Zanhasaponins A and B, Antiphospholipase A_2 Saponins from an Antiinflammatory Extract of *Zanha africana* Root Bark

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Received May 2, 1997[⊗]

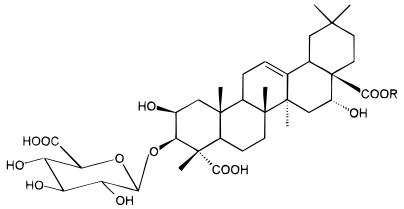
A MeOH extract from *Z. africana* was examined for topical antiinflammatory activity and proved to be active against arachidonic acid (AA) acute edema, 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced chronic inflammation, and oxazolone delayed-type hypersensitivity in mice. The extract also showed significant inhibitory activity of *Naja naja* phospholipase A_2 when a polarographic method was used. Two oleanane-type triterpene saponins, zanhasaponins A (**1**) and B (**2**), and the cyclitol pinitol (**4**), isolated from the extract, were active as inhibitors of PLA_2. A further saponin, zanhasaponin C (**3**) was inactive in this assay.

As a part of a study on the efficacy of topical herbal remedies combined with a search for novel structures with antiinflammatory activity, we have tested *Zanha africana* (Randlk.) Exell (Sapindaceae), which is used in traditional medicine in Southern Africa for the treatment of dysentery.[1] This screening revealed that the MeOH extract of this species possessed a marked effect against carrageenan and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) edemas in mice.[2] Thus far, *Z. africana* has been reported to contain triterpenoid derivatives belonging to the oleanane series,[1,3,4] as well as three new zanhic acid-based saponins [zanhasaponins A (**1**), B (**2**), and C(**3**)] and three cyclitols [bornesitol, quebrachitol, and pinitol (**4**)].[5]

The purpose of the present investigation was to study the effect of a crude extract and several constituents (**1**−**4**) of this species on other acute and chronic topical inflammatory in vivo models as well as their behavior as in vitro inhibitors of phospholipase A_2, an enzyme that is considered to play a major role in the pathogenesis of several inflammatory skin processes.[6]

The MeOH-soluble extract of *Z. africana* was first tested against arachidonic acid (AA)-induced edema. Topical treatment with 0.5 mg/ear of the extract displayed a weak effect, that is, a 26% decrease in ear thickness (Table 1).

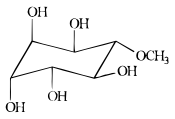
When evaluated in the phorbol ester (TPA)-induced chronic mouse skin inflammation model, the MeOH-soluble extract, topically applied, inhibited ear thickness (48%) and markedly lowered leukocyte infiltration, with a 66% decrease in myeloperoxidase (MPO) activity with respect to the TPA-treated controls. When the extract was assayed against oxazolone-induced delayed hypersensitivity, it only reduced the ear edema by 25% at 48 h and had little effect on the MPO activity (24%), an assay that is an end-point analysis for both chronic TPA inflammation and oxazolone delayed hypersensitivity (Table 1).



1 R = α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranosyl

2 R = β-D-xylopyranosyl (1→2)-α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranosyl

3 R = β-D-xylopyranosyl (1→3)-β-D-xylopyranosyl (1→2)-α-L-rhamnopyranosyl (1→2)-α-L-rhamnopyranosyl



4

A histological examination was carried out on ear sections after repeated topical applications of TPA. A severe edema and an increase in the epidermal thickness due to a noticeable cellular proliferation was observed. Margination and cellular infiltration occurred, mainly of polymorphonuclears (PMN) and lymphocytes (41−80 cells/field). Granulocyte infiltration was diffuse and affected the conjunctive tissue. Fibrosis and sometimes abscesses at the epidermal level were detected.

Significant decrease in swelling and cellular infiltration, practically recovering to the normal condition, were observed in the dexamethasone-treated ears; however,

Table 1. Inhibitory Effects of the MeOH Extract of *Z. africana* on Different Models of Inflammation in Comparison with Reference Drugs

extract/ compound	TPA (repeated)	AA	oxazolone 24 h	48 h	72 h	96 h	102 h
Z. africana MeOH extract[a]	48[e]	26[d]	0	25[d]	18	4	3
dexamethasone[b]	81[e]		76[e]	89[e]	93[e]	81[e]	84[e]
NDGA[c]		50[e]					

[a] MeOH extract: 0.5 mg/ear (AA test); 1 mg/ear (repeated TPA and oxazolone). [b] Dexamethasone: 0.05 mg/ear. [c] NDGA: 2 mg/ear. [d] Differences are significant, ($p < 0.05$). [e] Differences are significant, ($p < 0.01$) Dunnet's *t*-test.

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[⊗] Abstract published in *Advance ACS Abstracts,* October 1, 1997.

S0163-3864(97)00221-8 CCC: $14.00

fibrosis was still present. After applying *Z. africana* MeOH extract, a mild congestion and a reduction in the thickness of the epidermis and dermis were perceived. PMN infiltration was moderate (6−40 cells/field). Neither fibrosis nor hyperplasia were apparent.

The extract was also tested in vitro as an inhibitor of PLA_2 from snake venom using a polarographic method, and it exhibited an IC_{50} of 0.57 mg/mL. Zanhasaponins A (**1**) and B (**2**) were the most active inhibitors of the *Z. africana* constituents, with IC_{50} values of 0.47 and 0.44 mM, respectively, or some four times lower than that of mepacrine (IC_{50} 2.15 mM) in each case. Pinitol (**4**) showed inhibitory potency in the same range as mepacrine (IC_{50} 1.92 mM) (Table 2). Zanhasaponin C (**3**) and quebrachitol reached their highest inhibition (30 and 45%, respectively) at 0.8 and 1.3 mM, respectively, but it was not possible to establish their IC_{50} values.

Our study also demonstrated that the extract of *Z. africana* and its constituents were effective not only in the single-dose acute TPA test, as we have previously reported,[2] but also in a multiple-dose TPA inflammation assay. Apart from the swelling reduction, the histological data made evident that the *Z. africana* MeOH extract effectively inhibited many of the major signs of the skin inflammatory process and were congruent with the measurements of thickness and MPO activity. Given the fact that the decrease in MPO activity parallels that of swelling, it is reasonable to propose that this extract is able to reduce leukocyte recruitment and penetration into skin and non-selectively, the degranulation process leading the MPO release. In the AA-induced acute inflammation model, however, the inhibition of ear thickness is considerably lower than that obtained previously in the acute TPA ear edema (78% inhibition),[2] which means that although the antiinflammatory effect does exist, it does not seem to be directly related to an eventual interaction with AA metabolism.

This is the first time that saponins have been found to interact with PLA_2. The relatively more polar nature of these compounds, if compared with tritepene aglycons, could interfere with the interaction with the lipophilic surroundings of the active site of the enzyme. On the other hand, the physical characteristics of these molecules could obstruct the phospholipid enzyme hydrolysis due to the well-known fact that PLA_2 exerts its effect mainly on vesicles, liposomes, or other bilayer-forming aggregates of the substrate.[7]

Comparison of the effects of zanhasaponins A−C (**1**−**3**) on acute TPA-induced edema shows that their potency is inversely correlated with glycoside−sugar length chain, because a high polarity reduces the cutaneous penetration.

Experimental Section

Chemicals and Enzymes. TPA, AA, oxazolone, H_2O_2, phosphate buffer saline (PBS), *N*,*N*-dimethylformamide, tetramethylbenzidine (TMB), hexadecyltrimethylammonium bromide (HTAB), phosphatidyl choline substrate, soybean lipoxygenase, phospholipase A_2 from *Naja naja* venom, oleic acid, deoxycholic acid, 2-amino-2-methyl-1,3-propanediol (ammediol) buffer, mepacrine, and calcium chloride, and the reference drugs indomethacin, nordihydroguaiaretic acid (NDGA), and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO) and NaOAc from Panreac, Barcelona, Spain.

Table 2. Inhibitory Effect of Zanhasaponins A (**1**), B (**2**), and C (**3**) and Pinitol (**4**) on PLA_2 Activity

compound	IC_{50} (mM)[a]
zanhasaponin A (**1**)	0.47[b] (1.00−0.34)
zanhasaponin B (**2**)	0.44[c] (0.61−0.34)
zanhasaponin C (**3**)	nd[d]
pinitol (**4**)	1.92[e] (2.66−1.00)
mepacrine	2.15[f] (2.56−1.92)

[a] IC_{50} = 50% Inhibitory Concentration. 95% confidence limits given in parentheses. [b] $p = 0.0117$ (Anova test, significant). [c] $p = 0.0043$ (significant). [d] nd = not determined, 30% maximum inhibition at 0.8 mM. [e] $p = 0.0342$ (significant). [f] $p = 0.0413$ (significant).

Plant Material. The root bark of *Zanha africana* was collected on the Zomba Plateau in Malawi in November 1988. A specimen was deposited in the National Herbarium (no. 88246) of Malawi, Zomba.

Extraction and Isolation. Air-dried and powdered root bark of *Z. africana* was extracted with MeOH at room temperature. The MeOH extract was fractionated by diverse chromatographic techniques as previously described, and further purification yielded six compounds that were identified as zanhasaponins A−C (**1**−**3**), bornesitol, quebrachitol, and pinitol (**4**) by ^{1}H- and ^{13}C-NMR spectral analysis.[5]

Animals. Groups of six female Swiss mice weighing 25−30 g were used. All animals were fed a standard diet *ad libitum* and maintained in suitable environmental conditions throughout the experiments.

AA-Induced Mouse Ear Edema.[8] AA was dissolved in Me_2CO at a concentration of 100 mg/mL. An edema was induced on each right ear by topical application of 2 mg/ear of AA in Me_2CO. The left ear (control) received the vehicle (Me_2CO or 70% aqueous EtOH). The *Z. africana* MeOH extract and pure compounds (**1**−**4**), dissolved in 70% aqueous EtOH, were applied topically (0.5 mg/ear), 30 min before application of AA. The thickness of each ear was measured 1 h after induction of inflammation using a micrometer. The edema was expressed as an increase in the ear thickness due to AA application. A reference group was treated with NDGA at 2 mg/ear.

Mouse-Ear Edema Induced by Multiple Topical Applications of TPA.[9] Chronic inflammation was induced by topical application of 10 μL of TPA (2.5 μg/ear) to both the inner and outer surface of both ears of each mouse with a micropipete on alternate days. The MeOH extract was dissolved in 70% aqueous EtOH and applied topically (1 mg/ear) twice daily for the last four days, in the morning immediately after TPA application and 6 h later. Dexamethasone was used as the reference drug (0.05 mg/ear). The thickness of each ear was measured using a micrometer, before (day 0) and after (day 10) treatment; therefore, each ear served as its own control. The swelling was assessed in terms of the mean of the increase in the thickness of each ear.

Oxazolone-Induced Contact-Delayed Hypersensitivity in Mouse-Ear Edema.[8] Female mice were sensitized by topical application on the shaven ventral abdomen of 50 μL of a 2% (w/v) solution of oxazolone in Me_2CO on two consecutive days (days 1 and 2). Challenge was performed on day 6 by application of 30 μL of 2% oxazolone to both ears. The *Z. africana* MeOH extract and dexamethasone were applied (30 μL) to right ears 6 h after challenge (single application) and 24, 48, 72, and 96 h after challenge (repeated dosage). Ear-thickness measurements of treated and control groups

were performed with a micrometer just before drug application and 24, 48, 72, 96, and 102 h after challenge. The final measurement was performed immediately before sacrifice. The thickness of each ear was measured as described in the previous test.

Myeloperoxidase Assay.[10] Each biopsy was placed in an Eppendorf tube containing 0.75 mL of 0.5% HTAB in 80 mM sodium phosphate buffer (pH 5.4). After adding a second 0.75-mL aliquot, the sample was centrifuged at 12 000 *g* at 4 °C for 20 min. The supernatant (30 μL) was assayed by mixing it with 20 μL of TMB 18.4 mM and 15 μL of H_2O_2 (0.017%) in a 96-well microtiter plate. The mixture was incubated for 3 min at 37 °C. Enzyme activity was determined colorimetrically using a Labsystems Multiskan MCC/340 plate reader set to measure absorbance at 620 nm.

Histology. Biopsies were placed in 10% formalin. Each biopsy was cut longitudinally into equal halves. Half of each sample was set in paraffin, with the cut surface down. A histologic slide that included a section of the entire longitudinal cut surface (from base to margin) was prepared, stained with hematoxylin and eosin, and examined at low magnification and at × 100. The extent of edema in the papillary dermis and in the reticular dermis/subcutis was classified as absent (−), mild (+), moderate (++), or severe (+++). A representative area of the inflammatory cellular response was then selected for cell counting in a × 400 field. The total number of inflammatory cells, mononuclears (MN), and PMN was counted in the papillary and in the reticular dermis/subcutis layers. The mean total inflammatory cell count per treatment group was calculated and classified as <5 = normal, 6−40 = mild (+), 41−80 = moderate (++), and >80 = severe (+++).

Statistics. Percentages of edema reduction are expressed by the mean ± SEM. Dunnet's *t*-test for unpaired data was used for statistical evaluation.

PLA_2 Assay System.[11] Snake venom PLA_2 (8.3 units), 1.5 mL of ammediol−HCl buffer (pH 8.49) containing 10^{-4} M Ca^{2+} and 2.25 mg of soybean lipoxygenase (1.5 mg lipoxygenase/mL buffer) were then added to one of three oxygraph cells equipped with magnetic stirring bars. The oxygen probe of the oxygraph was inserted in the cell, and, after 3 min with stirring for temperature equilibration at 37 °C, 100 μL of phosphatidyl choline were added to the cell to initiate the reaction, which was monitored for oxygen consumption. The initial rates of oxygen incorporation were determined within the first minute of the reaction, during which a maximum of 30% of the total substrate was consumed. An oxygraph from the Yellow Springs Instrument Company coupled to a Merck-Hitachi recorder was used for measurements of oxygen consumption.

The extract and the pure compounds were dissolved in MeOH and added directly to the PLA_2 to ensure contact of the inhibitor with the enzyme. If an inhibition of the reaction was observed, 20 μL of linoleic acid was added to the incubation mixture to determine whether it was really an inhibition of PLA_2 or of lipoxygenase. The degree of inhibition was calculated from the disminution of the slopes when compared with noninhibited controls. The 50% inhibitory concentration (IC_{50}) was calculated from the concentration/response analysis at a range of concentrations between 0.25 and 1.0 mg/mL for the extract and between 0.22 and 3.9 mM for the pure compounds. The level of statistical significance was determined by Dunnet's *t*-test for unpaired samples.

Acknowledgment. This work was supported by the Comisión Interministerial de Ciencia y Tecnología of the Spanish Government (CICYT grant SAF-92-0643).

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NP970221R