

Topical antiinflammatory activity and chemical composition of the epicuticular wax from the leaves of *Eugenia beaurepaireana* (Myrtaceae)

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In order to verify the topical antiinflammatory effect of epicuticular wax from leaves of *Eugenia beaurepaireana*, it was tested in mice croton oil-induced inflammation. Our findings show that topical application of *Eugenia beaurepaireana* epicuticular wax was significantly active in inhibiting both oedema (Inhibitory dose 50 % (ID₅₀) = 0.31 (0.26 – 0.39) mg.ear⁻¹, inhibition = 79 ± 6 %) and tissue myeloperoxidase activity (indicative of polymorphonuclear leukocytes influx) (ID₅₀ = 0.34 (0.20 – 0.41) mg.ear⁻¹, inhibition = 77 ± 4 %) in mice ear treated with croton oil. Two main compounds were detected on epicuticular wax of *E. beaurepaireana*. These compounds were identified as α - and β -amyrin by flame ionization detection (GC–FID) and spectroscopic methods (IR, NMR ¹H and ¹³C). In conclusion, the results indicate a topical antiinflammatory activity for the *Eugenia* specie studied and, that, at least in part, α - and β -amyrin are responsible for this activity.

Uniterms: *Eugenia beaurepaireana*/pharmacognosy. α -amyrin. β -amyrin. Epicuticular wax/antiinflammatory activity. Ear oedema/experimental study.

A atividade antiinflamatória tópica da cera epicuticular das folhas de *Eugenia beaurepaireana* foi avaliada pelo modelo do edema de orelha induzido pelo óleo de cróton em camundongos. Os resultados do estudo mostram que a aplicação tópica da cera epicuticular de *Eugenia beaurepaireana* inibiu significativamente a formação do edema (Dose inibitória 50 % (DI₅₀) = 0,31 (0,26 – 0,39) mg.orelha⁻¹, inibição = 79 ± 6 %) e a atividade da mieloperoxidase tissular (indicativo do influxo de leucócitos polimorfonucleares) (DI₅₀ = 0,34 (0,20 - 0,41) mg.orelha⁻¹, inibição = 77 ± 4 %) em camundongos tratados com o óleo de cróton. Dois compostos majoritários foram detectados e isolados da cera epicuticular de *E. beaurepaireana*. Estes compostos foram identificados como os triterpenos α -amirina e β -amirina, através de técnicas cromatográficas (CG-FID) e espectroscópicas (IV, RMN ¹H e ¹³C). Em conclusão, os resultados indicam que a espécie *E. beaurepaireana* apresenta um efeito antiinflamatório tópico relevante, sendo os compostos α -amirina e β -amirina responsáveis, pelo menos em parte, por esta atividade.

Unitermos: *Eugenia beaurepaireana*/farmacognosia. α -amirina. β -amirina. Cera epicuticular/atividade antiinflamatória. Edema de orelha/estudo experimental.

INTRODUCTION

Epicuticular waxes are complex mixtures of long

chain aliphatic and cyclic components, including hydrocarbons, primary and secondary alcohols, aldehydes, ketones, esters, fatty acids, triterpenoids and flavonoids that cover the external side of the leaf epidermis of all higher plants (Alcerito *et al.*, 2002; Furlan *et al.*, 2006; Medina *et al.*, 2006). The physical and chemical properties of epicuticular wax determine functions vital for plant life. It restricts

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non-stomatal water loss, protects plants against ultraviolet radiation and reduces water retention on the surface of the plant thus minimizing deposition of dust, pollen and air pollutants. In addition, surface wax is believed to play important roles in plant defense against bacterial and fungal pathogens and has been shown to participate in a variety of plant-insect interactions (Kunst, Samuels, 2003).

The family Myrtaceae consists of around 129 genera and 4620 species. One important genera of this family is *Eugenia*, which is one of the larger genera with around 500 species. Several species of *Eugenia* have been reported for uses in folk medicine (Lee *et al.*, 1997; Revilla, 2002). *Eugenia beaurepaireana* (Kiaerskou) Legrand (Myrtaceae) is a tree popularly called “ingabaú” or “guamirim-ferro”. The use of *E. beaurepaireana* in South of America is recommended for the treatment of inflammatory and ulcerative diseases and used as astringent (Revilla, 2002). There have been no previous reports of isolated compounds and biological activities of this species. One study on the essential oils of this plant has demonstrated that bicyclogermacrene, Δ -cadinene, τ -cadinol and β -caryophyllene are the major compounds in *E. beaurepaireana* essential oil (Apel *et al.*, 2004).

Tissue injury triggers a homeostatic response in which various cells populations like as keratinocytes, neutrophils and mast cells release proinflammatory mediators such as cytokines, prostaglandins, and leukotrienes that are coordinated to achieve repair, defined as the reestablishment of vital functions (Puignero, Queralt, 1997; Kim, Wong, Coulombe, 2006). However, chronic inflammatory response that is characteristic of many skin diseases as psoriasis (Lebwohl, 2003). These diseases can be treated either topically or systemically with glucocorticoids, immunosuppressors, and more recently, with monoclonal antibodies and recombinant cytokines (Nickoloff, Stevens, 2006).

However, these therapeutic alternatives are usually aggressive and not effective in all cases (Gottlieb, 2005). Thus, many studies are being developed with the purpose of finding better treatment options with higher efficacy and fewer side effects. One of the strategies used for the medicine development is the use of medicinal plants (Calixto, Otuki, Santos, 2003).

In order to verify the topical antiinflammatory effect of *E. beaurepaireana*, its epicuticular wax from leaves was tested in mice skin inflammation model.

MATERIAL AND METHODS

Material

Croton oil, dexamethasone, hexadecyltrimethylammonium bromide, tetramethylbenzidine hydrogen

peroxide, formaldehyde, tween 80, phosphate-buffered saline eosin, hematoxylin were from Sigma, St. Louis, USA. Sodium acetate, dimethylformamide, acetone, formaldehyde, and absolute ethanol were from Merck, Darmstadt, Germany.

Wax extraction

Leaves of *E. beaurepaireana* were collected in the city of Santo Amaro da Imperatriz (SC), in September 2004. Voucher specimens were identified by Prof. Dr. Daniel de Barcellos Falkenberg (Botany Department, UFSC) and deposited at the Universidade Federal de Santa Catarina Herbarium at number FLOR-34674. Epicuticular wax was extracted from fresh and healthy leaves by wash in hexane for 5 min. Wax extract was filtered and evaporated to dryness at rotary evaporator under 40 °C.

Chemical analysis

Chemical composition of extracted epicuticular wax was analyzed by capillary gas chromatography flame ionization detection (GC-FID) using GC Shimadzu 14-B equipment with DB-1 column (30 m x 0.25 mm; film thickness 0.25 μ m). Hydrogen acted as carrier gas. The chromatographic conditions used were temperature program 60 °C to 300 °C, with increasing of 10 °C.min⁻¹. The temperatures of injector and detector were 300 °C and 290 °C, respectively.

Identification of wax compounds was done by GC retention index, by co-injection with standards of α -amyirin (1) and β -amyirin (2), and spectroscopic methods (IR, NMR ¹H and ¹³C), in comparison with published data (Mahato, Kundu, 1994).

Animals

Male Swiss mice (25–35 g) housed at 22 \pm 2 °C 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 1 h before testing and were used only once. Experiments reported in this study were performed after approval of the protocol n° 127 by the Institutional Ethics of UFPR University and were carried out in accordance with the “Principles of laboratory animal care” from NIH publication 85-23.

Ear oedema measurement

Oedema was expressed as the increase in ear thick-

ness due to the inflammatory challenge. Ear thickness was measured before and after induction of the inflammatory response by using a digital micrometer (Great, MT-045B). The micrometer was applied near the tip of the ear just distal to the cartilaginous ridges and the thickness was recorded in μm . To minimize variation due to technique, a single investigator performed the measurements throughout any one experiment. Epicuticular wax of *E. beaurepaireana* was topically applied in 20 μL acetone (Otuki *et al.*, 2005).

Croton oil-induced dermatitis

Oedema was induced on the right ear by topical application of 0.1 $\text{mg}\cdot\text{ear}^{-1}$ of croton oil dissolved in 20 μL of acetone. Epicuticular wax of *E. beaurepaireana* and dexamethasone (used as a positive control) was topically applied simultaneously with croton oil. The thickness of the ears was measured before and 6 h after the induction of inflammation (Swingle, Reiter, Schwartzmiller, 1981; Tragni *et al.*, 1985).

Tissue myeloperoxidase activity assay

The activity of tissue myeloperoxidase was assessed 24 h after croton oil 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 homogenized (45 s at 0 $^{\circ}\text{C}$) 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 $\times g$ at 4 $^{\circ}\text{C}$ for 15 min. Triplicate 30 μL samples of the

resulting supernatant were added to 96-well plates. For assay, 200 μL of a mixture containing 100 μL of 0.08 M 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 with addition of 20 μL of 18.4 mM tetramethylbenzidine HCl in dimethylformamide. Plates were incubated at 37 $^{\circ}\text{C}$ for 3 min and then placed on ice where the reaction was stopped by addition of 30 μL of 1.46 M sodium acetate, pH 3.0. Enzyme activity was determined colorimetrically using a plate reader (EL808 - BioTech Instruments, INC) set to measure absorbance at 630 nm and expressed as mOD/ mg tissue.

Statistical analysis

The results are presented as means \pm S.E.M (standard error of mean), except the ID_{50} values (i.e., the dose of epicuticular wax of *E. beaurepaireana* 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 post hoc test. $P < 0.05$ was considered as indicative of significance. The ID_{50} values were determined by linear regression from individual experiments using Graphpad Software (California, USA).

RESULTS AND DISCUSSION

Two main compounds were detected on epicuticular wax of *E. beaurepaireana*. They were identified as α - and β -amyrin (Figure 1) by GC relative retention index with available standards and co-injection with authentic standards. The identification was confirmed by NMR and the spectral data of these isolated compounds stayed in agreement with literature (Mahato, Kundu, 1994; Park *et al.*, 2004).

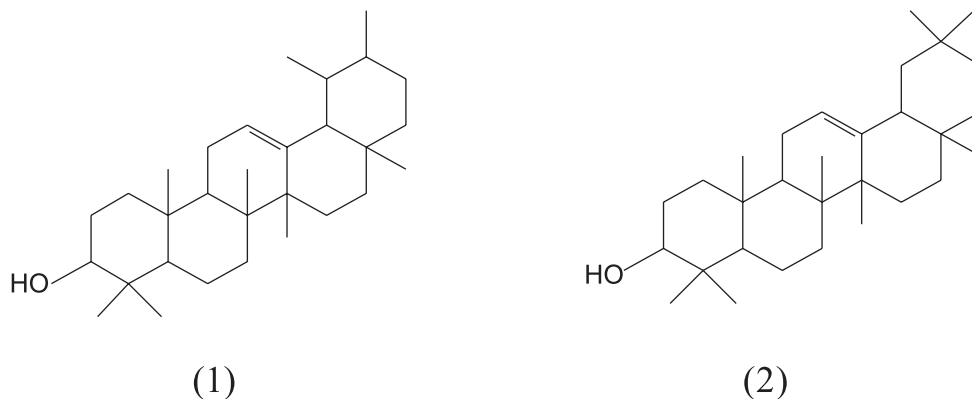


FIGURE 1 - Structures of α - (1) and β - amyrin (2).

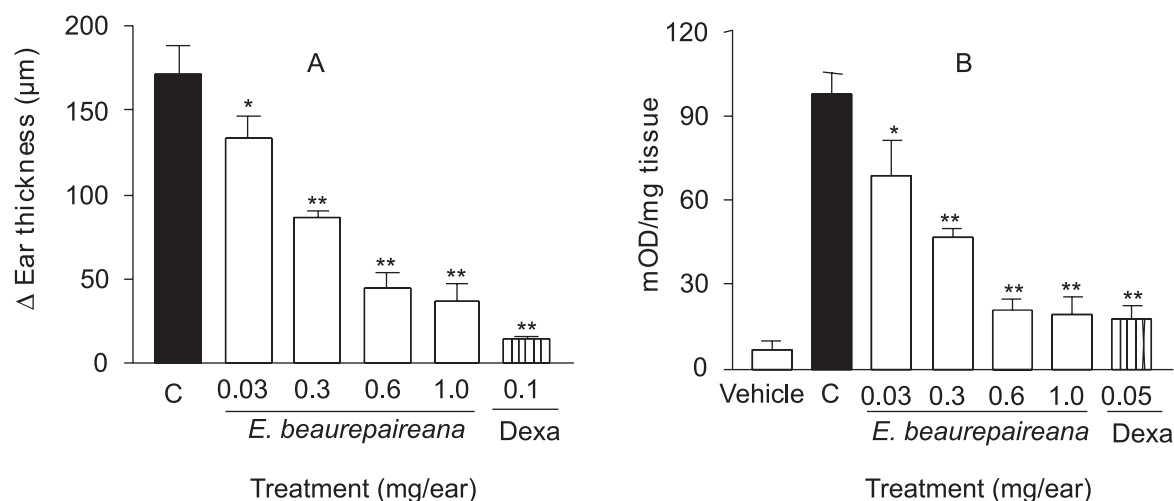


FIGURE 2 - Effect of *Eugenia beaurepaireana* and dexamethasone (Dexa) administered topically on (A) croton oil-induced ear edema and (B) myeloperoxidase activity in supernatants of homogenates from croton oil-treated ears. C = control; mOD = optical density (10^{-3}). Ear edema and myeloperoxidase activities were measured at 6 and 24 h after croton oil treatment, respectively. Each point represents the mean \pm S.E.M. for 6-8 animals. The asterisks denote the significance levels when compared with control groups. Significantly different from controls, * $P < 0.05$ and ** $P < 0.01$.

The relative apparent percentage of each compound was determined by area of the peak, α -amyryn (61.92 %) showed to be more abundant than β -amyryn (27.66 %).

Triterpenes derivatives of ursane, such as ursolic acid (Srivasta, Shaw, Kulshreshtha, 1995) and oleanane structure, such 3-cis-*p*-coumaroyloxy-2- α -oleanolic acid, were previously identified of *Eugenia* genus (Gu *et al.*, 2001). However, this is a first study showing the presence of α - and β -amyryn in *E. beaurepaireana*.

Topical application of croton oil has been used to screen for topically applied antiinflammatory steroids and nonsteroid agents and promotes events of inflammatory processes such as oedema, cell infiltration and proliferation, with the production of arachidonic acid metabolites, cytokines, and other proinflammatory mediators (Otuki *et al.*, 2005).

As shown in Figure 2, the topical application of the epicuticular wax from the leaves of *E. beaurepaireana* was capable to prevent two important events of inflammation, namely oedema (Figure 2A) and the increase of cell migration (Figure 2B) caused by topical administration of croton oil in mice. The estimated ID_{50} values from the oedema and myeloperoxidase activity (indicative of polymorphonuclear leukocytes influx) were 0.31 (0.26-0.39) and 0.34 (0.20-0.41) mg.ear⁻¹, with inhibition of 79 \pm 6 and 77 \pm 4%, respectively. Dexamethasone, the reference drug, also cause a significant inhibition of both parameters, 81 \pm 3 and 79 \pm 4%, for oedema and myeloperoxidase activity, respectively (Figure 2B). The above reported actions are interesting because infection or injury of the tissue leads

to an influx of neutrophils from blood and its accumulation plays a critical role in cutaneous inflammatory diseases such as dermatitis (Schaerli *et al.*, 2004).

The antiinflammatory activity of *E. beaurepaireana* is explained, at least in part, by the presence of pentacyclic triterpenes α - and β -amyryn. This fact is supported by the antiinflammatory activity of α - and β -amyryn demonstrated in literature (Otuki *et al.*, 2005). These works prove the efficacy of the topical application of α - and β -amyryn in models of skin inflammation induce by croton oil and TPA (12-O-tetradecanoylphorbol-acetate) (Della Loggia *et al.*, 1994; Recio *et al.*, 1995; Akihisa *et al.*, 1996; Otuki *et al.*, 2005). Furthermore, Otuki and collaborators (2005) also demonstrated that the topical application of α -amyryn is capable of reducing skin levels of IL-1 β , an important cytokine involved in skin inflammation. Thus, the present study demonstrates that the wax obtained from leaves of *E. beaurepaireana* possesses a topical antiinflammatory activity in mice, and this could be a source of the biologically active compounds and an alternative to search of a new medicine for the treatment of skin diseases.

CONCLUSIONS

The results show that topical application of *Eugenia beaurepaireana* epicuticular wax was significantly active in inhibiting both oedema and tissue myeloperoxidase activity in mice ear treated with croton oil. The results provide useful pharmacological information related to

antiinflammatory activity of this species, and suggests the possibility of the usage of this plant against different pathologies related to topical inflammatory process.

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