5% and 65 ± 5%, respectively, for 1 mg/ear). In addition, the TPA-induced increase in myeloperoxidase activity (MPO) in the ear was reduced (77 ± 8%) by the topical application of EEVS. In the chronic model, the EEVS reduced all parameters evaluated: oedema formation (31 ± 2%), epidermal hyperproliferation (histology) and MPO (25 ± 10%). However, the topical treatment of EEVS had no effect on N-acetyl-β-D-glucosaminidase activity. The EEVS effectively interfered in the ear oedema on the delayed-type hypersensitivity reaction induced by oxazolone. The topical treatment with EEVS performed on both phases or only on the elicitation phase caused the inhibition of the ear oedema-induced by oxazolone in 42.9% and 63.4%, respectively, when compared to control animals (sensitized and challenged).

Conclusions: The results suggest that EEVS is effective as a topical anti-inflammatory agent in acute and chronic inflammatory processes and that its action is markedly influenced by the inhibition of neutrophil migration into inflamed tissue as well as by epidermal hyperproliferation.

1. Introduction

Medicinal plants are widely used to treat skin disorders. The efficacy of certain plants, such as *Matricaria recutita*, *Hamamelis virginiana*, *Arnica montana* and *Calendula officinalis* has been established by pre-clinical and clinical studies (Bedi and Shenefelt, 2002).

Several species of genus *Vernonia* (Asteraceae) are used in traditional medicine to treat various ailments (Johri and Singh, 1997). Recent studies focusing on the anti-inflammatory (Mazumder et al., 2003), anti-pyretic (Gupta et al., 2003), anti-cancer (Izevbigie et al., 2004) and anti-malarial activities of several *Vernonia* species have been published. *Vernonia scorpioides* (Lam.) Pers. is a Brazilian herb that grows in poor and deforested soils all over the country (Leite et al., 2002), and it is used topically by native people to treat a variety of skin conditions, such as allergies, skin parasites, irritations, skin injuries, itching and chronic wounds, including ulcers of the lower limbs (Pagno et al., 2006). Pharmacological studies of *Vernonia scorpioides* indicated the fungicidal activity of the plant's crude extract as well as its chloroform and hexane fractions (Freire

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et al., 1996); mild wound healing effects for its ethanolic extract (Leite et al., 2002) and antitumoural activity for its dichloromethane fraction (Pagno et al., 2006).

Members of the genus *Vernonia* are sources of sesquiterpene lactones, typical constituents of the Asteraceae family, which have been identified as the active compounds in a variety of medicinal plants used in traditional medicine for the treatment of inflammatory diseases (Valério et al., 2003). In addition, phytochemical studies have reported the presence of compounds, such as flavonoids, steroids and polysaccharides, which contribute to the anti-inflammatory and immunomodulating activity of some medicinal plants from the genus *Vernonia* (Huang et al., 2003; Tchinda et al., 2003; Nergard et al., 2004). These compounds may act in several inflammatory pathways or interact with mediators involved in the inflammatory response, such as arachidonic acid metabolites, cytokines, nitric oxide, cyclooxigenase-2, phospholipase A_2, nuclear factor-κB and others (Calixto et al., 2003), making the plants of Asteraceae family promising as a new therapy for inflammatory skin conditions.

Skin is well-known for its functional role as a protective physical barrier. It is now clear that skin is far more than a mere container but rather is a dynamic organ that has other recognised functions, such as endogenous homeostasis, metabolism and sensory input. In addition, skin actively participates in immunological regulatory processes and inflammatory responses (Bos, 1997). However, some inflammatory or immunological reactions lead to chronic inflammation processes, such as psoriasis or to intolerable skin inflammation conditions, such as contact dermatitis, which requires medication (Robert and Kupper, 1999).

Considering the traditional use of *Vernonia scorpioides* on skin disorders and that no information is available about its topical anti-inflammatory properties, we investigated the topical anti-inflammatory effect of the ethanolic extract of *Vernonia scorpioides* (EEVS) on acute and chronic cutaneous inflammation models in mice.

2. Materials and methods

2.1. Plant material

Aerial components (leaves and flowers) of *Vernonia scorpioides* (Lam.) Pers. (Asteraceae) were collected in November 2005 from wild specimens in a "restinga" forest (a distinct type of coastal tropical and subtropical moist broadleaf forest) in Navegantes (SC), and the identity of the specimens was confirmed by Dr Ana Claudia Araújo (Universidade do Vale do Itajaí, Santa Catarina, Brazil). Voucher specimens [M. Biavatti 11 (15/03/01)] were deposited at the Herbário Barbosa Rodrigues (Itajaí, Santa Catarina, Brazil).

2.2. Chemicals

For this study, 12-O-tetradecanoylphorbol acetate (TPA), 99% arachidonic acid (AA), croton oil, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone), dexamethasone acetate, indomethacin, sodium phosphate-buffered saline (PBS), hexadecyltrimethylamonium bromide (HTAB), tetramethylbenzidine HCl (TMB), N,N-dimethylformamide (DMF), p-nitrophenyl-acetamide-μ-D-glucopyranoside, glycine and foetal bovine serum (FBS) were obtained from Sigma–Aldrich Co. (St. Louis, USA). Anti-mouse CD45/FICT was purchased from BD Biosciences Pharmingen (San Diego, USA), and acetone, absolute ethanol, dimethylformamide, formaldehyde, acetic acid, sodium citrate and sodium acetate were obtained from Merck Biosciences (Bad Soden, Germany). Hydrogen peroxide (H_2O_2), eosin, hematoxyline, floxine B and xylol were purchased from Vetec (Rio de Janeiro, Brazil).

2.3. Preparation of extract

The fresh leaves and flowers of the plant (3 kg) were crushed with bidistilled ethanol (6 L) in a domestic mixer and macerated for 7 days in the absence of light, and the extract obtained was filtered and dried under vacuum using a Rotary Vacuum Evaporator (Quimis, São Paulo, Brazil), resulting in 37 g of a crude extract (1.2%, w/w), named ethanol extract of *Vernonia scorpioides* (EEVS). An aliquot of the crude extract obtained was used to perform the pharmacological assays; each aliquot was properly diluted in acetone (20 μL) to reach the selected dose.

2.4. Pharmacological assays

2.4.1. Animals

Experiments were performed using groups of 5 male Swiss mice (20–30 g) that were kept at a controlled room temperature (22 ± 1 °C) and under a 12 h light/dark cycle (lights on at 07:00 h) with water and food given *ad libitum*. Experiments were performed in accordance with guidelines specified by the Ethics Committee on Animal Experimentation, and the experimental protocol was approved by the Institution Ethics Committee for Animal Use (protocol number 127, UFPR) in accordance with international guidelines.

2.4.2. TPA-induced mouse ear oedema

TPA-induced ear swelling in mice was performed according to the method described by Young et al. (1983). Ear oedema was induced on the right ear by topical application of 2.5 μg/ear of TPA dissolved in 20 μL acetone. Both EEVS (0.003–1 mg/ear) and dexamethasone (0.05 mg/ear), which was used as positive control, were also dissolved in 20 μL of acetone and were applied after the TPA. Ear thickness was measured before and 6 h after induction of inflammation using a digital micrometer (MT-045B, Shangai Metal Great Tools Co., Ltd., Shangai, China). Oedema was expressed as the difference between the basal ear thickness and the ear thickness after 6 h of TPA application. Animals were euthanised 24 h after TPA treatment, and tissue samples (6 mm) were removed from the animals' right ears and stored at −70 °C until use in the myeloperoxidase assay (MPO).

2.4.3. Multiple croton oil application-induced mouse ear oedema

Croton oil is a relatively crude mixture of many constituents, including phorbol esters, which elicit skin inflammation and hyperproliferative responses in animals. Chronic inflammation was induced by the application of 0.4 mg/ear of croton oil dissolved in 20 μL of acetone on alternated days for 9 days (Stanley et al., 1991). EEVS was topically applied (1 mg/ear) twice a day during the last 4 days of the experiment. Dexamethasone was used as the reference drug (0.05 mg/ear). Oedema was expressed as the change in ear thickness due to croton oil application, and ear thickness was measured every day over the 9-day experiment. The animals were euthanised on day 9, and ear samples (6 mm) were removed and stored at −70 °C for use in the following assays.

2.4.4. Tissue MPO assay

MPO is an enzyme present in the intracellular granules of neutrophils, and it can be used as a marker for the influx of polymorphonuclear leucocytes into inflamed tissues. MPO activity was evaluated according to the method proposed by Bradley et al. (1982) and modified by De Young et al. (1989). Mice were sacrificed 24 h after a single TPA application and 6 h after the last application of croton oil on day 9. Each ear sample (6 mm) was placed in 0.75 ml of 80 mM sodium phosphate buffer (PBS, pH 5.4) containing 0.5% hexadecyltrimethylamonium bromide (HTAB). Next, the sample was homogenised (45 s at 0 °C) in a motor-driven homogeniser.

Homogenate was decanted into a microfuge tube, and the washes from a second 0.75 ml aliquot of HTAB in PBS were added to the tube. The 1.5 ml mixture was centrifuged at 11,200 × *g* at 4 °C for 20 min. The supernatant samples (triplicates of 30 μL) were added to 96-well microtitre plates. For the assay, 200 μL of a mixture containing 100 μL of 80 mM PBS (pH 5.4), 85 μL of 0.22 M PBS (pH 5.4) and 15 μL of 0.017% H_2O_2 were added to the wells. The reaction was started by the addition of 20 μl of 18.4 mM tetramethylbenzidine HCl (TMB) in dimethylformamide. The mixture was incubated for 3 min at 37 °C, and the reaction was subsequently stopped by the addition of 30 μL of 1.46 M sodium acetate (NaOAc, pH 3.0). Enzyme activity was determined colourimetrically using a plate reader (EL808B, BioTech Instruments, INC, Winooski, VT, USA) to measure absorbance at 620 nm, and the results were expressed as mDO per biopsy.

2.4.5. Tissue N-acetyl-β-D-glucosaminidase (NAG) assay

According to the method used by Sanchez and Moreno (1999), ear samples (6 mm) were treated using the same method described for the MPO assay. The supernatant samples (triplicates of 25 μL) were added into 96-well microtitre plates. For the assay, 25 μL of p-nitrophenyl-acetamide-μ-D-glucopyranoside (2.24 mM) and 100 μL of 50 mM buffer citrate (pH 4.5) per well were used. The mixture was incubated for 60 min at 37 °C, and the reaction was stopped by the addition of 100 μL of 200 nM buffer glycine (pH 10.4). The enzyme activity was determined colourimetrically using a plate reader (EL808B, BioTech Instruments, INC, Winooski, VT, USA) to measure absorbance at 405 nm, and enzyme activity was expressed as mDO per biopsy.

2.4.6. Flow cytometric analysis of cell surface marker (CD_{45}) on leucocyte population

Ear tissue samples were placed in 1 ml of 80 mM sodium phosphate buffer (PBS, pH 5.4), homogenised in low temperature in a motor-driven homogeniser and filtered with a filcon filter (100 μm pore size). Pooled cells (10^6) were incubated with an antibody specific against anti-CD_{45}/FICT, a common leucocyte marker, in PBS/1% FBS for 30 min and washed three times with PBS. Ten thousand cells per sample were analysed by CellQuest software (BD, Franklin Lakes, USA) in a FACSCalibur (BD, Franklin Lakes, USA) flow cytometer, and the experiments were analysed using WinMDI 2.9 software (Miscellaneous software, The Scripps Research Institute, CA, USA).

2.4.7. Histology

Ear samples were fixed in ALFAC (formaldehyde, ethanol 80%, glacial acetic acid) solution. Each sample was cut longitudinally into equal halves. Half of each sample was embedded in paraffin, cut into 5 μm sections and stained with hematoxylin–eosin. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response at magnifications of 200× and 400×.

2.4.8. Arachidonic acid (AA)-induced mouse ear oedema

AA-induced ear oedema in mice was achieved according to the method used by Young et al. (1984). AA (2 mg) was dissolved in 20 μL of acetone and applied to the right ear of the animals. EEVS (0.03–1 mg/ear) and indomethacin (2 mg/ear, reference drug) were applied topically after the application of AA (2 mg/ear). Ear thickness was measured before and 1 h after induction of oedema. Oedema was measured as indicated above and expressed as an increase in the ear thickness due to AA application.

2.4.9. Oxazolone induced contact-delayed-type hypersensitivity (DTH)

Oxazolone-induced dermatitis induction was achieved according to the previously published method used by Fujii et al. (2002). To induce DTH, mice were sensitized by the application of 2% oxazolone in acetone (50 μL) on the shaved abdomen skin for two consecutive days, and this treatment was followed by the application of EEVS (1 mg/site, 50 μL) or the reference drug dexamethasone (0.05 mg/site, 50 μL) on the same region. After 6 days, the challenge was performed by the application of 30 μL of 2% oxazolone in acetone on both sides of the mouse ear (elicitation phase). EEVS (1 mg/ear) and dexamethasone (0.05 mg/ear) were topically applied (30 μL) 1, 24, 36, 48, 60, 72, 84 and 96 h after the challenge with oxazolone, and the ear thickness was measured each 24 h for 4 days using a digital micrometer as described above.

2.5. Statistical analysis

Results are presented as means ± S.E.M. except the ID_{50} values (i.e., the dose of EEVS that reduces the inflammatory response by 50%, relative to the control), which are reported as geometric means accompanied by their respective 95% confidence limits. Data were subjected to analysis of variance (ANOVA) followed by a post hoc Newman–Keuls test. The accepted level of significance for the test was $P < 0.05$. The ID_{50} values were determined by linear regression from individual experiments using the GraphPad Software (Prism version 3.0, San Diego, CA, USA).

3. Results

3.1. Effect of EEVS on TPA-induced mouse ear oedema and tissue MPO assay

Topical application of the vehicle (acetone) did not alter the ear thicknesses of the mice (data not shown), but the application of TPA promoted an increase in ear thickness. However, the application of EEVS caused significant and dose-dependent inhibition of the TPA-induced skin oedema. Calculated mean ID_{50} value for this EEVS inhibition was 0.24 (0.1–0.55) mg/ear with a maximal inhibition of 80 ± 5% (1 mg/ear). Topical application of EEVS also reduced the MPO activity with a maximum effect of 77 ± 8% (1 mg/ear). The reference drug dexamethasone (0.05 mg/ear) also caused a significant inhibition of 88 ± 1% and 80 ± 3%, respectively, for oedema and MPO activity (Fig. 1).

3.2. Effect of EEVS on the croton oil multiple application-induced mouse ear oedema and cellular infiltration

When tested after the establishment of skin inflammation, the chronic treatment with EEVS (1 mg/ear, 2× per day, 4 days) and dexamethasone (0.05 mg/ear, 2× per day, 4 days) promoted a significant inhibition of the oedema induced by croton oil on day 9 (31 ± 2% and 30 ± 9%, respectively) (Fig. 2A). Repeated treatment of EEVS also caused a moderate inhibition of MPO activity (25 ± 10%) compared to the standard substance dexamethasone (57 ± 8%) (Fig. 2C). As shown in Fig. 2B, the NAG activity was not modified by EEVS treatment; however, the activity of this enzyme was inhibited by dexamethasone treatment, undergoing a reduction to 25 ± 7% (Fig. 2B).

3.3. Flow cytometric analysis of leucocyte infiltration in the croton oil multiple applications model

EEVS's control over the cellular infiltration in a chronic inflammatory process was confirmed by the quantification of total leucocytes in the inflamed tissue. Flow cytometric analysis revealed a markedly increased leucocyte count in the control group compared to the vehicle group (36% vs 2.6%) as shown in Fig. 3A and B, respectively. This analysis also showed a reduction of leucocyte numbers in groups treated topically with EEVS and dexamethasone (27% and 23%, respectively).

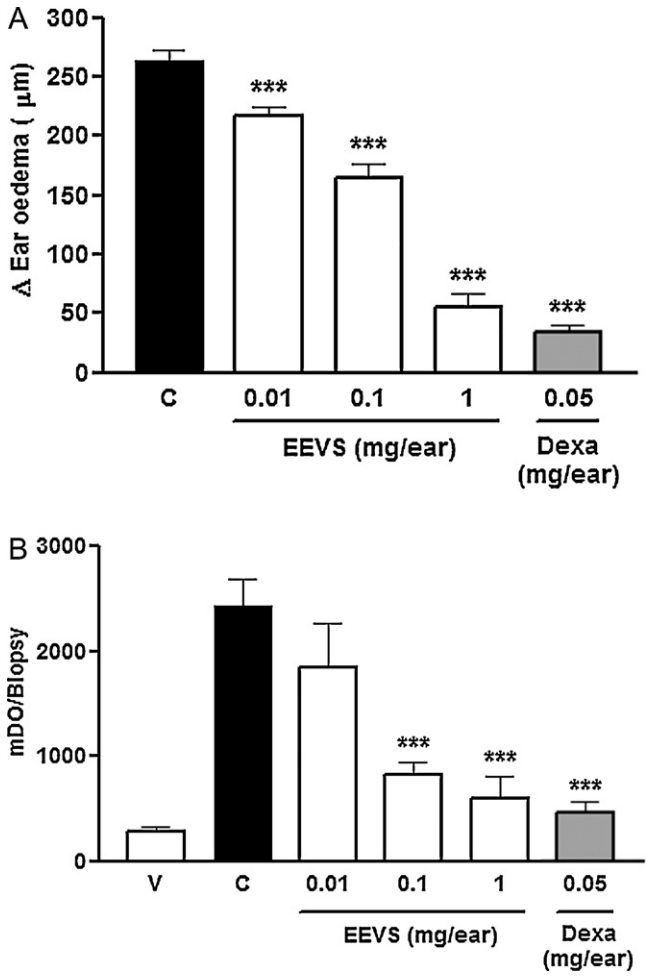

Fig. 1. Topical effect of ethanolic extract of *Vernonia scorpioides* (EEVS) and dexamethasone (Dexa) on TPA-induced ear oedema (A) and MPO activity on TPA-treated ears (B). Ear oedema and MPO activity were measured at 6 and 24 h after TPA treatment, respectively. The control group only received acetone topically after the challenge with TPA (C). The vehicle group (V) was only acetone applied. The bars represent the mean ± S.E.M. for 5–6 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from control (C), $^{***}p < 0.001$.

3.4. Histological analysis

Repeated treatment with the vehicle showed no alterations in cutaneous morphology in the histological analysis (Fig. 4A). Multiple applications of croton oil induced an intense increase in the ear diameter (oedema formation), epidermal hyperproliferation (acanthosis) and leucocyte infiltration into the dermis (Fig. 4B). The EEVS treatment as well as the positive control dexamethasone (0.05 mg/ear) markedly reduced the keratinocyte hyperproliferation caused by repeated croton oil application, thereby reducing acanthosis formation. Similar treatments of EEVS (1 mg/ear) or dexamethasone also suppressed other inflammatory parameters, such as oedema formation and leucocyte infiltration (Fig. 4).

3.5. Effect of the ethanolic extract on the AA-induced mouse ear oedema

Topical application of AA on the right ear produced significant oedema formation peaking at 1 h after challenge. The EEVS application exhibited a significant and dose-dependent inhibition of the AA-ear oedema formation. The estimated mean ID_{50} for EEVS derived from the oedema inhibition study was 0.68 (0.41–1.13) mg/ear with a maximal inhibition of 60 ± 8% (1 mg/ear). Similarly, the reference drug indomethacin (2 mg/ear) also caused a significant inhibition of AA-induced ear oedema, showing a maximal inhibition of 65 ± 5%. The results obtained are shown in Fig. 5.

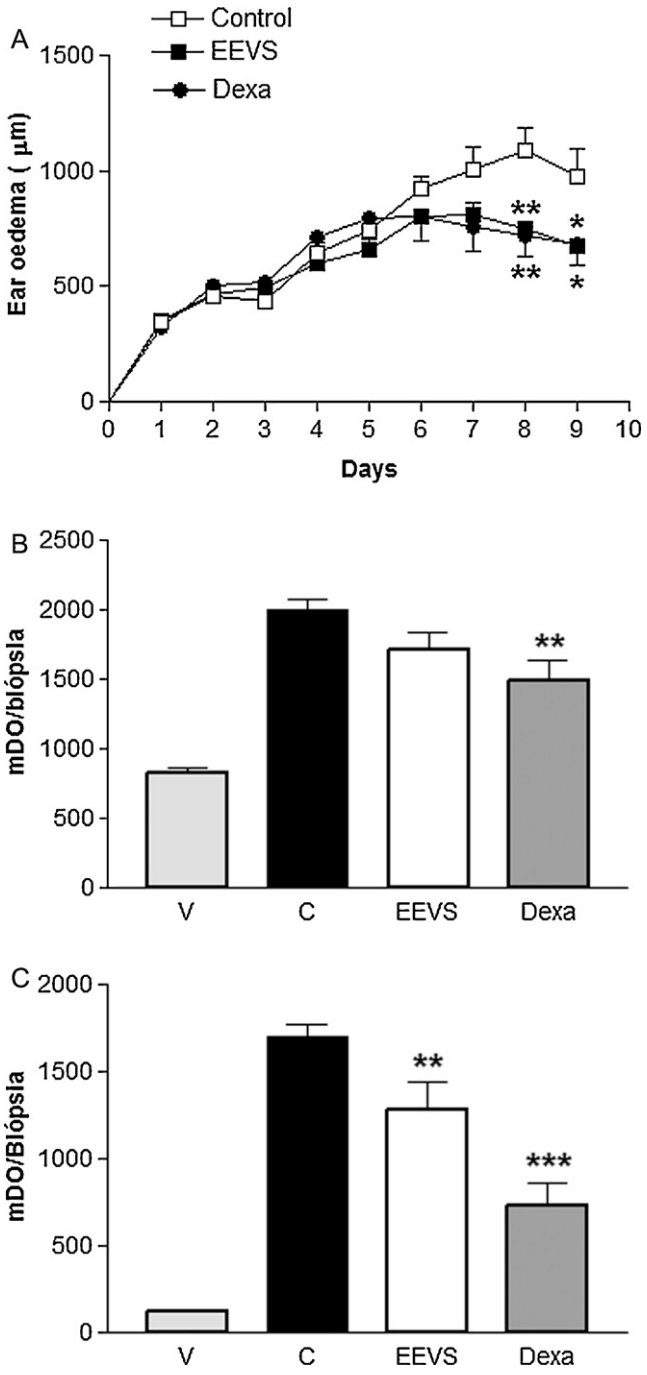

Fig. 2. Topical effect of *Vernonia scorpioides* ethanolic extract (EEVS) on the croton oil multiple application-induced mouse ear oedema. The chronic inflammation process was induced by multiple applications of croton oil (0.4 mg/ear) in alternated days during 9 days. The EEVS (1 mg/ear) and dexamethasone (0.05 mg/ear) were applied topically during the last 4 days (2× per day) of the experiment (indicated by the arrow), and the ear thickness was measured daily (A). In the last day of the experiment, tissue samples from the ears were collected to evaluate NAG (B) and MPO activity (C). The vehicle group (V) received only acetone. The bars represent the mean ± S.E.M. for 5–6 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from control (C), $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0,001$.

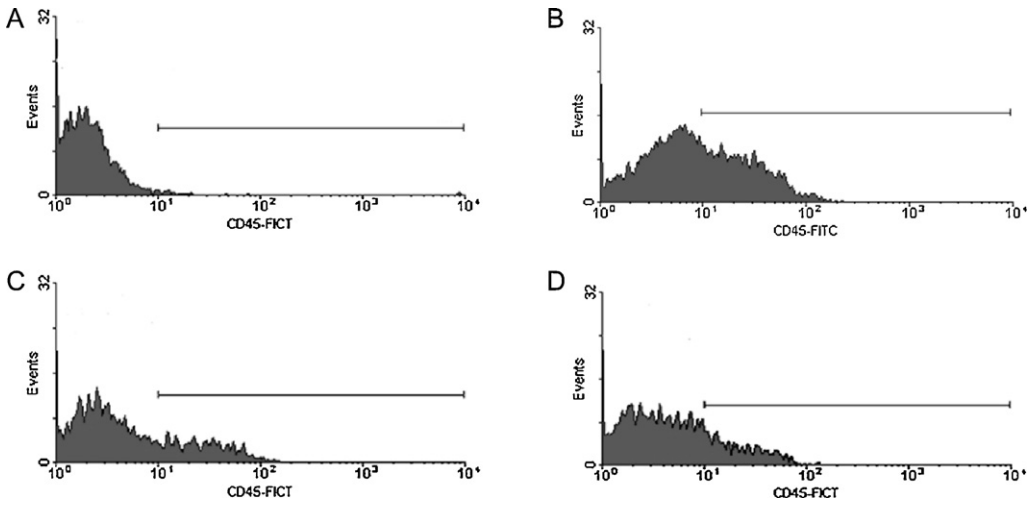

Fig. 3. Flow cytometric analysis of leukocytes infiltration in the ear caused by croton oil multiple applications. Ear tissue were collected on the last day of the experiment (day 9) and homogenised. Pool of the ears (10^{-6} cells/group, $n = 5$) were processed and incubated with an anti-CD_{45}/FICT and acquired by flow cytometer. The pool analysed showed that 2.6%, 36%, 27% and 23% among of cell types represents leukocytes in the vehicle group (A), control group (B), group topically treated with EEVS (1 mg/ear) (C) or dexamethasone (0.05 mg/ear) (D), respectively.

3.6. Effect of EEVS on oxazolone-induced contact-delayed-type hypersensitivity

Sensitisation with oxazolone elicited a marked increase in ear oedema that persisted until 96 h after the challenge; however, this effect was not observed in the non-sensitized group. Ear oedema was potently suppressed by EEVS (1 mg/ear), even in the first 24 h after challenge, showing an inhibition of 42 ± 9% with this effect lasting until 96 h. The positive control dexamethasone (0.05 mg/site) showed a similar effect (Fig. 6A). Unexpectedly, treatment with EEVS and dexamethasone at the same doses during the sensitisation phase alone was unable to avert the oedema formation when the animals were re-exposed to oxazolone (Fig. 6B). Treatment with these agents in the elicitation phase (challenge) alone was able to inhibit ear oedema formation, reproducing the results obtained when the animals were treated on both phases of oxazolone exposition (Fig. 6C).

4. Discussion and conclusion

For the first time, we demonstrate the topical anti-inflammatory activity of the EEVS in acute and chronic skin inflammation. The phlogistic agents used in our experiments have long been accepted

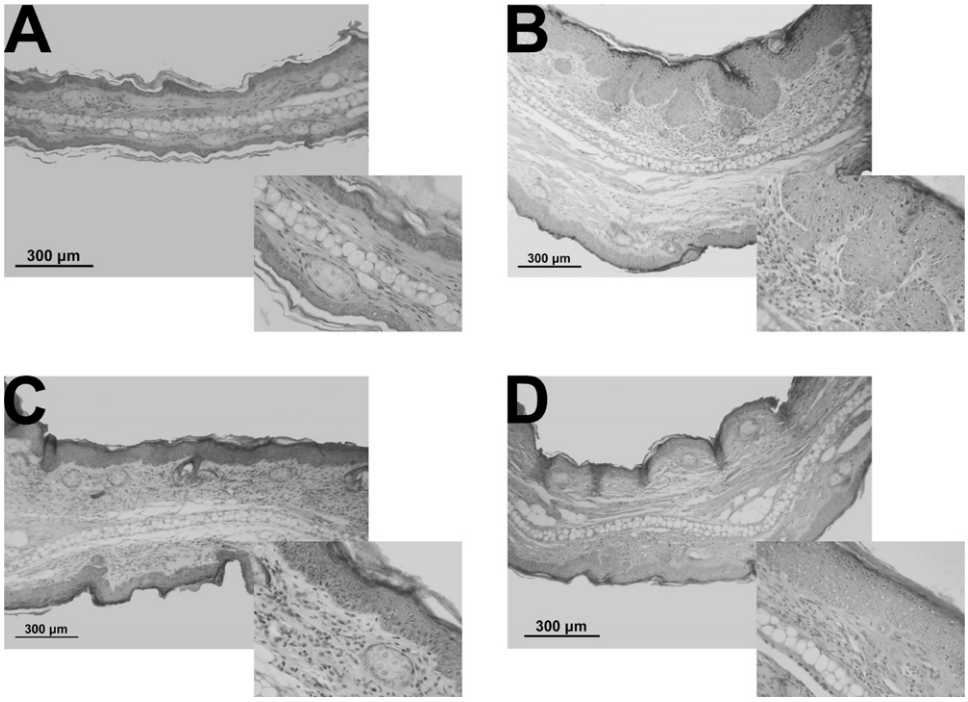

Fig. 4. Histological analysis of *Vernonia scorpioides* ethanolic extract effect on the croton oil multiple applications. Representative pictures of transversal slices from ears collected in the last day of experiment (day 9) and stained with hematoxylin–eosin (increase of 200× and 400×, scale 100 µm). Ears without treatment (A), treated with croton oil (B), croton oil plus EEVS (1 mg/ear) (C) and croton oil plus dexamethasone (0.05 mg/ear) (D). Five slices from each animal were analysed and a minimum of 3 animals for each treatment were taken.

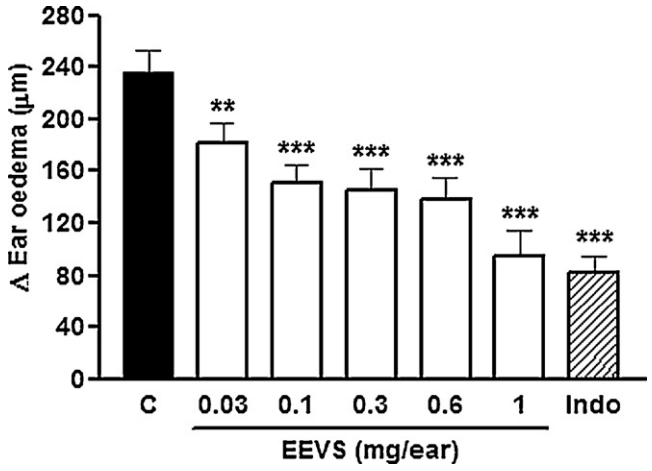

Fig. 5. Topical effect of *Vernonia scorpioides* ethanolic extract (EEVS) on AA-induced ear oedema. AA was administered topically (2 mg/ear) and immediately after EEVS or indomethacin (Indo, 2 mg/ear) were applied, and ear thickness was measured (μm) 1 h after. The bars represent the mean ± S.E.M. for 5–6 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from control (C), $^{**}p < 0.01$; $^{***}p < 0.001$.

as useful pharmacological tools for the investigation of new anti-inflammatory drugs, allowing the identification of potential drugs, including substances of plant origin and plant extracts (Gábor, 2000), to treat inflammatory skin disorders. TPA is one of the phorbol ester constituents of croton oil (Gábor, 2000), and its topical application triggered local inflammation with oedema formation, polymorphonuclear leucocytes infiltration and epidermal hyperproliferation as consequence of the production of inflammatory mediators, such as prostaglandin E_2, leucotrienes, histamine, serotonin and IL-1 (Furstenberger et al., 1994). Corticoid-like agents, phospholipase A_2 and cyclooxygenase inhibitors as well as 5-lipoxigenase inhibitors and LTB_4 antagonists are highly effective against the inflammation caused by TPA (Furstenberger et al., 1994; Gábor, 2000). With an extent of activity comparable to that of the standard drug dexamethasone, EEVS inhibited two important events related to the skin inflammatory response induced by a single application of TPA and multiple applications of croton oil: oedema formation and the migration of neutrophils, as determined by MPO activity assays and flow cytometry. Neutrophil activation results in increases in the leucotrienes and prostaglandins released in the skin, and the obstruction of the synthesis of these inflammatory mediators could explain the anti-inflammatory activity of certain plants used in the treatment of skin disorders (Bradley et al., 1982). In addition, compounds that inhibit neutrophil infiltration can also reduce oedema formation and eicosanoid production at the inflamed site (Sanchez and Moreno, 1999). According to our results regarding oedema and cell infiltration, the topical anti-inflammatory properties of *Vernonia scorpioides* proves to be interesting because the accumulation of neutrophils in the skin plays a critical role in cutaneous inflammatory diseases, such as dermatitis and psoriasis.

The inhibition of inflammatory oedema and leucocyte migration to ear skin challenged with TPA by different sesquiterpene lactones was demonstrated by Recio et al. (2000). Sesquiterpenes lactones, such as hirsutinolides and glaucolides, are commonly found in *Vernonia* species (Jakupovic et al., 1985; Kuo et al., 2003). Regarding the chemical composition of the EEVS, it has been exhaustively studied and the main compounds found were: the known triterpenes lupeol and lupeol acetate together with the widespread steroids β-sitosterol and stigmasterol, the sesquiterpene lactones (glaucolides and hirsutinolides) such as diacethylpiptocarphol and related hirsutinolides, flavonoids (luteolin and apigenin)

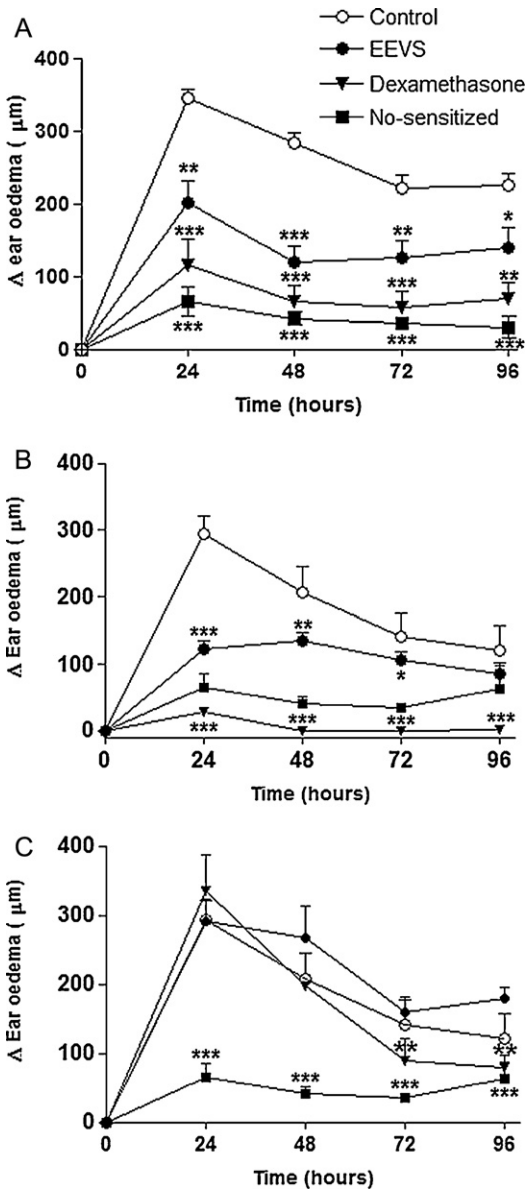

Fig. 6. Topical effect of *Vernonia scorpioides* ethanolic extract (EEVS) on the oxazolone induced contact-delayed-type hypersensitivity. The animals were sensitized and after 6 days they were re-exposed to oxazolone. The topical treatment with EEVS (1 mg/site) and dexamethasone (0.05 mg/site) was carried out on both phases (A), in the sensitization phase (B) and in the elicitation phase (C). A group of animals was treated just with vehicle (control) and another group was not previously sensitized with oxazolone (no-sensitized). The bars represent the mean ± S.E.M. for 5–6 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from control (C), $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

and cinnamic acid derivatives (caffeic acid and ethyl caffeate) (Buskuhl et al., 2010), and also a polyacetylene (Buskuhl et al., 2009).

The general action mechanism of sesquiterpene lactones involve the transcription factor NF-κB throughout the alkylation of the p65 subunit cysteine residue of the NF-κB complex (Buskuhl et al., 2009) or the inhibition of NF-κB activation by preventing the degradation of IκB. Both cases inhibited the interaction of NF-κB with DNA (Valério et al., 2003), thereby resulting in a reduction in the synthesis of inflammatory mediators, such as chemotatic cytokines. Certain hirsutinolides isolated from *Vernonia trifloscolosa* inhibited IL-8 production in *in vitro* studies (Kos et al., 2006). Given that IL-8 exerts a potent chemotatic action, the effect of EEVS on neutrophil infiltration might involve the inhibition of this cytokine synthesis

as consequence of the presence of sesquiterpene lactone. However, EEVS did not exert any action over the infiltration of mononuclear phagocytes, which represent the main cell type involved in the chronic inflammation. However, the reduction of neutrophil infiltration in the dermis can promote a relative inflammatory resolution because neutrophils collaborate in the release of free radicals, inflammatory mediators and proteolytic enzymes (Bradley et al., 1982).

One critical action of EEVS was the reduction of acanthosis incidence, which is an indicator of the hyperproliferation of epidermis keratinocytes. The repeated application of croton oil increases the levels of IL-1β and TNF-α as well as COX-2 expression (Stanley et al., 1991). The suppression of these cytokines and of prostaglandin E_2 production could be the mechanism through which EEVS abolishes the epidermal hyperproliferation, oedema formation and leucocyte infiltration. Again, the expression of these cytokines and COX-2 as well as the actions of IL-1β and TNF-α are all dependent of NF-κB activity, and, because nuclear factors can be inhibited by EEVS sesquiterpene lactones, the extract's ability to protect against hyperproliferative disorder is well explained. In addition, some studies have demonstrated that a reduction in the inflammation by the multidose-TPA test can only be produced by corticoids and LOX inhibitors (Cuéllar et al., 2001).

Increased levels of leucotrienes were detected in inflammatory and hyperproliferative skin diseases; therefore, inhibitors of 5-lipoxygenase display beneficial effects in inflammatory skin disorders (Gábor, 2000). Moreover, an inhibition of the AA-induced oedema is connected with a reduction in LOX activity. This finding is in agreement with the results obtained with the EEVS in the AA-induced oedema in which EEVS prevents ear oedema formation. The AA and TPA-induced ear oedema models may be useful for the detection of the *in vivo* activity of COX/LOX inhibitors (Gábor, 2000). Other studies have been shown that some antioxidants, histamine and serotonin antagonists also inhibit the AA-induced inflammation (Sanchez and Moreno, 1999).

Another model utilised in this work was the repeated application of the hapten oxazolone, which induces a contact-delayed-type hypersensitivity (DTH) resembling human allergic contact dermatitis and characterised by a sustained ear swelling and marked polymorphonuclear and T lymphocytes infiltration (Fujii et al., 2002). Some cytokines (IL-2, TNF-β and IFNγ) released by activated T lymphocytes in the elicitation phase promote the activation of macrophages that trigger an acute inflammation response by the release of TNF-α, IL-1, chemokines, prostaglandin and leucotrienes (Homey et al., 2006). Based on our results, we suggest that EEVS acts essentially as an anti-inflammatory agent and not as an immunosupressor, given that only the elicitation phase was responsive to the EEVS treatment in the DTH model. The anti-inflammatory effect of EEVS in this model might be a consequence of the suppression of important inflammatory mediators, such as arachidonic acid metabolites involved in the inflammatory response triggered after the elicitation phase.

Based on our results, we suggest that EEVS is effective against acute and chronic skin inflammatory processes as well as delayed hypersensitivity reaction and keratinocytes hyperproliferative disorder. Moreover, the probable mechanism of action through which EEVS exerts its effects could involve several targets, resulting in the reduction of important inflammatory mediators in the cutaneous tissue. Investigations into the mechanism of action of the anti-inflammatory activity and into the compounds responsible for the activity of EEVS are currently in progress.

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