Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **28** (4): 594-8 (2009)

Short Communication

Received: January 22, 2009
Accepted: March 30, 2009

Topical Anti-inflammatory Effect of Creams containing Kaurenoic Acid Isolated from *Wedelia paludosa* in Mice

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SUMMARY. **The aim of this work is to evaluate *in vivo* the anti-inflammatory effect of creams containing kaurenoic acid (KA), isolated from the acetonic extract of the *W. paludosa* (stems and roots). The herbal drug was incorporated into anionic cream (Lanette®) at 100 μg/g. Creams containing different permeation enhancers (urea, alpha bisabolol, isodecyl oleate, isopropyl myristate, soy lecithin) were prepared, and the *in vivo* topical anti-inflammatory effect was evaluated by the croton oil-induced ear edema method in mice using dexamethasone cream (5 mg/g) and Acheflan® (essential oil of *Cordia verbenacea* 5 mg/g) as positive control. The potential cutaneous irritation was evaluated by the agarose overlay assay. KA cream, KA and isopropyl myristate and soy lecithin cream and dexamethasone cream presented inhibition of ear edema of 61.73 ± 23.23%, 71.71 ± 15.77% and 64.45 ± 13.41%, respectively. These results suggest that KA incorporated in the cream showed a greater anti-inflammatory effect than positive control, while KA cream containing a concentration lower than dexamethasone cream presents a statistically similar ear edema reduction compared with the control, with no potential cutaneous irritation being observed.**

INTRODUCTION

Kaurenoic acid (KA, Fig. 1), is a diterpene found in several plants, including *Wedelia paludosa* [1,2], *Annona glabra* [3], *Copaifera langsdorffi* [4], *Xylopia frutescens* [5], *Mikania glomerata* and *Mikania laevigata* [6,7].

Wedelia paludosa DC (L.) Pruski (reclassified as *Acmela brasiliensis* and more recently as *Sphagneticola trilobata*), a member of the Asteraceae family, is a native Brazilian plant, which frequently grows in coastal regions and barren land. In Brazil it is popularly known as *pseudo-arnica, margaridão* or *picão da praia,* among other names, and in English, it is known as *trailing daisy* or *wedelia.* It is used in folk medicine to treat various disorders, including infections of the respiratory tract and inflammation [8]. It also presents antimicrobial [9], antiparasitic [10], analgesic [1,11] and oral hypoglycemic activity [2]. Our research group has demonstrated the presence of different compounds, such as KA, flavonoids [1] and a eudesmanolide lactone denominated paludolactone [12].

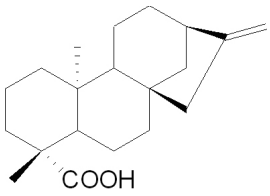

Figure 1. Structure of kaurenoic acid.

We have also shown the variation of KA concentrations in the leaves, flowers, stems and roots of the *W. paludosa* in different seasons [2]. It was observed that autumn is the season which presents the highest amount of KA, and it is present in larger amounts in the roots.

KA has presented *in vitro* trypanosomicidal, antimicrobial [9,13], antiparasitic [10], and analgesic [1] properties, among others, and is a promising natural compound with potential application as a phytodrug in the development of new medications.

KEY WORDS: Anti-inflammatory effect, Kaurenoic acid, Phytodrug, *Wedelia paludosa*.

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ISSN 0326-2383

The aim of this study was to incorporate KA in semi-solid formulations for topical use, with and without permeation enhancers, and to evaluate its *in vivo* topical anti-inflammatory activity and the potential for cutaneous irritation *in vitro*. Dexamethasone and Acheflan® creams were included for the purpose of comparison.

MATERIALS AND METHODS

Materials

W. paludosa was cultivated and collected in the Medicinal Garden of the University of Vale do Itajaí in Itajaí, Santa Catarina, Brazil, in March 2006. The plant was authenticated by Professor Renê Ferreira (UNIVALI) and a voucher was deposited at the Barbosa Rodrigues Herbarium (Itajaí), under the code V.C. Filho 002.

Anionic wax, alpha bisabolol, soy lecithin and isodecyl oleate of pharmaceutical grade were purchased from All Chemistry (São Paulo, SP, Brazil); dexamethasone of pharmaceutical grade was purchased from Galena (São Paulo, SP, Brazil); cream containing 0.5% essential oil of *Cordia verbenacea* was purchased from the Brazilian pharmaceutical market; Murine fibroblast cell L929 (Mus musculus C_3H/Na) was obtained from BCRJ, number CR020/ATCC CCL1; Heat Inactivated Fetal Bovine Serum was purchased from Sorale (São Paulo, SP, Brazil); Minimal essential medium (MEM), PSN antibiotic mixture and Tripsine were purchased from Gibco (São Paulo, SP, Brazil) and Neutral red was purchased from Sigma (São Paulo, SP, Brazil). All the other reagents used were of analytical grade.

KA isolation

KA was isolated from the stems and roots of *W. paludosa,* as described previously [1]. The KA isolation method gave a yield of 0.14%. KA was identified by direct comparison with an authentic sample previously isolated by our research group.

Preparation of creams containing KA and permeation enhancers

KA was incorporated in anionic creams containing permeation enhancers (urea, alpha bisabolol, isodecyl oleate or isopropyl myristate and soy lecithin), as described in Table 1. KA (100 µg/g) was incorporated in the formulations, after previous dissolution with propylene glycol (1 mg/mL).

Evaluation of anti-inflammatory effect in vivo

Animals

Male Swiss mice, 25-35 g, were kept in a temperature controlled environment (21 °C) and 12 h light/dark cycle, with free access to food and water, except during the experiments. Procedures in these animals were performed in accordance with protocol approved by the ethics committee in research from UNIVALI under number 130/07.

Croton oil induced mouse ear edema

Topical anti-inflammatory effect was evaluated according to the croton oil-induced ear edema method described previously [14], with minor modifications. The animals were divided into

Ingredients	Composition (%)				
	LO*	LA*	LU*	LUA*	LML*
Anionic Wax	15	15	15	15	15
Preservatives	0.2	0.2	0.2	0.2	0.2
Propylene glycol	5.0	5.0	5.0	5.0	5.0
EDTA	0.1	0.1	0.1	0.1	0.1
BHT	0.01	0.01	0.01	0.01	0.01
Water	qsp	qsp	qsp	qsp	qsp
Alpha-bisabolol	-	1.0	-	1.0	
Isodecyl oleate	5.0			-	-
Urea	-	-	10	10	
Isopropyl myristate	-	-	-	-	1.0
Soy lecithin		-	-	-	0.5

Table 1. Formulations of cream containing KA and permeation enhancers. *LO – cream containing isodecyl oleate; LA – cream containing alpha bisabolol; LU - cream containing urea; LUA – cream containing urea and alpha bisabolol; LML – cream containing isopropyl myristate and soy lecithin.

eight different groups (n = 6). The right ear was measured (basal measure) and the creams (100 mg) were applied to the internal surface: cream without KA (negative control), creams containing dexamethasone 0.5% (w/w) and Acheflan® as positive controls, and the samples of creams containing KA and permeation enhancers (alpha bisabolol, urea, isodecyl oleate, isopropyl myristate and soy lecithin). Thirty minutes after applying the creams, croton oil (2.5% in acetone) was applied, as an irritating agent, to the external surface. Six hours after administration of the oil, the ears were measured again and the ear edema was evaluated through the difference between the two measurements, expressed in mm.

Statistical analysis

The results were expressed as mean ± S.D. For the group comparisons, one-way layout analysis of variance was applied. The data were analyzed statistically using variance analysis followed Dunnett's Multiple Comparison test. P value less than 0.05 were considered as indicative of significance.

Potential of reactivity/cutaneous irritation analysis

The reactivity or cutaneous irritation test was carried out in the semi-solid preparations containing KA solution at a concentration of 100 µg/g (w/v) in the presence or absence of permeation enhancers through the agarose overlay or diffusion in agar tests, used to evaluate the sample reactivity with L929 cells [15].

L929 cells were trypsinized and 300.000 cel/mL, 3 mL/well transferred to a 6-well plate. After 24 hours, the culture medium was substituted by MEM containing 0.01% of neutral red (NR) and incubated for 15 min in the dark, at room temperature. The excess of vital coloring was removed and 3 mL of MEM were added in each well, then incubated for 1 h (37 °C)/5%CO_2 until the NR uptake. The culture medium was removed and substituted with a mixture of 1:1.2 of agarose:MEM (agarose overlay), maintained at 40 °C. It was transferred 3 mL/well of the overlay mixture, and the plates incubated for 2 h (37 °C)/5%CO_2.

The samples were incorporated in paper disks (0.54 cm), previously washed with PBS, dried and sterilized by autoclavation. As positive control, a SDS solution was used at 1% in water, and as negative control, PBS (pH 7.4) was used. The plates were incubated for 24 h in an atmosphere of 5%CO_2 (37 °C). At the end of the exposure period, the diameter of the affected area was recorded. The culture was also examined by phase contrast microscopy, to assess morphological damage. In agreement with United States Pharmacopoeia the samples were classified from 0 (no reaction) to 4 (severe reaction) [15].

RESULTS AND DISCUSSION

The creams containing KA and permeation enhancers presented conformity when physical (centrifugation, spreading and rheological properties) and chemical (KA concentration) stabilitywere analyzed for 90 days at room temperature, and at 40 °C (results not published).

Through the results obtained in the croton oil-induced ear edema test, the anti-inflammatory effect of KA was verified when compared with the negative control, saline solution. Topical application of croton oil is a common method for identifying topically applied anti-inflammatory steroids and non-steroidal agents. Its application promotes inflammatory events such as edema, cell infiltration and proliferation with the production of arachidonic acid metabolites, cytokines and other pro-inflammatory mediators [16,17]. In addition, croton oil induced inflammation process because activation of arachidonic acid cascade for inflammable constituents as phorbol esters, the same manner that increase the release of arachidonic acid increase too the expression of COX-2 [18]. Cream containing 100 µg/g of KA decreased the edema formed (Fig. 2) by 61.73% (± 23.23), while the cream with dexamethasone 0.5% do not present any significant difference ($p > 0.05$). In a previous study [19], creams containing extract of *W. paludosa,* all using the same method, presented inhibition of 59.84% of edema. The anti-inflammatory effect of the cream containing the extract was dose-dependent, and KA is possibly one of the main components responsible for this effect.

Naturally occurring diterpenoids, such as resinic acid, taxane nucleus precursor of taxol and derivatives of the abietane and kaurane nucleus are substances with promising pharmacological activity, mainly anti-inflammatory and antitumoral activities [20]. Using this same model of topical inflammation, Sosa *et al.* [21] demonstrated that many diterpenoid and triterpenoid compounds exhibited topical anti-inflammatory activity. These compounds were obtained from medicinal plants used in folk medicine to prepare decoctions against rheumatism.

The influence of different permeation en-

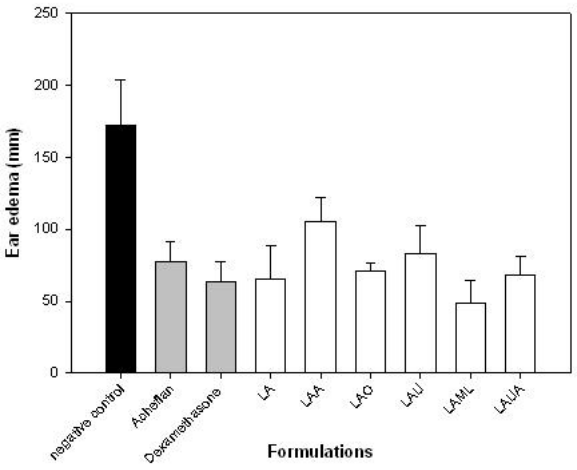

Figure 2. Anti-inflammatory effect of the kaurenoic acid (KA) creams for ear edema method. L – negative control (cream without KA); Acheflan® - positive control (cream containing 0.5% of essential oil of *Cordia verbenacea*); Dexamethasone – positive control (cream containing 0.5% of dexamethasone); LA – cream containing 100 µg/g of KA; LAA - containing KA 100 µg/g and alpha bisabolol; LAO - containing KA 100 µg/g and isodecyl oleate; LAU – containing KA 100 µg/g and urea; LAML - cream containing KA 100 µg/g, isopropyl myristate and soy lecithin; LAUA - containing KA 100 µg/g, urea and alpha bisabolol.** - significant difference ($p < 0.01$).

hancers (isodecyl oleate, isopropyl myristate + soy lecithin and urea + alpha bisabolol) was evaluated in the pharmacological activity of KA cream. KA cream containing permeation enhancer presented a similar effect to the positive control ($p > 0.05$), except for the creams with containing urea and alpha bisabolol.

When the KA creams (100 µg/g) were compared with and without permeation enhancers, it was observed that there was no significant difference among them ($p > 0.05$), except for the creams with the permeation enhancer urea ($p < 0.05$) and alpha bisabolol ($p < 0.01$), which presented smaller edema inhibition.

KA creams (100 µg/g) with and without permeation enhancer were compared with Acheflan®, an anti-inflammatory cream containing essential oil of *Cordea verbenacea,* and a significant difference was observed in the preparations containing isopropyl myristate + soy lecithin ($p < 0.05$), with these same higher anti-inflammatory effect.

It was also observed that the KA creams (100 µg/g) with urea and alpha bisabolol did not demonstrate any increased in the effect of KA, with lower absorption than for cream without permeation enhancer, but when the two permeation enhancers (urea + alpha bisabolol) were associated, an increase was observed in drug permeation.

A useful non-direct contact assay used to evaluate the *in vitro* reactivity of cosmetics and topical formulation is the agarose overlay assay, as a prediction of cutaneous irritation. The assay may be useful in assessing the irritation potential of test substances (*e.g.* surfactant-based products) as an alternative to the Draize rabbit eye test. The test of cutaneous irritation in agarose gel could be used to verify the irritation potential in emulsions and gels.

None of the semi-solid preparations with or without KA and in the presence of the different types of permeation enhancers presented any degree of reactivity in the murine fibroblast cells (L929), determined by the absence of cytotoxic halo and by inverted optical microscopy (Fig. 3). The positive control (SDS) presented a halo of 1.341 cm ± 0.06 cm, which was classified as 4 or severe reactivity, according to the USP classification [15].

Figure 3 shows the presence of viable cells in dark stains (dyed with neutral red) in cream with or without KA, indicating the absence of reactivity and ocular or cutaneous irritation. In the Figure 3 the absence of viable cells (absence of dark stains) in the samples containing the positive control (SDS) demonstrate reactivity and severe irritation.

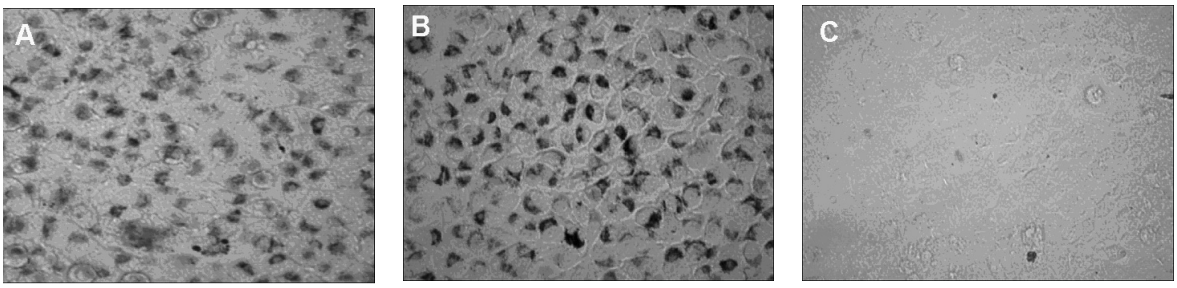

Figure 3. Irritation potential of creams containing kaurenoic acid (KA) by agarose overlay assay using L929 cells. (**A**) Cream with KA; (**B**) cream without KA; (**C**) positive control. 400X.

CONCLUSIONS

In summary, our results demonstrate that creams containing kaurenoic acid, isolated from the acetonic extract of *Wedelia paludosa,* presents a significant topical anti-inflammatory effect. This activity was optimized by the incorporation of two permeation enhancers (soy lecithin and isopropyl myristate) and preparation analyzed not present potential for cutaneous irritation.

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