In vivo anti-inflammatory activity of grandiflorenic acid and kaurenic acid isolated from Coespeletia moritziana and Espeletia semiglobulata.

Actividad antiinflamatoria in vivo del ácido grandiflorénico y ácido kaurénico obtenidos a partir de Coespeletia moritziana y Espeletia semiglobulata.

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RESUMEN
Los ácidos kaurénico (KA) y grandiflorénico (GA) fueron aislados de Espeletia semiglobulata y Coespeletia moritziana (Asteraceae), respectivamente, recolectadas en los páramos Andinos, Mérida, Venezuela. La actividad antiinflamatoria de estos compuestos fue evaluada in vivo en ratones BIO:NMRI, por los métodos siguientes: edema de oreja inducido por xilol y edema de pata inducido con carragenina, a dosis de 16, 32 y 64 mg/Kg. La actividad antiinflamatoria fue estudiada mediante comparación con fármacos de actividad antiinflamatoria reconocida: dexametasona (1 mg/Kg), diclofenac (25 mg/Kg) y ketoprofeno (10 mg/Kg). El índice del edema y el porcentaje de inhibición fueron determinados después del tratamiento con xileno o carragenina. La administración intramuscular del GA mostró mayor actividad antiinflamatoria que el KA a dosis de 64 mg/Kg. Este es el primer estudio del efecto antiinflamatorio in vivo del GA.

PALABRAS CLAVES
Ácido grandiflorénico, ácido kaurénico, edema de oreja, edema de pata, inflamación.

INTRODUCTION
Bioactive natural products can be considered as very promising starting points for the development of new therapeutic agents. Indeed, medicinal drug synthesis has not replaced naturally occurring compounds as essential components of our therapeutic arsenal, and many of the medicines prescribed today are natural products [1,2]. Among the most important natural compounds known for their medicinal value are the terpenoids [3,4]. Kaurenic acid (KA) and grandiflorenic acid (GA) are found in the aerial parts of several species of Espeletiinae, a family of resinous plants, popularly known as “Frailejon”, that grow above 2500 m of altitude in the Andes of Northern South America. These plants are used by high paramos inhabitants to treat asthma and rheumatic conditions [5-7]. Espeletia semiglobulata (ES) and Coespeletia moritziana (CM) grow above 3900 m of altitude at Paramo of Piedras Blancas, which is part of Sierra La Culata, located near the city of Mérida, Venezuela. Ent-kaurenes and many natural derivatives of these diterpenes display in vivo significant anti-inflammatory, anti-hypertensive, and diuretic biological effects, as well as in vitro antimicrobial, smooth muscle relaxant, and cytotoxic actions. Numerous findings
indicate that ent-kaurenines are potential anti-inflammatory agents, with a specific mechanism in which both the inhibition of nuclear factor kappa B (NF-kappa B) translocation and the consequent decrease of proinflammatory mediators are implicated [1,8].

Inflammation is the first response of the immunological defense system to microbial infections, burns, allergens, mechanical injuries and other noxious stimuli [9,10]. The anti-inflammatory activity of extracts isolated from natural sources, and pure secondary metabolites have been evaluated in vivo using different pharmacological models including those of acute inflammation: xylene-induced mouse ear edema and carrageenan-induced mouse paw edema [11]. This study has been carried out in order to evaluate the anti-inflammatory activity of GA and KA using in vivo experiments on animals.

MATERIAL AND METHODS

Plant material. Leaves of Espeletia semiglobulata Cuatrec. and Coespeletia moritziana (Sch. Bip. ex Wedd.) Cuatrec., were collected at 3900 m of altitude in Paramo of the Piedras Blancas and along the road to Piñango, about 13 km from Aguila’s Peak. Voucher specimens (AU30 and AU21) were deposited at the Herbarium of the Faculty of Pharmacy and Bioanalysis, University of the Andes (MERF Herbarium).

Extraction and isolation of kaurane diterpenes. Air-dried leaves of Espeletia semiglobulata (640 g) and Coespeletia moritziana (2.8 Kg) were pulverized and extracted at room temperature with hexane/diethyl ether (3:1, v/v) and hexane containing 2 % of ethyl acetate (EtOAc), respectively. The solvents were removed under reduced pressure and the solid residues were dissolved in hexane/EtOAc, and shaken with 5 % aqueous NaOH. The aqueous layer was acidified with diluted HCl and shaken with hexane to recover 18 g of an acidic fraction in the case of E. semiglobulata and 120 g of an acidic fraction in the C. moritziana case. This acidic fractions were submitted to flash chromatography to yield 3.2 g of GA (ent-kaur-9(11)-16-dien-19-oic acid), mp 155-157 °C, and 15.3 g of KA (ent-kaur-9(11)-16-en-19-oic acid), mp 178-180 °C, which was calculated using the formula [15].

Animals. Male BIO:NMRI mice weighing 20-30 g were obtained from the vivarium of University of Los Andes (ULA). All animals were housed in groups in a room under a 12-h light/12 h dark cycle (temperature 23-25 °C and humidity of 50-60 %), with sanitaries barriers food and water provided ad libitum. The animals were conditioned to the facility for at least two days before being used in the experiments. All animal-related experimental procedures were approved by Bioethics Committee of the ULA (Protocols numbers: CEBIOULA/82 and CEBIOULA/84). All experiments performed in this work followed the principles of laboratory animal care outlined by the Ethical Committee of the University of Los Andes.

Xylene-induced mice ear edema. The male BIO:NMRI mice were divided into groups of five mice each. Animals were treated intramuscularly with 7 mL/Kg [14] of a 0.9 % physiological solution (control group PS), a 7 % Tween 80 suspension (control group TW-80), Dexamethasone (1 mg/Kg), Ketoprofen (10 mg/Kg), Diclofenac sodium (25 mg/Kg), GA and KA (16, 32 and 64 mg/Kg). Thirty minutes after the treatment, ear edema was induced by applying 20 µL of xylene with micropipette on the inner surface of the right ear of each mice; the left ear was used as control. Three hours after xylene application, the mice were killed by cervical borer with a diameter of 6.0 mm and weighed. The difference between the right and left ear-disks weights was assessed as intensity of edema.

Edema index = W_e - W_i, where W_e and W_i represent the weight of the right ear and the left ear, respectively. The antiinflammatory activity was expressed as the percentage inhibition (%) of edema in comparison with control mice, which was calculated using the formula [15].

\[ \%I = \frac{W_e - W_i}{W_e} \times 100 \]

Carrageenan-induced mice paw edema. Mice hind paw edema was induced by carrageenan injection, as previously described [16,17]. The male BIO:NMRI mice were divided into groups of five mice each, and were treated intramuscularly with 7 mL/Kg [14] of a 0.9 % physiological solution (control group PS), a 7 % Tween 80 suspension (control group TW-80), Dexamethasone (1 mg/Kg), Ketoprofen (10 mg/Kg),

![Fig. 1. Structures of (1a) Grandiflorenic acid (ent-kaur-9(11),16-dien-19-oic acid) and (1b) Kaurenic acid (ent-kaur-16-en-19-oic acid).](image-url)
Diclofenac sodium (25 mg/Kg), GA and KA (16, 32 and 64 mg/Kg). Thirty minutes after the treatment, the mice received subplantar tissue injections (0.1 mL) of carrageenan at a concentration stock of 1 % (0.9 % saline solution) in the right hind paw. Paw edema was measured 1, 3, and 5 h after carrageenan injection with an electronic digital caliper (*RUN). The percentage edema inhibition (%I) was assessed with the increase of paw diameter (millimeters, mm), which were calculated using the following formula [18].

\[ \%I = \frac{(D_t - D_0)}{D_0} \times 100\% \]

where \(D_t\) and \(D_0\) represent the mean paw diameter of the group control at a given time and that of treated group at the same time.

Statistical analysis. Data were recorded as mean ± standard error of mean (S.E.M). Statistical analyses were performed with SPSS software. Statistical significance between the treated groups and the negative control was determined by one-way ANOVA followed by Sheffe’s multiple range test at significant level \(p<0.05\).

RESULTS AND DISCUSSION

Effects on xylene-induced mice ear edema. Xylene-induced ear edema model is partially associated with substance P, which is an undecapeptide that is widely distributed in the central and peripheral nervous system, and that functions as a neurotransmitter or a neuromodulator in a variety of physiological processes [19]. Release of substance P from the sensory neurons causes vasodilatation and plasma extravasations, suggesting its role in neurogenic inflammation, and thus causing the swelling of ear in mice [18]; it is also reported that xylene-induced ear edema is an acute inflammation model, which may involves inflammatory mediators such as histamine, serotonin and bradykinin. These mediators induce ear edema by promoting vasodilatation and increasing vascular permeability [20]. Severe vasodilatation, skin edema changes and infiltration of inflammatory cells are detected as signs of acute inflammation after topical application of xylene [21]. The xylene ear edema model permits the evaluation of anti-inflammatory steroids and is less sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) [22,23]. The results of the xylene-induced ear edema test in mice are presented in Table 1, and it can be seen that both anti-inflammatory steroids and NSAIDs produced antiinflammatory effects by this method. Intramuscular administration of GA and KA (16, 32 and 64 mg/Kg), 30 min after xylene application, significantly (\(p<0.05\)) inhibited the development of ear edema in mice in a dose-dependent manner. The inhibition produced by 64 mg/Kg of GA (69.85 %) results in a significant inhibition of edema, with a stronger effect than that of kaurenic acid (58.81 %).

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<th>TABLE 1</th>
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<td>Effects of grandiflorenic acid (GA) and kaurenic acid (KA) on xylene-induced ear edema in mice.</td>
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<td>Groups</td>
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<td>Control PS</td>
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<td>Dexamethasone</td>
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<td>Diclofenac sodium</td>
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Each value was represented as mean ± S.E.M of \(n=5\) \(p<0.05\) when compared with control (one-way ANOVA followed by Sheffe’s multiple range test). PS: Physiological solution. TW: Tween 80

Effects on carrageenan-induced paw edema in mice. Carrageenan-induced edema has been commonly used as an experimental model for acute inflammation and is believed to proceed in two phases. In general, the first phase (1-2 h) involves inflammation mediated by histamine, serotonin and increased synthesis of prostaglandins (PG) in the surrounding damaged tissues. On the other hand, the late phase (3-5 h) is sustained by prostaglandins and mediated by bradykinin and leukotrienes, which are produced by tissue macrophages [24, 25]. Carrageenan-induced inflammation in the rat paw is a classical model of edema formation and hyperalgesia that has been extensively used in the development of nonsteroidal antiinflammatory drugs and selective cyclooxygenase-2 (COX-2) inhibitors. Several lines of evidence indicate that
COX-2-mediated increases in PGE\textsubscript{2} production in the central nervous system contribute to the severity of the inflammatory and pain responses in this model [26]. The pre-treatment of mice with GA and KA (16, 32 and 64 mg/Kg), significantly reduces the diameter (mm) of the edema in the 1, 3 and 5 hours after administration of carrageenan, as compared with the control group. Greater effects were observed when the dose was 64 mg/Kg, which reduced the edema by 87.89 % (1 h), 67.03 % (3 h), 60.01 % (5 h), respectively, using GA and 79.13 % (1 h), 60.59 % (3 h), 58.59 % (5 h), respectively, using KA; Figure 2 shows the effects of GA and KA 1 h after the injection, which corresponds to the maximum activity of edema inhibition in a dose-dependent manner. The anti-inflammatory activity of NSAIDs tests on carrageenan-induced paw edema was most pronounced 1 h after the injection of the phlogistic agent. However, the effect of Dexamethasone was most pronounced 3 h after, while the highest activity of GA and KA was found 1 h after carrageenan administration, thus indicating that the use of both GA and KA is beneficial in the case of an acute inflammation.

Results gathered through this study are in accordance, to those previously reported for anti-inflammatory effects [8, 27].

**CONCLUSIONS**

Present study revealed that GA and KA are effective against both xylene-induced mice ear edema and carrageenan-induced paw edema. Results suggest that the inhibitory effect of both kaurene acids on both carrageenan-induced paw edema may be due to the release suppression of mediators including histamine, serotonin, and/or prostaglandins responsible for the first phase of acute inflammation induced by carrageenan. There are also evidences that carrageenan induced edema inhibitors are also effective in inhibiting the cyclooxygenase enzymes [26]. These two compounds are inhibitors of the exudative and proliferative phases of inflammation. Higher antiinflammatory activity of GA indicates that the additional C9-C11 double bond, that affects the stereochemical configuration of this kaurene, is important for antiinflammatory activity. Results indicate that these compounds could be a potential source for development of new anti-inflammatory drugs.

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