ACADEMIC PRESS

Pharmacological Research 50 (2004) 165–172

Pharmacological research

www.elsevier.com/locate/yphrs

Protection against septic shock and suppression of tumor necrosis factor alpha and nitric oxide production on macrophages and microglia by a standard aqueous extract of *Mangifera indica* L. (VIMANG®) Role of mangiferin isolated from the extract

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Accepted 22 December 2003

Abstract

The present study illustrates the effects of a standard aqueous extract, used in Cuba under the brand name of VIMANG®, from the stem bark of *Mangifera indica* L. on the production of tumor necrosis factor alpha (TNFα) and nitric oxide (NO) in in vivo and in vitro experiments. In vivo was determined by the action of the extract and its purified glucosylxanthone (mangiferin) on TNFα in a murine model of endotoxic shock using Balb/c mice pre-treated with lipopolysaccharide (LPS) 0.125 mg kg^{-1}, i.p. In vitro, *M. indica* extract and mangiferin were tested on TNFα and NO production in activated macrophages (RAW264.7 cell line) and microglia (N9 cell line) stimulated with LPS (10 ng ml^{-1}) and interferon gamma (IFNγ, 2 U ml^{-1}). *M. indica* extract reduced dose-dependently TNFα production in the serum (ED_{50} = 64.5 mg kg^{-1}) and the TNFα mRNA expression in the lungs and livers of mice. Mangiferin also inhibited systemic TNFα at 20 mg kg^{-1}. In RAW264.7, the extract inhibited TNFα (IC_{50} = 94.1 μg ml^{-1}) and NO (IC_{50} = 64.4 μg ml^{-1}). In microglia the inhibitions of the extract were IC_{50} = 76.0 μg ml^{-1} (TNFα) and 84.0 μg ml^{-1} (NO). These findings suggest that the anti-inflammatory response observed during treatment with *M. indica* extract must be related with inhibition of TNFα and NO production. Mangiferin, a main component in the extract, is involved in these effects. The TNFα and NO inhibitions by *M. indica* extract and mangiferin on endotoxic shock and microglia are reported here for the first time.

Keywords: *Mangifera indica* L.; Tumor necrosis factor; Nitric oxide; Endotoxic shock; Mangiferin

1. Introduction

Many natural compounds extracted from different parts of plants present anti-inflammatory and immunomodulatory activities [1–5].

Recently, a standard aqueous stem bark extract from selected species of *Mangifera indica* L. (Anacardiaceae), which has been used as food supplement in Cuba under the brand name of VIMANG®, has reported a potent in vivo and in vitro anti-inflammatory activity [6,7], immunomodulation on rat macrophages [8], and a strong in vitro and in vivo antioxidant effect [9,10]. Chemical studies performed with this extract have enabled the isolation and identification of phenolic acids, phenolic esters, flavan-3-ols, mangiferin, which is the predominant component of this extract [11], and micronutrients as selenium [12,13]. Various studies indicate that these compounds modulate the activities reported for this extract of *M. indica* [14–17] and inhibit the cytokine and NO production [14,18–22]. Cytokines mediate a vast array of biological effects, which include events involved in maintenance of immune homeostasis, as well as pathophysiologic events that occur in disease states. A number of disease states has been shown to be mediated by the overzealous action of pro-inflammatory cytokines, including TNFα and IL-1β, which occurs as a result of enhanced secretion of pro-inflammatory cytokines and/or inadequate inhibition of cytokine effects. Diseases include sepsis syndrome, rheumatoid arthritis and inflammatory bowel disease [23]. On the other hand, the relevance of NO as mediator of inflammation is more recent [24]. The role

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doi:10.1016/j.phrs.2003.12.020

of NO vary considerably in different inflammatory events, depending on the cell/tissue in which it is produced and the complex interactions with the different components of the inflammatory process. It has been reported in different cell types in vitro that inflammatory cytokines can have both enhancing and suppressing effects on the expression of inducible NO synthase (iNOS) and NO production [25] and activated macrophage is one of the most important effector cells in the inflammatory response that secrete NO [26].

Because *M. indica* extract has been described to be an antioxidant with anti-inflammatory and immunomodulatory activities, we tested the hypothesis that these effects are mediated through its modulation of pro-inflammatory cytokines and NO production. In this study, we report, for the first time, that *M. indica* aqueous stem bark extract blocks TNFα and inhibits the NO production in two activated macrophages cell lines (RAW264.7 and N9) and in vivo model of septic shock. Mangiferin, a glucosylxanthone isolated from the extract, is involved in these effects.

2. Materials and methods

2.1. Plant material

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 Academy of Sciences, guarded by the Institute of Ecology and Systematic from Ministry of Science, Technology, and Environmental, La Habana, Cuba. 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 [11]. The solid extract was dissolved in distilled water for pharmacological studies.

Mangiferin (2-β-D-glucopyranosyl-1,3,6,7-tetra-hydroxy-9*H*-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 [11].

2.2. Animals

Male Balb/c mice supply by National Center for Laboratory Animal Production (CENPALAB, La Habana, Cuba) of 20–25 g were used. Animals were housed in air-conditioned quarters (60% relative humidity, and 22 °C of temperature) with a 12 h light–dark cycle. 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.

2.3. Reagents

Lipopolysaccharide (LPS, from *Escherichia coli* Serotype: 055:B5), interferon gamma (IFNγ), TNFα (recombinant TNFα, specific activity: 10^7 U mg^{-1}), actinomycin D, and dexamethasone water soluble were purchased from Sigma Chemical Co. (St. Louis, MO). N^{ω}-Monomethyl-L-arginine (L-NMMA) was purchased from Cayman Chemical, Ann Arbor, MI.

2.4. Animal models of endotoxic shock

Mice were pre-treated with *M. indica* extract or mangiferin, suspended in sterile water (doses: 1–200 mg kg^{-1}) administered by gavage 1 h or during 7 days before LPS (0.125 mg kg^{-1}, i.p.) administration [27]. One hour after LPS arrest, blood of anesthetized mice was extracted by rupture of retro-orbital plexus. Serum was separated by centrifugation at 3603 × *g*, 4 °C, and frozen at −70 °C until TNFα determination.

The lungs and livers of mice with an acute treatment were extracted 30 min after of LPS administration for the Northern blot analysis of the expression of TNFα mRNA.

Dexamethasone 3 mg kg^{-1} p.o. was administered as reference drug.

2.5. Macrophage murine cell line

RAW264.7 cell line (kindly provided by Dr. J.C. Drapier, Institut de Chimie des Sustances Naturelles, France) was suspended in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO-BRL, Pisley, UK) supplemented with 10% fetal calf serum (FCS), 4.5 g l^{-1} glucose, 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin sulfate (GIBCO-BRL) and 4 mM glutamine and seeded into T-75 cm^2 culture flask at a concentration for 5 × 10^5 cells ml^{-1}.

2.6. Murine microglia cell cultures

The N9 clone of murine microglial cells was obtained by immortalization of embryonic brain cultures with the 3RV retrovirus carrying an activated v-*myc* oncogene [28], was kindly provided by P. Ricciardi Castagnoli (Centro di Cito-Farmacologia, CNR, Milano, Italy). Cells were cultured in T-75 cm^2 culture flask (Corning, Cambridge, MA) and maintained at 37 °C in a humidified incubator under 5% CO_2 atmosphere in RPMI 1640 (Seromed, Berlin, Germany) supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin sulfate (GIBCO-BRL), 10% heat-inactivated fetal bovine serum (FBS, Hyclone Lab. Inc., Logan, TU) and 1.5 g l^{-1} sodium bicarbonate, 12 mM Hepes until experiments were performed. Cells were used between the first and the tenth passage.

2.7. RAW264.7 and N9 activation

Sub-confluent microglial and RAW264.7 cells were washed twice with phosphate-buffered saline (PBS) and incubated with trypsin 0.025% and ethylenediaminetetraacetate (EDTA) 0.02% without calcium and magnesium for 3 min at 37 °C to detach the cells from the culture flask. Cells were them resuspended in medium and incubated in 24-well tissue-culture plates at a concentration of 2×10^5 cells ml^{-1} for 16 h in a humidified incubator (37 °C, 5% CO_2). Growth medium was removed and cell monolayers were stimulated with 10 ng ml^{-1} LPS plus 2 U ml^{-1} murine IFNγ. To test effects of *M. indica* extract and mangiferin, concentrations (1–200 μg ml^{-1}) were dissolved in medium and added to wells 10 min before treatment with LPS + IFNγ. Cell-free supernatants were harvested after 24 h incubation with the stimuli and assayed for TNFα and NO_2^-.

The *M. indica* extract and mangiferin concentration (<200 μg ml^{-1}) were non-cytotoxics according with previous study of cytotoxicity performed with each cellular line determining the viability of cells. It was assessed by dimethyl-diphenyl-tetrazolium (MTT) incorporation for each experimental condition (it was consistently >97%) [29].

Dexamethasone and L-NMMA were used as positive control for TNFα and NO inhibition, respectively. Tests were repeated in at least three independent experiments and assays were performed in triplicate.

2.8. TNFα determination

TNFα was measured in serum of mice treated with LPS and supernatants of cell cultures by standard cytotoxicity assay using L929 line cell in the presence of 1 μg ml^{-1} of actinomycin D [30]. Recombinant TNFα was used as standard (specific activity, 10^7 U mg^{-1}).

2.9. Nitrite determination

NO is rapidly oxidized to nitrite in culture medium, and nitrite (NO_2^-) concentration is an indicator of NO production. Cell-free culture supernatants were mixed with equal amounts of Griess reagent (1% sulfanilamide, 0.1% naphtylethylenediamide in 2.5% phosphoric acid) in 96-well ELISA plates [31]. Samples were incubated at room temperature for 10 min and absorbance was measured at 540 nm with the use of a microplate reader. Nitrite concentrations were calculated using a sodium nitrite standard curve.

2.10. Northern blot analysis

Total RNA from lung and liver of mice was isolated by the guanidium thiocyanate–phenol–chloroform method [32]. RNA was separated by electrophoresis on 1% agarosa-MOPS gel with 2.2 M formaldehyde and transferred to a nylon membrane by capillary action. Membranes were hybridized with a cDNA probe specific for murine TNFα [30] labeled with [^{32}P]dCTP using the Ready-To-Go DNA Labeling Kit (Pharmacia, Uppsala, Sweden). After overnight hybridization, blots were washed and exposed to and X-ray film (Hyperfilm, Amersham, UK) for 48 h. Blots were subsequently rehybridized with glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) cDNA probe as an internal control (Clontech Laboratories, Inc., Palo Alto, CA).

2.11. Statistical analysis

Effects of *M. indica* extract and mangiferin on TNFα and NO production represent the means ± standard error of means (S.E.M.) by repeated measures analysis of variance followed by Dunnet's *t* test for specific comparisons. Probability values less than 0.05 ($P < 0.05$) were considered significant. Regression analysis was used to calculate the effective dose 50 (ED_{50}), defined as the dosage of each drug necessary to produce a 50% of inhibition on TNFα in endotoxic shock of mice arrest with LPS. In the same manner was calculated the inhibitory concentration 50 (IC_{50}) for the in vitro experiments.

3. Results

3.1. M. indica extract and mangiferin, administered orally, inhibit the TNFα production during endotoxic shock

M. indica extract, administered orally 1 h before LPS, inhibited LPS-induced TNFα production in mice (Fig. 1A). A dose-dependent inhibition of TNFα production by *M. indica* extract was observed with $ED_{50} = 64.5$ mg kg^{-1}. In the same manner, the extract inhibited the TNF serum levels dose-dependently (Fig. 1B) with $ED_{50} = 37.4$ mg kg^{-1} when it was administered p.o. during 7 days before LPS arrest, with a maximal inhibition of 80.1%. Mangiferin (a glucosylxanthone isolated from the extract, 20 mg kg^{-1}) also inhibited the TNFα (75.2%, respect to control group treated with vehicle).

Northern blots analysis showed that the inhibitory action of *M. indica* extract on TNFα production was at the level of mRNA in lungs and livers of mice treated with LPS (Fig. 2).

M. indica extract, mangiferin, dexamethasone and L-NMMA were not cytotoxic at any of the concentrations tested (1–200 μg ml^{-1}) in the in vitro assays.

3.2. M. indica extract and mangiferin inhibit TNFα and NO production in RAW264.7 cell line activated with LPS and IFNγ

Fig. 3A shows a marked reduction on TNFα production when macrophages RAW264.7 stimulated with LPS and IFNγ were pre-incubated with *M. indica* extract ($IC_{50} = 94.1 \pm 0.1$ μg ml^{-1}). The extract also inhibited the NO_2^- production in this cell line with $IC_{50} = 69.4 \pm 0.3$ μg ml^{-1}

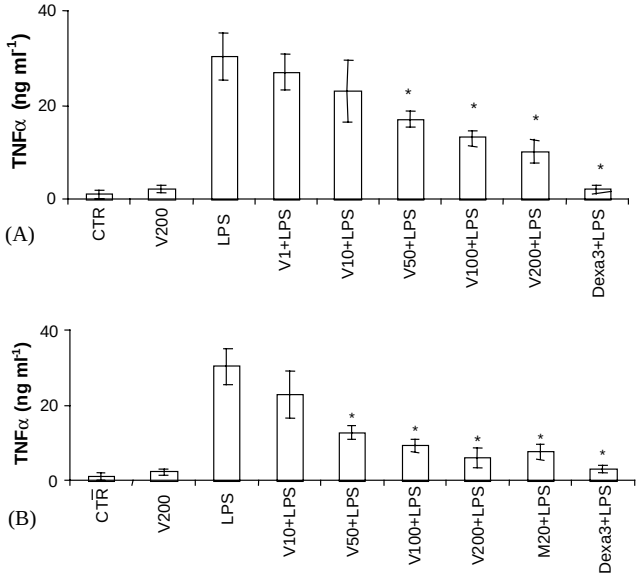

Fig. 1. Effects of *M. indica* L. extract and mangiferin isolated from the extract on LPS-induced serum TNFα production. *M. indica* extract (V: 1–200 mg kg^{-1}) and DEXA: dexamethasone (3 mg kg^{-1}, used as reference drug) were administered to mice 1 h (A) or during 7 days (B) before LPS (0.125 mg kg^{-1}, i.p.). Mangiferin (M, 20 mg kg^{-1}), was administered 7 days before LPS arrest. The control group (LPS) was treated with distilled and sterile water (vehicle). The drugs and vehicle were administered by gavage. CTR-animals without treatment. Each group represents the mean ± S.E.M. of six animals. $^{*}P < 0.05$ statistical significance compared with LPS group (ANOVA followed by Dunnet's *t* test).

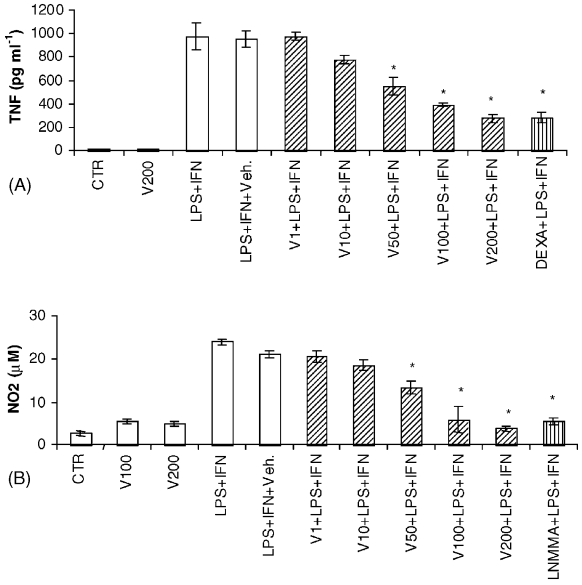

Fig. 3. Effect of *M. indica* extract on TNFα production (A) and total nitrites (B) in RAW264.7 cells. RAW264.7 cells (10^5 cells ml^{-1}) were activated with LPS (10 ng ml^{-1}) and IFNγ (2 U ml^{-1}). V: *M. indica* extract 1, 10, 50, 100, and 200 μg ml^{-1}. DEXA: dexamethasone 1 mM and L-NMMA 1 mM were the reference drugs. Each group represents the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ statistical significance compared with the group treated only with LPS plus IFNγ (ANOVA followed by Dunnet's *t* test). Veh. (vehicle in which *M. indica* extract was dissolved, DMSO 0.4% in PBS).

(Fig. 3B). Mangiferin also inhibited TNFα and NO production with IC_{50}s higher than the extract. In this case, TNFα was blocked with $IC_{50} = 159.2 \pm 0.7\,\mu g\,ml^{-1}$ (Fig. 4A) and NO_2^- with $IC_{50} = 381.0 \pm 0.9\,\mu g\,ml^{-1}$ (Fig. 4B).

Maximal inhibitions, at maximal doses tested, on TNFα production were 71.8% (*M. indica* extract) and 53.8% (mangiferin). Meanwhile, maximal inhibition on NO_2^- production were 83.8% (*M. indica* extract) and 33.8% (mangiferin).

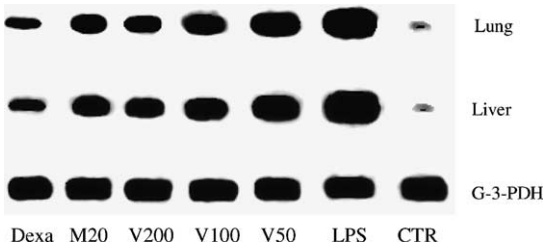

Fig. 2. Northern blots analysis of the expression of TNFα mRNA in lung and liver of mice treated with *M. indica* extract and them arrest with LPS. Northern blot analysis showed the inhibitory influence of *M. indica* extract (V, 50–200 mg kg^{-1}), mangiferin (M, 20 mg kg^{-1}) and dexamethasone (Dexa, 3 mg kg^{-1}) on TNFα production in lungs and livers of mice arrested with LPS (0.125 mg kg^{-1}, i.p.) CTR-control group without LPS. mRNA was extracted from lung and liver, according with Section 2, 30 min after of LPS administration. Signals for G-3-PDH mRNA for each lane are shown as controls.

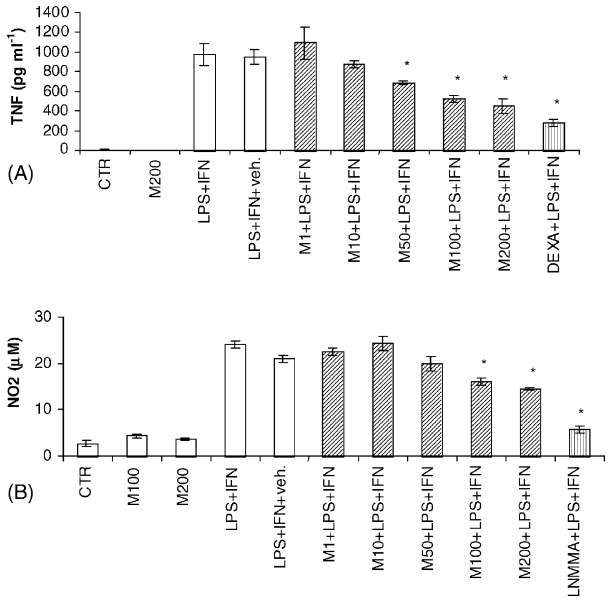

Fig. 4. Effect of mangiferin on TNFα production (A) and total nitrites (B) in RAW264.7 cells. RAW264.7 cells (10^5 cells ml^{-1}) were activated with LPS (10 ng ml^{-1}) and IFNγ (2 U ml^{-1}). M: mangiferin 1, 10, 50, 100, and 200 μg ml^{-1}. DEXA: dexamethasone 1 mM and L-NMMA 1 mM were the reference drugs. Each group represents the mean ± S.E.M. of three independent experiments. $^{*}P < 0.05$ statistical significance compared with the group treated only with LPS plus IFNγ (ANOVA followed by Dunnet's *t* test). Veh. (vehicle in which mangiferin extract was dissolved, DMSO 0.4% in PBS).

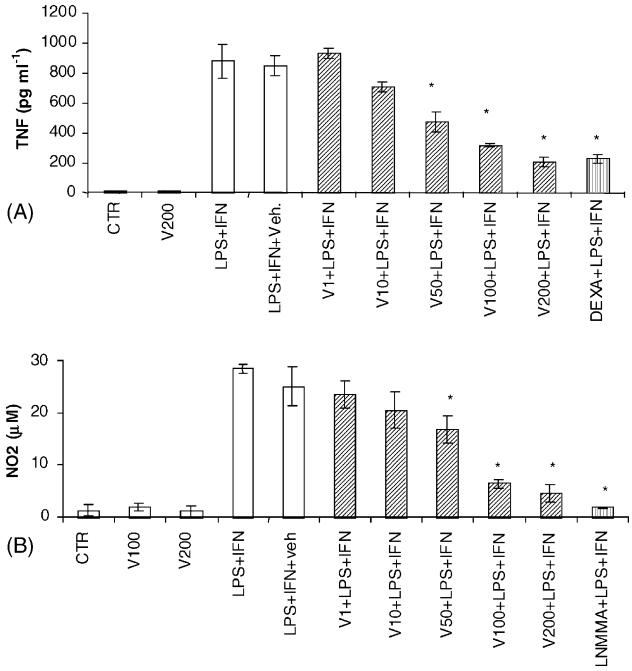

Fig. 5. Effect of *M. indica* extract on TNFα production (A) and total nitrites (B) in murine microglial cells (N9). Microglia cells (10^5 cells ml^{-1}) were activated with LPS (10 ng ml^{-1}) and IFNγ (2 U ml^{-1}). V: *M. indica* extract 1, 10, 50, 100, and 200 μg ml^{-1}. DEXA: dexamethasone 1 mM and L-NMMA 1 mM were the reference drugs. Each group represents the mean ± S.E.M. of three independent experiments. $^*P < 0.05$ statistical significance compared with the group treated only with LPS plus IFNγ (ANOVA followed by Dunnet's *t* test). Veh.: vehicle in which *M. indica* extract was dissolved, DMSO 0.4% in PBS.

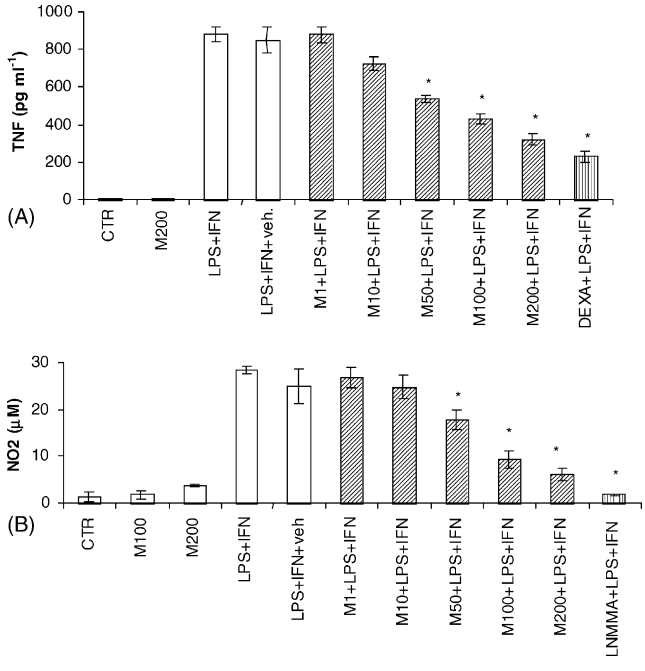

Fig. 6. Effect of mangiferin on TNFα production (A) and total nitrites (B) in murine microglial cells (N9). Microglia cells (10^5 cells ml^{-1}) were activated with LPS (10 ng ml^{-1}) and IFNγ (2 U ml^{-1}). M: mangiferin 1, 10, 50, 100, and 200 μg ml^{-1}. DEXA: dexamethasone 1 mM and L-NMMA 1 mM were the reference drugs. Each group represents the mean ± S.E.M. of three independent experiments. $^*P < 0.05$ statistical significance compared with the group treated only with LPS plus IFNγ (ANOVA followed by Dunnet's *t* test). Veh.: vehicle in which mangiferin exract was dissolved, DMSO 0.4% in PBS.

3.3. M. indica extract and mangiferin inhibit TNFα and NO production in microglia cell line activated with LPS and IFNγ

The pre-treatment with *M. indica* extract in microglia inhibited the production of TNFα (Fig. 5A) and total NO_2^-, as measurement of NO (Fig. 5B), with $IC_{50} = 76.0 \pm 0.5$ μg ml^{-1} and 84.0 ± 0.1 μg ml^{-1}, respectively.

Fig. 6 shows that treatment with mangiferin. This xanthone also inhibited the TNFα production (Fig. 6A) and total NO_2^- (Fig. 6B) with $IC_{50} = 63.6 \pm 0.7$ μg ml^{-1} and 78.8 ± 0.9 μg ml^{-1}, respectively.

Maximal inhibitions on TNFα production were 71.8% (*M. indica* extract) and 63.6% (mangiferin). Meanwhile, maximal inhibition on NO_2^- production were 83.8% (*M. indica* extract) and 78.8% (mangiferin).

4. Discussion

In this study, the anti-inflammatory activity of a standard *M. indica* extract (used in Cuba as a food supplement) and its principal component (mangiferin), and their abilities to reduce the production of pro-inflammatory mediators (NO and TNFα) have been investigated both in vivo and in vitro. Firstly, we have demonstrated that the extract protected by overproduction of TNFα in endotoxic shock and this inhibition was regulated at TNFα mRNA level. The inhibition of TNFα was showed at the same manner when the mice were treated 1 h or during 7 days with the extract before of LPS arrest.

Experimental and clinical studies demonstrate that exposure to endotoxin (LPS) results in the release of various inflammatory mediators although particular attention has been paid to the pro-inflammatory cytokines, e.g. TNFα, and the reactive oxygen (ROS) and nitrogen species, such as NO, which currently are considered to be a key mediators of tissue injury and mortality in septic shock [33,34].

Recently, it has been reported that the standard aqueous extract obtained from the stem bark of selected *M. indica* species, has antioxidant and ROS scavenging properties [9,10] and also exerts anti-inflammatory activity in experimental models of inflammation, in which NO, TNFα and arachidonic acid metabolites are strongly involved in the inflammatory response [6]. In our study, the anti-inflammatory activity and the inhibitory effects of *M. indica* extract and mangiferin on TNFα levels in the serum of mice treated with endotoxin are consequence primarily elicited by the antioxidant properties of this extract, and its glucosylxanthone [10,35], although their inhibitory effects on eicosanoids pro-

duction may contribute to the effects on endotoxic shock [7]. This view is scientifically supported by the fact that ROS are strongly involved in the induction and development of the inflammatory process, in arachidonic acid metabolism (e.g. prostaglandins and leukotrienes production) and in the pathogenesis of endotoxic shock [36].

On the other hand, various antioxidants with ROS scavenging properties, leukotriene antagonists and lipoxygenase inhibitors protected mice against endotoxin-mediated organ injury and reduced TNFα levels in blood serum [19,37]. The effects of antioxidant agents have been ascribed by some authors to inhibition of activation of the nuclear transcription factor NF-κB, which is activated by ROS with the subsequent induction and expression of various cytokines (such as TNFα) and enzymes (i.e. iNOS), respectively [3,20,25], which are involved in the induction and development of endotoxic shock. As occur with other antioxidants, *M. indica* extract might exert its effect on endotoxic shock by inhibition of the NF-κB activated by TNFα. Recently, we have demonstrated that the extract inhibits the TNFα-activated NF-κB in HeLa and Jurkat cells and peritoneal macrophages (data not shown). This could explain the inhibitory effects of the extract on TNFα serum level and TNFα mRNA in lung and liver of mice.

It has been demonstrated that LPS and TNFα represent the most potent stimulus for iNOS, which contributes to the enhanced generation of related nitrooxy radicals and subsequent alteration of organ and cellular physiology at all levels [38]. Previous work indicated that ROS play a role in LPS toxicity. Treatment with LPS is associated with an increase in lipid peroxidation in the liver, i.e. one of the toxic consequences of oxidative damage. It was recently shown that oxidative injury might be implicated in the toxicity of TNFα in rats, as well as in its cytotoxic action on tumor cells. These reports have showed that depletion of the endogenous glutathione, a major component of antioxidant system of cells, is associated with increased sensitivity to TNFα [39]. The beneficial effects demonstrated by *M. indica* extract could be given by its protective activity on the lipid peroxidation in the liver and brain [9,10] and the selenium content in the extract [12]. The selenium is an essential cofactor for glutathione peroxidase, enzyme that maintains the glutathione levels and reduces hydrogen peroxide and organic hydroperoxides [22,40]. It has been also demonstrate that *M. indica* extract (250 mg kg^{-1}, p.o.) increases, until normal levels, the decrease of glutathione peroxidase when the mice are arrested with TPA, an inductor of lipid peroxidation [10]. Moreover, recent studies demonstrate that endogenous glutathione plays an important role in reducing vascular hyporeactivity and endothelial dysfunction in response to peroxynitrite and endotoxic shock, as well as in acute inflammation [41,42]. In fact, we have shown that *M. indica* extract inhibits NO in macrophage cell line and these findings could be related with the improvement of mice with endotoxic shock tested in this study. *M. indica* extract may represent an alternative directed toward the prevention of the loss vascular potency in shock and inflammation.

These results suggest that polyphenols present in *M. indica* extract (like mangiferin and others) are probably responsible for the inhibition of TNFα production in septic shock. This activity is very important for the anti-inflammatory actions of this natural extract, in correspondence with others investigations of natural inhibitors on this cytokine in the inflammatory process [43,44].

Like TNFα, an excessive production of NO is linked to neuronal toxicity [45]. Because activated microglia and brain macrophages are the main source of TNFα and NO within the CNS, molecules that effectively reduce brain inflammation should inhibit activity of these central phagocytes. In our study, microglial cells were subjected in vitro to activation of LPS plus IFNγ because the combined action of these two agents resulted in a robust production of NO and TNFα [46]. This overproduction was inhibited similarly by *M. indica* extract and mangiferin in a manner dependent of the dose. It is another possible explication for the protective effect exerted by *M. indica* extract on ischaemia and reperfusion in Gerbil brain [47] in which these mediators are considered to be deleterious.

As can be seen in our study, *M. indica* extract also inhibited the overproduction of NO and TNFα in RAW264.7 cells. It has been reported that some polyphenols structurally related to some compounds present in *M. indica* extract inhibited lymphocyte proliferation and cytokine production [35,48]. As far as TNFα is concerned, it was found that flavonoids, isolated from the extract of *Amorpha fruticosa*, significantly inhibited TNFα production in LPS-stimulate RAW264.7 cell line. The inhibitory activity was comparable or higher than that of standard flavonoids, as genistein and silybin [49]. More recently, the effects on NO production, TNFα secretion and NF-κB activity have been described for polyphenols pine bark extract [50] and other compounds like those present on *M. indica* extract [3,14,18,19].

These findings suggest that the anti-inflammatory response observed during treatment with *M. indica* extract must be related with inhibition of TNFα and NO production. Mangiferin, a glucosylxanthone isolated from the extract, is involved in these effects.

The TNFα and NO inhibitions by the extract and mangiferin in endotoxic shock and microglia are reported here for the first time. Because the excessive production of NO, parallel with overproduction of TNFα, is thought that contribute to the pathogenesis of various diseases, (including CNS disorders, infections, cancers, autoimmune diseases and inflammation) [51,52] it could be expected that *M. indica* extract might be useful for the prevention of some of these diseases.

Further experimental and clinical studies will be needed to clarify such protective effect of *M. indica* extract (VIMANG®).

Acknowledgements

This work was partially supported by the Project CITMA No. 00403238 (CITMA, Cuba). Specially thanks to Dr. Anna Catania (Ospedale Maggiore di Milano, Italy) and Dr. Manuela Mengozzi (Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy) for their important scientific assistance and collaboration.

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