Protective effects of a standard extract of *Mangifera indica* L. (VIMANG®) against mouse ear edemas and its inhibition of eicosanoid production in J774 murine macrophages

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Abstract

A standard aqueous extract of *Mangifera indica* L., used in Cuba as antioxidant under the brand name VIMANG®, was tested in vivo for its anti-inflammatory activity, using commonly accepted assays. The standard extract of *M. indica*, administered orally (50–200 mg/kg body wt.), reduced ear edema induced by arachidonic acid (AA) and phorbol myristate acetate (PMA) in mice. In the PMA model, *M. indica* extract also reduced myeloperoxidase (MPO) activity. In vitro studies were performed using macrophage cell line J774 stimulated with pro-inflammatory stimuli lipopolysaccharide-interferon gamma (LPS-IFNγ) or calcium ionophore A23187 to determine prostaglandin PGE2 or leukotriene LTB4 release, respectively. The extract inhibited the induction of PGE2 and LTB4 with IC50 values of 21.7 and 26.0 µg/ml, respectively. Mangiferin (a glucosylxanthone isolated from the extract) also inhibited these AA metabolites (PGE2, IC50 value = 17.2 µg/ml and LTB4, IC50 value = 2.1 µg/ml). These results represent an important contribution to the elucidation of the mechanism involved in the anti-inflammatory and anti-nociceptive effects reported for the standard extract of *M. indica* VIMANG®.

Keywords: *Mangifera indica*; Mangiferin; Eicosanoids; Ear edema

Introduction

*Mangifera indica* L. (Anacardiaceae) grows in the tropical and subtropical regions and its parts are used commonly in folk medicine for a wide variety of conditions (Coe and Anderson, 1996). Chemical studies performed using a standard aqueous extract of the stem bark from *M. indica*, which has been used in pharmaceutical formulations in Cuba under the brand name VIMANG®, have enabled the isolation and identification of phenolic acids (gallic acid, 3,4 dihydroxy benzoic acid, benzoic acid), phenolic ester (gallic acid methyl ester, gallic acid propyl ester, benzoic acid propyl ester), flavan-3-ols (catechin and epicatechin) and the xanthone mangiferin, which is the predominant component of this extract (10%). The extract is also rich in fatty acids such as myristic, palmitic, stearic, oleic-linoleic and eicosaatrienoic (Núñez-Sellés et al., 2002). The anti-inflammatory activity of many of these compounds, which occur commonly in several plants, is well known (Bremner and Heinrich, 2002). On the other hand, it has been suggested that many anti-inflammatory drugs may exert some of their effects by scavenging oxidants, and decreasing formation of reactive oxygen species (ROS)
by activated phagocytes (Arouma, 1996). Recently, the first analgesic and anti-inflammatory effects from a standard extract of M. indica were reported. This extract inhibited the abdominal constriction induced by acetic acid and formalin-induced licking in mice, and the edema induced by carrageenan and formalin in mice, rats and guinea-pigs (Garrido et al., 2001). Additionally, it has been demonstrated that the extract shows a powerful scavenger activity against hydroxyl radicals and hypochlorous acid. This extract also presented a significant inhibitory effect on the peroxidation of rat-brain phospholipid and inhibited DNA damage by bleomycin or copper phenanthroline systems (Martínez et al., 2000). By comparison with mangiferin and other well-known antioxidants against tetradecanoylphorbol acetate-induced oxidative damage in serum, liver, and brain, as well as in the hyper-production of ROS by peritoneal macrophages, and it could be concluded that the stem bark extract of M. indica prevented the production of ROS and the oxidative tissue damage in vivo and it was more active than vitamin C, vitamin E, mangiferin and β-carotene (Sánchez et al., 2000).

Taking these findings into account, we decided to test the potential anti-inflammatory activity of a standard aqueous extract of M. indica stem bark (VIMANGs) in several experimental models, in order to characterize roles relevant to the inflammatory process of this extract. Mangiferin, the glucosylxanthone isolated from the extract, was also tested in the in vitro models as the constituent most responsible for its anti-inflammatory activity.

**Materials and methods**

**Drugs**

M. indica was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code: 41722) were deposited at the Herbarium of the Academy of Sciences, maintained by the Institute of Ecology and Systematic, Ministry of Science, Technology, and Environmental, La Habana, Cuba. The stem bark extract of M. indica was prepared by decoction for 1 h. The extract was concentrated by evaporation and spray dried to obtain a fine brown powder, which was used as the standardized active ingredient of VIMANG® formulations. It melts at 210–215 °C with decomposition. The chemical composition of this extract has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry and UV/VIS spectrophotometry (Núñez-Sellés et al., 2002). The solid extract was dissolved in distilled water for pharmacological studies.

**Mangiferin** (2-β-D-glucopyranosyl-1,3,6,7-tetra-hydroxy-9H-xanthen-9-one) was supplied by the Laboratory of Analytical Chemistry, Center of Pharmaceutical Chemistry (Cuba). It was purified from M. indica stem bark standardized extract by extraction with methanol and its purity (90%) was assessed (Núñez-Sellés et al., 2002).

**Chemicals**

Arachidonic acid (AA), phorbol myristate acetate (PMA), indomethacin, dexamethasone, dimethyl sulfoxide (DMSO), interferon gamma (IFNγ), bacterial lipopolysaccharide (LPS), calcium ionophore A23187, O-dianisidine, nordihydroguaiaretic acid (NDGA), and all other reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA).

**Animals**

Male OF-1 mice weighing 25–30 g each were obtained from Centro para la Producción de Animales de Laboratorio (CENPALAB, La Habana, Cuba) and used in these studies. They were kept in a temperature-controlled environment (23 °C) with a 12 h light-dark cycle, relative humidity 40–70%, with food and water ad libitum, fasted overnight (18 h) before the day of the experiments. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the Center of Pharmaceutical Chemistry.

**AA-induced mouse ear edema**

The method described by Romay et al. (1998) was followed. Inflammation was induced by topical application of AA (2 mg/20 μl acetone) to both surfaces of the right ear of each mouse. Left ear (control) received the vehicle. The standard extract of M. indica was administered orally (50, 100 and 200 mg/kg body wt. in water) 1 h before AA. Two control groups were used: a group with an application of AA to the right ear and a positive control group receiving dexamethasone (30 mg/kg body wt., p.o.). Inflammation was followed for 1 h and thereafter animals were killed by cervical dislocation. A 6-mm section from each ear was removed with a metal punch and weighed. Ear edema was calculated by subtracting the weight of the left ear (vehicle) from the weight of the right ear (treatment), and was expressed as edema weight. Inhibition percentage was expressed as a reduction in weight with respect to the control group.
PMA-induced mouse ear edema

According to a modified method of Griswold et al. (1998), 4 μg/ear of PMA, in 20 μl of acetone, was applied to both surfaces of the right ear of each mouse. The left ear (control) received the vehicle (acetone 20 μl). The extract of M. indica was administered orally (50, 100 and 200 mg/kg body wt. in water) 1 h before PMA application. Two control groups were used: a control group with application of PMA to the right ear and a reference group treated with indomethacin (10 mg/kg body wt.). Six hours after PMA application, mice were killed by cervical dislocation and a 6-mm diameter disc from each ear was removed with a metal punch and weighed. Ear edema was calculated by subtracting the weight of the left ear (vehicle) from right ear (treatment), and was expressed as edema weight. Inhibition percentage was expressed as a reduction in weight with respect to the control group.

MPO assay

Tissue samples of each ear, from the PMA model, were assessed biochemically with the neutrophil marker enzyme, myeloperoxidase (MPO), using the method of Bradley et al. (1982), with minor modifications. All the ear tissue was homogenized in 50 mM K2HPO4 buffer (pH 6) containing 0.5% hexadecyl trimethylammonium bromide (HTBA) using a Polytron (Ultra-turrax T-25) homogenizer. After freeze-thawing 3 times, the samples were centrifuged at 2500 g for 30 min at 4 °C. The resulting supernatant assayed spectrophotometrically for MPO determination. In brief, 40 μl of sample were mixed with 960 μl of 50 mM phosphate buffer pH 6, containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured using a Spekol 220 spectrophotometer (Carl Zeiss, Germany). MPO activity data are presented as units per mg of tissue. One unit of MPO activity was defined as that degrading 1 μm of peroxide per minute at 25 °C.

Cell viability

To assess the correlation between cytotoxicity and suppression of eicosanoid release by test compounds, preliminary cytotoxicity studies were performed. Cell viability was assessed using a MTT-based colorimetric assay (Diaz-Lanza et al., 2001). In all, 8 × 105 cells diluted with Dulbecco’s modified Eagle’s medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS) were pipetted into 96-well microtiter plates, and were incubated overnight at 37°C and 5% CO2. The cells were exposed to various concentrations of samples for 3 h under the same conditions of incubation. MTT solution dissolved in phosphate-buffered saline was added to the wells at 1 mg/ml final concentration. After carefully aspirating the medium, 100 μl of DMSO were added for the dissolution of formazan crystals. The absorbance of each well was then read at 520 nm using a microplate reader. The values of the maximum non-toxic concentration obtained were used in PGE2 and LTB4 determination.

Assay of PGE2 and LTB4 release in activated macrophages

The murine monocyte/macrophage cell line J774 was suspended in DMEM supplemented with 10% FCS, and seeded into 24-well plates at a concentration of 5 × 105 cells/ml. After adhering to plates (24 h at 37 °C in an atmosphere of 5% CO2), non-adherent cells were washed off, and the cells (90% of adherent cells) received fresh DMEM (without FCS). Cells were pretreated for 1 h at 37 °C with the test compounds or vehicle, and then stimulated for a further 4 h by adding calcium ionophore A23187 (final concentration 10⁻⁶ M, for LTB4 release) or 24 h with LPS-IFNγ (100 ng/ml and 10 U/ml, for PGE2 release), respectively, according to the method of Hulckower et al. (1996). Controls contained only DMSO (basic level of released eicosanoid) or reference compound (total inhibition of eicosanoid release). The contents of all dishes were frozen at −20 °C and retained for analysis for PGE2 and LTB4 by means of radioimmunoassay, according to the procedure described by the manufacturer (Amersham).

Statistical analysis

PGE2 and LTB4 concentrations were assessed directly from a standard curve. The results are presented as the mean ± s.e.m. and statistical significance between the groups was determined by one-way analysis of variance (ANOVA) followed by unpaired Student’s t-test to determine statistical significance. p-Values less than 0.05 (p < 0.05) were considered as indicative of significance. Regression analysis was used to calculate the effective dose 50 (ED50 value), defined as the dosage of each drug necessary to produce 50% inhibition of AA- and PMA-induced ear edema. The inhibitory concentration (IC50 value) was calculated in the same manner.

Results

AA-induced mouse ear edema

The standard extract of M. indica significantly attenuated AA-induced edema 1 h after AA application.
(p<0.05; Table 1) with an ED\textsubscript{50} value of 144.6 mg/kg body wt. Dexamethasone also inhibited the edema.

**PMA-induced mouse ear edema and MPO activity**

As shown in Table 1, PMA-induced inflammation was also inhibited by the extract when it was administered orally at 200 mg/kg body wt., 6 h after PMA application. The extract also inhibited significantly MPO activity induced by PMA (p<0.05; Table 2). Indomethacin also inhibited inflammatory and MPO responses in this model.

**Effects of PGE\textsubscript{2} and LTB\textsubscript{4} release**

*M. indica* extract and the isolated compounds mangiferin, indomethacin and NDGA were not cytotoxic at any of the concentrations tested (1–100 \textmu g/ml).

As shown in Table 3, addition of LPS-IFN\textgamma and calcium ionophore A23187 to J774 macrophages causes the generation of nanogram amounts of eicosanoids via both cyclooxygenase (COX) and lipoxygenase (LOX) pathways. This effect was measured in terms of immunoassayable PGE\textsubscript{2} and LTB\textsubscript{4}, respectively.

**Discussion**

VIMANG\textsuperscript{®} is an extract obtained from the stem bark of selected varieties of *M. indica* and contains a defined mixture of components: polyphenols, terpenoids, steriods, fatty acids and microelements (Núñez-Sellés et al., 2002). Mangiferin (the main polyphenol of this extract, 10\%) has been tested in vitro for its antioxidant (Sato et al., 1992; Rouillard et al., 1998; Markus, 1996),

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>AA-induced ear edema</th>
<th>PMA-induced ear edema</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Edema weight (mg)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>AA</td>
<td>2 mg/ear</td>
<td>17.8±1.3</td>
<td>—</td>
</tr>
<tr>
<td>PMA</td>
<td>4 \textmu g/ear</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>M. indica</em></td>
<td>50 mg/kg body wt.</td>
<td>13.5±1.7*</td>
<td>24.2</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg body wt.</td>
<td>11.8±3.4*</td>
<td>33.7</td>
</tr>
<tr>
<td></td>
<td>200 mg/kg body wt.</td>
<td>5.8±1.1*</td>
<td>67.4</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10 mg/kg body wt.</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>30 mg/kg body wt.</td>
<td>4.3±0.9*</td>
<td>—</td>
</tr>
</tbody>
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*M. indica* extract was administered orally 1 h before AA or PMA. Inflammation was followed for 1 or 6 h after application of AA or PMA, respectively, and animals were thereafter killed by cervical dislocation. Ear edema was calculated by subtracting the weight of biopsies of the left ear (vehicle) from right ear (treatment), and was expressed as edema weight. Each group represents the mean ± s.e.m. of 7–10 animals. *p<0.05 indicates statistical significance compared with AA or PMA groups.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>MPO (U/mg tissue)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>4 \textmu g/ear</td>
<td>2.9±0.4</td>
<td>—</td>
</tr>
<tr>
<td><em>M. indica</em></td>
<td>50 mg/kg body wt.</td>
<td>2.2±0.4*</td>
<td>24.1</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg body wt.</td>
<td>1.8±0.2*</td>
<td>37.9</td>
</tr>
<tr>
<td></td>
<td>200 mg/kg body wt.</td>
<td>1.6±0.1*</td>
<td>44.8</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>10 mg/kg body wt.</td>
<td>0.7±0.2*</td>
<td>75.8</td>
</tr>
</tbody>
</table>

Right ears of mice have been stimulated with PMA to induce respiratory burst and MPO release. *M. indica* extract and indomethacin were administered orally 1 h before PMA. Inflammation was followed for 6 h after application of PMA and animals thereafter were killed by cervical dislocation. Biopsies were extracted from both ears and MPO was determined, using the method of Bradley et al. (1982), according to the description in the “Materials and methods”. Each group represents the mean ± s.e.m. of 7–10 animals. *p<0.05 indicates statistical significance compared with PMA group.

Table 1. Effect of the standard extract of *M. indica* on AA- and PMA-induced mouse ear edemas

Table 2. Effect of the standard extract of *M. indica* on MPO induction in PMA-induced mouse ear edema
immuno-stimulating and antiviral properties (Ritchey et al., 1981). Mangiferin aglycone (norathyriol) was also tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalnine-induced respiratory burst in rat neutrophils (Hsu et al., 1997).}

The extract could also exert this dual action because of its powerful antioxidant activity (Martinez et al., 2000; Sánchez et al., 2000), as it has been shown that the treatment of mouse skin with protein kinase C (PKC) promoters, such as PMA, induces formation of free radicals in vivo. It has also been demonstrated that the pre-treatment of mouse skin with antagonists of PKC may suppress inflammation and ROS formation (Wei and Frenkel, 1992). In the PMA model, M. indica extract also reduced MPO activity. MPO is an enzyme present in neutrophils and macrophages. It is well-known that the level of MPO activity is directly proportional to the neutrophil concentration on the inflamed tissue (Bradley et al., 1982), by which measurement of the enzyme activity has been considered a quantitative and sensitive marker of chemotaxis and neutrophil infiltration in the inflammatory process (Smith, 1994).

The inhibitory effects of M. indica extract and mangiferin are presented in this study on in vitro, eicosanoid-releasing systems in the J774 macrophage cell line. Many different studies have suggested that macrophages are a potent source of AA metabolites generated via COX and LOX pathways (Samuelsson et al., 1978). Moreover, macrophages participate in host defense, immunity and inflammatory responses, where they are potently activated resulting in the production of cytokines, oxygen and nitrogen species and eicosanoids.

The results of our study were consistent with these findings, and the standard extract of M. indica showed an inhibitory effect on AA- or PMA-induced edemas when these agents were administered topically (Table 1); however, the orally administered extract is more potent in the model of AA. It could be that its action in vivo on leukotrienes is favored.
of genes encoding pro-inflammatory proteins, resulting in cytokine release and the synthesis of enzymes such as COX-2 and 5-LOX, respectively. The inducible isof orm COX-2 and 5-LOX would be responsible for the high prostanoid generation during inflammatory responses (Herencia et al., 1999). In the current investigation the extract of M. indica and mangiferin inhibited the release of PGE₂ and LTB₄ from calcium ionophore- and LPS-IFNγ-stimulated murine macrophage cell line and these inhibitions were more potent for mangiferin purified from the extract.

Taken together, these current findings show that both COX and LOX pathways of arachidonate metabolism were inhibited, suggesting that M. indica extract and mangiferin might be classified as dual inhibitors. The present data support the inhibition of AA metabolism as one of the biochemical mechanisms which may contribute to the anti-inflammatory activity of VIMANG®. Its main component, mangiferin, is responsible for this activity. We concluded that those natural products interact with the eicosanoid system to exert the anti-inflammatory effect. This activity is most potent in its inhibitory action against MPO activity.

Acknowledgments

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References


G. Garrido et al.


