Topically Applied Diterpenoids from *Egletes viscosa* (Asteraceae)
Attenuate the Dermal Inflammation in Mouse Ear Induced by
Tetradecanoylphorbol 13-Acetate- and Oxazolone

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The diterpene compounds, centipede acid (CA) and 12-acetoxyhawtriwaic acid lactone (AHAL, tanabalin) isolated from the flower buds of *Egletes viscosa* Less. (Asteraceae) were evaluated on acute and chronic models of mouse ear dermatitis. A single topical application of CA (0.125; 0.25 and 0.5 mg/ear) or AHAL (0.125, 0.25, 0.5 mg/ear) immediately before 12-O-tetradecanoylphorbol-13-acetate (TPA, 2.5 μg/ear) caused a dose-related significant inhibition of ear inflammatory edema and influx of polymorphonuclear cells, as evidenced by a decrease in ear thickness and reduced myeloperoxidase (MPO) activity and tumor necrosis factor-α (TNF-α) in ear tissue homogenates. The maximal obtained inhibition for both ear edema and neutrophil influx were almost similar to that of topically applied dexamethasone (0.05 mg/ear). The extent of inhibitions for the respective treatments of CA (0.5 mg/ear), AHAL (0.5 mg/ear), or dexamethasone (0.05 mg/ear) were in the order of 63%, 61% and 81% for the ear edema, and 90%, 95% and 95% for the neutrophil influx. Also, at similar doses, both diterpenes and dexamethasone effectively inhibited the delayed-type hypersensitivity reaction induced by repeated topical application of 1% oxazolone (OXA, 20 μl/ear), as evidenced by significant decreases in ear thickness and interferon-γ (INF-γ) levels in ear tissue. Histopathological analysis revealed a marked decrease in epidermal hyperplasia and neutrophil infiltration in animals pretreated with CA or AHAL, in a manner similar to dexamethasone. These data provide evidence for the anti-dermatitis effect of *Egletes viscosa* diterpenes, by mechanisms that involve a reduced neutrophil influx and decreased production of inflammatory cytokines, TNF-α and IFN-γ.

Key words  *Egletes viscosa*; diterpene; experimental dermatitis; anti-inflammatory activity

Over the past three decades, the prevalence of atopic dermatitis and allergic or irritant contact dermatitis has been increasing significantly in the general population, causing considerable economic costs and decreased quality of life.1—3 Topical corticosteroids have been the first-choice therapy for treatment of these inflammatory skin diseases such as eczema, atopic and seborrheic dermatitis, and psoriasis. While effective in many patients, this form of therapy carry the concern of local and systemic adverse effects and may induce skin atrophy, especially after long-term use.4,5 In contrast to topical corticosteroids, the more recently introduced calcineurin inhibitors like tacrolimus and pimecrolimus do not induce skin atrophy, even after long-term use but may induce adverse effects such as burning, erythema and pruritus and are contraindicated in patients younger than two years or in those who are immunosuppressed.6,7 Thus, the available drugs, although have efficacy they are associated with adverse effects and therefore the development of new and safe anti-inflammatory topical agents for the treatment of dermatitis is needed.

Studies reveal that a large percentage of patients use some form of complementary and alternative medicine for the treatment of atopic and contact dermatitis, which include herbal remedies.8—10 In the recent past, several diterpene compounds of plant origin such as abietic acid from *Pimenta racemosa* var. *grissea*, hypoestoxide from *Hypoestes rosea* and marrubiin from *Marrubium vulgare* were shown to be effective in the mouse model of ear edema induced by several phlogestogens like histamine, bradykinin, capsaicin, prostaglandin E2, Croton oil, TPA (12-O-tetradecanoylphorbol 13-acetate) or oxazolone.11—13 Centipede acid (CA) and 12-acetoxyhawtriwaic acid lactone (AHAL, Tanabalin) are the naturally occurring diterpenes isolated from the aerial parts of *Egletes viscosa* L. (Asteraceae), a traditional medicinal plant.10 Previously we reported on the gastroprotective and antiinociceptive properties of these diterpenes as well as an anti-edematogenic activity of tanabalin against ear edema induced by capsaicin.5,10 However, the anti-inflammatory efficacy of these two diterpene compounds was not tested in the models of ear edema induced by either TPA or oxazolone, the most commonly used ones for analysing the agents effective against contact or atopic dermatitis.17,18 Thus as a part of our continuation study, we describe here for the first time the anti-dermatitis effects of these two natural diterpenes from *Egletes viscosa* using the experimental models of acute and chronic dermatitis induced by TPA and oxazolone, respectively, in the mouse ear.

**MATERIALS AND METHODS**

**Plant Material and Isolation of Diterpenoids**
The aerial parts (2.5 kg) of *Egletes viscosa* Less. (Asteraceae) were collected from the experimental plantation pertaining to the Department of Agronomy of this University, after its authentication and the voucher specimen (#16327) was retained in Prisco Bezerra Herbarium of the same University. Centipedic
acids (CA) and 12-acetoxy-hawtriwaic acid lactone (AHAL) were extracted and isolated from the dried plant material as per procedures described earlier.\(^1,^2\) (Figs. 1A, B). For experiments, CA and AHAL were suspended in aqueous Tween 80 solution (2%) and control groups were treated with this vehicle in a similar volume as for test groups.

**Chemicals and Drugs** The chemicals and drugs used in the study were the 12-O-tetradecanoylphorbol-acetate (TPA), oxazolone, dexamethasone, hexadecyltrimethylammonium bromide, o-dianisidine dihydrochloride, hydrogen peroxide, formaldehyde, Tween 80, Tween 20, eosin, hematoxylin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TP A was dissolved in acetone. Oxazolone was dissolved in 10 ml of 1.5% oxazolone in ethanol or in a mixture of acetone and hexadecyltrimethylammonium bromide,\(^2\)\(^4\) Ears were excised from the animal groups sensitized by application of 100 \(\mu\)l of 1% oxazolone in ethanol and fixed in 10% buffered formalin solution, embedded in paraffin by standard methods, cut into 5 \(\mu\)m sections, stained with hematoxylin–eosin, and then assessed under light microscopy.

**Histopathological Study** Mouse ears were excised 4 h after TPA administration and 6 h after the last application of oxazolone.\(^2\)\(^3\) Ears were excised from the animal groups treated with vehicle, CA (0.5 mg/ear), AHAL (0.5 mg/ear) or dexamethasone (0.05 mg/ear), and homogenized with 1 ml of 0.1% Triton X-100. The supernatant (0.1 ml) was mixed with 2.9 ml of 50 \(\mu\)M phosphate buffer, pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 470 nm was then measured for 5 min using a Beckman spectrophotometer (Beckman DU 640B).

**Measurement of Tumor Necrosis Factor-\(\alpha\) (TNF-\(\alpha\))** The TNF-\(\alpha\) in mouse ear tissue was assessed 4 h after TPA.\(^1,^8\) Ears were excised from the animal groups treated with vehicle, CA (0.5 mg/ear), AHAL (0.5 mg/ear) or dexamethasone (0.05 mg/ear) or dexamethasone (0.05 mg/ear), and homogenized in 50 \(\mu\)M Tris–HCl buffer (pH 7.5) with 1 mM EDTA, and their homogenates was incubated on ice for 15 min with the freezing and thawing procedure repeated once, then were sonicated for 15 s and centrifuged for 5 min at 13000 \(\times g\) for 10 min. TNF-\(\alpha\) in the supernatant was measured by ELISA using the commercial kit for TNF-\(\alpha\) (Quantikine® , R&D Systems, Minneapolis, U.S.A.). The assay was performed according to the manufacturer’s instructions.

**Measurement of Interferon-\(\gamma\) (IFN-\(\gamma\))** The IFN-\(\gamma\) in mouse ear tissue was assessed 6 h after the last application of oxazolone.\(^2\)\(^3\) Ears were excised from the animal groups treated with vehicle, CA (0.5 mg/ear), AHAL (0.5 mg/ear) or dexamethasone (0.05 mg/ear), and homogenized with 1 ml of 0.1% Tween 20 in phosphate-buffered saline (PBS; pH 7.4). Samples were frozen at \(-30^\circ\)C for 30 min, thawed in a 37 \(^\circ\)C water bath for 15 min with the freezing and thawing procedure repeated once, then were sonicated for 15 s and centrifuged for 5 min at 13000 \(\times g\). The supernatants were collected and kept at \(-30^\circ\)C until measurement of cytokines. IFN-\(\gamma\) levels were determined using the commercial kit of ELISA (Quantikine®) from R&D Systems (Minneapolis, U.S.A.). The assay was performed according to the manufacturer’s instructions.

**Statistical Analysis** The results are expressed as mean±S.E.M. from 8 mice per group. For statistical analysis, ANOVA followed by Student Newman Kuel’s post \(\text{hoc}\) test was used. A \(p<0.05\) was considered statistically significant.
RESULTS

TPA (2.5 μg/ear) in the mouse ear induced an edematogenic response as evidenced by a marked increase in ear thickness and as well as a marked increase in MPO activity, a marker of neutrophil influx at 4 h following its topical application (Figs. 2A, B). Animal groups that received topical pre-treatments with either CA or AHAL (0.125; 0.25, 0.5 mg/ear) showed significantly less ear edema to the extent of 33%, 50%, 63%, and 45%, 55% and 61%, respectively (Fig. 2A). MPO activity was also markedly reduced in animal groups that received either of these diterpenes and the inhibitions were in the order of 76%, 85%, 90% for CA, and 81%, 86% and 95% for AHAL, respectively (Fig. 2B). The corticosteroid dexamethasone (0.05 mg/ear) also significantly suppressed the ear edema and MPO activity by 81% and 95%, respectively. TPA significantly enhanced the TNF-α in the ear tissue of vehicle-treated mice (Fig. 2C). The TPA-associated increase in TNF-α levels were significantly low in animal groups treated with CA (0.5 mg/ear), AHAL (0.5 mg/ear) or dexamethasone (0.05 mg/ear).

In the oxazolone challenged group, the ear thickness significantly increased from day 4 onwards and stayed all throughout the experimental period, an indication of chronic dermatitis. CA (0.25, 0.5 mg/ear/d), AHAL (0.5 mg/ear/d) and dexamethasone (0.05 mg/ear) significantly suppressed the oxazolone-induced increase in ear thickness (Figs. 3A, B). Oxazolone significantly enhanced the interferon-γ in the ear tissue of vehicle-treated mice (Fig. 3C). The oxazolone-associated increase in IFN-γ levels were significantly low in animal groups treated with CA (0.5 mg/ear), AHAL (0.5 mg/ear) or dexamethasone (0.05 mg/ear).

Histological analysis of representative mouse ear 4 h after application of TPA (2.5 μg/ear) showed intense dermal edema and inflammatory cell infiltration (Fig. 4B), compared to the one treated with vehicle (Fig. 4A). Figure 4D details the TPA-induced intense dermal edema and much cell infiltration with mononuclear and polymorphonuclear cells as compared with vehicle treatment (Fig. 4D). These events were markedly reduced in ear tissues of animal groups treated with dexamethasone (0.05 mg/ear), CA (0.5 mg/ear), or AHAL (0.5 mg/ear) (Figs. 4E, F, G, respectively). Repeated applications of oxazolone (1%, 20 μl/ear) induced an intense dermal edema, hyperplasia, and inflammatory cell infiltration with mononuclear and polymorphonuclear cells in ear tissues of vehicle-treated controls (Fig. 5A). Treatment with dexamethasone (0.05 mg/ear) prevented both dermal edema and the inflammatory cell infiltration (Fig. 5B). While CA (0.5 mg/ear) reduced prominently the cell infiltration, its
The anti-edema effect was partial and inferior to that of dexamethasone (Fig. 5C). AHAL treatment resulted in only smaller reductions of dermal edema and inflammatory cell infiltration (Fig. 5D).

**DISCUSSION**

The results of this investigation provide evidence that the diterpenes centipedic acid (CA) and 12-acetoxy-hawtriwaic acid lactone (AHAL) from *Egletes viscosa* are topically active in the attenuation of acute dermatitis induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) or chronic dermatitis-
induced by oxazolone. Both compounds (0.125; 0.25, 0.5 mg/ear) caused a dose-dependent suppression of TPA-associated inflammatory edema, polymorphonuclear leukocyte migration, and the increase in tissue levels of TNF-α, in a manner similar to dexamethasone, the reference anti-inflammatory drug. Topical application of TPA, the well-characterized protein kinase C activator and tumor promoter, is a valid model to screen compounds effective for potential topical anti-inflammatory therapy. A single application of TPA induces oxidative stress, cutaneous inflammation and epidermal hyperplasia due to enhanced keratinocyte proliferation. TPA induces TNF-α production and the formation of LTB4 with a resultant increase in vascular permeability and neutrophil influx.18) Myeloperoxidase (MPO) is an enzyme found in neutrophils, which is commonly used as an index of granulocyte infiltration, and its inhibition is indicative of an antiinflammatory action.20) Therefore in characterizing the topical anti-inflammatory potential of diterpenes under investigation, we examined their effects on TPA-associated increase in TNF-α and on MPO activity in mouse ear homogenates. The topical application of CA and AHAL, similar to the antiinflammatory drug dexamethasone, resulted in marked inhibition of TNF-α and MPO activity, and consequently the edema formation and migration of polymorphonuclear leukocytes induced by TPA. Our histological analysis of the ear tissue clearly confirmed that the diterpenes and dexamethasone inhibited the influx of polymorphonuclear cells to the mouse ear skin following TPA application.

The mouse ear edema test allows not only to identify the potential allergens on the basis of challenge-induced increases in ear thickness in sensitized animals, but also to take a subsequent study on potential inhibitory agents.25) The potential allergens on the basis of challenge-induced ear skin following TP A application.

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tory events and the induction of skin hypertrophy

due to an increase in keratinocyte proliferation.35,36) In this
case, a previous study described the antiproliferative ac-

REFERENCES