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Anti-inflammatory Evaluation and Phytochemical Characterization of some Plants of the *Zanthoxylum* genus

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SUMMARY. This study examines the anti-inflammatory activity of some species of *Zanthoxylum* genus. We evaluated 4 ethanolic extracts from stem bark of *Zanthoxylum elephantiasis* Macfd., *Z. fagara* (L.) Sargent., *Z. martinicense* (Lam.) DC, and from fruits of *Z. coriaceum* A. Rich. species. We used phorbol myristate acetate (PMA) and arachidonic acid (AA)-induced mouse ear oedema as models of acute inflammation. The extracts of *Z. coriaceum* and *Z. fagara* (1-3 mg/ear) were active against the AA and PMA application on mouse oedema. *Z. elephantiasis* extract (0.5-2 mg/ear) exhibited an anti-inflammatory effect in AA application. In the PMA model it was also effective, at all assayed doses. Ethanolic extract of *Z. martinicense* (1-3 mg/ear) was active on AA induced oedema however; it wasn't effective in the PMA model. Considering the relevant anti-inflammatory effect exhibited by *Z. elephantiasis* extract we decided to analyze the chemical composition of extract by gas chromatography coupled to mass spectrometry (GC-MS). Among others, 3 alkaloids, 1 coumarin, 1 lignan, 3 amides and 5 steroids were found in analyzed fractions.

RESUMEN. "Evaluación Anti-inflamatoria y Caracterización Fitoquímica de algunas Plantas del género *Zanthoxylum"*. El presente estudio centra su atención en 4 especies cubanas, pertenecientes al género *Zanthoxylum*. Se evaluaron 4 extractos etanólicos provenientes de la corteza del tronco de *Zanthoxylum. elephantiasis,* Macfd., *Zanthoylum fagara* (L.) Sargent., *Zanthoxylum martinicense* (Lam.) DC., y de los frutos de *Zanthoxylum coriaceum,* A. Rich, mediante el uso del modelo pro-inflamatorio del edema inducido en la oreja del ratón con el empleo de dos agentes irritantes, forbol miristato acetato (PMA) y ácido araquidónico (AA). Todos los extractos demostraron un efecto anti-inflamatorio marcado, dependiente de la dosis para los dos agentes irritantes utilizados, con excepción del extracto de *Z. martinicense* que no presentó una actividad anti-inflamatoria notable frente al edema inducido por PMA, sin embargo frente al agente irritante AA exhibió una potente acción. En el caso de los extractos de *Z. coriaceum* y *Z. fagara* el efecto anti-inflamatorio fue similar frente a ambos agentes irritantes. El extracto de *Z. elephantiasis* mostró el mayor efecto al usar PMA como agente irritante siendo este resultado el mayor observado. Por esta razón se analizó la composición química del extracto por cromatografía gaseosa acoplada a espectrometría de masa (GC-MS). En las fracciones analizadas se encontraron 3 alcaloides, 1 cumarina, 1 lignano, 3 amidas y 5 esteroides entre otros compuestos.

INTRODUCTION

The use of medicinal plants is very widespread in Cuba. *Zanthoxylum* genus (Rutaceae) is represented by 25 species, 15 of which are endemic [1] and six have been reported as medicinal or potential medicinal plants [2]. These Cuban plants have been used to treat a wide range of physical ailments, such as asthma, ulcers, rheumatism, and earache [3], all of them related with the inflammatory process.

Inflammation is normally a localized protective response aimed to destroy, eliminate or wall-off both, the inflammatory agent and the injured tissue. Inflammation is a coordinated response of cells such as macrophages, lymphocytes, leukocytes and mast cells. A large number

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PALABRAS CLAVE: GC-MS Edema oreja, Inflamación, *Zanthoxylum coriaceum, Zanthoxylum elephantiasis, Zanthoxylum fagara, Zanthoxylum martinicense.*

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of mediators produced by these cells play a key role. Among these mediators are included arachidonic acid, prostaglandins and leukotrienes (LTs), reactive oxygen species, hydrolytic enzymes, histamine, nitric oxide, and cytokines [4].

Plants of the *Zanthoxylum* genus are known to produce a variety of biologically active secondary metabolites including alkaloids, lignans and coumarins [5], compounds that can be involved in the possible anti-inflammatory properties found for the *Zanthoxylum* species [6,7].

Taking into consideration these precedents, the objective of the present study is to determine the anti-inflammatory effects of ethanolic extracts from the fruits of *Zanthoxylum coriaceum* A. Rich and from the stem bark of *Z. elephantiasis* Macfd., *Z. fagara* (L.) Sargent. and *Z. martinicense* (Lam.) DC. We also carry out the preliminary characterization of *Z. elephantiasis* stem bark by GC-MS.

In this paper, we have evaluated for the first time the *in vivo* anti-inflammatory effects of some *Zanthoxylum* species in models of mice ear inflammation.

MATERIALS AND METHODS

Plant material

All the samples (fruits of *Z. coriaceum* and stem bark of *Z. elephantiasis, Z. martinicense* and *Z. fagara)* were collected from the province of Pinar del Río in April *(Z. coriaceum)* and November (the others) 2001. Professor Armando Urquiola, Director of the Herbarium of the Pedagogic Institute and the Botanic Garden of Pinar del Río, Cuba, identified them. Relevant voucher specimens are kept for reference in the Department herbarium.

Preparation of extracts

The raw materials *(Z. elephantiasis, Z. martinicense* and *Z. fagara,* fresh stem bark, 65, 45 and 65 g, respectively) were powdered and successively macerated with 95 % ethanol (200, 160 and 300 ml, respectively) for 12 days with replacements of solvent every three days. All extracts were properly stored at 4 °C. The raw materials corresponding to *Z. coriaceum* (air-dried fruits, 30 g) were powdered and extracted with 95 % ethanol (150 ml) by a reflux system during 1 h. Each extract was concentrated on a rotatory evaporator R-121 (Büchi, Switzerland) coupled to a hole system and a water bath. Preliminarily, the extracts were analyzed by thin layer chromatography (TLC), using hexane/ethyl acetate (2:1, v/v) as mobile phase. Spots were observed at 254 nm and revealed with vainillin/sulphuric acid mixture as well. Extracts were dissolved in 100% ethanol for pharmacological experiments.

GC-MS analysis

Previously to GC-MS, oily ethanolic extract from *Z. elephantiasis* was successively fractionated by liquid partitioning in dichloromethane (Fraction A) and acetone (Fraction B). Finally, to the insoluble portion of crude extract HCl 0.1 N was added and twice re-extracted with chloroform (Fraction C).

Analysis by GC-MS was carried out in a Hewlett Packard High Resolution GC-MS equipped with a HP1-MS capillary column (30 m x 0.25 mm x 0.25 µm). Chromatographic run was performed with programmed temperature from 50 °C (3 min) up to 300 °C at 4 °C/min. Injection port and carrier gas flow were set at 270 °C and 1 ml/min, respectively. Mass cuadrupolar detector was operated in EI+ at 70 eV.

Reagents

All solvents were of analytical grade and provided by Merck (Darmstat, Germany). AA, PMA, nimesulide and indomethacin for pharmacological assays were from Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Male OF-1 mice (25-30 g) were used. All animals were housed in standard environmental conditions (25 ± 1 °C, 12 h light/dark cycle) with free access to a standard commercial diet and water *ad libitum*. All experiments were carry out in accordance with the ethical guidelines for research in laboratory animals.

AA-induced mouse ear oedema

The method described by Romay *et al.* [8] was followed. Ethanolic extracts of *Z. coriaceum, Z. fagara, Z. martinicense* (1.0, 2.0, 3.0 mg/ear) and *Z. elephantiasis* (0.5, 1.0, 2.0 mg/ear) were applied topically simultaneously with AA (1 mg/ear). The reference group was treated with nimesulide (2 mg/ear). After 1 h, the animals were killed by cervical dislocation and disks of 6 mm diameter were removed from each ear and the weight was determined. The swelling was measured as the difference in weight between the punches from right and left ears, and expressed as an increase in ear thickness.

PMA-induced mouse ear oedema

An oedema was induced on the right ear by

topical application of PMA in acetone according to Griswold *et al.* [9], while the left ear (control) received only the vehicle (acetone or ethanol 100%). Ethanolic extracts of *Z. coriaceum, Z. fagara, Z. martinicense* and *Z. elephantiasis* at the same doses were applied topically simultaneously with PMA (2 µg/ear). The reference group was treated with indomethacin (3 mg/ear). After 4 h, the animals were killed by cervical dislocation. Disks of 6 mm diameter were removed from each ear and the weight was determined. The swelling was measured as the difference in weight between the punches from right and left ears, and expressed as an increase in ear thickness.

Statistical analysis

Oedemas are expressed as mean ± SEM Inhibition percentages arise from differences between treated and non-treated tissues, and are referred to the control treated only with the inflammatory agent. One-way analysis of variance (ANOVA) followed by Mann-Whitney test for unpaired data was used for statistical evaluation ($p< 0.01$).

	a) Dichloromethane extract (Fraction A)
1	Lauric acid; Mw = 200
2	Myristic acid; Mw = 228
3	9-Octadecenamide; Mw = 281
4	Tetradecanenitrile; Mw = 209
5	Neophytadiene; Mw = 278
6	Palmitic acid; Mw = 256
7	Amide
8	3-Phenyl-N-(2-phenylethyl)-2-propenamide; Mw = 251
9	3β-ergost-5-en-3-ol; Mw = 400
10	3β,24S-Stigmaste-5-en-3-ol; Mw = 414
11	Stigmast-5-en-3-one; Mw = 412
	b) Acetone extract (Fraction B)
1	4-vinyl-2-methoxiethanol; Mw = 150
2	3,4,5-trimethoxyphenol; Mw = 184
3	Bravelin; Mw = 258
4	Canthin-6-one; Mw = 220
5	5-metoxicanthin-6-one; Mw = 250
6	DL-Canadine; Mw = 339
7	Sesamin; Mw = 354
8	Steroid (stigmastenol derivative)
9	Stigmastenol; Mw = 414
10	Stigmast-4-en-3-one; Mw = 412
	c) Chloroformic extract (Fraction C)
1	Valencene; Mw = 204
2	Bravelin; Mw = 258
3	Canthin-6-one; Mw = 220
4	DL-Canadine; Mw = 339
5	Asarinin; Mw = 354

Table 1. Compounds identified in extracts from of *Z. elephantiasis* stem bark.

RESULTS

Phytochemical screening of the extracts

Crude extracts from specified organs of *Z. coriaceum, Z. fagara, Z. martinicense* and *Z. elephantiasis* applied to TLC plate were resolved in bands which revealed under UV (254 nm) and vainillin/sulphuric acid with colours from red through green or blue to yellow, indicative of probable occurrence of alkaloids, coumarins and lignanes, among other secondary metabolites typical of *Zanthoxylum* genus.

Fatty acids, amides, benzophenanthridine alkaloids, steroids, coumarins among others, were the predominant family of secondary metabolites occurring in the analysed extracts. Table 1 lists compounds identified by GC-MS in fractions obtained from ethanolic extract of *Z. elephantiasis* stem bark.

AA-induced mouse ear oedema

The ethanolic extracts of *Z. coriaceum, Z. fagara* and *Z. martinicense* were active against the arachidonic acid (AA) application on mouse oedema at doses 1, 2 and 3 mg/ear showing a dose dependent fashion (Tables 2 and 3) in the range of doses evaluated (ED_{50} were 1.3, 1.0 and 1.8 mg/ear respectively). *Z. elephantiasis* extract showed a ED_{50} =1.4 mg/ear. Maximal inhibitions on oedema were 74.9 *(Z. coriaceum* extract), 69.1 *(Z. elephantiasis* extract), 75.9 *(Z. fagara* extract) and 73.3% *(Z. martinicense* extract). The reference drug nimesulide (1 mg/ear) also inhibited the oedema (66.7%).

PMA-induced mouse ear oedema

Phorbol myristate acetate (PMA)-induced acute inflammation in the ear of mice was inhibited by topical administration of ethanolic extracts of *Z. coriaceum* and *Z. fagara* at doses 1, 2 and 3 mg/ear (Tables 2 and 3). Ethanolic extract of *Z. elephantiasis* also showed an anti-inflammatory activity at doses of 0.5, 1 and 2 mg/ear (Table 2) while ethanolic extract of *Z. martinicense* didn't have an anti-inflammatory effect at the assayed doses (Table 3). ED50 were 1.5, 0.2 and 0.8 for *Z coriaceum, Z. elephantiasis,* and *Z. fagara*. Maximal inhibitions were found at higher doses for *Z. coriaceum* and *Z.*

elephantiasis extracts 4 h after PMA application (66.0 and 70.9%). The ethanolic extract of *Z. fagara* exhibited the greatest effect at 2 mg/ear dose (77.7%). Indomethacin (3 mg/ear) was used as the reference drug and also inhibited PMA-induced oedema (98.6%).

DISCUSSION

AA and PMA are tumor-promoting agents, and protein kinase C activators are widely used to induce cutaneous inflammation in experimental animal models. The initiation of inflammatory responses by metabolites of AA and suppression of acute inflammatory responses by inhibitors of cyclooxygenase (COX) and lypoxygenase (LOX) establish an important role for metabolites of AA in acute inflammation induced by AA and PMA [10].

The *in vivo* model of AA mouse ear inflammation is very suitable and sensitive although not specific to test inhibitors of LOX, and the direct topical application of AA results in the rapid onset of oedema formation [11] and is most probably related to LTC_4 [12] and LTB_4 release [13]. In contrast, topical application of phorbol esters (like PMA) induce a long lasting inflammatory response associated whit transient increase in prostanoid production and marked cellular influx. This high prostaglandin level is very likely due to COX induction [14].

Few studies have demonstrated the pharmacological activity of ethanolic extracts of *Zanthoxylum* species studied in this paper. We demonstrated its inhibitory effects on the ear inflammation models induced by AA and PMA, except for the ethanolic extract of *Z. martinicense* which didn't show a significant activity in PMA induction. Ethanolic extracts of *Z. coriaceum* and *Z. fagara* had a similar effect in AA and PMA models; it could be thought that the extracts act in both leukotriene and prostaglandin pathways, probably by inhibition of LOX and COX enzymes respectively. The extracts could have an inhibitory effect on pospholipase A_2 (PLA_2).

On the other hand, the anti-inflammatory effect of *Z. elephantiasis* extract was higher in the case of oedema induced by PMA, it could be acting more specifically on the leukotrienes process and the extract could show more affinity to the LOX enzyme. However, ethanolic extract of *Z. martinicense* demonstrated a great activity against the oedema induced by AA but didn't exhibit any effect on PMA induction, this behavior could be related with a direct action on LOX enzyme or leukotrienes as mediators obtained by its action.

The presence of coumarins (braylin), alkaloids (canadine and canthinone), alkamides (3-phenyl-*N*-(2-phenylethyl)-2-propenamide and 9-octadecenamide) and steroids, among others constituents in *Z. elephantiasis* should be taking into account in order to justify its high anti-inflammatory activity. Although further work should be made to achieve precise correlation between anti-inflammatory effect of assayed *Zanthoxylum* species and chemical composition of its extracts, it is interesting to remark that

Treatment		PMA-induced ear oedema		AA-induced ear oedema	
	Dose (mg/ear)	Oedema weight (mg)	Inhibition (%)	Oedema weight (mg)	Inhibition (%)
PMA	0.002	8.2 ± 0.6	-	-	-
AA	1	-	-	7.3 ± 0.2	-
Zanthoxylum coriaceum A. Rich. (dried fruits)	1	4.3 ± 1.4*	47.5	3.9 ± 0.9*	47.0
	2	4.1 ± 1.0*	50.2	3.0 ± 0.5*	58.7
	3	2.8 ± 0.7*	66.0	1.8 ± 0.3*	74.9
Zanthoxylum elephantiasis Macf. (stem bark)	0.5	3.5 ± 1.2*	57.5	6.2 ± 0.6*	14.7
	1	2.9 ± 0.8*	64.4	4.5 ± 0.4*	38.1
	2	2.4 ± 0.9*	70.9	2.2 ± 0.5*	69.1
Indomethacin	3	0.1 ± 0.2*	98.6	-	-
Nimesulide	1	-	-	2.7 ± 0.2*	66.7

Table 2. Effect of *Z. coriaceum* A. Rich. and *Z. elephantiasis* Macfd., administered topically, on oedema induced by PMA and AA in mouse ear. Each group represents the mean ± SEM of 7-8 animals.
*$p < 0.01$ statistical significance compared with PMA and AA groups respectively. Oedema weight was assessed as the increase in the weight of the right ear punch biopsy over that of the left ear. The oedema was measured 4 h after PMA application and 1 h after AA application.

Treatment		PMA-induced ear oedema		AA-induced ear oedema	
	Dose (mg/ear)	Oedema weight(mg)	Inhibition (%)	Oedema weight (mg)	Inhibition (%)
PMA	0.002	13.7 ± 1.1	-	-	-
AA	1	-	-	8.2 ± 0.9	-
Zanthoxylum fagara,	1	6.9 ± 0.7*	49.3	4.2 ± 0.7*	48.6
Sarget.	2	2.8 ± 0.6*	79.3	2.9 ± 0.6*	65.1
(stem bark)	3	2.6 ± 0.8*	77.7	2.0 ± 0.5*	75.9
Zanthoxylum. martinicense	1	11.8 ± 0.7	13.7	5.2 ± 0.6*	37.2
(Lam.) D.C.	2	7.4 ± 0.6	24.4	3.9 ± 0.9*	53.0
(stem bark)	3	10.6 ± 0.7	19.4	2.0 ± 0.5*	73.3
Indometacin	3	0.1 ± 0.2*	98.6	-	-
Nimesulide	1	-	-	2.7 ± 0.2*	66.7

Table 3. Effect of *Z. fagara* (L.) Sarget and *Z. martinicense* (Lam.) DC. administered topically, on oedema induced by PMA and AA in mouse ear. Each group represents the mean ± SEM of 7-8 animals.
*$p < 0.01$ statistical significance compared with PMA and AA groups respectively. Oedema weight was assessed as the increase in the weight of the right ear punch biopsy over that of the left ear. The oedema was measured 4 h after PMA application and 1 h after AA application.

there are reports about anti-inflammatory properties of benzophenanthridines alkaloids, coumarins, steroids, fatty acids and some other metabolites found in the tested extract [4,6]. Analgesic properties of canadine have been demonstrated and also chelerythrine (the *N*–methylated analog of *N*–norchelerythrine) exert analgesic and anti-inflammatory activities [15]. Osthol, coumarin isolated from *Zanthoxylum* species [16] demonstrated its selectivity to inhibit 5-LOX *in vitro* [17]. Seselin, pyranocumarin structurally related to bravelin, exhibited potent activity in carrageenan-induced inflammation assay [18]. Other coumarins have inhibited generation of LB_4 [6]. Also, alkamides have been found in *Zanthoxylum* species [19,20] and some of them have proved their inhibitory potential on COX and LOX enzymes; due to the structural similarity between the alkamides and the AA, it is probable that these compounds act as similar of the AA and compete for the interaction with the enzymes [21].

Alkaloids with benzophenanthridine moiety and coumarins appear commonly in *Zanthoxylum* genus [22-24] and it could expect the occurrence of alkaloidal and coumarin analogs, among others, in extracts of above evaluated species.

CONCLUSIONS

A significative effect of the ethanolic extracts was observed on the ear oedema model and it was supposed that the demonstrated anti-inflammatory response could be directly related to alkaloids, coumarin and some others constituents dominating chemical composition of *Zanthoxylum* species.

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