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Combretum leprosum Mart. (Combretaceae): Potential as an antiproliferative and anti-inflammatory agent

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ABSTRACT

Ethnopharmacological relevance: *Combretum leprosum* is a species that is popularly used in Brazil as a healing agent to treat skin problems and lesions. In this study we investigated the possible potential of this extract to treat inflammatory and hyperproliferative skin conditions.
Materials and methods: Classical models of skin inflammation such as TPA- and croton oil-induced mouse ear oedema were applied in order to verify the potential topical anti-inflammatory activity of the ethanolic extract from flowers of *Combretum leprosum*.
Results: Topical application of ethanolic extract promoted a dose-dependent inhibition of phorbol ester-induced ear oedema, reduced myeloperoxidase activity and IL-6 tissue levels with inhibition comparable to dexamethasone (positive control). Histological and immunohistochemical analysis revealed that ethanolic extract also suppressed cell infiltration. Ethanolic extract altered inflammatory parameters on a chronic skin inflammation model induced by repeated applications of croton oil, decreasing ear oedema, epidermal hyperproliferation and cell infiltration. In addition, immunohistochemical analysis showed that the extract decreased PCNA expression on the epidermis.
Conclusion: Taken together, these results suggest that the extract from flowers of *Combretum leprosum* could be considered as a new potential tool for the treatment of several skin inflammatory diseases since it reversed the skin inflammatory and hyperproliferative process in a very significant manner. Further investigations are needed in order to verify the cellular mechanism and safety of *Combretum leprosum* extract.

1. Introduction

Skin is the body organ responsible for a direct interaction between the environment and the organism, since it is localised on the body surface. Therefore, its main function is to form an effective barrier protecting the organism from several external stimuli, such as pathological agents, processes and events. Thus, as a mechanism of defence, the skin is able to recognise, discriminate, and integrate specific signals from the environment and generate appropriate responses aimed at preserving body homeostasis (Debenedictis et al., 2001). However, this response is usually marked with the presence of pro-inflammatory mediators which are released by skin cells promoting an inflammatory process which can cause inflammatory skin diseases when it is not properly controlled (Serhan and Petasis, 2011).

Skin disorders can be initiated by either exogenous or endogenous stimuli and usually are marked by a disruption of the barrier, sensitisation, inflammation, changes in epidermal proliferation and differentiation (Proksch et al., 2008). The most common inflammatory dermatoses are psoriasis and atopic dermatitis, which have a high impact on the patient's life. Psychological disturbances, such as embarrassment, worry, stigmatisation, depression, and problems with self-esteem and body image are very common in dermatological patients. All of these feelings can impair several aspects of life, such as personal relationships, sports, sexuality, self-care actions, and activities at work or school (Tejada Cdos et al., 2011).

Most of the chronic skin inflammatory conditions, such as psoriasis, have no aetiology or pathophysiology elucidated. Thus, current therapeutic treatments are not effective or can show undesirable side effects when effective. The lack of ideal therapy

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supports the necessity of searching for safe and effective interventions which could prevent oedema, plasma extravasation, and the recruitment of inflammatory mediators to combat excessive inflammatory reactions (Stern et al., 2004). An interesting alternative would be the use of medicinal plants that have been used since ancient times to treat skin disorders and wounds. As well as presenting a cheaper and easily accessible alternative when compared with synthetic medicines, studies have proven the effectiveness of medicinal plants with regard to altering immune functions and modulating inflammatory processes without causing side effects such as immunosuppression (Plaeger, 2003; Saklani and Kutty, 2008). In fact, some medicinal plants, such as Aloe vera (*Aloe barbadensis)* and the Chinese herb indigo naturalis (*Baphicacanthus cusia*), have been employed in clinical trials and have demonstrated efficacy comparable to the drugs currently used in the treatment of psoriasis (Choonhakarn et al., 2010; Lin et al., 2008). Furthermore, several plant-derived compounds are established in dermatologic therapy, as seen with dithranol, an anthracene derivative isolated from *Andira araroba,* which shows great efficacy when employed as an anti-psoriatic therapy (Reuter et al., 2010).

Combretum leprosum Mart. (Combretaceae) is a species that is commonly found in northeast Brazil where it is popularly known as "mufumbo" (Lira et al., 2002). Several parts of this plant, such as the leaves and flowers, are used in folk medicine as a healing agent, for the prevention of rashes, and to clean wounds. In addition, the plant is also used for the containment of bleeding, and as a sedative, anti-diarrhoeal, expectorant, and antitussive (Agra et al., 2007; De Albuquerque et al., 2007). Pharmacological studies with extracts and isolated compounds from different parts of the plant suggested that the biological activities of *Combretum leprosum* include anti-inflammatory, antinociceptive, anticholinesterase and anti-ulcerogenic effects (Facundo et al., 2005; Nunes et al., 2009; Pietrovski et al., 2006). According to phytochemical analysis, *Combretum leprosum* is rich in compounds such as cycloartanes, triterpenes (arjunolic and mollic acid, and 3β,6β,16β-trihidroxilup-20(29)-ene), and flavonoids (3-*O*-methylquercetin, and quercetrin), and some of these substances have a proven biological activity (Facundo et al., 1993).

Although *Combretum leprosum* is popularly used as a medicinal plant to treat skin problems and lesions (Facundo et al., 2005), there is no scientific data proving the effectiveness of its possible topical anti-inflammatory activity. Thus, in this study, we used classical models of skin inflammation such as TPA- and croton oil-induced mouse ear oedema, in order to verify the potential topical anti-inflammatory activity of the ethanolic extract (EE) from flowers of *Combretum leprosum*.

2. Material and methods

2.1. Plant material and preparation of EE

Botanical material was collected in May 2007 at Viçosa, Ceará State, Brazil, and was classified by Dr. Afrânio Fernandes (Universidade Federal do Ceará, Fortaleza) as *Combretum leprosum* Mart. A voucher specimen of this plant was deposited in the Herbarium Prisco Bezerra of the Biology Department, Universidade Federal do Ceará, Brazil, under number 12446 (May 2007). The dried flowers (2.7 kg) were powdered and extracted using ethanol (5 L), which was stirred and macerated at room temperature (24 ± 3 °C) for approximately 24 h. This procedure was repeated three times. The solvent was fully evaporated under reduced pressure, and the extract (yield 58.3 g) was lyophilised and stored in a freezer at -20 °C until use.

2.2. Drugs and reagents

The following drugs were used to execute the experimental protocols: 12-*O*-tetradecanoylphorbol-acetate (TPA), croton oil, arachidonic acid (AA), indomethacin, dexamethasone, tetramethylbenzidine (TMB), 4-nitrophenyl *N*-acetyl-β-D-glucosaminide, hexadecyltrimethylammonium bromide (HTAB), Triton-X, mifepristone and 3-(4,5-dimetylyhiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (all from Sigma Chemical Co, St Louis MO), dimethylformamide, acetone, formaldehyde, glacial acetic acid, phosphate-buffered saline (PBS), paraffin (all from Merck Biosciences, Germany), bovine serum albumin, Cohn fraction *V* (BSA) (Inlab, Brazil), hydrogen peroxide, absolute ethanol, methanol, eosin, sodium acetate, glycine, hematoxylin and xylene (all from Vetec, Rio de Janeiro, Brazil). Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum were obtained from Cultilab (Brazil).

2.3. Animals

Experiments were performed on female Swiss mice (25–35 g) which were housed in an animal room under conditions of 22 ± 2 °C, and a 12-h light/dark cycle, with free access to food and water. The animals were allowed to adapt to the laboratory for at least 1 h before testing and were used only once. All animal procedures were performed after approval of the protocol by the Institutional Ethics of our University (protocol number 296) and were carried out in accordance with current guidelines for the care of laboratory animal.

2.4. Irritative contact dermatitis models

Skin dermatitis was induced by TPA or AA and the inflammatory response was evaluated through oedema formation. Oedema was expressed as the increase in mice ear thickness (μm). Ear thickness was assessed near the medial edge of the ear by using a digital micrometer (MT-045B, Shangai Metal Great Tools Co., Ltd., Shangai, China). Measures were taken before and after induction of the inflammatory process. The phlogistic agents were dissolved in 20 μL of acetone while the extract was dissolved in 20 μL of ethanol-acetone (3:7 v/v) and applied to the right ear of each mouse.

Oedema was induced by topical application of TPA (2.5 μg/ear) or AA (2 mg) in the right ear of the mice. Plant extract (0.01–1.0 mg/ear) and dexamethasone (0.1 mg/ear) or indomethacin (2 mg/ear) were applied as reference drugs immediately after the phlogistic agents in experimental groups. To verify the possible involvement of glucocorticoid receptors, animals were pre-treated with mifepristone (50 mg/kg, s.c.) dissolved in polyethylene glycol 400, 15 min prior to EE (0.6 mg/ear) or dexamethasone (0.001 mg/ear). Ear thickness was measured before and 6 or 1 h after challenge with TPA or AA, respectively. Ear samples (6 mm circles of tissue) were collected 24 h after the application of TPA and subjected to histological analysis and assessment of the enzyme myeloperoxidase (MPO) activity and interleukin (IL)-6 tissue levels.

2.5. Croton oil-induced chronic skin inflammation

The chronic inflammatory process was induced by application of croton oil (0.4 mg/ear) on alternate days for nine days. The extract (0.6 mg/ear) and dexamethasone (0.1 mg/ear, positive control), were administered from the fifth day of the trial and applied topically for the last four days (twice a day). On the ninth day of the experiment, the animals were sacrificed and 6 mm circles of ear tissue were collected, weighed and submitted to the

assessment of the MPO and *N*-acetyl-β-D-glucosaminidase (NAG) activities, as well as histological analysis.

2.6. MPO and NAG enzyme activity assay

To assess enzyme activity, the methodology of Bradley et al. (1982) modified by De Young et al. (1989) was used. The biopsies (6 mm circles of tissue) were added to 0.75 mL of 80 mM PBS pH 5.4 containing 0.5% of HTBA, and were homogenised (45 s at 0 °C) in a motor-driven homogeniser. The homogenate was decanted into microtubes and added to 0.75 mL of buffer, as previously described. The samples (1.5 mL) were placed in microfuge tubes and centrifuged at 11,200*g* at 4 °C for 20 min. MPO activity was achieved with triplicates of 30 μL of the supernatant placed on 96-well plates, where 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% hydrogen peroxide was subsequently added into each well. The addition of 20 μL of 18.4 mM TMB in dimethylformamide promoted the start of the reaction. The plate was then incubated at 37 °C for 3 min and the reaction was stopped by the addition of 30 μL of 1.46 M sodium acetate, pH 3.0. NAG activity was reached with triplicates of 25 μL of supernatant placed on 96-well plates, followed by the addition of 100 μL of 50 mM citrate buffer, pH 4.5. The reaction was initiated by the addition of 25 μL of 2.24 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide. The plate was incubated at 37 °C for 1 h and the reaction was stopped by the addition of 30 μL of 200 nM glycine buffer, pH 10.4. The enzymatic activity was determined colourimetrically using a plate reader (EL808; BioTech Instruments, INC) set to measure absorbance at 630 nm for MPO or 405 nm for NAG. The results are expressed as mOD/mg tissue.

2.7. Measurement of IL-6 levels

Amounts of IL-6 in homogenates of ear tissue samples were quantified using a mouse IL-6 ELISA kit (eBioscience, San Diego, USA) according to the manufacturer's instructions. Levels of this cytokine in each supernatant were normalised to total protein content, which was determined using a Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Histological assessment of skin tissue

Ear samples were fixed in a solution containing ethanol 80%, formalin 40%, and glacial acetic acid (ALFAC solution). The ears were subsequently embedded in paraffin, sectioned to 5 μm and stained with hematoxylin–eosin. The infiltration of leukocytes and epidermis thickness were evaluated in representative areas selected with 10× and 40× objectives. The quantification of leukocytes in the dermis was performed by counting these cells per field, and five fields from three distinct histological sections of each group were analysed. To reduce the probability of error, the researcher did not know which group he was investigating.

2.9. Immunohistochemical evaluation of proliferating cell nuclear antigen (PCNA) levels

Sections (5 μm) of tissue previously fixed in ALFAC and embedded in paraffin were placed onto silanised glass slides and deparaffinised twice with xylene, followed by rehydration through a graded alcohol bath. To block radical aldehyde, each section was treated with glycine (0.1 M) and with 3% hydrogen peroxide in methanol to block endogenous peroxidase. Slices were treated with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to diminish non-specific staining. For the detection of PCNA, slides were incubated with 1:50 dilutions of a polyclonal anti-PCNA antibody (Santa Cruz Biotechnology, Inc, USA) in PBS/BSA 1% at room temperature in a moist chamber for 2 h and washed with PBS/BSA 1%. Subsequently, the sections were incubated using a secondary antibody IgG HRP (Santa Cruz Biotechnology, Inc, USA) diluted 1:50 in PBS/BSA 1% at room temperature in a moist chamber for 1 h. The peroxidase-binding sites were detected by staining with DAB substrate Kit (BD Bioscience, California, USA), and incubating for 15 min. Finally, slices were counterstained with Mayer's hematoxylin and then dehydrated and mounted.

2.10. Cell culture

The human keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum and 1% penicillin/streptomycin (10,000 U/100 μg/ml) at 37 °C with 5% CO_2 in a humidified atmosphere.

2.11. Cell proliferation assay

HaCaT cells (7×10^3) were seeded in each well of a 96-well plate and incubated at 37 °C for a period of 24 h. The media were then replaced with 200 μl of fresh media containing varying concentrations of EE (5, 10, 15, 20, 25 and 30 μg/mL). The plate was then re-incubated, maintaining the same conditions, for 24, 48 and 72 h, after which cell viability and cell density were verified by MTT and CyQuant assays, respectively. Following

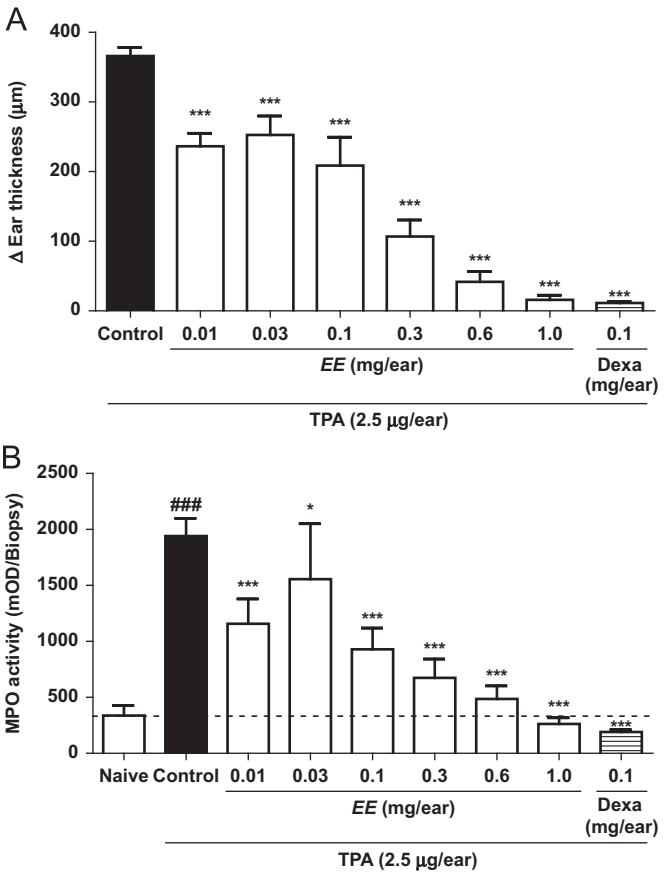

Fig. 1. Effect of ethanolic extract from *Combretum leprosum* (EE) and dexamethasone (Dexa) administered topically on TPA-induced ear oedema (a) and myeloperoxidase activity in supernatants of homogenates from TPA-treated ears (b). Ear oedema and MPO activities were measured at 6 and 24 h after TPA treatment, respectively. All tested drugs were applied after TPA application. Each bar represents the mean ± S.E.M. for 5–10 animals. The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, $^{*}P < 0.05$, $^{**}P < 0.01$ and *** and $^{\#\#\#}P < 0.001$.

incubation, MTT solution (0.5 mg/mL) was added and incubated at 37 °C for 4 h. After removing the supernatant, 150 μL of ethanol was added to each well to dissolve formazan crystals, and optical density was detected at 470 nm using a plate reader (EL808B, BioTech Instruments, Inc., Winooski, VT, USA). Cell density was estimated using a cell proliferation assay (CyQuant kit; Invitrogen Molecular Probes), which relies on a fluorescent dye, and exhibits a strong increase in fluorescence when bound to DNA. Following incubation, the medium was removed and the plate was frozen at −70 °C. 200 μL of the CyQuant dye-cell lysis buffer was then incubated for 2–5 min at room temperature and the fluorescence intensity, which was related to the number of cells present, was measured at 485/535 nm.

2.12. Statistical analysis

The results were expressed as mean ± S.E.M., except for the ID_{50} values (dose required to reduce by 50% the responses of the groups treated relative to the control group), which were represented as the geometric mean accompanied by their 95% confidence intervals. Data were evaluated by one-way analysis of variance (ANOVA) followed by the Newman–Keuls post-hoc test when appropriate. $P < 0.05$ was considered as indicative of significance. The values were obtained using the Statistical software GraphPad Prism version 3.00, San Diego California, USA.

3. Results

Topical application of TPA promoted an increase in the thickness of the ear and in the tissue MPO activity, while EE applied topically alone did not alter any of these parameters (data not shown). However, EE caused a dose-dependent inhibition of both of the inflammatory parameters, oedema and cell migration, induced by TPA (Fig. 1). EE presented an ID_{50} value of 0.11 (0.10–0.13) and 0.13 (0.11–0.16) mg/ear and the maximal inhibitions observed at a dose of 1 mg/ear were 96 ± 2% and 97 ± 2% for the oedema and MPO activity, respectively, (Fig. 1a and b). In

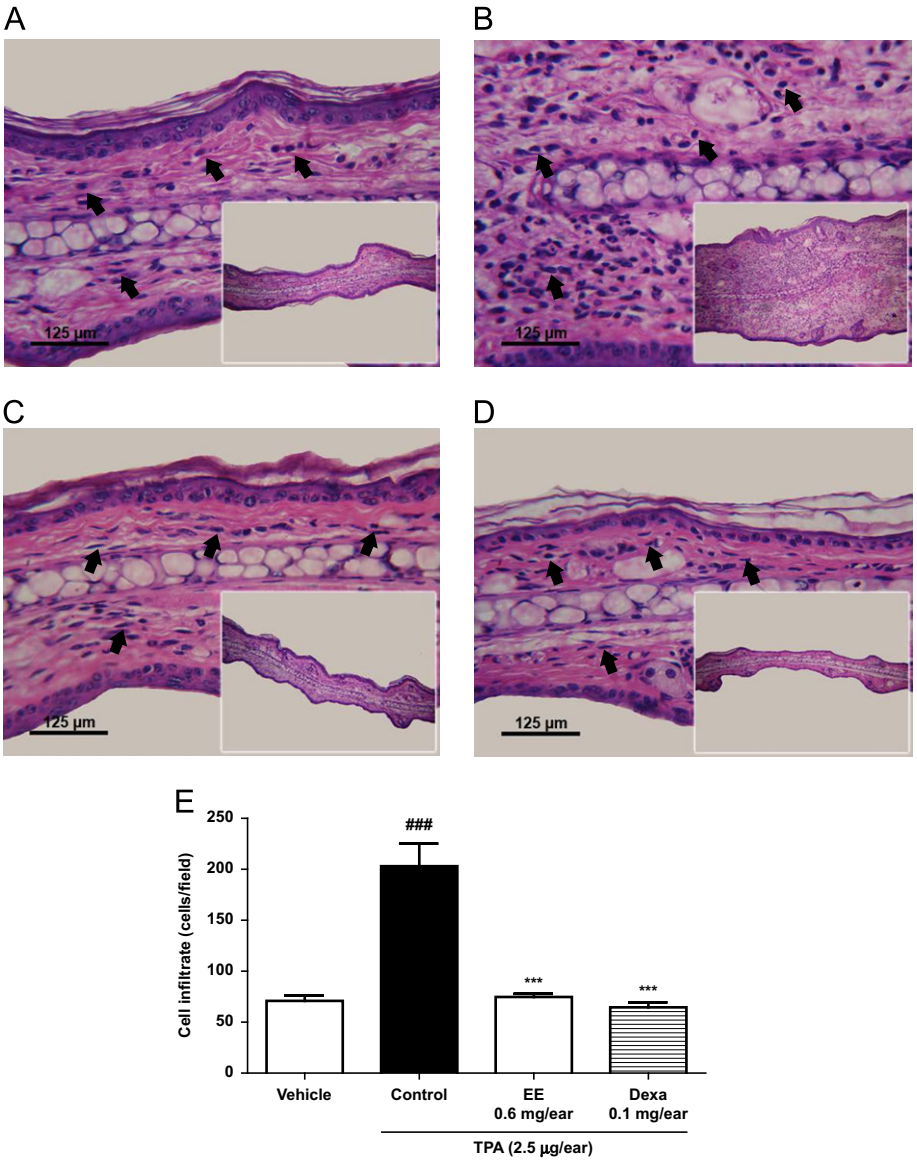

Fig. 2. Representative pictures of histological sections from mice ears stained with hematoxylin–eosin (40 ×, scale 125 μm), 24 h after TPA application and quantification of leukocytes (f). (a) vehicle, (b) control, (c) treatment with ethanolic extract from *Combretum leprosum* (EE) (0.6 mg/ear) and (d) dexamethasone (0.1 mg/ear). Arrows indicate the infiltrated leukocytes. Each bar represents the mean ± S.E.M. for 3–4 sections. The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, *** and $^{###}P < 0.001$.

these tests the use of the reference drug dexamethasone (0.1 mg/ear) showed inhibition values of 97 ± 1% and 99 ± 0.3% for oedema and MPO activity, respectively.

Fig. 2 shows the analysis of HE-stained ear sections from TPA-treated mice. TPA application resulted in a marked increase in ear thickness, with clear evidence of oedema, and substantial inflammatory cell infiltration in the dermis (Fig. 2b). EE treatment remarkably reduced cell infiltration with an inhibition of 96 ± 2% (Fig. 2c), which was comparable to the positive control dexamethasone to an extent, (Fig. 2d) which showed an inhibition of 100.0 ± 0.4%.

Levels of IL-6 in skin tissue were significantly higher in ears submitted to TPA application as compared to naïve or vehicle (acetone) groups (Fig. 3). EE treatment promoted a decrease of IL-6 concentration with a maximum inhibition of 93.4 ± 3.8% (0.3 mg/ear), while dexamethasone inhibited the cytokine concentration by 66.4 ± 10.1%.

Topical application of AA promoted rapid and intense inflammatory response, as verified by oedema formation. Topical EE treatment inhibited oedema formation in all of the tested concentrations with a maximum inhibition of 72 ± 2% (1.0 mg/ear), while the reference drug, indomethacin, promoted inhibition of 82 ± 4% (Fig. 4).

As shown in Fig. 5a, both the EE and dexamethasone treatments were able to revert the oedema formation even after the establishment of the inflammatory process, which was shown by the ear weight, evaluated on the ninth day, presenting an inhibition of 63 ± 3% and 77 ± 2%, respectively.

Histological analysis enabled the assessment of some parameters, such as hyperproliferation of epidermal keratinocytes.

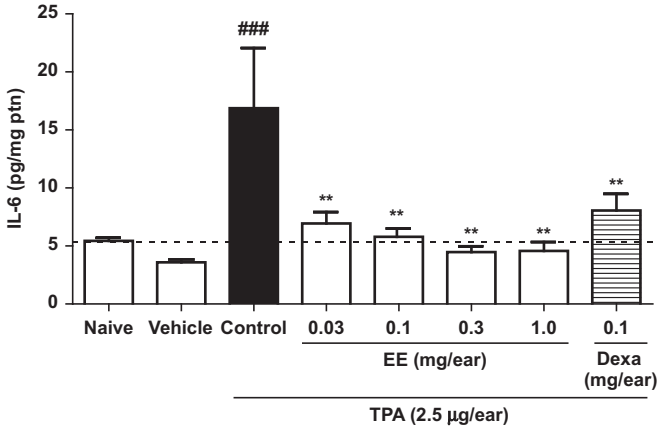

Fig. 3. Interleukin-6 levels in supernatants of homogenates from TPA-treated ears after treatment with EE (0.03–1.0 mg/ear) or dexamethasone (0.1 mg/ear). Measurements were performed with a commercial ELISA kit. Each bar represents the mean ± S.E.M. for 3–4 sections. The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, $^{**}P < 0.01$ and $^{###}P < 0.001$.

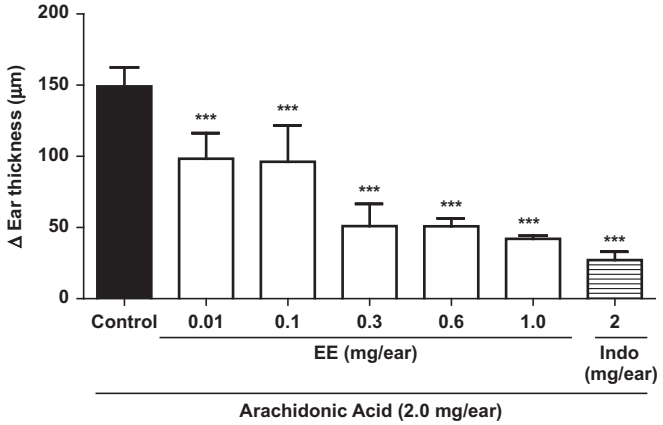

Fig. 4. Effect of ethanolic extract from *Combretum leprosum* (EE) and indomethacin (Indo) administered topically on AA-induced ear oedema. Ear oedema was measured 1 h after AA treatment. All tested drugs were applied after AA application. Each bar represents the mean ± S.E.M. for five animals. The graphic symbols denote the significance levels when compared with control group. Significantly different from control $^{***}P < 0.001$.

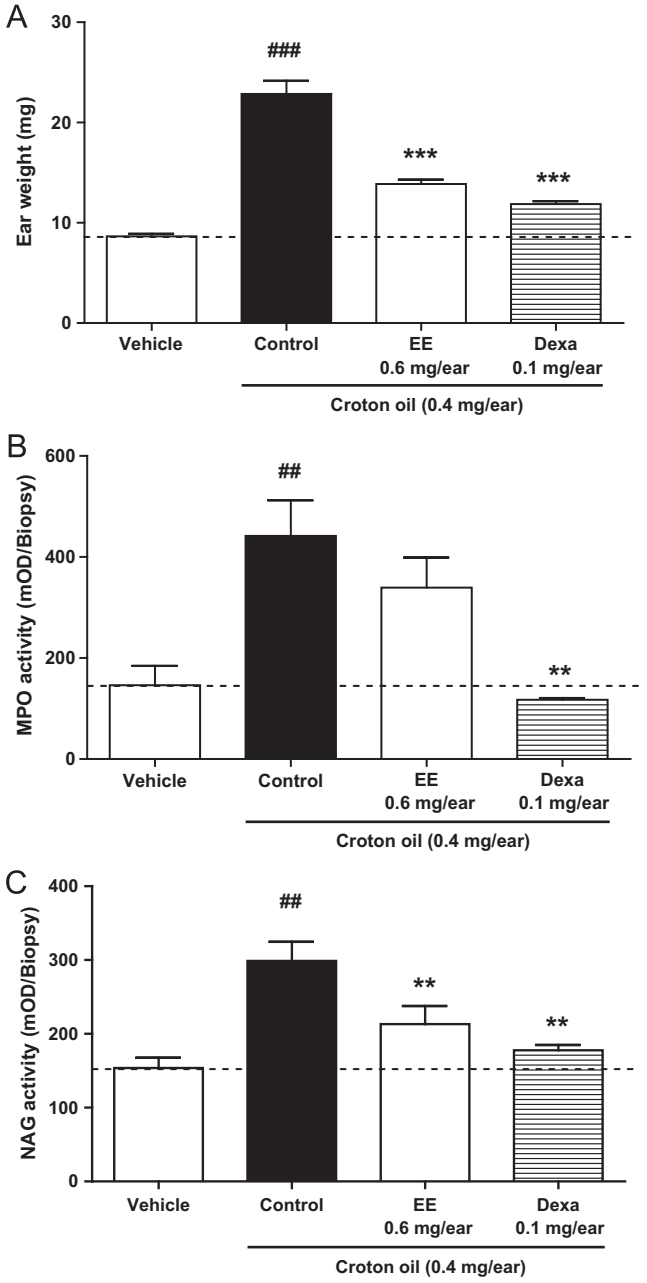

Fig. 5. Effect of ethanolic extract from *Combretum leprosum* (EE) and dexamethasone (Dexa) administered topically on croton oil-induced ear oedema (a), MPO activity (b) and NAG activity (c) in supernatants of homogenates from croton oil-treated ears. Croton oil was applied on alternate days for nine days and the tested drugs were applied after the fifth day for four days, twice a day. Ear oedema and enzymatic activities were measured on the ninth day of croton oil treatment. In the vehicle animal (*V*), only acetone was applied. Each bar represents the mean ± S.E.M. for 5–10 animals. The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, ** and $^{##}P < 0.01$ and *** and $^{###}P < 0.001$.

Multiple applications of croton oil promoted an increase of epidermis thickness by approximately five times that of the control (Fig. 6). Both EE and dexamethasone applications were effective in reducing the epidermal hyperproliferation by 50 ± 4% and 70 ± 2%, respectively (Fig. 6f).

Concerning neutrophil migration in the chronic skin inflammation, the treatment with EE was not able to modify MPO activity (Fig. 5b), unlike dexamethasone, which promoted the complete inhibition of enzyme activity. However, EE inhibited NAG activity, like dexamethasone, causing a reduction in 76 ± 4% and 83 ± 5%, respectively (Fig. 5c). Inhibition of cell infiltration was confirmed by the quantification of leukocytes in the dermis through histological analysis, and EE and dexamethasone treatments were able to reduce the quantity of cells in 61 ± 4% and 74 ± 10%, respectively (Fig. 6e).

As depicted in Fig. 7, PCNA-positive cells were detected in all groups. However, the vehicle group demonstrated few labelled cells as an indication of a normal basal proliferation activity (Fig. 7a). The challenge with repeated croton oil treatment promoted an increase in the number of proliferative cells, with some of these proliferative cells being observed outside of the basal layer (Fig. 7b). The epidermis hyperproliferation induced by croton oil was significantly reduced by treatment with EE, as well as with dexamethasone, presenting a decrease of PCNA-positive cells in 27 ± 12% and 65 ± 6%, respectively (Fig. 7c and d). Moreover, an anti-proliferative effect was also observed in cultured HaCaT keratinocytes, as determined by MTT and CyQUANT assays (Fig. 8). EE reduced cell viability, which was shown by an increase in the efficacy at higher concentrations and a slight increase with the higher duration of exposure. At the higher concentration of 30 μg/mL, an efficacy of 93.1 ± 2.7% was observed after 72 h of incubation. Similar results were achieved with the CyQUANT cell proliferation assay, where EE reduced the cell density at higher concentrations and longer durations of exposure. After 72 h of incubation, the higher concentration (30 μg/mL) showed an efficacy of 96.8 ± 3.2%.

In an attempt to verify a possible mechanism of action, mifepristone, a glucocorticoid receptor antagonist, was applied before treatments. Pre-treatment with the antagonist was able to abolish the anti-oedematogenic effect of EE and dexamethasone (Fig. 9).

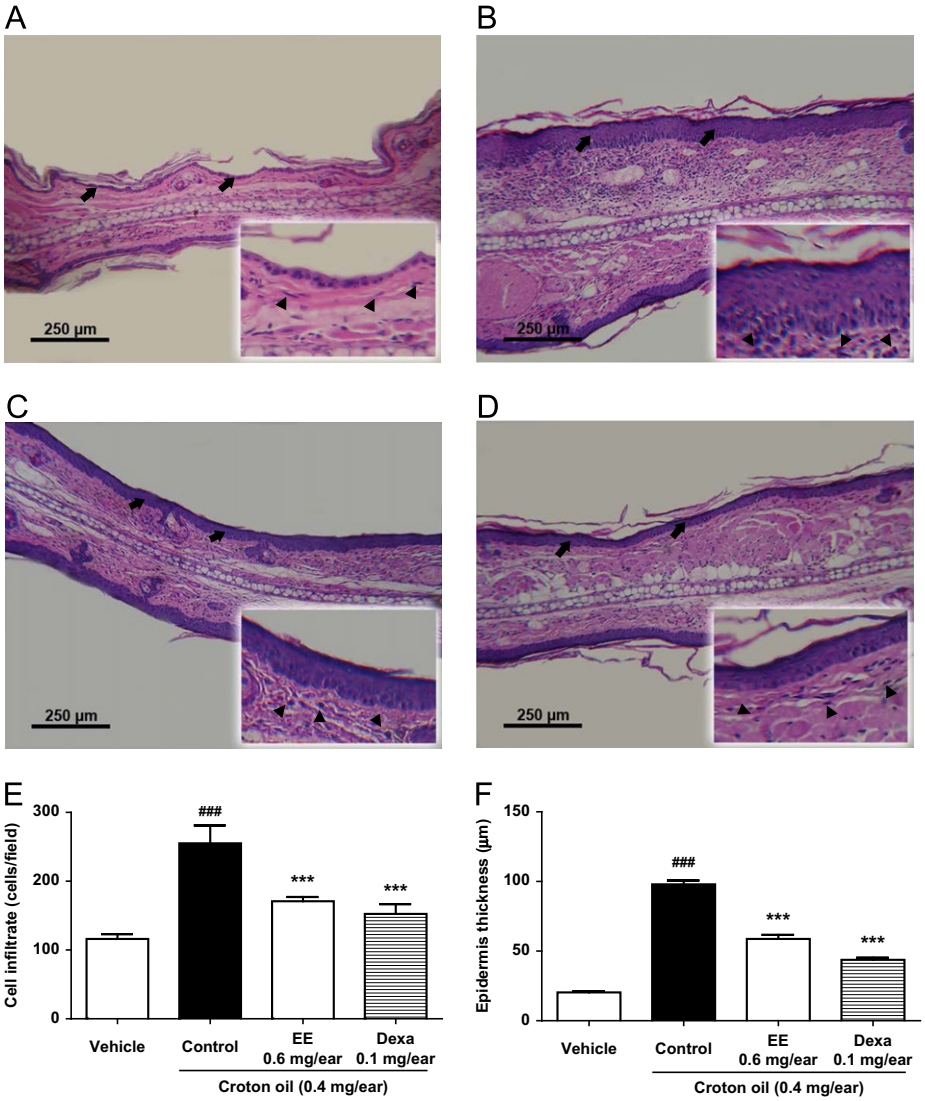

Fig. 6. Representative pictures of histological sections from mice ears stained with hematoxylin–eosin (10 ×, scale 250 μm), after multiple croton oil application, quantification of leukocytes (e) and measurement of epidermis thickness (f). (a) vehicle, (b) control, (c) treatment with ethanolic extract from *Combretum leprosum* (EE) (0.6 mg/ear), (d) dexamethasone (0.1 mg/ear). Arrows indicate the epidermis and arrowheads indicate the infiltrated leukocytes. Each bar represents the mean ± S.E.M. for 3–4 sections. The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, *** and $^{###}P < 0.001$.

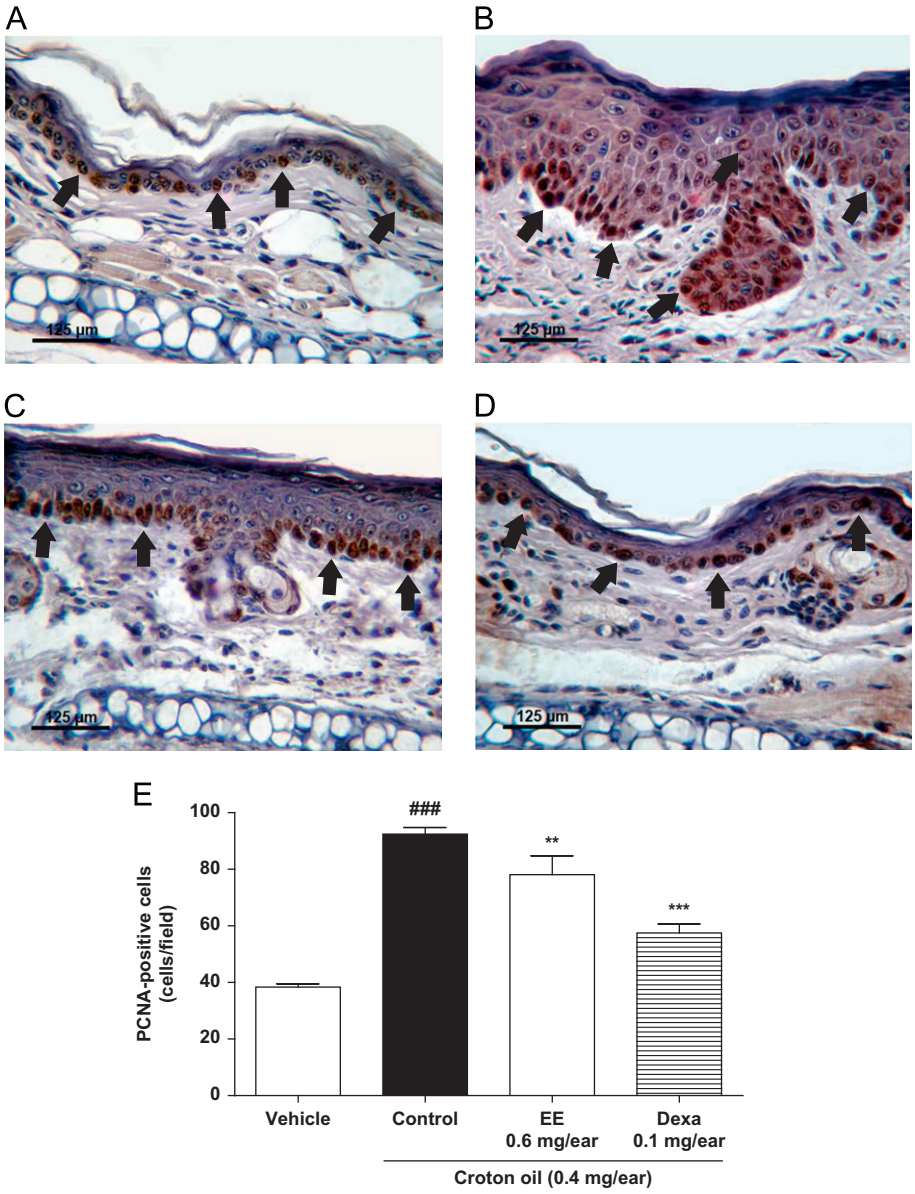

Fig. 7. Representative pictures of immunohistochemical analysis from histological sections of mice ears counterstained with hematoxylin (40 ×, scale 125 µm), after multiple croton oil application and quantification of PCNA-positive cells (e). (a) vehicle, (b) control, (c) treatment with ethanolic extract from *Combretum leprosum* (EE) (0.6 mg/ear), (d) dexamethasone (0.1 mg/ear). Arrows indicate PCNA-positive cells. Each bar represents the mean ± S.E.M. for 3–4 sections. The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, $^{**}P < 0.01$, *** and $^{###}P < 0.001$.

4. Discussion

The present work provides evidence that flowers of *Combretum leprosum* possess an anti-inflammatory effect when topically applied to the skin. As first evidence, EE demonstrated efficacy at reducing inflammatory parameters, such as oedema and cell migration, in a dose-dependent manner in the model of TPA-induced ear oedema. TPA is a phorbol ester, which, once topically applied, promotes an acute inflammatory response that displays vasodilation, polymorphonuclear leukocyte tissue infiltration and oedema formation. All of these events appear to arise from the direct activation of protein kinase *C* (PKC) which promotes an increase in activity of the enzyme phospholipase A_2 (PLA_2), increasing the levels of arachidonic acid and its metabolites, such as prostaglandins and leukotrienes. These metabolites, as well as cytokines, are mediators of inflammatory pathways, and are responsible for triggering and maintaining inflammation (Stanley et al., 1991). Thus, the constituents of the *Combretum leprosum* extract are probably negatively interacting with molecules such as cytokines in some of these steps during the formation of oedema, and can therefore be targets for the development of new topical anti-inflammatory therapeutic agents.

Inflammatory skin diseases are usually characterised by intense neutrophil infiltration, which are cells that are considered the first line of defence against pathogens. Neutrophils possess rich machinery, containing mainly reactive oxygen species (ROS), which are capable of degrading pathogen proteins. This defence system is harmful not only to pathogens, but can also have a detrimental effect on components of host tissues (Nemeth and Mocsai, 2012). Although polymorphonuclear cells (PMNs) are particularly involved in acute inflammatory responses, in some chronic immune diseases, such as psoriasis, there is an important accumulation of PMN. Neutrophils in psoriatic lesions secrete cytokines, proteolytic enzymes and ROS, which can stimulate T cells and keratinocytes to maintain an inflammation-sustaining loop (Terui et al., 2000). Thus, compounds which are able to

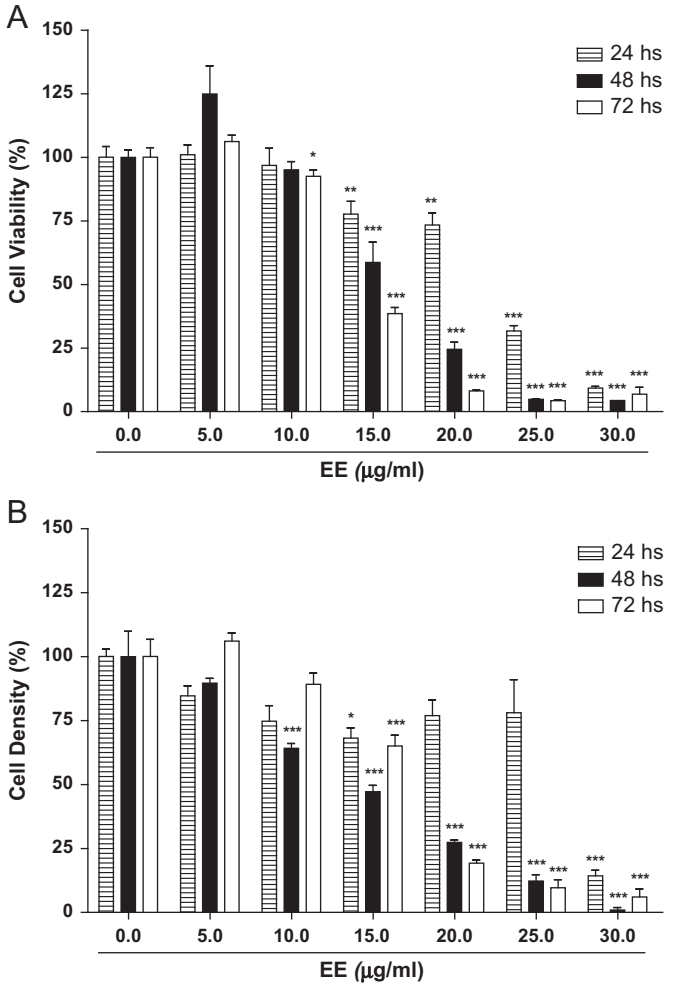

Fig. 8. Effect of ethanolic extract from *Combretum leprosum* (EE) on the viability and proliferation of HaCaT cells. HaCaT cells were exposed to EE (0–30 µg/mL) and incubated for 24, 48 or 72 h. For cell viability, surviving cells were measured by MTT assay (a). Cell density was measured by CyQUANT Cell Proliferation Assay (b). Results were expressed as the percentage of the control group (0—receiving just fresh medium). The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

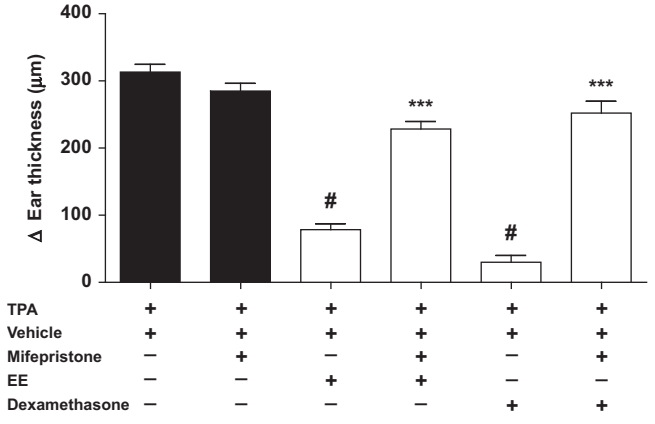

Fig. 9. Reversal of the anti-oedematogenic activity of EE and dexamethasone by mifepristone. EE and dexamethasone was applied immediately after TPA. Mifepristone (50 mg/kg s.c.) was administered 15 min prior to EE (0.6 mg/ear) or dexamethasone (0.001 mg/ear). Each bar represents the mean ± S.E.M. for five animals. The graphic symbols denote the significance levels when compared with control groups. Significantly different from controls, *** and $^{\#}P < 0.001$.

reduce PMN tissue infiltration normally reduce inflammatory parameters and are potential anti-inflammatory tools. MPO is known as a direct marker of neutrophil infiltration; therefore, the inhibition of its activity can be used as an indicator of anti-inflammatory action (Bradley et al., 1982). In our results, it was evidenced that EE was able to decrease the activity of this enzyme, suggesting possible interference in cell migration during the inflammatory process. The histological analysis clearly confirmed that EE, like dexamethasone, inhibited the TPA-induced influx of PMN to the mouse ear skin.

Another evidence of the EE anti-inflammatory activity in the acute inflammatory model was the reduction of IL-6 tissue levels. IL-6 is rapidly expressed during an inflammatory stimulus and acts synergistically with IL-1 and Tumor Necrosis Factor-α in promoting and maintaining acute inflammatory processes. In addition, IL-6 is a modulator of the switch from innate to adaptative immune response thus the reduced level of IL-6 promoted by EE could justify, at least in part, the inhibition of leukocyte tissue infiltration in the EE-treated tissue. (Scheller et al., 2011). Actually, it is clear that defects in the normal IL-6 activity are noticed in the pathogenesis of a number of inflammatory disorders, especially autoimmune diseases. However, increased levels of this cytokine are usually observed in patients with psoriasis and other immune disorders (Ishihara and Hirano, 2002). Nowadays, the blockade of the IL-6 pathway has been considered an interesting target for the treatment of autoimmune diseases. Therefore, in the research for the development of therapies based on IL-6 downregulation, the current challenge is to establish how to interfere with this pathway, and in which conditions it would be beneficial (Jones et al., 2011).

A time-course evaluation of anti-oedematogenic topic activity of the EE showed a long-lasting effect for at least twelve hours (data not shown). *Combretum leprosum* extract seems to act as a prophylactic agent and, most importantly, as a potential option for treatment after the establishment of the inflammatory process, since it was effective even when applied three hours after the TPA stimulus. These results suggested that the extract could be effective in pre-established inflammatory processes. Thus, the model of multiple applications of croton oil allowed the evaluation of the EE in a pre-existent inflammatory process. This model is a reliable tool to assess the response of anti-inflammatory and anti-proliferative compounds on an established chronic inflammatory skin process, characterised by an increase in tissue (ears) weight, intense cell infiltration, and epidermal hyperproliferation (increasing in epidermis measurement with development of acanthosis). These events are similar to those observed in some chronic inflammatory skin diseases like psoriasis (Stanley et al., 1991). Skin inflammation is a prominent pathological feature of psoriasis, which is characterised immunologically by the migration and accumulation of neutrophils and mononuclear cells in the epidermis. Indeed, this condition shows a marked thickening of this layer, due to the uncontrolled proliferation of keratinocytes, also presenting thin downward projections into the dermis. In addition, there is marked infiltration of mononuclear leukocytes in the dermis, which can gain entry to skin parenchyma by transmigration through reactive vessels (Lowes et al., 2007). Therefore, in the model of chronic applications of croton oil, the EE of *Combretum leprosum* was able to reduce all inflammatory parameters evaluated, such as oedema, cell migration and epidermal hyperproliferation.

In chronic inflammation animal models, the major type of infiltrated cells are mononuclear, which are evaluated through of the measurement of the activity of the enzyme *N*-acetyl-β-D-glucosaminidase (NAG). When activated, mononuclear cells degranulate and sequentially promote the release of inflammatory mediators, including cytokines, chemokines, as well as lipid

mediators, that work together to promote the recruitment and activation of other inflammatory cells (Lawrence and Gilroy, 2007). Thus, by inhibiting mononuclear cell migration the extract can contribute to the relief of symptoms trigged by those inflammatory cells and mediators, which is an important deleterious mechanism of chronic inflammatory diseases. Last but not least, EE topical treatment was also able to inhibit the epidermal hyperproliferation induced by croton oil. Since the hallmarks of psoriasis lesions are plaques covered with silvery scales generated by hyperproliferation and altered differentiation of epidermal keratinocytes (Wolf et al., 2012), the efficacy of EE in reducing this occurrence suggests a potential option to treat skin diseases such as psoriasis and seborrheic dermatitis. In addition, our results from *in vitro* experiments showed that EE substantially reduced the proliferation of keratinocytes, which are the main cellular type involved in psoriasis pathogenesis. This effect demonstrates that the extract does not reduce the proliferation of epidermal cells indirectly just by inhibiting tissue inflammation, but also act directly in the epidermal cell viability.

Pre-treatment with the glucocorticoid receptor antagonist mifepristone reversed the inhibitory effect of EE and dexamethasone on TPA-induced ear oedema, suggesting a glucocorticoid-like effect for EE. Interaction with glucocorticoid receptors could explain both the anti-inflammatory and antiproliferative effects of EE, since glucocorticoids are known as great anti-proliferative and anti-inflammatory agents, and are extensively used in anti-psoriatic therapy.

In view of the fact that the Brazilian population uses *Combretum leprosum* as a topical remedy for wound healing and snake bites, based on this preliminary study using *in vivo* animal models of skin inflammation, it is possible to sustain its folk usage; since *Combretum leprosum* reduced inflammatory parameters in acute model it is possibly able to alleviate symptoms of skin injuries. In summary, these results suggest that *Combretum leprosum* can be effective as an anti-inflammatory when topically applied, even though the molecular mechanism whereby *Combretum leprosum* acts has not been completely identified. However, it is necessary to continue the investigations regarding this plant and its compounds to demonstrate its effectiveness, safety, and elucidate the mechanism of action.

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