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Determination of topical anti-inflammatory activity of the essential oil and extracts of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae), using the model of mouse ear edema induced by TPA and AA

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ABSTRACT - The topical anti-inflammatory properties of hydro-alcoholic and diethyl ether extracts, as well as the pure essential oil of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae), were studied. The mouse ear-inflammation-induction model was applied, using 12-O-tetradecanoylphorbol-13-acetate (TPA) and arachidonic acid (AA) as flogistic agents. Both hydro-alcoholic and diethyl ether extracts of *L. alba*, as well as the essential oil, showed topical anti-inflammatory activity. Topical administration of the essential oil showed a significant amount of anti-inflammatory activity in the TPA induced ear edema model and a lower potency in the AA induced edema. The anti-inflammatory effect of the hydro-alcoholic and diethyl ether extracts was best observed in the mouse ear edema model induced by AA (79.51% and 74.92% of inflammation respectively). The major components of the essential oil of the leaves are carvone (67.9%) and limonene (26.8%).

KEY WORDS - *Lippia alba*, hydro-alcoholic and diethyl ether extracts, essential oil, anti-inflammatory activity, carvone, limonene.

INTRODUCTION

Lippia alba (Mill.) N.E. Brown (Verbenaceae) has traditionally been used in many countries of the American continent for its anti-spasmodic, anti-pyretic, carminative and sedating properties (1,2); it is also employed as a remedy for colds, grippe (influenza), bronchitis, coughs and asthma (3,4). It is an aromatic shrub, 1.5 to 2 m high, bushy, with long, strangling, drooping branches, more or less minutely downy. The leaves are opposite or in whorls of 3, ovate to oblong, 2 to 8 cm long, 0.9 to 2 cm wide, crinkled, finely scalloped, covered with very short hairs on both surfaces; veins are prominent in the underside. The flowers are purple, lavender or white, tubular, 4 to 5 mm long, in rounded or oblong heads 8 to 12 mm long, usually in pairs on short stalks (to 1.5 cm long) in the leaf axils. It is a deciduous plant native of the American continent commonly grown in tropical countries (3). This plant shows great phytochemical variability and it has been classified in different chemotypes according to its main components (5-10).

The material used in ethnomedicine is obtained by collecting the leaves at wild fields or at domestic orchards (11). Although some authors have studied the analgesic and anti-inflammatory activity of extracts of *L alba* (12,13), we are interested in providing information regarding the topical anti-inflammatory effect, because this activity could justify the use of this plant as phytotherapeuticals for external use. The topical anti-inflammatory properties were studied using the model of mouse ear edema induced with 12-O-tetradecanoylphorbol-13-acetate (TPA) and arachidonic acid (AA), as described by Carlson *et al.* (14).

MATERIALS AND METHODS

Plant material

The botanical material was collected at the *Bougainvillea Botanical Garden* in Limón, Costa Rica, during the months of May, June and November. The plant was identified at source by ethnobotanist R. Ocampo. Leaves were dried at 40°C for 3 days and then ground. A voucher specimen was deposited at the Herbarium of the University of Costa Rica (USJ-70741).

Preparation of L. alba leaf extracts

Preparation of the hydro-alcoholic and diethyl ether extracts

The hydro-alcoholic extract was obtained through percolation of the dry and ground material (981 g) in an 8:2

ethanol/water solution, for 72 hours. The extract was evaporated and lyophilized (freeze-dried). The diethyl ether extract was obtained by extraction of the dry and ground leaves in three 24 hour periods. The pooled extracts were concentrated *in vacuo* and stored at 4-8 ºC until used.

Essential oil

The essential oil was obtained through hydro-distillation of the dried leaves, for 3 hours, using a modified Clevenger type apparatus. The distilled oil was collected and dried over anhydrous sodium sulfate and stored in a freezer (0-10ºC). The oil yield was 0.2% (w/w).

Animals used

Swiss Webster mice (28-32 g) were supplied by the Animal Care Unit of the University of Costa Rica (LEBi). All animals had free access to food and water and were kept under controlled light conditions during the experiment. Each group studied consisted of eight animals.

TPA- and AA-induced ear edema

TPA was used at a concentration of 0.125 g/l, while AA was used at a 0.1 mg/l. Positive control groups were treated with dexamethasone (4 g/l), and indomethacin (0.025 mg/l). The negative control group was treated with 0.9% saline solution. The hydro-alcoholic and diethyl ether extracts were applied topically at a concentration of 0.15 mg/l; the essential oil was used undiluted.

Determination of the topical anti-inflammatory effect was carried out using the model of mouse ear edema induced by TPA and AA (15). In brief, 10 µl of *L. alba* extract was applied at the inner side and 10 µl at the outer side of the right ear with the help of a micropipette tip. Five minutes later, either TPA or AA solution was applied (10 µl on the inner side and 10 µl on the outer side). Animals treated with TPA were sacrificed by cervical dislocation four hours later and those treated with AA one hour later. Circles of the ear (6 mm in diameter) where the application was made were collected and weighed. Left ears were treated likewise except that instead of the extracts, the corresponding solvent was used.

Inflammation percentage was calculated with the following formula:

$$\% = \frac{(RE-LE)}{LE} * 100$$

Where: RE is the disc weight obtained from the right ear.
LE is the disc weight obtained from the left ear.

Average inflammation percentages were statistically analyzed with the SPSS® program. Their statistical differences with respect to the negative control were determined with the "t" student test, and also the factorial variance analysis ANOVA was applied.

Significance level was established at $p < 0.05$.

The level of inflammation achieved by treatment with the anti-inflammatory substances was compared with that of the negative control. Percentage of topical anti-inflammatory effect (%AIE_t) was calculated according to the following formula:

$$\%AIE_t = \frac{(\text{Control weight} - \text{Sample weight})}{\text{Control weight}} * 100$$

Gas Chromatography/mass spectrometry analysis

The GC/MS analyses were performed using a Shimadzu GCMS-QP5050 apparatus and CLASS 5000 software with Wiley139 computer database. The data were obtained on a 5% methyl phenylpolysiloxane fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 µm). Operation conditions were: carrier gas He, flow 1.0 mL/min; oven temperature program: 60-240 °C at 3 °C/min; sample injection port temperature: 250 °C; detector temperature: 260 °C; ionization voltage: 70 eV; ionization current 60 µA; scanning speed 0.5 s over 38-400 amu range; split 1:70.

Identification of the components of the oil was performed using the retention indices on DB-5, and by comparison of their mass spectra with those published in the literature (16-18) or those of our own database.

RESULTS

Inflammation induced with TPA

The highest percentage of inflammation was observed on the saline solution control group (155.66 ± 20.84 %). The indomethacin-treated group showed an inflammation of 69.55 ± 22.19 %. Dexamethasone-treated group showed an inflammation of 30.87 ± 6.67 %. The groups treated with the hydro-alcoholic and diethyl ether extracts presented very similar inflammation percentages (125.53 ± 16.36 % and 127.02 ± 11.88 % respectively). The group treated with essential oil showed an inflammation of 24.62 ± 4.67 % (Table 1 and Figure 1).

When analyzing percentages of topical anti-inflammatory effect (AIE_t) it was observed that the highest AIE_t was for the group treated with essential oil (86.67 %), superior than the groups treated with dexamethasone (82.22%) and indomethacin (38.93 %) (Table 2). The groups treated with the hydro-alcoholic and diethyl ether extracts showed anti-inflammatory effects of 19.97 % and 20.99 %, respectively (Figure 2).

Inflammation Induced with Arachidonic Acid

The groups treated with dexamethasone and with indomethacin showed very different responses. The inflammation for dexamethasone was 103.39 ± 9.65 % and for indomethacin 8.54 ± 5.58 %.

Table 1: Effect of Lippia alba fractions on inflammation induced by TPA and AA in the mouse ear edema model

Substance	TPA Percentage of inflammation ± sd	AA Percentage of inflammation ± sd
Saline Solution	155.66 ± 20.84	134.13 ± 7.79
Dexamethasone	30.87 ± 6.67	103.39 ± 9.65
Indomethacin	69.55 ± 22.19	8.54 ± 5.58
Essential oil	24.62 ± 4.67	48.79 ± 7.88
Hydro-alcoholic Extract	125.53 ± 16.36	79.71 ± 10.36
Diethyl ether Extract	127.02 ± 11.88	74.92 ± 19.20

Table 2: Topical anti-inflammatory effect (%AIE$_t$) of Lippia alba fractions on the mouse ear edema model induced with TPA and AA.

SUBSTANCE	**TPA** % AIE$_t$	**AA** % AIE$_t$
Saline solution	Control	Control
Dexamethasone	82.22	29.02
Indomethacin	38.93	92.02
Essential oil	86.67	63.46
Hydroalcoholic Extract	20.99	39.72
Etheral Extract	19.97	39.75

Table 3: Percentage composition of Lippia alba leaf essential oil.

Compound[a]	RI[b]	Percentage	Method[b]
α-pinene	933	t[c]	1, 2, 3
camphene	948	t	1, 2
sabinene	976	t	1, 2
β-pinene	979	t	1, 2, 3
myrcene	995	0.6	1, 2, 3
p-cymene	1027	t	1, 2, 3
limonene	1031	26.8	1, 2
(*Z*)-β-ocimene	1039	t	1, 2
(*E*)-β-ocimene	1049	0.2	1, 2
linalool	1106	0.2	1, 2, 3
borneol	1177	0.1	1, 2
trans-carveol	1218	t	1, 2
carvone	1242	67.9	1, 2, 3, 4
piperitone	1252	0.1	1, 2
piperitenone	1344	0.1	1, 2
β-bourbonene	1384	0.4	1, 2
β-cubebene	1390	t	1, 2
β-elemene	1391	0.2	1, 2
β-caryophyllene	1418	0.4	1, 2, 3
β-copaene	1431	0.1	1, 2
germacrene D	1480	0.8	1, 2
γ-cadinene	1514	0.3	1, 2
δ-cadinene	1525	t	1, 2, 3
(*E*)-nerolidol	1571	0.1	1, 2

[a] *Compounds listed in order of elution from 5% phenyl methylpolysiloxane column.*
[b] *RI = Retention index relative to n-alkanes on the 5% phenyl methylpolysiloxane column.*
[c] *t = Traces (<0.05%).*
[d] *Method: 1 = Retention Index in 5% phenyl methylpolysiloxane column; 2 = MS spectra; 3 = Standard; 4 = FTIR; ^{1}H-NMR*

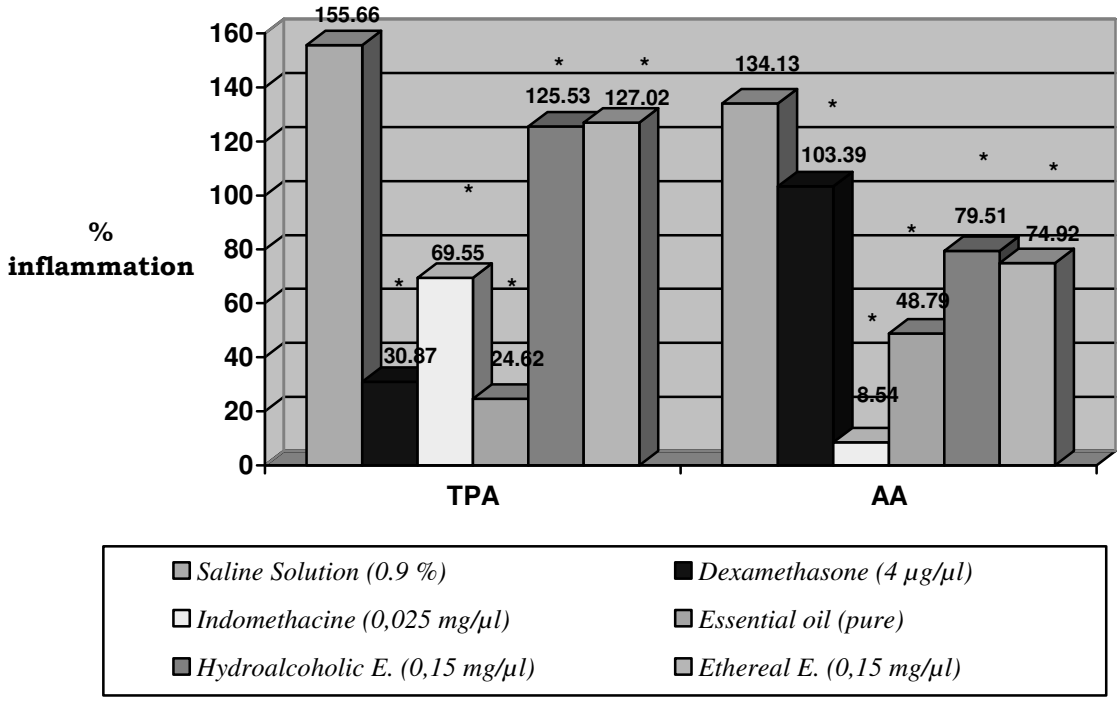

Figure 1. Inflammation percentage for the diferent substances studied when using the muose ear edema model induced by TPA and AA.
**** p < 0,05***

Essential oil showed the least inflammation percentage (48.79 ±7.88 %), whereas hydroalcoholic and diethyl ether extracts behaved in a similar manner (79.71 ± 10.36 % and 74.92 ± 19.20 % respectively) (Table 1 and Figure 1). When analyzing percentage of topical anti-inflammatory effect (AIEt) it was observed that the AIE_t for indomethacin was 92.02 % and that shown by dexamethasone was 29.02 %. For the group treated with essential oil, AIE_t was 63.46 %, for the hydro-alcoholic extract 39.72 % and for the diethyl ether extract 39.75 % (Figure 2 and Table 2). After application of the essential oil, an unusual behavior of self-cleansing and a little sedation was observed.

Composition of the essential oil

The detailed chemical composition of the essential oil from the leaves of *L. alba* from Costa Rica (corresponding to **carvone-limonene** chemotype) was recently described (19). The chemical composition of the essential oil used in this work is summarized in Table 3. Twenty-four compounds were identified accounting for over 98% of the composition of the oil. The monoterpenoids account for about 96% with minor percentages of sesquiterpenoids (2.3%). Carvone (67.9%) and limonene (26.8%) were the major components of the oil.

DISCUSSION

The main characteristics of TPA induced acute inflammation are increase of vascular permeability and vasodilatation, which results in edema and migration of inflammatory cells, mainly neutrophils, and moderate synthesis of eicosanoids. Changes induced by TPA start later but they last longer if compared with the AA response (20).

The significant increase in ear weight, when using TPA as the flogistic agent (Fig. 1), is due to hyperemia, cellular infiltration as well as accumulation of exuded serum (21).

The ability of TPA to cause inflammation is based on the release of AA dependent on the stimulation of $cPLA_2$ through phosphokinase C (PKC) and activation of MAP-kinase (21). This action is accompanied by multiple cellular phenomena which induce edema formation (22).

These changes seem to depend greatly on LTB_4 liberation since it is proposed that this potent chemotactic agent mediates a change in vascular permeability, inducing degranulation of mastocytes (20). In addition, prostaglandin or mediators like histamine, bradikinin and PAF can play a role in this response (23).

Inflammation percentage and topical anti-inflammatory effect percentage using indomethacin and dexamethasone

are different (Figures 1 and 2), with dexamethasone being more effective than indomethacin in attenuating changes in vascular permeability induced by TPA because of their different mechanism of action (24). This model seems to be dependent on PGE2 and COX inhibitors decrease edema with little effect over cell migration (25). Both AA and TPA induce a marked increase in vascular permeability and subsequent edema formation.

Indomethacin is an effective inhibitor of inflammation induced by TPA (26), but it reduces the process generated by AA more significantly. Indomethacin has no effect on the LTB_4 levels but it is said that this NSAI agent is very efficient in diminishing the 6-keto-$PGF_{1\alpha}$ (27). A significant increase in the concentration of this molecule is induced by AA as measured by the mouse ear edema model (23). This explains the high inflammation percentage when edema was induced by TPA.

L. alba essential oil, applied in its pure form, showed the highest anti-inflammatory activity. This extract produced a statistically significant anti-inflammatory effect in the ear edema induced by TPA and in that induced by AA; however, it reduced inflammation to a greater degree in the TPA model. Cyclo-oxygenase and lipo-oxygenase inhibitors are known to reduce AA and TPA induced ear edema , with differences in potency (28).

The anti-inflammatory action of *L. alba* essential oil is compatible with inhibition of the $cPLA_2$ activity, according to the TPA inflammation induction model. The anti-inflammatory effect of the hydro-alcoholic and diethyl ether extracts can be explained by inhibition of PGs production (25). It is important to underline that both extracts have a statistically significant anti-inflammatory activity and, in addition, their extraction yield is higher (hydro-alcoholic 9.36%, diethyl ether 5.43%) than that of essential oil (0.23%).

Among the three preparations, *L. alba* essential oil shows the best topical anti-inflammatory effect using the model of mouse ear edema induced by TPA and AA.

Concerning the change in animal behavior after the application of the essential oil, it is known that carvone, one of the major components of the essential oil, does not have any significant effect on the central nervous system, and therefore, we should not expect alteration of behavior induced by this substance (29). Nevertheless, the sedative effect of the *L alba* oil is also known, which could explain this effect (30). Limonene, on the other hand, has been found to have CNS depressant activity (31), including sedative as well as motor relaxant effects, despite a previous negative report (32).

The potential use of this essential oil as phyto-pharmacological agent is being studied in our laboratory.

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