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 PGE_2 or leukotriene LTB_4 release, respectively. The extract inhibited the induction of PGE_2 and LTB_4 with IC_{50} values of 21.7 and 26.0 μg/ml, respectively. Mangiferin (a glucosylxanthone isolated from the extract) also inhibited these AA metabolites (PGE_2, IC_{50} value = 17.2 μg/ml and LTB_4, IC_{50} 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, estearic, oleic-linoleic and eicosatrienoic (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)

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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 (VIMANG®) 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 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 K_2HPO_4 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 and 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 (Díaz-Lanza et al., 2001). In all, 8×10^5 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% CO_2. 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 PGE_2 and LTB_4 determination.

Assay of PGE_2 and LTB_4 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 5×10^5 cells/ml. After adhering to plates (24 h at 37 °C in an atmosphere of 5% CO_2), non-adherent cells were washed off, and the cells (90% of adherent cells) received fresh DMEM (without FCS). Cells were pre-treated 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 LTB_4 release) or 24 h with LPS-IFNγ (100 ng/ml and 10 U/ml, for PGE_2 release), respectively, according to the method of Hulkower 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 PGE_2 and LTB_4 by means of radioimmunoassay, according to the procedure described by the manufacturer (Amersham).

Statistical analysis

PGE_2 and LTB_4 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 (ED_{50} value), defined as the dosage of each drug necessary to produce 50% inhibition of AA- and PMA-induced ear edema. The inhibitory concentration (IC_{50} 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_{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_2 and LTB_4 release

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

As shown in Table 3, addition of LPS-IFNγ 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_2 and LTB_4, respectively. Validation of this system for the identification of inhibitors of the two divergent pathways of arachidonate metabolism was performed using indomethacin, a well-characterized COX inhibitor (93.2% inhibition of PGE_2 at 100 μM) and NDGA, a known inhibitor of 5-LOX (93.1% inhibition of LTB_4 at 25 μM).

The compounds tested showed considerable activity as inhibitors of eicosanoid release from LPS-IFNγ and calcium ionophore A23187 to J774 macrophages. In the PGE_2-release assay, the IC_{50} values were 21.7 μg/ml (*M. indica* extract) and 17.2 μg/ml (mangiferin). In the LTB_4-release assay the IC_{50} values were 26.0 μg/ml (*M. indica* extract) and 2.1 μg/ml (mangiferin).

Discussion

VIMANG® is an extract obtained from the stem bark of selected varieties of *M. indica* and contains a defined mixture of components: polyphenols, terpenoids, steroids, 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 1. Effect of the standard extract of *M. indica* on AA- and PMA-induced mouse ear edemas

Treatment	Dose	AA-induced ear edema		PMA-induced ear edema	
		Edema weight (mg)	Inhibition (%)	Edema weight (mg)	Inhibition (%)
AA	2 mg/ear	17.8 ± 1.3	—	—	—
PMA	4 μg/ear	—	—	13.4 ± 0.7	—
M. indica	50 mg/kg body wt.	13.5 ± 1.7*	24.2	12.8 ± 1.3	4.1
	100 mg/kg body wt.	11.8 ± 3.4*	33.7	12.0 ± 0.5	10.2
	200 mg/kg body wt.	5.8 ± 1.1*	67.4	10.1 ± 0.6*	24.4
Indomethacin	10 mg/kg body wt.	—	—	5.1 ± 1.4*	58.4
Dexamethasone	30 mg/kg body wt.	4.3 ± 0.9*	76.4	—	—

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 2. Effect of the standard extract of *M. indica* on MPO induction in PMA-induced mouse ear edema

Treatment	Dose	MPO (U/mg tissue)	Inhibition (%)
PMA	4 μg/ear	2.9 ± 0.4	—
M. indica	50 mg/kg body wt.	2.2 ± 0.4*	24.1
	100 mg/kg body wt.	1.8 ± 0.2*	37.9
	200 mg/kg body wt.	1.6 ± 0.1*	44.8
Indomethacin	10 mg/kg body wt.	0.7 ± 0.2*	75.8

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 3. Effect of the standard extract of *M. indica* and mangiferin on PGE_2 and LTB_4 biosynthesis by J774 macrophage, stimulated by LPS + IFNγ and A23187

	Concentration	PGE_2 (ng/ml)	Inhibition (%)	LTB_4 (ng/ml)	Inhibition (%)
LPS + IFNγ	100 ng/ml + 10 U/ml	45.9 ± 5.9	0	—	—
A23187	10^{-6} M	—	—	18.9 ± 2.6	0
M. indica	10 µg/ml	30.4 ± 3.5*	34.0	11.3 ± 1.5*	40.2
	50 µg/ml	18.4 ± 2.7*	59.9	9.7 ± 2.7*	48.7
	100 µg/ml	9.7 ± 1.9*	78.9	4.3 ± 0.6*	77.2
Mangiferin	1 µg/ml	28.5 ± 2.5*	37.9	14.1 ± 2.5*	25.4
	10 µg/ml	16.5 ± 2.1*	64.1	10.4 ± 2.1*	45.0
	20 µg/ml	2.3 ± 1.0*	95.0	5.0 ± 1.0*	73.0
Indomethacin	100 µM	3.1 ± 1.2*	93.2	—	—
NDGA	25 µM	—	—	1.3 ± 0.7*	93.1

J774 cells were pre-treated for 1 h at 37 °C with *M. indica* extract, mangiferin, indomethacin, NDGA and vehicle (DMSO), and stimulated for a further 4 h by calcium ionophore A23187 (10^{-6} M) for LTB_4 release or 24 h by LPS (100 ng/ml) – IFNγ (10 U/ml) for PGE_2 release, according to the procedure described in the "Materials and methods". LTB_4 and PGE_2 were determined by radioimmunoassay. All data values represent means ± s.e.m. ($n = 6$). *$p < 0.05$ indicates statistical significances as compared with control groups.

immuno-stimulating and antiviral properties (Ritchey et al., 1981). Mangiferin aglycone (norathyriol) was also tested in vitro as an inhibitor of the formylmethionyl-leucyl-phenylalanine-induced respiratory burst in rat neutrophils (Hsu et al., 1997). Norathyriol has also been tested on A23187-induced pleurisy and analgesia in mice (Wang et al., 1994) and it was demonstrated that this aglycone inhibits AA metabolites like PGs and LTs in these models. The results suggest that norathyriol inhibits both COX and 5-LOX pathway, and that this action probably accounts for its anti-inflammatory and analgesic effects.

The metabolism of AA by COX leads to the generation of PGs and thromboxanes that mediate pain and edema associated with inflammation (Samuelsson et al., 1978). The LOX pathway utilizes AA to produce LTs, including the leukocyte chemoattractant LTB_4. Owing to the contribution of LTs to the pathogenesis of many inflammatory processes, they also represent an important target for therapeutic regulation (Piper, 1984).

The in vivo model of AA mouse ear inflammation is very suitable and sensitive, although not specific, to test inhibitors of LOX. The direct topical application of AA results in the rapid onset of edema formation most probably related to LTC_4 (Inoue et al., 1988) and LTB_4 synthesis (Griswold et al., 1991). In contrast, topical application of phorbol esters (like PMA) induce a long-lasting inflammatory response associated with a transient increase in prostanoid production and marked cellular influx. This high prostaglandin level is very likely due to COX induction (Muller-Decker et al., 1995).

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.

The extract could also exert this dual action because of its powerful antioxidant activity (Martínez 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 (at a much lower concentration) in monocyte 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. In macrophages, LPS alone or in combination with cytokine-like IFNγ, and calcium ionophore A23187 are the best-characterized stimuli to induce the transcription

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 isoform 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 PGE_2 and LTB_4 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.

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