JPP Journal of Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY

JPP Journal of Pharmacy and Pharmacology

JPP 2011, 63: 1336–1345

Received September 7, 2010
Accepted June 15, 2011
DOI
10.1111/j.2042-7158.2011.01328.x
ISSN 0022-3573

Research Paper

Mangifera indica L. extract (Vimang) and mangiferin reduce the airway inflammation and Th2 cytokines in murine model of allergic asthma

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Abstract

Objectives The aim was to study the effects of *Mangifera indica* extract and its major component mangiferin on lung inflammation response and Th2 cytokine production using a murine experimental model of allergic asthma.
Methods BALB/c mice were intraperitoneally sensitized with 10 μg of ovoalbumin (OVA) adsorbed on aluminium hydroxide on days 0, 7 and 14. Seven days after the last injection, the mice were challenged with 2% aerosolized OVA inhalation for 30 min beginning on day 21 and continuing until day 24. To evaluate the protective effect, mice were orally treated with *M. indica* extract (50, 100 or 250 mg/kg) or mangiferin (50 mg/kg) from days 0 to 24. Anti-OVA immunoglobulin E, interleukin (IL)-4 and IL-5 were determined by ELISA and lungs were analysed by histology.
Key findings *M. indica* extract and mangiferin produced a marked reduction of airway inflammation around vessels and bronchi, inhibition of IL-4 and IL-5 cytokines in bronchoalveolar lavage fluid and lymphocyte culture supernatant, IgE levels and lymphocyte proliferation.
Conclusion This is the first pre-clinical report of the anti-inflammatory properties of *M. indica* extract and mangiferin in experimental asthma and it could be an important part of pre-clinical requirement necessary for its use to complement the treatment of this complex disease.
Keywords asthma; *Mangifera indica* L.; mangiferin; Vimang

Introduction

Allergic asthma is a disorder characterized by chronic lung inflammation, reversible airway obstruction and increases in airway hyper-responsiveness to nonspecific stimuli. In the last three decades, the prevalence of asthma has significantly increased and it has been attributed to certain aspects of Western culture, including outdoor and indoor air pollution, childhood immunizations and cleaner living conditions, but no single cause has been identified.[1]

Allergic inflammation in asthma is characterized by the influx of leukocytes into the airway and lung and many cells are involved, including lymphocytes (mainly B and Th2 cells), eosinophils, mast cells, macrophages, basophiles and endothelial cells among others. Allergen-specific Th2 lymphocytes are the key orchestrators of this inflammation, initiating and propagating inflammation through the release of their cytokines interleukin (IL)-4, IL-5 and IL-13. IL-5 recruits and activates eosinophils, which contribute to airway inflammation and release eosinophil cationic protein (ECP) which has become one of the most important markers of the disease. Mast cells release vasoactive mediators, such as histamine, and B cells produce high serum levels of immunoglobulin E (IgE).[2–4]

As result of the complexity of the disease and its clinical heterogeneity, treatment approaches need to be individualized to obtain disease control over time. In this respect, the complementary or alternative medicines (CAM), based on natural products, acupuncture, homeopathy and nutritional therapy, among other approaches, have become an increasingly appealing component of standard medical care for bronchial asthma in several countries.

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Nevertheless, it is recognized that more pre-clinical evidence and randomized controlled trials of good methodological quality are required to support the use of the CAM in bronchial asthma.[5]

Vimang is the brand name of formulations containing the stem bark aqueous extract of *Mangifera indica* L. (Anacardiaceae) traditionally used in Cuba for its anti-inflammatory, analgesic, antioxidant and immunomodulatory properties.[6–8] The Cuban population uses this extract for the treatment of inflammatory disorders, cancer and some other diseases and, recently, a report of two asthmatic patients treated with Vimang tablets was published.[9]

The phytochemical investigation of this mango stem bark extract has led to the isolation of seven phenolic constituents: mangiferin, gallic acid, 3,4-dihydroxy benzoic acid, gallic acid methyl ester, gallic acid propyl ester (+)-catechin (−)-epicatechin, benzoic acid and benzoic acid propyl ester.[10] The extract also contains triterpenes, phytosterols, fatty acids and microelements. Quantitative analysis of the compounds has been performed by HPLC, and mangiferin was found to be the predominant component.[10] Mangiferin is a C-glucosylxanthone molecule with antiviral, anti-tumour and antioxidant activity.[11–13]

Recently, the anti-allergic properties of *M. indica* extract and mangiferin have been described.[14] *M. indica* extract and mangiferin produced a significant dose-dependent inhibition of: (a) IgE production in mice and anaphylactic reaction in rats; (b) histamine-induced vascular permeability and histamine release from rat mast cells; and (c) lymphocyte proliferative response as evidence of the reduction of the amount of B and T lymphocytes able to contribute to allergic response. Moreover, the inhibitory effect of *M. indica* extract on eosinophil migration was studied but using a parasitic murine model of *Toxocara canis* infection,[15] which induces an IL-5-dependent systemic eosinophilia[16] and mimics the features observed in asthma, such as high levels of serum IgE and airway inflammation.[17] However, the inhibitory effects of *M. indica* extract in allergic asthma are still poorly studied in animal models. Therefore, taking into account the previous evidence and the relationship between asthma, allergy and inflammation, the goal of this research was to study the effects of *M. indica* extract and its major component mangiferin on lung inflammation response and Th2 cytokine production using a murine experimental model of allergic asthma.[18]

We found a reduction of airway inflammation, Th2 cytokines production and IgE levels after treatment with *M. indica* extract and mangiferin. These findings constitute the first pre-clinical report of the anti-inflammatory properties of *M. indica* extract and mangiferin in a murine model of allergic asthma.

Materials and Methods

Reagents

Chicken egg ovoalbumin (OVA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), dexamethasone, histopaque, RPMI-1640 culture medium, fetal bovine serum (FBS), antibiotic and other reagents were obtained from Sigma (St Louis, USA). IL-4 and IL-5 ELISA kits were purchased from MABTECH (Mabtech AB, Nacka Strand, Sweden). Sheep anti-mouse IgE and horseradish peroxidase-conjugated rabbit anti-sheep IgG were purchased from Calbiochem (Calbiochem, Merck KGaA, Darmstadt, Germany).

Gallic acid, 3,4-dihydroxybenzoic, catechin, epicatechin, propyl gallate and benzoic acid used as reference standards for HPLC analysis were purchased from Sigma Aldrich Corporation (Sigma-Aldrich Co, St. Louis, MO, USA). Mangiferin and homomangiferin were house standards. These were isolated from the aqueous extract and characterized by spectroscopic methods.

Preparation and chemical characterization of *M. indica* extract and mangiferin isolation

Mangifera indica L. bark 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 Academy of Sciences, guarded by the Institute of Ecology and Systematic from Ministry of Science, Technology and Environment, Havana, Cuba. Stem bark extract from *M. indica* was prepared by decoction with water for 1 h and then it was concentrated by evaporation and spray-dried to obtain a fine homogeneous brown powder with a particle size of 30–60 μm.[10] The lot used in this study was analysed in the Quality Department of the Pharmaceutical Chemistry Center (Havana, Cuba) and was found to have the following content: moisture <10%, water-soluble substances >50%, total phenol (in anhydrous base) >30% and mangiferin >10%, according to the quality specification established.

High-performance liquid chromatography–mass spectrometry analysis of the extract (LCMS)

LCMS analysis of *M. indica* L extract was carried out on a Shimadzu UFLC system consisting of a two LC20AD pump, a column oven CTO20A, an autosampler SIL20AC and DAD-coupled to an LCMS model–IT–TOF (Shimadzu Co., Kyoto, Japan). The chromatographic separation was performed on a LiChrospher RP 18, 5 μm (250 × 4.6 mm i.d.; Merck, Darmstadt, Germany) with the column temperature set at 30°C. The mobile phase was 2.5% acetic acid aqueous solution–methanol (72 : 28, v/v) and the flow rate 1.0 ml/min. The detection was carried out at 254 nm. Negative ion mass spectra of the eluate were recorded in the range m/z 50–1000. Nitrogen was used as drying gas at a flow rate of 10 l/min. The nebulizer temperature was set at 300°C. Helium was used as the collision gas at 1.2×10^{-5} mbar. Fragmentation experiments were performed in auto MS scan mode. Only mass spectra of first and second order (MS/MS) were used to identify the compounds.

Preparation of sample and standards

One hundred milligrams (100 mg) were accurately weighed into 100 ml conical flasks and extracted three times with 25.0 ml of 85% v/v methanol at 40°C with magnetic stirrer for 20 min each time. The extractions were combined and the volume adjusted to 100 ml with methanol 85% to have a final concentration of 1 mg/ml. The standards were prepared to 0.1 mg/ml by weighing an appropriate amount of each one and dissolving in methanol. Samples and standards were filtered through a membrane filter of 0.45 μm (Sartorious, Goettingen, Germany) before the HPLC analysis.

Mangiferin isolation

Mangiferin (1,3,6,7-tetrahydroxyxanthone-C2-b-D-glucoside) was supplied by the Laboratory of Analytical Chemistry, Center of Pharmaceutical Chemistry (Cuba) and had been isolated from *M. indica* stem bark standardized extract by extraction with methanol. Mangiferin is a yellow powder and it was obtained at 90% purity as determined by HPLC.[10] It was pharmacologically tested because it is the major compound of the extract.

Animals

Female Balb/c mice, 8–10 weeks old, 18–20 g, were purchased from the National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba). They were housed in Macrolon cages (Panlab, Barcelona, Spain), in a standard bio-clean animal room, and kept under a 12-h light–dark cycle at 22–24°C. The mice had free access to food and tap water, and were allowed to acclimatize for one week before the experiments.

All experiments were carried out in accordance with the Institutional Animal Ethics Committee guidelines. The experimental protocols were approved by this committee subordinated to the Quality Department of the institution. Each mouse was used just once and all efforts were made to use the minimum number of mice required to obtain consistent experimental data. The dead mice were removed following the established protocols.

Antigen sensitization, challenge, and treatments

The experimental schedule was based on a murine model of allergic asthma.[18] Seven groups of ten mice were conformed. Six groups were intraperitoneally sensitized with 0.2 ml of saline solution containing 10 μg OVA adsorbed on 2 mg aluminium hydroxide on days 0, 7 and 14. Seven days after the last intraperitoneal injection, the mice were subjected to aerosolized OVA (2%) inhalation for 30 min beginning on day 21 and continuing until day 24. Mice were placed in a plexiglass chamber ($20 \times 20 \times 10$ cm) and exposed to an aerosol generated from a nebulizer with an airflow rate of 7 l/min. Control group mice received intraperitoneal injections of 0.2 ml saline containing 2 mg aluminium hydroxide and were challenged with saline alone.

To evaluate the protective effect, mice were orally treated with *M. indica* L. extract (doses of 50, 100 or 250 mg/kg) or mangiferin (50 mg/kg) from days 0 to 24. Distilled water was used as vehicle and the extract and mangiferin were administered at a dose of 10 ml/kg. Dexamethasone (3 mg/kg) used as reference drug was orally administered only between days 18 and 24, and it was included as experimental control for the murine model, not for comparison with the extract or mangiferin.

Collection of bronchoalveolar lavage fluid (BALF) and blood

When the treatments and experimental schedule were completed (on day 25), the mice were bled via the retro-orbital plexus and sera were separated by centrifugation at 3000 rpm for 10 min and kept at −70°C until analysis for OVA-specific IgE. Then, the mice were sacrificed using an overdose of sodium pentothal (100 mg/kg, i.p.). The trachea was cannulated and 0.5 ml of saline solution was used per lavage and repeated four times for each mouse. About 1.5 ml of BALF was recovered per mouse. The BALF was centrifuged (400*g*, 4°C, 10 min) and the supernatant was kept at −70°C until analysis for cytokines. The BALF cells were washed three times with phosphate-buffered saline (PBS) and the pellet was resuspended in 200 μl cold PBS. The total number of BALF cells was counted using a haemocytometer.

Histological examination and morphometry

After removal of BALF, lungs were isolated and prepared for histology study. They were slowly perfused with 10 ml PBS via the right ventricle, and then perfused with 4% paraformaldehyde and immersed in fixative solution overnight before being embedded in paraffin. Representative sections of lung were obtained by taking three 5-μm sections every 100 μm. Sections were stained with haematoxylin and eosin (H & E) and slides were evaluated by microscopy.

Inflammation was scored in a double-blind screen with two independent researchers who specialized in pathology. The degree of peribronchial and perivascular inflammation was evaluated by a subjective scale of 0–3 points, as described elsewhere.[19] A value of 0 was assigned when no inflammation was detectable, a value of 1 indicated occasional cuffing with inflammatory cells, a value of 2 indicated that most bronchi or vessels were surrounded by a thin layer (one to five cells) of inflammatory cells and a value of 3 indicated that most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells.

For semi-quantitative evaluation of the total lung inflammation, the pathologist counted the number of bronchi or vessels with positive inflammatory reaction (grade 2 or 3) in the total lung section, and the results were expressed as a percentage of perivascular and peribronchial positive reaction with respect to the total vessels or bronchi in the section.

Ovoalbumin-specific immunoglobulin E enzyme-linked immunosorbent assay

OVA-specific IgE levels were analysed by enzyme-linked immunosorbent assay (ELISA) following the assay previously described,[20] using OVA to capture the antibodies, sheep anti-mouse IgE as the secondary antibody, and HRP-conjugated rabbit anti-sheep IgG as the tertiary antibody. The sera samples were diluted 100 times in appropriate buffer and were individually evaluated in duplicate. The results are shown as absorbance units at 492 nm. Sera from OVA-sensitized/challenged mice served as a positive control and pooled normal mouse sera served as a negative control.

Antigen-specific lymphocyte proliferative response

Lymphocytes were obtained from the spleens of mice previously killed and were purified by histopaque density gradient centrifugation. The cells were suspended in complete culture medium (RPMI 1640 containing 10% FBS, 1% penicillin-

streptomycin, and 1% glutamate). The cellular suspension was adjusted to a working dilution of 2×10^6 cells/ml. Lymphocytes were cultured in flat-bottom 96-well microtitre plates (Corning Inc., Corning, NY, USA) in the presence or absence of OVA 10 µg/ml for 72 h, at 37°C under 5% CO_2. At the end of the time point, the supernatant was collected for cytokine determination and the lymphocyte proliferation was assessed by MTT assay.[21] This is a colorimetric assays based on the mitochondrial enzymatic reduction of the reagent MTT to purple formazan in living cells. Briefly, 15 µl of MTT (final concentration 0.5 mg/ml) were added into the wells and the cells were incubated for 4 h. Then, the formazan crystals were dissolved using HCl 0.01 M/SDS 10% solution. The absorbance of this coloured solution was quantified by measuring at 540 nm on a spectrophotometer. The experiment was done in duplicate, and the values are expressed as proliferation index, calculated as a percentage with respect to the proliferation of stimulated cells from OVA control group (considered 100%).

Interleukin-4 and interleukin-5 quantification

The cytokines IL-4 and IL-5 were measured (in triplicate) in the BALF and in the lymphocyte supernatant by ELISA kits (MabTech AB, Nacka Strand, Sweden) according to the manufacturer's protocol and the results were reported in pg/ml for each protein.

Statistics

All statistical analyses were performed using Graph Pad Prism 4.03 (San Diego, USA). Results were expressed as the means ± SD for $n = 10$ mice. The significant differences between the groups were determined by the Kruskal–Wallis non-parametric test, and when significant differences were obtained, the Dunn's post-test was used. For all tests, $P < 0.05$ was considered significant.

Results

Identification of 11 compounds from the *M. indica* L. extract

A typical chromatogram of *M. indica* L. extract is shown in Figure 1. Compounds **2**, **3**, **6**, **8**, **9**, **10** and **11** were identified as gallic acid, catechin, epicatechin, mangiferin, homomangiferin and propyl esters of gallic acid and benzoic acid, respectively, by comparison of their UV spectra and MS pattern of their peaks with those of authentic reference compounds. These compounds have been previously reported as components of this extract.[10] The other compounds were tentatively identified by comparison of the MS spectra with those reported in the literature. Compound **1**, with a retention time of 2.25 min, showed a spectrum UV spectrum similar to that of gallic acid, with absorption maxima at 230 and 265 nm. It gave an [M-H] ion at m/z 331 on MS analysis with fragment ions at m/z 241 [M-H-90] and 169 [M-H-162] in the MS-MS experiment. The loss of 90 Da and 162 Da indicates the presence of glycosidic residue in the molecule. Since the m/z 169 ion suggests the presence of a galloyl residue, this compound was tentatively identified as monogalloyl glucose (mw 331.062 g/mol).

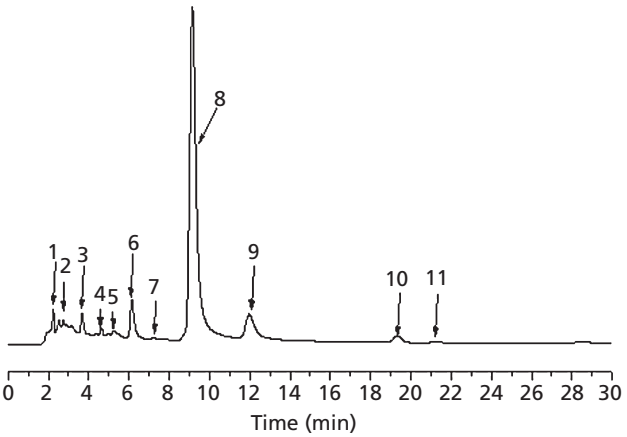

Figure 1 LC chromatogram of *Mangifera indica* L. extract. The peaks identified are corresponding to: monogalloyl glucose (**1**), gallic acid (**2**), catechin (**3**), maclurin 3-(2-galloyl)-β-D-glucoside (**4**), iriflofenone 3-C-β-D-glucoside (**5**), epicatechin (**6**), mangiferin derivative (**7**), mangiferin (**8**), homomangiferin (**9**), propyl gallate (**10**) and benzoic acid (**11**).

Compound **4** with retention time of 4.64 min had a UV spectrum with absorption maxima at 230, 260 and 295 nm. It gave an [M-H] ion m/z 575 in the MS experiment and fragment ions at m/z 423 [M-H-152] and m/z 405 [M-H-170], that correspond with the loss of galloyl from gallic acid, m/z 313[M-H-152-110] and m/z 303 [M-H-152-120] due to the loss of glucose. The fragment m/z 285 is due to the loss of one water molecule from fragment m/z 303 and the fragment m/z 261 corresponds to the molecular ion of benzophenone. This compound was unambiguously identified as maclurin 3-(2-galloyl)-β-D-glucoside (MW: 576.11 g/mol).

Compound **5** with a retention time of 5.23 min showed a UV spectrum with absorption maxima at 230, 290 and 340 nm. It gave an [M-H] ion m/z 407 and other fragments at m/z 317 [M-H-90] and m/z 287 [M-H-120] that correspond to the loss of glucose. In the mass spectrum besides the previous molecular ions reports was observed the fragment m/z 245 that correspond at the ion of the benzophenone. This compound was identified as iriflophenone 3-C-β-D-glucoside (MW: 408.07 g/mol).

The above two compounds showed the same fragmentation patterns previously reported by Berardini *et al*. and Barreto *et al*. who found these compounds in various extracts derived from fruits, kernel, pulp and bark of various species of *Mangifera indica* L. cultivated in Brazil.[22,23]

Compound **7**, with a retention time of 7.24 min, had a UV spectrum similar to mangiferin, with absorption maxima at 240, 260, 320 and 365 nm. It gave an [M-H] ion at m/z 583 with fragments at m/z 493[M-H-90] and m/z 463 [M-H-120] in the MS-MS experiment caused by the loss of one hexose. The loss of the second hexose was demonstrated by the presence of fragment ions at m/z 403 [M-H-90-90] and m/z 373 [M-H-120-120]. This compound was tentatively identified as a mangiferin derivative. Two derivatives of mangiferin were reported in the literature: neomangiferin[24] and glucomangiferin.[25] These compounds may have a similar fragmentation pattern; thus to determine the final structure it is necessary to isolate compound 7.

M. indica extract and mangiferin reduce the inflammatory lung response

The inflammatory lung response is one of the pathological features of bronchial asthma. Histological analysis of H&E-stained lung tissue sections demonstrated a marked airway inflammation in OVA-sensitized/challenged mice (Figure 2a and 2b). This inflammation was qualitative and semi-quantitatively evaluated using an inflammation scores as described in the Materials and Methods section. We found marked peribronchial and perivascular inflammatory infiltrates in lung sections of OVA-sensitized/challenged mice compared with non-sensitized/challenged mice (Figure 2a and 2b), indicative of a significant inflammatory response induced by the experimental procedure. Figures 2c–2f show the lung sections of treated mice, and the reduction of inflammation brought about by *M. indica* extract, mangiferin and dexamethasone is evident. Only two representative pictures of *M. indica* extract-treated mice are shown, corresponding to the lowest and highest doses.

The inflammation score in non-treated groups was around 3, indicating a majority of bronchi and vessels surrounded by a thick layer with more than five inflammatory cells. This score was reduced to 2 or less when the mice were treated with the extract and mangiferin. The highest dose of the extract (250 mg/kg) and mangiferin (50 mg/kg) had similar effects to dexamethasone, used as positive control (Figure 3a).

We established our own scoring system in order to evaluate semi-quantitatively the lung perivascular and peribronchial reaction. The bronchi and vessels with positive inflammation reaction were counted and the percentage was calculated with respect to the total bronchi and vessels in the complete lung section. Following this methodology, we found a 93% and 76% vascular and bronchial positive inflammatory reaction in OVA-sensitized/challenged mice, respectively, while the non-sensitized/challenged mice had minimal percentage in both. The treatment with *M. indica* extract, mangiferin and dexamethasone significantly reduced the percentage of bronchi and vessels affected by inflammatory reaction in the mouse lung

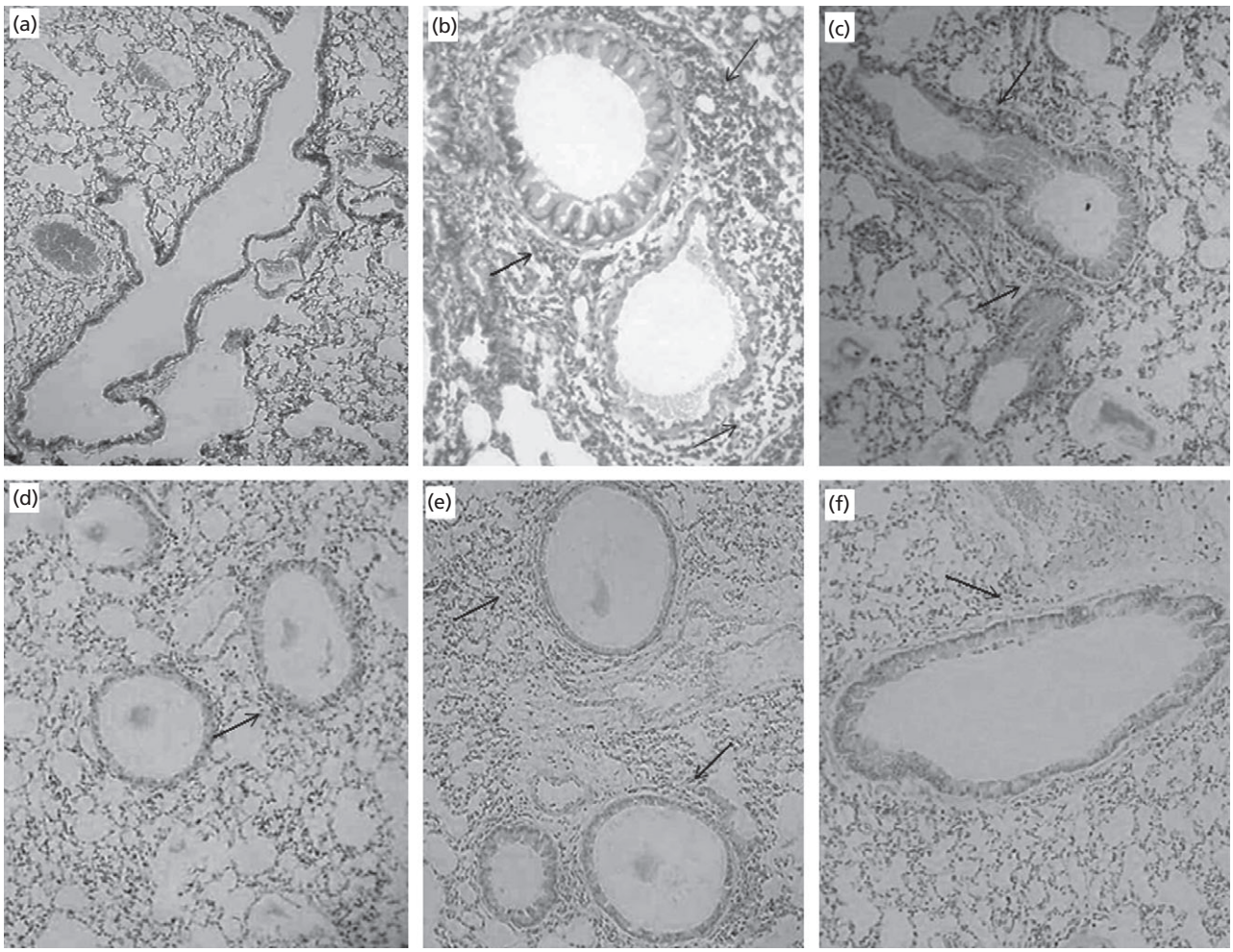

Figure 2 Qualitative evaluation of anti-inflammatory effects of *M. indica* extract and mangiferin on airway inflammation in mice pre-treated for 24 days. Histological study of lung sections (representative H&E-stained) from: (a) control group; Ovoalbumin (OVA)-sensitized/challenged groups treated with (b) saline solution (OVA group), (c) *M. indica* extract 50 mg/kg, (d) *M. indica* extract 250 mg/kg (e) Mangiferin 50 mg/kg (f) Dexamethasone 3 mg/kg. Objective lens × 100. Arrow indicates areas of inflammation. Only a representative picture is shown for each group; and for *M. indica* extract treatment only those corresponding to the lowest and highest doses are shown.

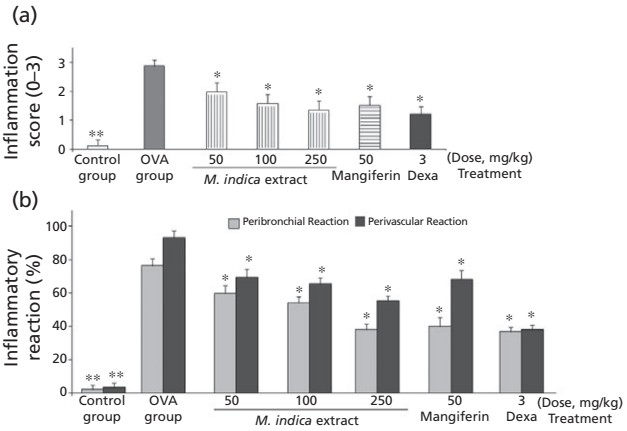

Figure 3 Effect of *M. indica* extract and mangiferin on airway inflammation in ovoalbumin (OVA)-sensitized/challenged mice pre-treated for 24 days. (a) Qualitative analysis of airway inflammation based on inflammation scores (0–3 points). (b) Semiquantitative analyses (as % of perivascular and peribronchial inflammatory reaction). Dexamethasone was used as positive control. Both methods are described in Materials and Methods section. Data are represented as mean ± SD from ten mice in each group. $*P < 0.05$, compared with OVA-sensitized/challenged group (OVA group).

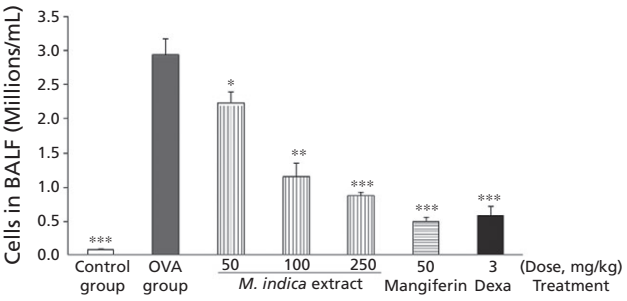

Figure 4 Effect of *M. indica* extract and mangiferin on inflammatory cell recruitment into the lung after ovoalbumin (OVA)-sensitization/challenge period, in mice pre-treated for 24 days. Dexamethasone was used as positive control. Data are represented as mean ± SD from ten mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with OVA-sensitized/challenged group (OVA group).

(Figure 3b). Also, the number of inflammatory cells recruited into the lung was reduced by *M. indica* extract, mangiferin and dexamethasone treatment (Figure 4). With extract treatment, the reduction of the recruitment of inflammatory cells into BALF was dose dependent. The results of histopathological examination correspond to the dramatically reduced BALF cell counts in treated mice. These results demonstrate that *M. indica* extract and mangiferin inhibit the OVA-induced inflammation in the lungs of OVA-sensitized/challenged mice.

M. indica extract and mangiferin reduce ovoalbumin-specific IgE in sera

To investigate the effect of *M. indica* extract and mangiferin on serum IgE levels, we determined the OVA-specific IgE levels in treated mice. The IgE levels increased markedly (five

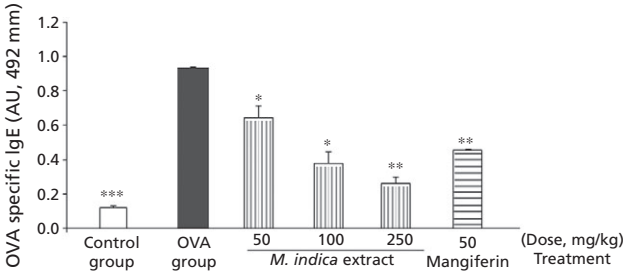

Figure 5 Effect of *M. indica* extract and mangiferin on ovoalbumin (OVA)-specific IgE serum levels, in OVA-sensitized/challenged mice pre-treated for 24 days. IgE levels were determined by ELISA. Data are represented as mean ± SD from ten mice in each group, and are represented as absorbance units (AU). $*P < 0.05$, $**P < 0.01$, compared with OVA-sensitized/challenged group (OVA group).

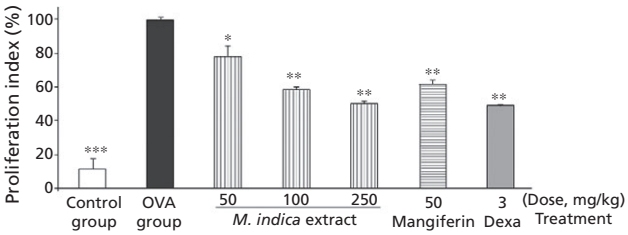

Figure 6 Effect of *M. indica* extract and mangiferin on lymphocyte proliferation in response to ovoalbumin (OVA), in OVA-sensitized/challenged mice pre-treated for 24 days. Dexamethasone was used as positive control. Proliferation index was determined by MTT assay. Data are represented as mean ± SD from ten mice in each group. $*P < 0.05$, $**P < 0.01$, compared with OVA-sensitized/challenged group (OVA group).

fold) in the OVA-sensitized/challenged mice as compared with non-sensitized/challenged mice (Figure 5). The oral administration of *M. indica* extract (50, 100 and 250 mg/kg) and mangiferin (50 mg/kg) during the OVA-sensitization/challenge period, prevented significantly the rise in serum OVA-IgE levels. The IgE levels were reduced in dose-dependent manner by the *M. indica* extract, with an inhibition of 31–71%, and 50% with mangiferin.

M. indica extract and mangiferin reduce the lymphocyte proliferation specific to ovoalbumin

The lymphocytes from the spleen of OVA-sensitized/challenged mice had an important proliferative response when they were stimulated *in vitro* for 72 h with 10 µg/ml of the same antigen (OVA) used during the sensitization period, while the lymphocytes from non-immunized mice did not respond to OVA (Figure 6). The lymphocyte culture from any of the mice groups proliferated in the absence of OVA stimulation (data not shown). Interestingly, when the OVA-sensitized/challenged mice were pre-treated with different doses of *M. indica* extract (50, 100 and 250 mg/kg), mangiferin (50 mg/kg) and dexamethasone (3 mg/kg), the OVA-specific proliferative capacity of lymphocytes was significantly reduced. *M. indica* extract (50, 100 and 250 mg/kg) reduced lymphocytes' proliferative response by 20–50%, while mangiferin caused 60% inhibition.

M. indica extract and mangiferin reduce interleukin-4 and interleukin-5 production

The levels of IL-4 and IL-5 in BALF and lymphocyte culture supernatant were determined by ELISA, and the values of different groups were compared. As shown in Table 1, the levels of IL-4 and IL-5 were significantly increased in BALF and lymphocyte culture supernatant from OVA-sensitized/challenged group of mice as compared with the sham-sensitized group, (typical response Th2 profile). The production of IL-5 was higher than IL-4 in BALF and lymphocyte supernatant as well. Treatment with *M. indica* extract, mangiferin and dexamethasone during the sensitization/challenge period reduced the production of both Th2 cytokines from immune cells (Table 1). The inhibitory effect of *M. indica* L. was dose-dependent between 50 and 250 mg/kg, while IL-5 production in lymphocyte culture supernatant was the highest, inhibited to 78.2%. Mangiferin produced lower inhibition than the extract, between 43 and 58% for both cytokines in BALB or lymphocyte supernatant.

Discussion

Complementary or alternative medicine is one of the new approaches proposed for the treatment of bronchial asthma, in most cases as a complement to conventional therapies. The aim of this study was to determine whether the natural extract obtained from stem bark of *M. indica* had an inhibitory effect in a murine model of this disease. Additionally, we studied the effects of mangiferin, a polyphenol forming the majority of the chemical composition of the extract. Previously, it was reported to have beneficial effects in two asthmatic patients treated with Vimang tablets (from *M. indica* L.),[9] and some pre-clinical studies demonstrated the anti-allergic properties of the extract,[14] but its pre-clinical effect on animal models of asthma have still not been reported.

The experimental schedule was based on a common well-described experimental model of asthma, which involves intraperitoneal immunization with OVA precipitated with aluminium hydroxide (sensitization period), followed by repetitive challenge with OVA by aerosol (challenge period) in Balb/C mice. These conditions produce a vigorous Th2 response, characterized by an inflammatory response that is typically distributed around bronchi and vascular structures, higher levels of allergen-specific IgE, and greater levels of induced airway hyperresponsiveness (AHR), as the main shared features with human bronchial asthma.[26] Therefore, we studied the effects of *M. indica* extract and mangiferin on airway inflammation, serum IgE levels, lymphocyte proliferation and Th2 cytokine production using this experimental model.

Our first finding was the inhibitory effect on airway inflammation shown by *M. indica* extract and mangiferin. We examined structural changes at the level of the conducting airways and found a marked influx of inflammatory cells into the lung around blood vessels and bronchi during the period of OVA-sensitization/challenge. This peribronchial, perivascular and total inflammation was significantly reduced when the mice were pretreated with *M. indica* extract, mangiferin and dexamethasone. Dexamethasone is a corticosteroid well-

Table 1 Effect of *M. indica* extract and mangiferin on interleukin-5 and interleukin-4 levels in bronchoalveolar lavage fluid and ovoalbumin-stimulated lymphocyte culture supernatant from mice were pre-treated for 24 days

Groups, treatment and doses	BALF				OVA-stimulated lymphocyte culture supernatant			
	IL-5		IL-4		IL-5		IL-4	
	Cytokine concn (pg/ml)	% Inhibition	Cytokine concn (pg/ml)	% Inhibition	Cytokine concn (pg/ml)	% Inhibition	Cytokine concn (pg/ml)	% Inhibition
Control group	76.5 ± 1.3**	–	42.5 ± 7.1**	–	106.2 ± 1.7**	–	82.5 ± 7.8*	–
OVA group	278.5 ± 16.7	–	171.1 ± 4.1	–	458.7 ± 27.1	–	191.1 ± 1.9	–
M. indica extract 50 mg/kg	218.7 ± 4.7*	21.4	114.4 ± 5.5*	33	238.7 ± 8.8*	47.9	95 ± 1.6*	50.3
M. indica extract 100 mg/kg	176.5 ± 12.4*	36.6	82.22 ± 6.4**	52	157.5 ± 28.3**	65.6	92.2 ± 1.9*	51.7
M. indica extract 250 mg/kg	99.5 ± 12.1**	64.2	65.6 ± 5.5**	61.6	100 ± 18.4*	78.2	74.4 ± 5.8*	61
Mangiferin 50 mg/kg	156 ± 5.3**	43.9	84.7 ± 2.8**	50.4	190 ± 3.5*	58.5	104.7 ± 3.9*	45.2
Dexamethasone 3 mg/kg	96.25 ± 7.8**	65.4	76.4 ± 2.5**	55.3	86.3 ± 12.9**	81.2	104.4 ± 5.8*	45.3

BALF, bronchoalveolar lavage fluid; OVA, ovoalbumin; IL, interleukin. IL-4 and IL-5 were determined by ELISA. Results are shown as concentration of cytokines (pg/ml) and % of inhibition with respect to OVA group. Concentration data represents the means ± SD from ten mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with OVA-sensitized/challenged group (OVA group).

known as a potent inhibitor of airway inflammation and it is frequently used in murine models of asthma as a positive control.[27]

The anti-inflammatory effects of *M. indica* extract and mangiferin have been widely studied and published. It has been demonstrated that they are able to inhibit the arachidonic acid pathway, reducing the activation of phospholipase A2 and COX-2 expression,[8,28] and consequently production of prostaglandins and leukotrienes, two important mediators in asthma.[29]

Additionally, *M. indica* extract and mangiferin modulate the nuclear factor (NF)κB pathway, inhibiting IκBα degradation and some other step in NFκB pathway in macrophages and T lymphocytes.[8,30] NFκB plays a pivotal role in asthma because of its transcriptional control over different pro-inflammatory proteins such as cytokines, chemokines and adhesion molecules. Some clinical and pre-clinical evidence supports this role: (a) increased NFκB activity in epithelial cells and alveolar macrophages from asthmatic patients have been demonstrated;[31,32] (b) during an allergen-induced asthma model with knockout mice (deficient for p50 component of NFκB pathway), the mice showed reduced levels of the Th2 cytokine IL-5 and eotaxin, involved in the recruitment and survival of eosinophils;[33] (c) some of the effects of glucocorticosteroids used in the treatment of asthma and other inflammatory diseases are mediated through the inhibition of NF-κB activation.[34] Therefore, the inhibition of the NFκB pathway previously demonstrated for the extract and mangiferin could be beneficial for the control of the inflammatory response during OVA challenge in our model.

IgE is the most important immunoglobulin in allergic diseases and asthma. Since 1966, IgE has been recognised as having a causal role in arming mast cells to respond to antigenic stimulation by mediator secretion,[35] but now, its biology is better characterized. There is new evidence regarding the ability of IgE to regulate the receptor FcεRI, the presence of high affinity receptors on several cell types and the role IgE may play on other cell types different to mast cells and basophiles.[36] Therefore, the implication of IgE in allergic responses and bronchial asthma has increased in the last years. Taking into account aforementioned reasons, we studied the effects of *M. indica* extract and mangiferin on IgE levels, and we determined that both treatments were able to reduce the serum levels of IgE induced in mice during the OVA-sensitization/challenge period. This is not the first report of inhibition of IgE by *M. indica* extract and mangiferin, but is the first one in a pre-clinical asthma model. In 2003, we described the inhibition of IgE induced by *T. spirallis* (helminthic parasite) when rats were treated with *M. indica* extract and mangiferin, and more recently, the inhibitory effects of both on OVA-specific IgE production in a murine allergic model were reported.[14,37]

For IgE synthesis, two signal types are required for B lymphocytes. The first is provided by the cytokines' IL-4 interacting with IL-4R, which activate transcription at a specific immunoglobulin locus. The second signal is provided by ligation of CD40L molecule on B cells with CD40 on Th2 cell, which in turn activates DNA switch recombination and induces the expression of the ε chain for IgE synthesis, which is followed by secretion of allergen-specific IgE.[38,39] We found that *M. indica* extract and mangiferin inhibited IL-4 production in BALF and lymphocyte culture supernatant as well. This result suggests that *M. indica* extract and mangiferin could reduce IgE levels mediated by IL-4 inhibition.

The effects of *M. indica* extract and mangiferin on OVA-specific lymphocyte proliferation were also evaluated. As expected, when lymphocytes of OVA-sensitized/challenged mice were stimulated *in vitro* with OVA, the specific clones that were activated during the sensitization period responded to antigen through an increase in the number of specific lymphocytes to develop secondary immune response. The treatment with *M. indica* extract and mangiferin demonstrated a reduction in the proliferative lymphocyte response, as evidence of the reduction of lymphocytes able to contribute to immune response. The inhibition of lymphocyte proliferation could also explain the inhibitory effects of *M. indica* extract and mangiferin on IgE production, because, in addition to IL-4 inhibition, they would reduce the number of B cells able to produce anti-OVA specific IgE.

One of the most important features of this murine model of bronchial asthma is the perivascular and peribronchial eosinophilia, due to the concerted actions of IL-5 and the chemokine eotaxin-1.[26] IL-5 is a canonical Th2 cytokine involved in airway eosinophilia; it regulates most aspects of eosinophil behaviour, such as growth, maturation, apoptosis, adhesion and secretion. So IL-5 has been targeted for novel asthma treatments using anti-IL-5 antibodies.[40,41] In human asthma, eotaxin is produced in high concentrations in the airway epithelium. The chemokine eotaxin also acts on eosinophils, binding to a single receptor, CCR3, which is highly expressed on these cells. After receptor interaction, eotaxin induces the binding of eosinophils to endothelium via an integrin-mediated mechanism.[42] More recently, it has been demonstrated that eotaxin might contribute to the airway remodelling seen in asthma by enhancing the number and survival of airway smooth muscle cells.[43]

Previously, we described the inhibitory effects of *M. indica* extract (50 mg/kg) on eosinophil generation in bone marrow, blood eosinophilia, and IL-5 production in serum and eotaxin in lung homogenates in *T. canis*-infected mice.[15] Herein, we found inhibitory effects of *M. indica* extract and mangiferin on IL-5 production, both in BALF and lymphocytes culture supernatant from OVA-sensitized/challenged mice. This result, in correspondence with the previous ones, suggests that *M. indica* extract and mangiferin could be able to modulate the eosinophils' function, and in this way, contribute to the control of airway inflammation.

There is some other previous pre-clinical evidence regarding the pharmacological activity of *M. indica* extract and mangiferin that could contribute to the inhibition of airway inflammation. For example, *M. indica* extract and mangiferin are able to reduce the histamine-induced vascular permeability and the histamine release from rat mast cells induced by compound 48/80.[14] Also, the extract has antioxidant activity[6] and the contribution of reactive oxygen species (ROS) to asthma physiopathology is well established,[44] even if it has demonstrated a reduced antioxidant capacity in serum and BALF from asthmatic patients.[45] Then, if ROS contribute to asthma development, enhancement of the antioxidant defences would be expected to have beneficial effects in this

disease, and treatment with *M. indica* extract could contribute to it.

M. indica extract has a major composition of polyphenols.[10] In general, these natural compounds reduce asthma inflammation through antioxidant, anti-allergic and anti-inflammatory actions. They are scavengers of nitric oxide[46] and can inhibit histamine release, arachidonic acid metabolism and cytokine production,[47] and down-regulate the transcription factor NFκB.[48] Particularly, some of the compounds found in the *M. indica* extract have pharmacological actions related to inflammation or allergy inhibition. Epicatechin suppressed IL-4-stimulated eotaxin secretion in human alveolar epithelial cells to reduce eosinophil recruitment and alleviate eosinophilic-driven airway inflammation.[49] Gallic acid attenuated compound 48/80 or immunoglobulin E (IgE)-induced histamine release from mast cells and the effects were mediated by the modulation of cAMP and intracellular calcium and it additionally decreased gene expression and production of TNFα and IL-6 in human mast cells.[50] Furthermore, herein we found enough evidence of the effects of mangiferin on airway inflammation. Taking into account our results, the anti-asthmatic activity of *M. indica* extract could be partially attributable to its polyphenol fraction, and particularly to mangiferin as its major component.

Conclusion

Herein, we demonstrated the inhibitory effects of *M. indica* extract on the asthmatic response induced in mice by ovoalbumin sensitization and challenge. The effect is characterized by a reduction in airway inflammation, Th2 cytokine production and IgE levels. Mangiferin, the major component in the extract, also has the same properties and thus contributes to the effects of the extract.

This is the first pre-clinical report of the anti-inflammatory properties of *M. indica* extract in a murine asthma model. These findings are an important part of the pre-clinical requirement for its use as a complement in the treatment of this complex disease.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported by Project No. 00403238 from the Ministry of Science, Technology and Environment, Havana, Cuba. This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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