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

Lucía MÁRQUEZ 1, Juan AGÜERO 1, Ivones HERNÁNDEZ 1, Gabino GARRIDO 1*, Ioanna MARTÍNEZ 1, Rodrigo DIÉGUEZ 1, Sylvia PRIETO 1, Yabelis RIVAS 1, Jorge MOLINA-TORRES 2, Massimo CURINI 3 & René DELGADO 1.

1 Laboratorio de Farmacología, Departamento de Investigaciones Biomédicas, Centro de Química Farmacéutica, A.P. 16042, Ciudad de La Habana, Cuba.
2 Departamento de Biotecnología y Bioquímica, CINVESTAV-IPN, Unidad Irapuato, México.
3 Dipartimento di Chimica e Tecnologia del Farmaco, Sezione di Chimica Organica, Università degli Studi, Via del Liceo, Perugia I-06123, Italy

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 Macf., Z. fagara (L.) Sarg., 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.

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

KEY WORDS: Ear oedema, inflammation, Zanthoxylum coriaceum, Zanthoxylum elephantiasis, Zanthoxylum fagara, Zanthoxylum martinicense.

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

* Autor a quien dirigir la correspondencia. E-mail: gabino.garrido@inomed.sld.cu, gabinocl@yahoo.com

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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.

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

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 Madcl., 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. Preliminary, 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 vanillin/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 quadrupolar 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 carried out in accordance with the ethical guidelines for research in laboratory animals.

AA-induced mouse ear oedema

The method described by Romay et al. 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).

**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 vanillin/sulphuric acid with colours from red through green to 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 (ED50 were 1.3, 1.0 and 1.8 mg/ear respectively). Z. elephantiasis extract showed a ED50 =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 lipoxygenase (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 LTC4 12 and LTB4 release 13. In contrast, topical application of phorbol esters (like PMA) induces a long lasting inflammatory response associated with 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. martiniense 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 A2 (PLA2).

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. martiniense 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), alkaloids (3-phenyl-N-(2-phenylethyl)-2-propenamide and 9-octadecenamide) and steroids, among others constituents in Z. elephantiasis should be taken 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PMA-induced ear oedema</th>
<th>AA-induced ear oedema</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dose (mg/ear)</td>
<td>Oedema weight (mg)</td>
</tr>
<tr>
<td>PMA</td>
<td>0.002</td>
<td>8.2 ± 0.6</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>4.3 ± 1.4*</td>
</tr>
<tr>
<td>coriaceum A. Rich.</td>
<td>2</td>
<td>4.1 ± 1.0*</td>
</tr>
<tr>
<td>(dried fruits)</td>
<td>3</td>
<td>2.8 ± 0.7*</td>
</tr>
<tr>
<td>Zanthoxylum elephantiasis Macf.</td>
<td>0.5</td>
<td>3.5 ± 1.2*</td>
</tr>
<tr>
<td>(stem bark)</td>
<td>1</td>
<td>2.9 ± 0.8*</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2.4 ± 0.9*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3</td>
<td>0.1 ± 0.2*</td>
</tr>
<tr>
<td>Nimesulide</td>
<td>1</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Effect of Z. coriaceum A. Rich. and Z. elephantiasis Macf., 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–nor-chelerythrine) 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.

Acknowledgements. This work was partially supported by the Ministry of Public Health, Republic of Cuba (Project MINSAP/Cuba 0008001) and Integral Project CONACYT/México E120,941/2002 and J200.265/2003. Special thanks to MSc. Enrique Ramirez (CINVESTAV-IPN, Unidad Irapuato, México); Mr. José Antonio Valdés (Center of Pharmaceutical Chemistry, Cuba) and NAPRALERT Database for free access to information.

REFERENCES


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<th>AA-induced ear oedema</th>
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<tbody>
<tr>
<td></td>
<td>Dose (mg/ear)</td>
<td>Oedema weight (mg)</td>
</tr>
<tr>
<td>PMA</td>
<td>0.002</td>
<td>13.7 ± 1.1</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>6.9 ± 0.7*</td>
</tr>
<tr>
<td>Zanthoxylum fagara, Sarget. (stem bark)</td>
<td>2</td>
<td>2.8 ± 0.6*</td>
</tr>
<tr>
<td>Zanthoxylum. martinicense (Lam.) D.C. (stem bark)</td>
<td>3</td>
<td>2.6 ± 0.8*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3</td>
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<tr>
<td>Zanthoxylum. martinicense (Lam.) D.C. (stem bark)</td>
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<td>7.4 ± 0.6</td>
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<tr>
<td>Zanthoxylum fagara, Sarget. (stem bark)</td>
<td>3</td>
<td>10.6 ± 0.7</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>3</td>
<td>0.1 ± 0.2*</td>
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<tr>
<td>Nimesulide</td>
<td>1</td>
<td>-</td>
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</table>

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.

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