Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 507 (2005) 253–259

www.elsevier.com/locate/ejphar

Topical antiinflammatory effects of the ether extract from *Protium kleinii* and α-amyrin pentacyclic triterpene

Michel F. Otuki[a], Fabiana Vieira-Lima[b,c], Ângela Malheiros[b,d], Rosendo A. Yunes[b], João B. Calixto[a,*]

[a]*Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário Trindade, 88049-900, Florianópolis, SC, Brazil*

[b]*Departamento de Química, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil*

[c]*Universidade do Sul de Santa Catarina, Curso de Farmácia, Tubarão, SC, Brazil*

[d]*Núcleo de Investigações Químico-Farmacêuticas-NIQFAR/CCS, Universidade do Vale do Itajaí, Itajaí, SC, Brazil*

Received 13 October 2004; accepted 11 November 2004
Available online 15 December 2004

Abstract

Protium kleinii (Burseraceae), a native Brazilian medicinal plant is claimed to be useful to treat some inflammatory states. Now we reported that topical application of either the ether extract or the main active constituent from *P. kleinii* the pentacyclic triterpene α-amyrin, all caused a dose-related inhibition of both ear oedema (ID_{50} values are 0.55 and 0.31 mg/ear, respectively) and influx of polymorphonuclear cells (ID_{50} values are 0.72 and 0.45 mg/ear, respectively) in response to topical application of 12-*O*-tetradecanoylphorbol-acetate (TPA) in the of mice ear. In terms of the efficacy, the maximal obtained inhibition for both ear oedema and neutrophil influx was very similar to that produced by the topical application of the steroidal antiinflammatory drug dexamethasone (DE; with inhibition of 70±5%, 66±3%, and 87±4% for oedema and 83±6%, 73±5%, and 91±3% for neutrophil influx, for the ether extract, α-amyrin, and dexamethasone, respectively). Likewise, both the ether extract and α-amyrin given topically dose-dependently prevented the increase of the proinflammatory cytokine interleukin-1β levels in response to topical application of TPA. The calculated mean ID_{50} values are 1.81 and 0.53 mg/ear, respectively. Again, the efficacy of the extract and α-amyrin was very similar to that produced by dexamethasone (63±6%, 61±5%, and 74±5%, respectively). In marked contrast to phenidone, a lipo and cyclooxygenase inhibitor, neither the ether extract nor the α-amyrin inhibited arachidonic acid-mediated ear oedema in mice. Collectively, these results indicate that the active constituents present in the ether extract of *P. kleinii* including the pentacyclic triterpene α-amyrin are good candidates to develop a skin permeable antiinflammatory drug.

Keywords: Skin inflammation; *Protium kleinii*; Burseraceae; α-Amyrin; Triterpenes; Ear oedema

1. Introduction

As our primary interface with the external environment, the skin is constantly subject to injury and invasion by pathogens, as a result of which various inflammatory disorders can occur over the course of a lifetime (Murphy et al., 2000; Chi et al., 2003). While some skin inflammatory disorders are well treated, normally, it is not possible to successfully treat chronic inflammatory diseases, such as psoriasis and atopic dermatitis (Chi et al., 2003). Cutaneous inflammation is produced and maintained by the interaction of various cell populations (resident macrophages, keratinocytes, fibroblasts, mast cells, platelets, and endothelial cells) that reach the inflammatory focus by chemotaxis (neutrophils, monocytes, lymphocytes) in response to the release of soluble proinflammatory mediators from these cells such as cytokines, prostaglandins, leukotrienes, and

* Corresponding author. Tel.: +55 482 319491; fax: +55 482 329139.
E-mail addresses: calixto@farmaco.ufsc.br, calixto3@terra.com.br (J.B. Calixto).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.11.012

platelet-activating factor (Puignero and Queralt, 1997; Lee et al., 2003; Briganti and Picardo, 2003). Following keratinocyte injury, the skin has the ability to mount an immediate, cytokine-driven, antigen-independent cutaneous inflammatory response mainly mediated by the release of interleukin-1 (Debenedictis et al., 2001).

The plants belonging to the genus *Protium* (Burseraceae) are known for their ability to produce oleoresin exudates that occur as a result of insect stings, broken branches, or other injuries to their bark (Siqueira et al., 1995). Among the reported medicinal uses of these plants are their folk uses for the management of certain inflammatory diseases (Duwiejua et al., 1993). A systemic antiinflammatory action for the essential oil from the leaves and resins of some *Protium* species has been reported (Siani et al., 1999). The resins and leaves of *Protium* species are also commonly used in Brazilian folk medicine for the healing of ulcers and for the treatment of inflammatory complaints (Corrêa, 1984). In addition, we have previously shown a systemic antinociceptive action for the ether extract in inflammatory models of nociception in mice (Otuki et al., 2001).

Phytochemical analysis carried out with the ether extract from *Protium kleinii* has revealed the presence of several pentacyclic triterpenes such as brein (urs-12-ene-3β-16β-diol) and α-amyrin (Recio et al., 1995).

In the present study, we assessed the topical anti-inflammatory properties of the ether extract obtained from the medicinal plant *P. kleinii* and also that of its constituent the ursane pentacyclic triterpene α-amyrin, against models of skin inflammation as TPA (12-*O*-tetradecanoylphorbol-acetate)-induced ear oedema. Topical application of TPA has been used to screen for topically applied antiinflammatory steroids and nonsteroid agents and promotes events of the inflammatory processes such as oedema, cell infiltration, and proliferation had the production of arachidonic acid metabolites, cytokines, and other proinflammatory mediators.

2. Materials and methods

2.1. Preparation of extract, isolation, and chemical identification of the active compound

The botanical material was collected in Morro do Baú, State of Santa Catarina, Brazil, and was classified by Dr. Ademir Reis as being *P. kleinii*, a plant belonging to the Burseraceae family. A voucher specimen of *P. kleinii* (number VC Filho 22) was deposited in the "Herbarium Barbosa Rodrigues" (Itajaí-SC). The resinous bark of *P. kleinii* (50 g) was ground to a powder and extracted with diethyl ether in the proportion of 1:10 (w/v), being stirred and macerated at room temperature for approximately 2 weeks. The solvent was fully evaporated under reduced pressure and the extract (33.42 g) was subjected to chromatography (14.42 g) on a silica gel column eluted successively with hexane, hexane–ethyl acetate, ethyl acetate, ethyl acetate–methanol, methanol, and water, respectively. The fraction eluted with hexane-ethyl acetate (1:1) gave a crystalline solid (120 mg), which was identified as being a mixture of the triterpenes α-amyrin and β-amyrin based on its spectral data. This mixture was obtained in a 1:1 proportion by gas chromatography. The chemical identification was confirmed by using the ^{1}H and ^{13}C NMR (Nuclear Magnetic Resonance) spectra.

2.2. Animals

Male Swiss mice (25–35 g), housed at 22±2 °C (60–80% humidity) under a 12-h light/12-h dark cycle and with access to food and water ad libitum, were used in these experiments that were performed during the light phase of the cycle. The animals were allowed to adapt to the laboratory for at least 2 h before testing and were used only once. Experiments reported in this study were carried out in accordance with current guidelines for the care of laboratory animals and ethical guidelines for the investigation of experimental pain in conscious animals (Zimmermann, 1983).

2.3. Ear oedema measurement

Oedema was expressed as the increase in ear thickness due to the inflammatory challenge. Ear thickness was measured before and after induction of the inflammatory response by using a micrometer (Mitutoyo Series 293). The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges and the thickness was recorded in μm. To minimise variation due to technique, a single investigator performed the measurements throughout any one experiment. Compounds and extract were applied topically in 20 μl acetone.

2.4. Arachidonic acid-induced mouse ear oedema

Ether fraction (3 mg/ear), α-amyrin (1 mg/ear), and phenidone (1 mg/ear; a dual inhibitor of cyclooxygenase/lipoxygenase, used as a positive control) were applied topically, 30 min before the application of arachidonic acid (2 mg/ear) to the right ear. The thickness of the ears was measured before and 1 h after induction of inflammation (Young et al., 1984; Crummey et al., 1987).

2.5. 12-O-tetradecanoylphorbol-acetate (TPA)-induced mouse ear oedema

Oedema was induced on the right ear by topical application of 2.5 μg/ear of TPA dissolved in 20 μl of acetone. Ether fraction (0.3–3.0 mg/ear), α-amyrin (0.1–1.0 mg/ear), or dexamethasone (DE; 0.05 mg/ear, used as a positive control) was applied topically simultaneously with TPA. The thickness of the ears was measured before and 6h after induction of inflammation (De Young et al., 1989).

2.6. Tissue myeloperoxidase assay

The activity of tissue myeloperoxidase was assessed 24 h after TPA application to the mouse ear according to the technique reported by Suzuki et al. (1983) and modified by De Young et al. (1989). A biopsy (6 mm ear tissue punch) was placed in 0.75 ml of 80 mM phosphate-buffered saline (PBS) pH 5.4, containing 0.5% hexadecyltrimethylammonium bromide, then homogenised (45 s at 0 °C) in a motor-driven homogenizer. The homogenate was decanted into a microfuge tube, and the vessel was washed with a second 0.75 ml aliquot of hexadecyltrimethylammonium bromide in buffer. The wash was added to the tube and the 1.5 ml sample was centrifuged at 12 000×*g* at 4 °C for 15 min. Triplicate 30-μl samples of the resulting supernatant were added to 96-well microtitre plates. For 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% hydrogen peroxide were added to the wells. The reaction was started by the addition of 20 μl of 18.4 mM tetramethylbenzidine HCl in 8% aqueous dimethylformamide. Plates were incubated at 37 °C for 3 min and then placed on ice where the reaction was stopped by the addition of 30 μl of 1.46 M sodium acetate, pH 3.0. Enzyme activity was determined colorimetrically using a Bio-Tek Ultra Microplate reader (EL 808) set to measure absorbance at 630 nm and expressed as mOD/biopsy.

2.7. Measurement of the ear levels of interleukin-1β

The tissue cytokine levels were determined 6 h after TPA application to the mouse ear using a standard sandwich ELISA (enzyme-linked immunosorbent assay) method similar to that described previously (Pinheiro and Calixto, 2002). The tissue was homogenised in PBS containing 0.05% Tween 20, 0.1 mM phenylmethylsulphonyl fluoride, 0.1 mM benzamethonium chloride, 10 mM EDTA and 20 Kl aprotinin A, and centrifuged at 3000×*g* for 10 min before being stored at −70 °C until further analysis. Interleukin-1β levels were measured using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

2.8. Histology

Ear samples (were fixed in 4% neutral buffered formalin. Each sample was cut longitudinally into equal halves, one of which was embedded in paraffin wax, sectioned at 3–4 μm and stained with haematoxylin–eosin. A representative area was selected for qualitative light microscopic analysis of the inflammatory cellular response with a 40× objective (Recio et al., 2000). To minimise a source of bias, the investigator did not know the group that he was analyzing.

2.9. Statistical analysis

The results are presented as means±S.E.M., except the ID_{50} values (i.e., the dose of compound or ether fraction reducing the inflammatory response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. Data were subjected to analysis of variance (ANOVA) or *t*-test and complemented by Dunnett's or Newman Keul's post hoc test when appropriate. $P<0.05$ was considered as indicative of significance. The ID_{50} values were determined by linear regression from individual experiments using GraphPad Software (California, USA).

2.10. Drugs

The following substances were used: 12-*O*-tetradecanoylphorbol-acetate (TPA), arachidonic acid, α-amyrin, dexamethasone, phenidone, hexadecyltrimethylammonium bromide, tetramethylbenzidine hydrogen peroxide, formaldehyde, Tween 20, Tween 80, phenylmethylsulphonyl fluoride, benzamethonium chloride, EDTA, aprotinin, phosphate-buffered saline eosin, hematoxylin (all from Sigma, St. Louis, USA), sodium acetate, dimethylformamide, acetone,

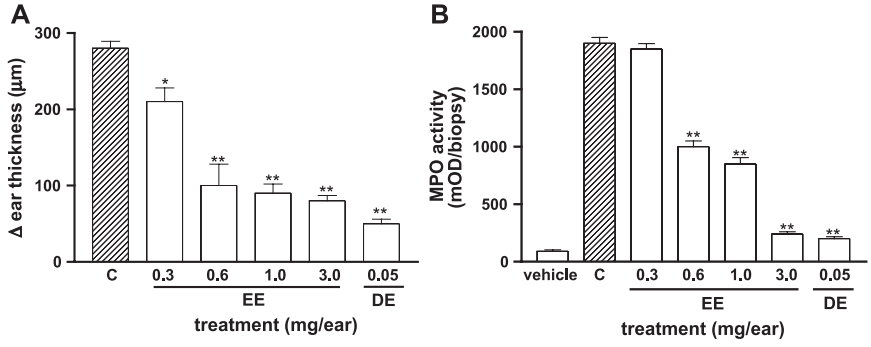

Fig. 1. Effect of ether extract (EE) and dexamethasone (DE) administered topically on (A) TPA-induced ear edema and (B) myeloperoxidase activity in supernatants of homogenates from TPA-treated ears. Ear edema and myeloperoxidase activities were measured at 6 and 24 h after TPA treatment, respectively. Each point represents the mean±S.E.M. for 6–10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, **P*<0.05 and ***P*<0.01.

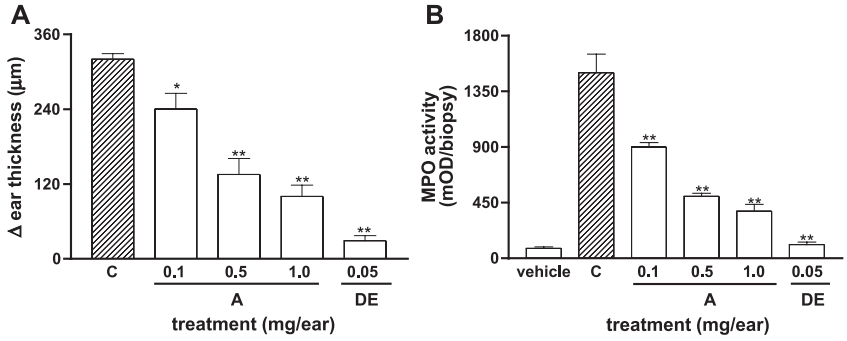

Fig. 2. Effect of α-amyrin (A) and dexamethasone (DE) administered topically on (A) TPA-induced ear edema and (B) myeloperoxidase activity in supernatants of homogenates from TPA-treated ears. Ear edema and myeloperoxidase activities were measured at 6 and 24 h after TPA treatment, respectively. Each point represents the mean±S.E.M. for 6–10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, *P<0.05 and **P<0.01.

formaldehyde, and absolute ethanol (Merck, Darmstadt, Germany).

3. Results

3.1. 12-O-tetradecanoylphorbol-acetate (TPA)-induced mouse ear oedema and tissue myeloperoxidase assay

Topical application of the ether extract of *P. kleinii* and its main active principle, the pentacyclic triterpene α-amyrin, caused a significant and dose-dependent inhibition of the TPA-induced skin inflammation, i.e., both oedema and cell migration. The estimated mean ID_{50} values from the oedema inhibition for ether extract and α-amyrin were 0.55 (0.38–0.64) and 0.31 (0.21–0.6) mg/ear, with inhibition of 70±5% and 66±5%, respectively. In contrast, the myeloperoxidase activity (indicative of polymorphonuclear leukocytes influx) peaked at 24 h after TPA application and then slowly decreased (De Young et al., 1989). Application of the ether extract of *P. kleinii* and the triterpene α-amyrin to the ear caused dose-dependent inhibition of myeloperoxidase activity. The calculated mean ID_{50} values were 0.72 (0.65–0.87) and 0.45 (0.28–0.59) mg/ear, with inhibition of 83±6% and 75±3%, respectively. The used reference drug dexamethasone (0.05 mg/ear) also caused a significant inhibition of both parameters, 87±4% and 91±3%, for oedema and myeloperoxidase activity, respectively (Figs. 1 and 2).

3.2. Histological analysis

Optical microscopic analysis of the mice' ears, 24 h after application of TPA, revealed epidermal hyperplasia and marked infiltration of inflammatory cells associated with dilated blood vessels. These events were greatly reduced after topical application of either ether extract (3 mg/ear) or α-amyrin (1 mg/ear) as well as by the positive control dexamethasone (0.05 mg/ear; Fig. 3).

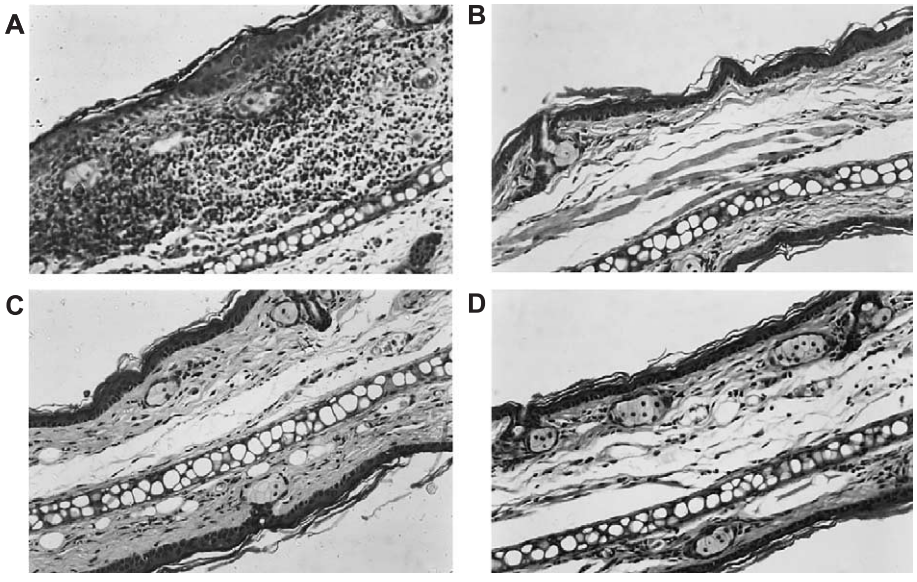

Fig. 3. Histology of vertical sections of mouse ears (16×, HE-stained) were evaluated 24 h after TPA application. Ears treated with TPA (A) and TPA plus ether extract of *P. kleinii* (3 mg/ear), (B); α-amyrin (1 mg/ear), (C); dexamethasone (0.05 mg/ear), (D).

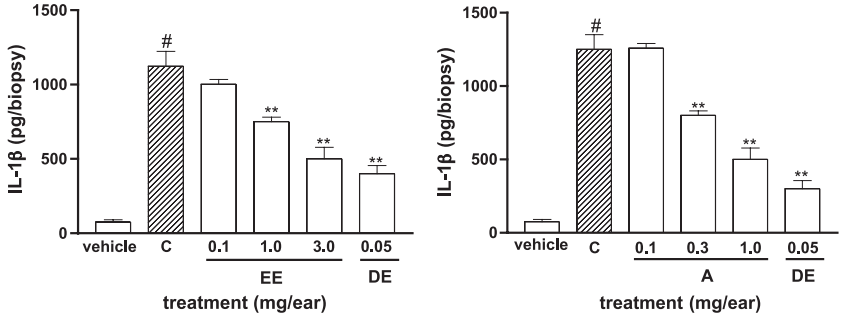

Fig. 4. Effect of ether extract (EE), α-amyrin (A), and dexamethasone (DE) administered topically on TPA-induced interleukin-1β production. Interleukin-1β level was measured at 6 h after TPA treatment. Each point represents the mean±S.E.M. for 6–10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, *P<0.05 and **P<0.01.

3.3. Measurement of tissue levels of interleukin-1β

Having shown that both the ether extract and the main active compound α-amyrin produced marked inhibition of the ear inflammatory response caused by topical application of TPA, we next investigated whether or not this inhibition involved a reduction in the level of the proinflammatory cytokine interleukin-1β. Topical application of TPA to the ear caused a dramatic increase (about eight-fold) in the tissue level of interleukin-1β, 6 h after challenge. The increase in the level of interleukin-1β in the ear was inhibited in a dose-dependent manner by topical application of either the ether extract from *P. kleinii* or the triterpene α-amyrin. The mean ID_{50} values were 1.81 (1.62–1.97) and 0.53 (0.42–0.73) mg/ear with inhibition of 63±6% and 61±5% for the ether extract and α-amyrin, respectively. Dexamethasone (0.05 mg/ear) also caused a marked inhibition (74±5%) of the increase in tissue levels of interleukin-1β (Fig. 4).

3.4. Arachidonic acid-induced mouse ear oedema

Both cyclooxygenase and lipoxygenase metabolites derived from the arachidonic acid pathway are involved in the inflammatory processes. Furthermore, topical application of arachidonic acid provokes a rapid and intense inflammatory response in the mouse ear that was blocked by lipoxygenase inhibitors (Young and De Young, 1989). Thus, we next investigated whether topical application of ether extract or α-amyrin would inhibit arachidonic acid-induced ear oedema in mice. In contrast to the dual cyclooxygenase/lipoxygenase inhibitor phenidone, both the ether extract and α-amyrin completely failed to interfere with arachidonic acid-induced mouse ear oedema (Fig. 5).

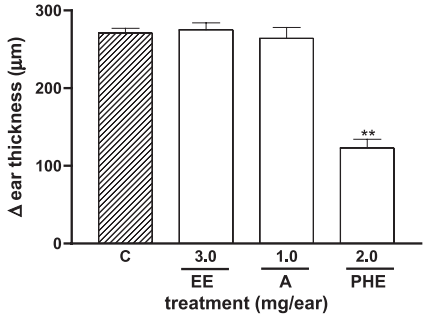

Fig. 5. Effect of ether extract (EE), α-amyrin (A), and phenidone (PHE) administered topically on AA-induced ear edema. Ear edema was measured at 1 h after AA treatment. Each point represents the mean±S.E.M. for 6–10 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, *P<0.05.

4. Discussion

The main finding of the present study is that topical application of the ether extract from *P. kleinii* or its main active principle, the pentacyclic triterpene α-amyrin, similarly to the antiinflammatory drug dexamethasone, resulted in a marked inhibition of three important events related to the skin inflammatory response induced by TPA, namely oedema formation, the migration of polymorphonuclear leukocytes, and increase in tissue IL-1β levels. Myeloperoxidase is an enzyme found in the azurophilic granules of neutrophils and other cells of myeloid origin, which is commonly used as an index of granulocyte infiltration, and its inhibition is indicative of an antiinflammatory action (Bradley et al., 1982; Ajuebor et al., 2000). Our histological analysis of the ear clearly confirmed that the ether extract and α-amyrin inhibited the influx of polymorphonuclear cells to the mouse ear skin following application of TPA. Therefore, taken together, these results consistently support the notion that the active principle present in *P. kleinii* possesses topical antiinflammatory properties.

The above reported actions are interesting because the accumulation of neutrophils plays a critical role in cutaneous inflammatory diseases such as dermatitis, indeed the presence of neutrophils in the lesion may bear a relationship to the pathological mechanism (Bradley et al., 1982; Katz and Strober, 1978). Moreover, it has been reported that skin leukocyte accumulation induced by TPA application is necessary for the progression of the inflammatory reaction as well as for the overexpression of the enzyme cyclooxygenase-2 (Sanchez and Moreno, 1999).

As reported for both ear oedema and myeloperoxidase inhibition, topical application of both the ether extract and

α-amyrin from *P. kleinii* resulted in a dose-dependent inhibition, with similar potencies, of the increase in interleukin-1β in the mouse ear following topical application of TPA. The efficacies of both the ether extract and α-amyrin were very close to that observed for the steroidal antiinflammatory drug dexamethasone. Such results strongly support the notion that the marked inhibition caused by the ether extract and α-amyrin from *P. kleinii* of ear oedema and myeloperoxidase activity in response to topical application of TPA may be related to their ability to inhibit the release of the proinflammatory cytokine interleukin-1β. In fact, cells in the injured skin such as dermal dendritic cells, epidermal Langerhans cells, melanocytes, fibroblasts, and migrating leukocytes are known as source and target of interleukin-1β (Grone, 2002). Moreover, interleukin-1β pathway is the induced transcription of a series of genes, including adhesion molecules, chemokines, secondary cytokines, nitric oxide synthase, and cyclooxygenase, all relevant to skin inflammation (Murphy et al., 2000).

The antiinflammatory effects of α-amyrin herein reported confirm and extend data previously reported in the literature (Recio et al., 1995; Akihisa et al., 1996). However, the proposed mechanism of the antiinflammatory action by interference in proinflammatory cytokine is in unpublished works. These results could be explained either in the in vivo antiinflammatory effect of other pentacyclic triterpenes, principally the ursane triterpenoid skeleton ursolic acid (Recio et al., 1995; Baricevic et al., 2001; Tapondjou et al., 2003) and uvaol (Recio et al., 1995; Yasukawa et al., 1996), which has very similar chemical structure. In addition, several plants as such *Himatanthus sucuuba*, *Sideritis taurica*, *Calendula officinalis* (used in cosmetic preparations) having antiinflammatory effects (Della Logia et al., 1994; Akihisa et al., 1996; De Miranda et al., 2000; Aboutabl et al., 2002) by amyrin triterpenes content.

A final interesting result was the complete lack of topical antiinflammatory activity for both the ether extract and α-amyrin from *P. kleinii* when assessed against ear oedema induced by application of arachidonic acid in a situation where phenidone, a dual inhibitor of cyclooxygenase/lipoxygenase, caused a marked inhibition of ear oedema. In fact, a marked difference exists in the mechanisms of the inflammatory response induced by topical application of TPA and arachidonic acid in the mouse. For example, phospholipase A_2 and cyclooxygenase inhibitors have little or no effect on arachidonic acid-induced ear oedema, but are highly effective against the inflammation caused by croton oil or TPA. For many years, the arachidonic acid-induced ear inflammation was regarded as a suitable screen for detecting lipoxygenase inhibitors in vivo. However, recently, several studies have shown that the arachidonic acid-induced inflammation can be inhibited by mechanisms other than cyclooxygenase/lipoxygenase enzyme inhibition, as modulation of histamine and serotonin response (Chang et al., 1985; Pignat et al., 1986; Sanchez and Moreno, 1999; Gabor, 2000). Thus, the identification of the antiinflammatory mechanisms of the ether extract of *P. kleinii* and α-amyrin in these models is very complex.

A major drawback for the development of percutaneous drug delivery is normally related to the low penetration of such drugs through the skin. Nevertheless, skin penetration studies carried out in vivo have shown that topically administered triterpenes can be easily absorbed through the skin surface and penetrate into the epidermis and dermis (Trotta et al., 2002). The results of the present study clearly show that the ether extract from *P. kleinii* and its main active constituent, the pentacyclic triterpene α-amyrin, are of potential interest for the development of low-molecular-weight drugs easily permeably through the skin and of interest in the treatment of skin inflammatory states.

At this stage of our work, it is difficult to speculate about the precise mechanism through which α-amyrin exerts such an effect. One or more targets could be involved including proteins that play a role in the genesis of the inflammatory response, namely cyclooxygenase-1 and cyclooxygenase-2, as well as an interaction with the nuclear transcription factor κB or with the release and/or action of several cytokines, chemokines or adhesion molecules (for recent reviews, see Calixto et al., 2003, 2004).

Experiments are currently in progress in our laboratory aimed at investigating the cellular and molecular mechanisms by which α-amyrin exerts its antiinflammatory action.

Acknowledgements

The authors are indebted to Dr. Ademir Reis for botanical classification of *Protium kleinii*. This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (FINEP) and by Programa de Apoio aos Grupos de Excelência (PRONEX) Brazil. Michel Fleith Otuki is a PhD student in pharmacology and thanks CNPq and CAPES for fellowship support.

References

Aboutabl, E.A., Nassar, M.I., Elsakhawy, F.M., Maklad, Y.A., Osman, A.F., El-Khrisy, E.A., 2002. Phytochemical and pharmacological studies on *Sideritis taurica* Stephan ex Wild. J. Ethnopharmacol. 82, 177–184.

Ajuebor, M.N., Singh, A., Wallace, J.L., 2000. Cyclooxygenase-2-derived prostaglandin D(2) is an early anti-inflammatory signal in experimental colitis. Am. J. Physiol.: Gasterointest. Liver Physiol. 279, G238–G244.

Akihisa, T., Yasukawa, K., Oinuma, H., Kasahara, Y., Yamanouchi, S., Takido, M., Kumaki, K., Tamura, T., 1996. Triterpene alcohols from the flowers of compositae and their anti-inflammatory effects. Phytochemistry 43, 1255–1260.

Baricevic, D., Sosa, S., Della Logia, R., Tubazo, A., Simonovska, B., Krasna, A., Zupancic, A., 2001. Topical anti-inflammatory activity of *Salvia officinalis* L. leaves: the relevance of ursolic acid. J. Ethnopharmacol. 75, 125–132.

Bradley, P.P., Priebat, D.A., Christensen, R.D., Rothstein, G., 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J. Invest. Dermatol. 78, 206–209.

Briganti, S., Picardo, M., 2003. Antioxidant activity, lipid peroxidation and skin diseases. What's new. J. Eur. Acad. Dermatol. Venereol. 17, 663–669.

Calixto, J.B., Otuki, M.F., Santos, A.R., 2003. Anti-inflammatory compounds of plant origin. Part I: Action on arachidonic acid pathway, nitric oxide and nuclear factor kappa B (NF-kappaB). Planta Med. 69, 973–983.

Calixto, J.B., Campos, M.M., Otuki, M.F., Santos, A.R., 2004. Anti-inflammatory compounds of plant origin. Part II: modulation of pro-inflammatory cytokines, chemokines and adhesion molecules. Planta Med. 70, 93–103.

Chang, J., Lamb, B., Marinari, L., Kreft, A.F., Lewis, A.J., 1985. Modulation by hydroxyeicosatetraenoic acids (HETEs) of arachidonic acid metabolism in mouse resident peritoneal macrophages. Eur. J. Pharmacol. 107, 215–222.

Chi, Y.S., Lim, H., Park, H., Kim, H.P., 2003. Effects of wogonin, a plant flavone from *Scutellaria radix*, on skin inflammation: in vivo regulation of inflammation-associated gene expression. Biochem. Pharmacol. 66, 1271–1278.

Corrêa, P., 1984. Dicionário de plantas úteis do Brasil e das Exóticas cultivadas. Imprensa Nacional, vol. 1. Ministério da Agricultura, Rio de Janeiro, Brasil, p. 82.

Crummey, A., Harper, G.P., Boyle, E.A., Mangan, F.R., 1987. Inhibition of arachidonic acid-induced ear oedema as a model for assessing topical anti-inflammatory compounds. Agents Actions 20, 69–76.

Debenedictis, C., Joubeh, S., Zhang, G., Barria, M., Ghohestani, R.F., 2001. Immune functions of the skin. Clin. Dermatol. 19, 573–585.

Della Logia, R., Tubazo, A., Sosa, S., Becker, H., Saar, S., Isaac, O., 1994. The role of triterpenoids in the topical anti-inflammatory activity of *Calendula officinalis* flowers. Planta Med. 60, 516–520.

De Miranda, A.L., Silva, J.R., Rezende, C.M., Neves, J.S., Parrini, S.C., Pinheiro, M.L., Cordeiro, M.C., Tamborini, E., Pinto, A.C., 2000. Anti-inflammatory and analgesic activities of the latex containing triterpenes from *Himatanthus sucuuba*. Planta Med. 66, 284–286.

De Young, L.M., Kheifets, J.B., Ballaron, S.J., Young, J.M., 1989. Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. Agents Actions 26, 335–341.

Duwiejua, M., Zeitlin, I.J., Waterman, P.G., Chapman, J., Mhango, G.J., Provan, G.J., 1993. Anti-inflammatory activity of resins from some species of the plant family Burseraceae. Planta Med. 59, 12–16.

Gabor, M., 2000. Mouse Ear Inflammation Models and their Pharmacological Applications. Akadémiai Kiadó, Budapest, HU.

Grone, A., 2002. Keratinocytes and cytokines. Vet. Immunol. Immunopathol. 88, 1–12.

Katz, S.I., Strober, W., 1978. The pathogenesis of dermatitis herpetiformis. J. Invest. Dermatol. 70, 63–75.

Lee, J.L., Mukhtar, H., Bickers, D.R., Kopelovich, L., Athar, M., 2003. Cyclooxygenases in the skin: pharmacological and toxicological implications. Toxicol. Appl. Pharmacol. 192, 294–306.

Murphy, J.E., Robert, C., Kupper, T.S., 2000. Interleukin-1 and cutaneous inflammation: a crucial link between innate and acquired immunity. J. Invest. Dermatol. 114, 602–608.

Otuki, M.F., Lima, F.V., Malheiros, A., Cechinel-Filho, V., Delle Monache, F., Yunes, R.A., Calixto, J.B., 2001. Evaluation of the antinociceptive action caused by ether fraction and a triterpene isolated from resin of *Protium kleinii*. Life Sci. 69, 2225–2236.

Pignat, W., Kienzle, R., Bottcher, I., 1986. How specific is the arachidonic acid-induced mouse ear oedema for lipoxygenase (LO)- and cyclo-oxygenase (CO)-inhibitors? Agents Actions 19, 368–370.

Pinheiro, R.M., Calixto, J.B., 2002. Effect of the selective COX-2 inhibitors, celecoxib and rofecoxib in rat acute models of inflammation. Inflamm. Res. 51, 603–610.

Puignero, V., Queralt, J., 1997. Effect of topically applied cyclooxygenase-2-selective inhibitors on arachidonic acid- and tetradecanoylphorbol acetate-induced dermal inflammation in the mouse. Inflammation 21, 431–442.

Recio, M.C., Giner, R.M., Manez, S., Rios, J.L., 1995. Structural requirements for the anti-inflammatory activity of natural triterpenoids. Planta Med. 61, 182–185.

Recio, M.C., Giner, R.M., Uriburu, L., Máñez, S., Cerdá, M., De la Fuente, J.R., Ríos, J.L., 2000. In vivo activity of pseudoguaianolide sesquiterpene lactones in acute and chronic inflammation. Life Sci. 66, 2509–2518.

Sanchez, T., Moreno, J.J., 1999. Role of leukocyte influx in tissue prostaglandin H synthase-2 overexpression induced by phorbol ester and arachidonic acid in skin. Biochem. Pharmacol. 58, 877–879.

Siani, A.C., Ramos, M.F., Menezes-de-Lima Jr., O., Ribeiro-dos-Santos, R., Fernadez-Ferreira, E., Soares, R.O., Rosas, E.C., Susunaga, G.S., Guimarães, A.C., Zoghbi, M.G., Henriques, M.G., 1999. Evaluation of anti-inflammatory-related activity of essential oils from the leaves and resin of species of *Protium*. J. Ethnopharmacol. 66, 57–69.

Siqueira, J.B.G., Zoghbi, M.G.B., Cabral, J.A., Filho, W.W., 1995. Lignans from *Protium tenuifolium*. J. Nat. Prod. 58, 730–732.

Suzuki, K., Ota, H., Sasagawa, S., Sakatani, T., Fujikura, T., 1983. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal. Biochem. 132, 345–352.

Tapondjou, L.A., Lontsi, D., Sondengam, B.L., Choi, J., Lee, K.T., Jung, H.J., Park, H.J., 2003. In vivo anti-nociceptive and anti-inflammatory effect of the two triterpenes, ursolic acid and 23-hydroxyursolic acid, from *Cussonia bancoensis*. Arch. Pharm. Res. 26, 143–146.

Trotta, M., Peira, E., Debernardi, F., Gallarate, M., 2002. Elastic liposomes for skin delivery of dipotassium glycyrrhizinate. Int. J. Pharm. 241, 319–327.

Yasukawa, K., Akihisa, T., Oinuma, H., Kasahara, Y., Kimura, Y., Yamanouchi, S., Kumaki, K., Tamura, T., Takido, M., 1996. Inhibitory effect of Di- and trihydroxy triterpenes from the flowers of compositae on 12-*O*-tetradecanoylphorbol-13-acetate-induced inflammation in mice. Biol. Pharm. Bull. 19, 1329–1331.

Young, J.M., De Young, L.M., 1989. Cutaneous models of inflammation for evaluation of topical and systemic pharmacological agents. In: Spector, J., Back, N. (Eds.), Pharmacological Agents in Pharmacological Methods in Control of Inflammation. Alan R. Liss, New York, NY, pp. 215–231.

Young, J.M., Spires, D.A., Bedord, C.J., Wagner, B., Ballaron, S.J., De Young, L.M., 1984. The mouse ear inflammatory response to topical arachidonic acid. J. Invest. Dermatol. 82, 367–371.

Zimmermann, M., 1983. Ethical guidelines for investigation of experimental pain in conscious animals. Pain 16, 109–110.