An Anacardiaceae preparation reduces the expression of inflammation-related genes in murine macrophages

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Abstract

This study investigated the effects of an aqueous extract of the stem bark of *Mangifera indica* L. (Anacardiaceae; Vimang®), which contains a defined mixture of components including polyphenols (principally mangiferin, MA), triterpenes, phytosteroids, fatty acids and microelements, on expression of inflammation mediators in inflammatory murine macrophages after stimulation in vitro with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ). In vitro treatment with Vimang at 4 μg/ml reduced levels of NOS-2 mRNA and NOS-2, while treatment at 40 μg/ml also reduced levels of COX-2 mRNA, COX-2, and prostaglandin E_2 (PGE$_2$). Results suggested that MA is involved in these effects. In vitro treatment with Vimang at 40 μg/ml also inhibited mRNA levels of the proinflammatory cytokines interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α) and colony-stimulating factor (GM-CSF), but did not affect mRNA levels of IL-6 or tumor growth factor-beta (TGF-β). Extracellular release of TNF-α by inflammatory macrophages was inhibited by in vitro treatment with Vimang at the same concentrations that showed inhibition of TNF-α mRNA levels. The inhibition of TNF-α production appears to be at least partially attributable to MA. Vimang at 4 μg/ml decreased mRNA levels of nuclear factor-kappaB (NF-κB) but did not affect expression of the NF-κB inhibitor (IκB). These data indicate that the potent anti-inflammatory effects of Vimang are due to selective modulation of the expression of inflammation-related genes, leading to attenuation of macrophage activation.
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Keywords: Mangifera indica extract; Pro-inflammatory cytokines; NOS-2; COX-2; mRNA; Murine macrophages

1. Introduction

Macrophages are critical cellular effectors of innate host defense that perform a variety of functions other than phagocytosis [1]. The phagocytic process is accompanied by intracellular signals that trigger cellular responses as diverse as cytoskeletal rearrangement, alterations in membrane trafficking, activation of microbial killing mechanisms, production of pro- and anti-inflammatory cytokines and chemokines, activation of apoptosis, and production of molecules required for efficient antigen presentation to the adaptive immune system [2]. Additionally, activated macrophages are capable of releasing an array of mediators, such as growth factors, bioactive lipids, hydrolytic enzymes and reactive oxygen and nitrogen intermediates (ROI and RNI), which are essential for microbe killing but also potentiate inflammatory reactions; regulation of this production is therefore critical to kill pathogens without inducing tissue injury [3]. The reactive nitrogen intermediate NO is a major mediator of inflammation [4], and the production of NO by mammalian macrophages constitutes a metabolic pathway know as the L-arginine/NO pathway. The enzyme responsible for NO production is the NO synthase (NOS), which in macrophages is calcium-independent and induced (iNOS, NOS-2 or type-II NOS) [5]. Expression of NOS-2 by macrophages after stimulation with bacterial lipopolysaccharide (LPS) and other cytokines is accompanied by the release of other inflammatory mediators, such as prostaglandin E_2 (PGE_2) and prostacyclin, via arachidonic acid metabolism by the cyclooxygenase (COX) pathway [6]. COX occurs in two forms (COX-1 and -2) and is the rate-limiting enzyme in the generation of prostaglandin H(2). COX-1 is produced as a steady-state enzyme, while COX-2 is involved in inflammation and tumorigenesis [7]. Several studies have focused on the role of NO and proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-12, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) in the expression and/or activity of the inducible form of COX (COX-2), in a number of cell types including macrophages [8]. Others cytokines, such as IL-10, IL-4 and transforming growth factor-beta (TGF-β), are involved in negative feedback mechanisms, which protect the organism from an "overshoot" of proinflammatory cytokines and other products of activated macrophages with tissue-damaging potential [9]. Oxidants and inflammatory mediators activate transcription factors such as nuclear factor-kappaB (NF-κB) and activator protein-1 (AP-1), leading to the expression of proinflammatory genes [10].

Vimang® is an aqueous extract of the stem bark of selected varieties of *Mangifera indica* L. (Anacardiaceae) which contains a defined mixture of components including polyphenols, triterpenes, phytosterols, fatty acids and microelements [11,12]. These compounds have been reported to have cytotoxic, antineoplastic, antioxidant, anti-inflammatory, antimicrobial, spasmolytic, analgesic and antipyretic activities [13]. In a previous study, we demonstrated that components of Vimang have inhibitory effects on macrophage functions including phagocytosis, chemotaxis in response to inflammatory stimuli, and the respiratory burst (production of ROI and RNI) [13]. To date, however, the molecular-level details of these effects of Vimang remain unknown, and in the present study we thus investigated the effects of Vimang on the responses of murine inflammatory peritoneal macrophages to LPS and IFN-γ, specifically effects on mRNA and protein levels of (a) the inducible inflammatory enzymes NOS-2 and COX-2 and their mediator PEG_2, (b) proinflammatory cytokines and their mediator TNF-α, and (c) proteins such as those of the NF-κB/IκB complex that promote the transcription of the NOS-2, COX-2 and proinflammatory cytokine genes.

2. Materials and methods

2.1. Animals

We used BALB/c mice supplied by Harlan OLAC (Oxon, England) and bred in our animal facilities. They were housed (groups of 10) in macrolon cages (Panlab, Barcelona, Spain) on poplar shaving bedding (B&K Universal, G. Jordi, Barcelona, Spain) in a standard bio-clean animal room, illuminated from 8:00 to 20:00 h (12 h light–12 h dark cycle) and maintained at a temperature of 22–24 °C. The animals had free access to food pellets (B&K Universal) and tap water, and were allowed to acclimatize for 1 week before the experiments. In all

experiments groups of five mice aged 8–10 weeks were used.

All experiments were carried out in accordance with European regulations on animal protection (Directive 86/609), the Declaration of Helsinki and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela.

2.2. Plant extract and treatments

M. indica L. was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant were deposited en the Department of Technology, Center of Pharmaceutical Chemistry, La Habana, Cuba. Stem bark extract of *M. indica* was prepared by decoction with a polar solvent for 1 h. The extract was concentrated by evaporation and spray dried to obtain a fine brown powder, which was used as the 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 [11,12]. The lot used by us was analyzed in the Quality Department of the Pharmaceutical Chemistry Center (Havanna, Cuba) and this analysis showed that the Vimang lot had the following content: moisture <10%, water-soluble substances >50%, total phenol (in anhydrous base) >30%, mangiferin (MA) >10%. We also assayed the effects of MA, a C-glucosylxanthone (1,3,6,7-tetra-hydroxyxanthone-C2-β-D-glucoside) that is present in significant quantities in Vimang [11]. The MA used in this study was purchased from Sigma and its purity (determined by thin-layer chromatography) was 100%. Stock solutions of Vimang (400 mg/ml), MA (100 mM), dexamethasone (DX, 100 mM; Sigma) or indomethacin (10 mg/ml; Sigma) were prepared in dimethylsulfoxide (DMSO). In in vitro experiments the culture medium contained Vimang at 4, 40 or 400 μg/ml, MA at 100 μM ($\approx$ 40 μg/ml), DX at 100 μM ($\approx$ 40 μg/ml) or indomethacin at 10 μg/ml.

2.3. Isolation and activation of murine peritoneal-exudate macrophages

For induction of inflammatory responses, mice were injected intraperitoneally with 0.5 ml of 3% thioglycollate broth, and peritoneal exudate was extracted 3 days later. Mouse resident and inflammatory peritoneal macrophages were obtained as previously described [13]. Briefly, 5 ml of Dulbecco's Modified Eagle's medium (DMEM) was then injected into the peritoneal cavity using a syringe with a 19-G needle. After gentle abdominal massage, about 3 ml of peritoneal fluid was extracted using the same syringe and transferred to 10-ml sterile polypropylene tubes on ice. A 20-μl aliquot was then extracted for cell counting in a hemocytometer and the cells were washed once by centrifugation at $400 \times g$ and resuspended to a concentration of 10^6 cells/ml. The number of viable cells was estimated by the trypan blue exclusion test. Aliquots (1000 μl) of the cell suspension were added to the wells of six-well microculture plates (Sterilin, England) and left for 90 min in a humidified incubator (37 °C, 5% CO_2) to allow adhesion. Non-adherent cells were then removed by gently washing with DMEM. More than 97% of the adherent cells showed nonspecific esterase activity, indicating that they were macrophages [13].

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Aliquots (1000 μl) of 10^6 peritoneal inflammatory cells stimulated in vivo 3 days previously with thioglycollate were incubated for 2 h at 37 °C in DMEM containing 10 U/ml of IFN-γ and 100 ng/ml of LPS, together with Vimang (4, 40 and 400 μg/ml), MA (100 μM), DX (100 μM) or no drug (controls). Isolation of total RNA from macrophage samples was done with a monophasic solution of phenol and guanidine thiocyanate (TriPure Isolation Reagent, Roche). Briefly, 1 ml of TriPure was added to each well (area 10 cm²), and the cells were lysed by passing the suspension through a pipette several times. The lysate was incubated for 5 min at room temperature (RT) to ensure complete dissociation of nucleoprotein complexes. Subsequently 0.2 ml of chloroform was added for each 1 ml of TriPure, and the tube was vortexed vigorously for 15 s, incubated at RT for 5 min, then centrifuged at

$12000 \times g$ for 15 min at 4 °C. The colorless aqueous upper phase was removed and RNA precipitated with isopropanol; the resulting RNA pellet was dried and dissolved in diethylpyrocarbonate (DEPC)-treated Rnase-free water at a concentration of 1μg/ml. cDNA synthesis (25 μl/reaction) was achieved using 1.25 μM random hexamer primers (Roche), 250 μM of each deoxyribonucleotide triphosphate (dNTPs), 10 mM DTT, 20 U of RNase inhibitor, 2.5 mM MgCl$_2$, 200 U of MMLV (murine leukemia virus) reverse transcriptase (Promega) in 30 mM Tris and 20 mM KCl, pH 8.3, and 2 μg of sample RNA. The cycling parameters for the RT step were: hybridization for 10 min at 25 °C and reverse transcription for 60 min at 42 °C. One μl of RT reaction mixture was amplified by PCR using the following mouse-specific forward/reverse primer pair: 5′-TGGAAGCCGTAACAAAG-GAAA-3′/5′-ACCACTCGTACTTGGGATGCT (NOS-2, GenBank of the National Center for Biotechnology Information, NCBI, accession number U03699); 5′-TGGTGGATGCCTTCTCTCG-3′/5′-AACAGATGGGATTCCCTAGGA-3′ (COX-1; NCBI, accession number BC005573); 5′-TGATCG-AAGACTACGTGCAAC-3′/5′-TCATCTCTCT-GCTCTGGTCAA-5′ (COX-2; NCBI, accession number NM_011198); 5′-TAATCCTTGGGAGTG-GAGCAA-3′/5′-TGTTGGAGTGTGGCTGGAGT-3′ (NF-κB; NCBI, accession number U20527); 5′-ATTGCTGAGGCACTTCTGAA-3′/5′-TTGACAT-CAGCACCCAAAGT-3′ (IκB; NCBI, accession number U36277); and 5′-ACCGCATCTTCTTGTG-CAGT-3′/5′-GCCAAAGTTGTCATGGATGA-3′ (glyceraldehyde-3-phosphate dehydrogenase, GAPDH; NCBI, accession number AF106860), as a control of gene expression. These primers amplified a fragment of 563 bp (NOS-2), 303 bp (COX-1), 345 bp (COX-2), 182 bp (NF-κB), 250 bp (IκB) and 544 bp (GAPDH). The 25-μl optimized reaction mixture contained reaction buffer (10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl$_2$, pH 9.0), 0.2 mM of each dNTP, 0.4 μM of each primer and 1.5 U of rTaq DNA polymerase (Roche). Thermal cycling in an automatic thermal cycler (GeneAmp PCR System 2400, Perkin-Elmer, Norwalk, USA) was as follows: initial denaturing at 94 °C for 5 min; then 35 cycles at 94 °C for 30 s, 55 °C (NOS-2, COX-1, COX-2, NF-κB and IκB) or 53 °C (GAPDH) for 45 s, and 72 °C for 1 min; and finally a 7-min extension phase at 72 °C. In all experiments we performed controls without RNA or without reverse transcriptase; in no case were amplification products obtained. PCR products (20 μl aliquots) were separated on a 2% agarose gel in TBE buffer stained with 0.5 μg/ml of ethidium bromide, and photographed with a digital camera under a Spectroline 312 variable-intensity UV transilluminator (Spectroline). mRNA amounts in each band were quantified using a densitometry analysis software (ImageMaster Total Lab, ver. 2.00; AmershamPharmaciaBiotech), expressed with respect to mRNA amount in the band corresponding to the control gene GAPDH, run in the same gel.

2.5. Slot-blot assay

The levels of the enzymes COX-2 and NOS-2 were determined by slot-blot assay in soluble extracts. Cultures of mouse peritoneal inflammatory macrophages (10^7 cells/ml) were incubated in vitro for 6 h in DMEM containing 10 U/ml of IFN-γ and 100 ng/ml of LPS, together with Vimang at 4, 40 or 400 μg/ml, MA at 100 μgM or no drug (controls), at 37 °C under humidified 5% CO$_2$. Cells were washed with TBS buffer (50 mM Tris, 0.15 M NaCl; pH 7.4) and resuspended in 0.4 ml of TBS and 0.1 ml of protease inhibitor cocktail (Sigma) containing 104 mM 4-(2-amino-ethyl)benzenesulfonyl fluoride, 80 μM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A and 1.4 mM E-64 [*N*-(*trans*-epoxysuccinyl)-L-leucine-4-guanidinobutylamine]. Cells were then sonicated in ice at 60 W for 15 s, then centrifuged for 5 min at $13\,000 \times g$; protein concentration in the supernatant was measured by the Bradford method (1976) using bovine serum albumin as standard. Equal amounts of total protein (60 μg in 100 μl) were transferred to hydrophobic polyvinylidene difluoride membranes (PVDF, Hybond-P, Amersham Biosciences) using a slot-blot filtration manifold (Amersham Biosciences). Membranes were blocked with 5% non-fat milk powder in TBS containing 0.2% Tween 20 for 1 h, and COX-2 and NOS-2 were immunodetected by incubation for 1 h at room temperature with a rabbit polyclonal antisera against murine COX-2 (Cayman Chemical) or NOS-2 (Alpha Diagnostic International) diluted 1:1000 in dilution buffer (TBS containing 0.05% Tween 20 and 1% non-fat dry milk). After

incubation with primary antibody, the membranes was washed five times with washing buffer (TBS containing 0.05% Tween 20), incubated for 1 h at room temperature with a 1:3000 dilution in dilution buffer of secondary antibody (horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig; Amersham Biosciences) and washed five times with washing buffer. Finally, antibody binding was visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences) following the supplier's instructions, using an autoradiography film (Hyperfilm ECL, Amersham Biosciences). Densitometric analysis was performed using a laser scanner and bands were quantified in TIF images using densitometry analysis software (ImageMaster Total Lab, ver. 2.00; Amersham Biosciences), with ovine COX-2 (Cayman Chemical) and mouse NOS-2 peptide (a 19 amino acid peptide sequence designated INOS22-P; control peptide; 1125–1144 aa; Alpha Diagnostic International) as standards.

2.6. RT multiplex PCR (RT-MPCR)

RT multiplex PCR (RT-MPCR) is a very sensitive and accurate method for measuring expression levels of multiple genes [14]. cDNA was obtained by RT as described above, and cleared by phenol extraction and ethanol precipitation. RT-MPCR was done using a CytoXpress™ kit (Rat Inflammatory Set 1, Biosource International), following the manufacturer's instructions as described previously [15]. This kit is designed to direct the simultaneous amplification of specific ORF regions of mouse GAPDH, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-1beta (IL-1β, IL-6 and transforming growth factor-beta (TGF-β genes. A 50-µl RT-MPCR mixture was made up with 10.5 µl of distilled water, 5.0 µl of 10x RT-MPCR buffer, 5.0 µl of 10x RT-MPCR enhancer, 5.0 µl of 10× RT-MPCR primers, 4.0 µl of dNTP (3.12 mM), 0.5 µl of rTaq DNA polymerase (5 U/µl) (Roche), and 20 µl of the clean cDNA. Thermal cycling was as follows: initial denaturing at 96 °C for 1 min; two cycles with a denaturing step of 96 °C for 1 min and annealing step of 60 °C for 4 min; then 33 cycles at 94 °C for 1 min and 60 °C for 2.5 min; and finally a 10-min extension phase at 70 °C, followed by a soak at 25 °C. RT-MPCR products (10-µl aliquots) were again analyzed by 2% agarose gel electrophoresis, with ethidium bromide staining.

The gel was photographed with a digital camera under a UV transilluminator, and the bands were quantified in TIF images using densitometry analysis software (ImageMaster Total Lab, ver. 2.00; AmershamPharmaciaBiotech), with respect to mRNA amount in the band corresponding to the control gene GAPDH in the same gel.

2.7. Enzyme-linked immunosorbent assay (ELISA) for quantification of TNF-α

Aliquots (100 µl) of the macrophage suspension (10^6 peritoneal macrophages per ml, obtained after prestimulation with thioglycollate as indicated above) were incubated for 6 h with LPS (100 ng/ml in DMEM) or LPS plus IFN-γ (10 U/ml in DMEM), and Vimang at 4, 40 or 400 µg/ml in DMEM or MA or DX at 1, 10 or 100 µM, or without drug (controls), in 96-well microculture plates at 37 °C under 5% CO_2 in a humidified incubator. The plates were then centrifuged at $400 \times g$ for 5 min and TNF-α was quantified in the supernatant using a sandwich ELISA from R&D Systems. Briefly, PVC microtitre plates (Costar) were coated with 100 µl per well of capture antibody (goat anti-mouse TNF-α) at 0.8 µg/ml in PBS by incubation overnight at room temperature. The plates were then washed five times with wash buffer (0.05% Tween 20 in PBS, pH 7.2–7.4) and blocked by adding 300 µl per well of blocking buffer (1% BSA, 5% sucrose in PBS with 0.05% sodium azide), then incubated for 2 h at 37 °C. After a washing step (five times with wash buffer) and the addition of 100 µl of culture supernatant (see above), or standard solution (seven serial twofold dilutions of a 2000 pg/ml stock solution of recombinant rat TNF-α in reagent diluent, i.e. PBS containing 1% BSA, pH 7.2–74), the plates were then incubated for 2 h with detection antibody (biotinylated goat anti-mouse TNF-α at 300 ng/ml in reagent diluent. The plates were again washed five times with wash buffer, adding 100 µl of a 1/200 working dilution of streptavidin conjugated to horseradish peroxidase for 20 min at room temperature. After washing with wash buffer, peroxidase activity was detected by adding 100 µl of substrate solution [*o*-phenylenediamine (Sigma) at a concentration of 0.04% in phosphate–citrate buffer (pH 5.0) containing 0.001% H_2O_2]. After 20 min incubation at room temperature, the reaction was

stopped with 25 μl of 3 N H_2SO_4, and optical density at 492 nm was measured with an ELISA reader (Titertek Multiscan, Flow Laboratories).

2.8. Determination of PGE$_2$ in culture supernatants

Aliquots (100 μl) of the macrophage suspension (10^7 peritoneal macrophages per ml, obtained after prestimulation with thioglycollate as indicated above) were incubated for 3 h with LPS (100 ng/ml in DMEM) plus IFN-γ (10 U/ml in DMEM), and Vimang at various concentrations (4, 40 or 400 μg/ml in DMEM), indomethacin (a known prostaglandin synthetase inhibitor, at 10 μg/ml) or without drug (controls) in 96-well microculture plates at 37 °C under 5% CO_2 in a humidified incubator. The plates

were centrifuged at $400 \times g$ for 5 min and PGE$_2$ levels were determined in the supernatant using a competitive binding immunoassay from R&D Systems. Briefly, 100 μl of PGE$_2$ standard (dilutions from 1000 to 7.8 pg/ml in assay buffer containing blocking proteins and preservative, buffer ED1) or samples (in assay buffer) was incubated for 18–24 h at 2–8 °C with 50 μl of PGE$_2$ HS conjugate (PGE$_2$ conjugated to alkaline phosphatase) and 50 μl of PGE$_2$ HS antibody solution (mouse monoclonal antibody to PGE$_2$). After three washes with wash buffer, 200 μl of pNPP (p-nitrophenylphosphate in a buffered solution) was added to each well, incubated for 1 h at 37 °C, and stopped with 50 μl of TSP (trisodium phosphate solution). The optical density (OD) of each well was determined immediately using a microplate reader
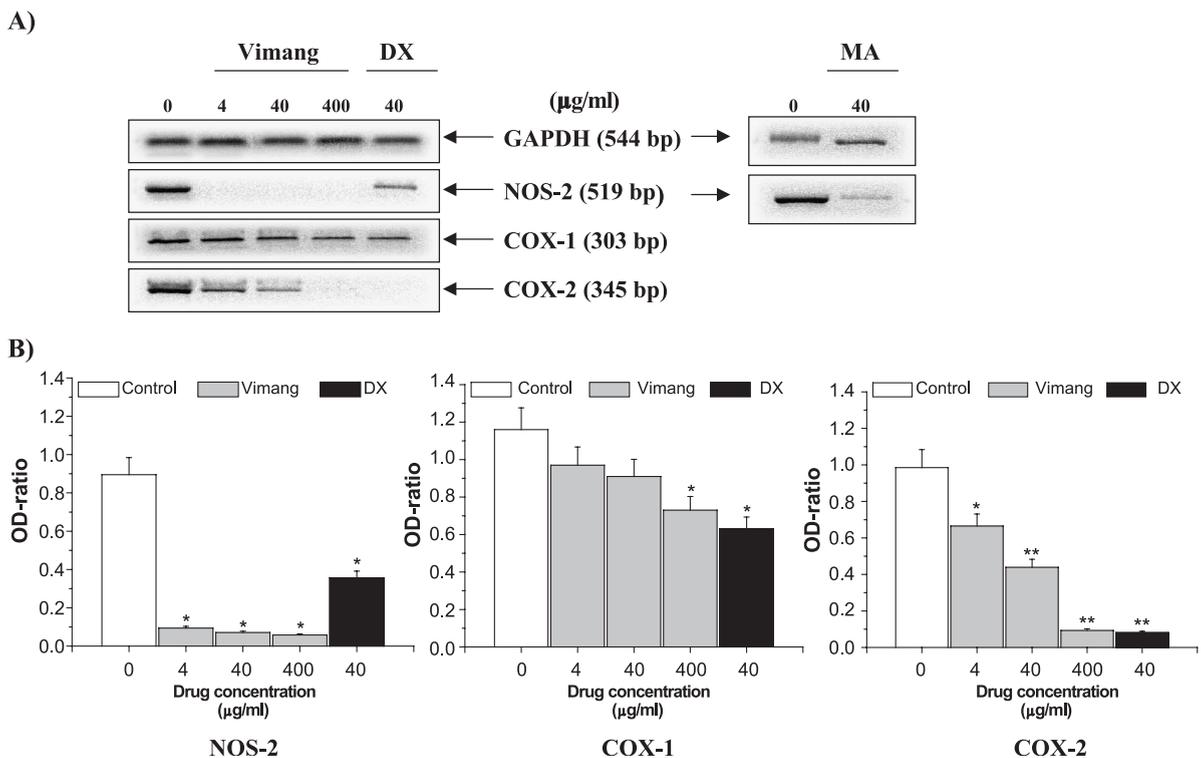

Fig. 1. In vitro effects of Vimang, mangiferin (MA) and dexamethasone (DX) on levels of NOS-2, COX-1 and COX-2 mRNAs in inflammatory peritoneal macrophages, as determined by semiquantitative RT-PCR. Inflammatory macrophages were stimulated with LPS (100 ng/ml) and IFN-γ (10 U/ml), in the absence or presence of Vimang (4–400 μg/ml), DX (100 μM ≈ 40 μg/ml) or MA (100 μM ≈ 40 μg/ml). (A) 2% gel agarose analysis of RT-PCR products using primers for NOS-2 (519 bp), COX-1 (303 bp), COX-2 (345 bp) or the control gene GADPH (544 bp); (B) mRNA levels as quantified by densitometric analysis. Bars show means ($n=3$) ± S.E.M. of [OD of NOS-2, COX-1 or COX-2 band]/ [OD of corresponding band in analysis using primer for GAPDH], where OD = optical density. *Significantly different from control, $P < 0.01$.

(Titertek Multiscan, Flow Laboratories) at 405 nm, with wavelength correction between 570 and 590 nm. For estimation of PGE_2 concentrations in samples, we constructed a calibration curve of PGE_2 concentration against $100 \times B/B_0$ (B = average OD for that concentration, minus non-specific binding; B_0 = average OD in wells without PGE_2 HS conjugate, minus non-specific binding; non-specific binding = average OD in wells without either PGE_2 HS conjugate or anti-PGE_2 antibody).

2.9. Statistics

Data are shown in the figures as means ± S.E.M. Statistical significances ($\alpha = 0.05$) were determined by one-way analysis of variance (ANOVA) fol-lowed by the Tukey–Kramer test for multiple comparisons.

3. Results

3.1. Vimang, mangiferin and dexamethasone do not affect macrophage viability

To rule out the possibility that the Vimang, MA or DX concentrations used were toxic for macrophages, we first investigated viability on the basis of trypan blue exclusion, following stimulation with LPS or IFN-γ and incubation for 2 h with maximal concentrations of Vimang (400 μg/ml), MA or DX (100 μM ≈ 40 μg/ml). Mean viability after 2 h of incuba-
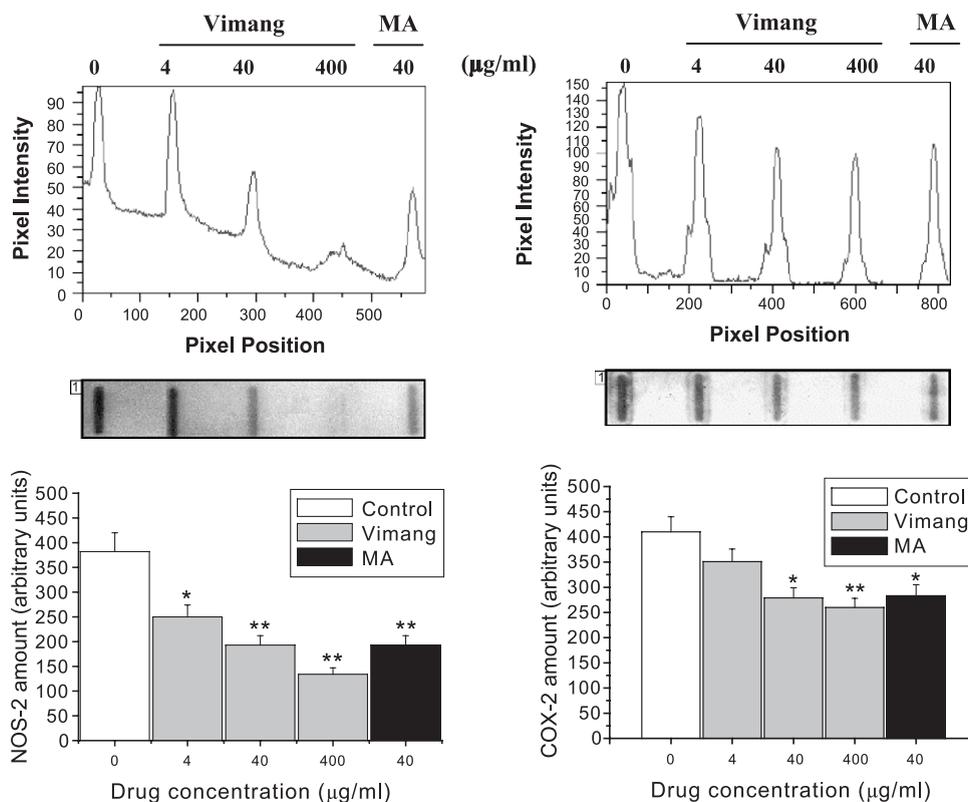
Fig. 2. In vitro effects of Vimang (4–400 μg/ml) and mangiferin (MA) (100 μM ≈ 40 μg/ml) on NOS-2 and COX-2 protein synthesis by inflammatory macrophages, as determined by slot-blot analysis of soluble extracts of cells. Macrophages were stimulated with 100 ng/ml of LPS and 10 U/ml of IFN-γ. Results show a representative blot (one of three experiments) and mean ± S.E.M. of NOS-2 or COX-2 amount (arbitrary units) as determined by densitometry. *$P < 0.05$ and **$P < 0.01$ with respect to control.
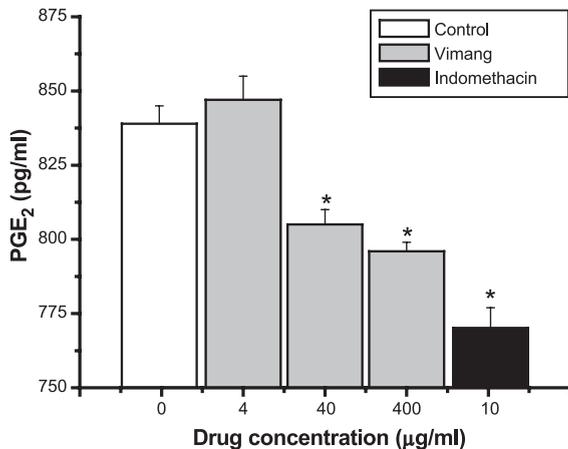
Fig. 3. In vitro effects of Vimang (4–400 μg/ml) and indomethacin (10 μg/ml) on PGE_2 production by inflammatory macrophages stimulated with LPS (100 ng/ml) and IFN-γ (10 U/ml). Bars show means ($n=3$) ± S.E.M. *$P<0.05$ with respect to control.

tion was $93 \pm 4\%$ (Vimang), $94 \pm 3\%$ (MA), $94 \pm 4\%$ (DX), and $95 \pm 5\%$ (control, no drug) ($n=5$, $P>0.05$).

3.2. Effect of Vimang on NOS-2 expression

We initially used semiquantitative RT-PCR to assess the effect of Vimang at different concentrations (4, 40 and 400 μg/ml) on NOS-2 mRNA levels in macrophages stimulated with LPS or IFN-γ (Fig. 1). Vimang very significantly reduced mRNA levels even at the lowest concentration tested (4 μg/ml, Fig. 1A). Vimang at 40 μg/ml led to reduction of both NOS-2 mRNA levels (Fig. 1) and NOS-2 protein levels (Fig. 2), and these effects were greater than those produced by DX (a drug controlling NOS-2 mRNA expression; see [16]) at the same concentration (Fig. 1A,B).

To assess whether the principal polyphenolic component of Vimang (MA, accounting for about 10% of polyphenols present in the extract; [13]) may be responsible for the observed inhibition of NOS-2 mRNA synthesis, we used semiquantitative RT-PCR and slot-blot analyses to confirm the inhibitory capacity of MA 100 μM (≈ 40 μg/ml) on NOS-2 expression (Figs. 1A and 2).

3.3. Effect of Vimang on COX expression

The effects of Vimang (4, 40 and 400 μg/ml) on COX expression were evaluated by RT-PCR. We also

performed assays with the control drug DX (a known inhibitor of COX mRNA synthesis; [17]) at 100 μM (≈ 40 μg/ml). Neither Vimang nor DX strongly reduced COX-1 mRNA levels: significant reductions in COX-1 mRNA levels were observed only with 400 μg/ml Vimang and 100 μM DX (Fig. 1). By contrast, both Vimang and DX significantly reduced COX-2 mRNA levels at all concentrations tested (Fig. 1), with DX showing somewhat higher activity. Likewise, both Vimang (4, 40 and 400 μg/ml) and MA (40 μg/ml) significantly reduced COX-2 protein levels (Fig. 2).

PGE_2, an active product of the COX pathway in murine peritoneal macrophages, is synthesized from arachidonic acid by the action of COX isoforms [18]. To determine whether Vimang affects the synthesis of PGE_2, a competitive-binding immunoassay was performed. Treatment with Vimang (40 μg/ml) caused a significant decrease in PGE_2 levels (Fig. 3).
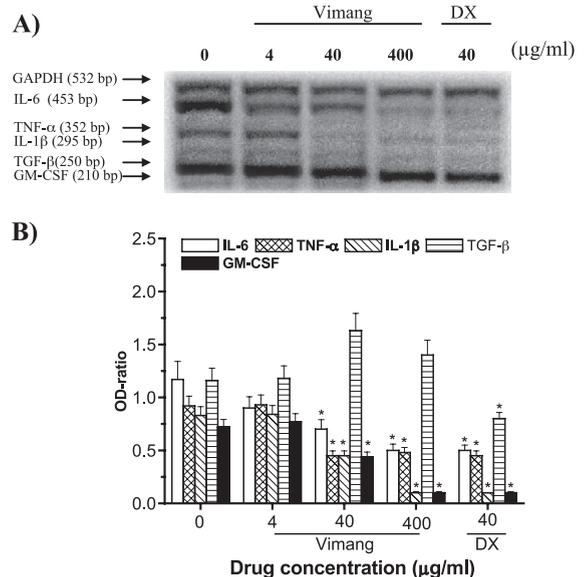

Fig. 4. In vitro effects of Vimang (4–400 μg/ml) on mRNA levels of the pro-inflammatory cytokines GM-CSF (210 bp), TGF-β (250 bp), IL-1β (295 bp), TNF-α (352 bp) in inflammatory macrophages as determined by semiquantitative multiplex RT-PCR (RT-MPCR). Macrophages were stimulated with LPS (100 ng/ml) and IFN-γ (10 U/ml), in the absence or presence of Vimang (4–400 μg/ml). (A) Samples were run on a 2% agarose gel and (B) mRNA levels were quantified by densitometric analysis, in all cases expressed with respect to the value for GAPDH obtained in same gel. Bars shows means ($n=3$) ± S.E.M. *$P<0.01$ with respect to control. Cytokines whose mRNA levels were reduced by Vimang are indicated in bold.
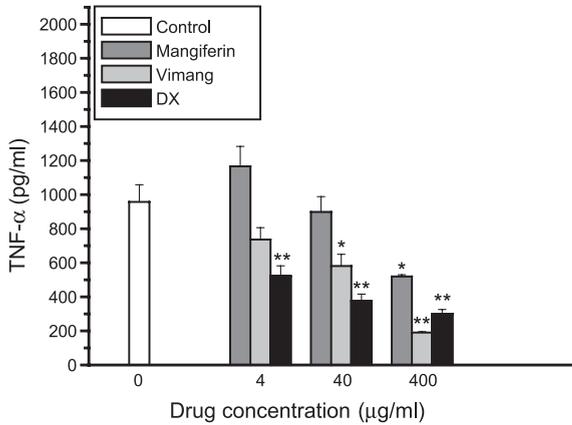
Fig. 5. In vitro effects of Vimang, mangiferin (MA) and dexamethasone (DX) on TNF-α levels, as determined by sandwich ELISA of culture supernatant of inflammatory macrophages stimulated for 6 h with LPS (100 ng/ml) and IFN-γ (10 U/ml). The assay contained Vimang (4, 40 or 400 μg/ml) or MA (1, 10 or 100 μM ≈ 4, 40 or 400 μg/ml) or DX (1, 10, 100 μM ≈ 4, 40 or 400 μg/ml). Bars represent means ($n = 3$) ± S.E.M. *$P < 0.05$ and **$P < 0.01$ with respect to control.

3.4. Effect of Vimang on proinflammatory cytokine mRNA levels

Using multiplex RT-PCR we evaluated the effects of Vimang on the levels of various proinflammatory cytokines produced by macrophages, including IL-1β, IL-6, GM-CSF, TGF-β and TNF-α. These effects were compared with that of DX, which has a known inhibitory effect on transcription of the mRNA of various pro-inflammatory cytokines [19] (Fig. 4). Vimang at concentrations above 40 μg/ml significantly reduced mRNA levels of TNF-α, IL-1β and GM-CSF, but not mRNA levels of IL-6 and TGF-β (Fig. 4A,B). At the same concentration, DX significantly reduced mRNA levels of all cytokines except TGF-β (Fig. 4B).

3.5. Effect of Vimang on TNF-α production

We next used ELISA to investigate the effects of Vimang (4, 40 and 400 μg/ml) on the production of TNF-α by activated macrophages (Fig. 5). We also assayed the effects of MA and DX (Fig. 5). Vimang
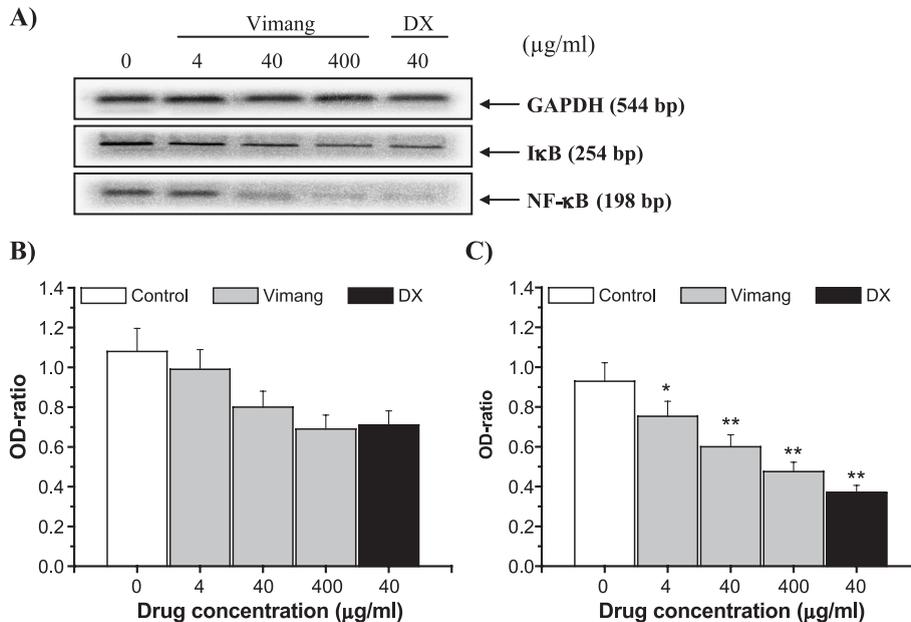

Fig. 6. In vitro effects of Vimang (4–400 μg/ml) and dexamethasone (DX) (40 μg/ml ≈ 10 μM) on levels of NF-κB and IκB in inflammatory murine peritoneal macrophages stimulated with LPS (100 ng/ml) and IFN-γ (10 U/ml), as determined by RT-PCR. (A) 2% gel agarose analysis of RT-PCR products using primers for NF-κB (198 bp), IκB (254 bp) or the control gene GAPDH (544 bp); (B and C) mRNA levels quantified by densitometric analysis for IκB and NFκB, respectively. In all cases expressed with respect to the value for GAPDH obtained in the same gel. Bars show means ($n = 3$) ± S.E.M. *$P < 0.05$ and **$P < 0.01$ with respect to control.

significantly reduced TNF-α levels at concentrations above 40 μg/ml. MA significantly reduced TNF-α levels at concentrations above 100 μM. DX significantly reduced TNF-α levels at concentrations above 1 μM (Fig. 5).

3.6. Effect of Vimang on mRNA of transcription factors

Finally, we used semiquantitative RT-PCR to investigate the effects of Vimang on expression of genes coding proteins of the NF-κb/IκB complex, a family of transcription factors induced by proinflammatory cytokines [20]. Vimang was assayed at 4, 40 and 400 μg/ml, and DX (an inhibitor of NF-κb [21] at 100 μM. Vimang significantly reduced NF-κb mRNA levels at concentrations above 4 μg/ml (Fig. 6A,C); however, to obtain reductions similar to those obtained with DX at 100 μM, about 10-fold-higher Vimang concentrations were required (Fig. 6B). At the concentrations tested, neither Vimang nor DX reduced IκB mRNA levels (Fig. 6B).

4. Discussion

This study presents findings that may explain the potent anti-inflammatory effects of Vimang. Previously, we have reported that Vimang at between 1 and 50 μg/ml significantly reduces extracellular ROI and RNI production by resident and thioglycollate-elicited macrophages stimulated in vitro with LPS and IFN-γ [13]. Although ROI and RNI are involved in host defence, overproduction of these species may contribute to the pathogenesis of inflammatory diseases [22]. A role for NO as a major mediator of inflammation has been clearly demonstrated, and there is extensive evidence implicating NO in the pathophysiology of inflammatory processes [23]. Three genes encoding NO synthases (NOS, EC 1.14.13.39) are expressed in mammals (NOS-1 = nNOS = ncNOS; NOS-2 = iNOS; and NOS-3 = eNOS = ecNOS) [24]. Broadly speaking, the "low-output" production of NO by NOS-1 or NOS-3 corresponds to physiologic functions of NO in the healthy host, while the "high-output" production of NO by NOS-2 (the only NOS expressed by macrophages) occurs during inflammation and infection [4]. Numerous cytokines and microbial products, often acting in synergistic pairs, stimulate expression of NOS-2; one such pair is LPS and IFN-γ [25]. The present results demonstrate that Vimang, at concentrations as low as 4 μg/ml, markedly reduces both NOS-2 mRNA levels and NOS-2 protein levels in inflammatory peritoneal macrophages stimulated in vitro with LPS and IFN-γ. Indeed, Vimang had these effects at lower concentrations than DX, which we used as control inhibitor of NOS-2 expression. The effect of Vimang on NOS-2 expression appears to involve its principal polyphenolic component MA, although other polyphenols present in Vimang, such as (−)-epicatechin, may also play a part [12]. In this connection, it has been demonstrated that various plant polyphenols (including MA) dose-dependently inhibit the expression of NOS-2 in several types of cell, including macrophages [13,15,25–29]. Our results are also in line with previous results indicating that both Vimang and NO inhibit NO production by inflammatory peritoneal macrophages stimulated in vitro with LPS and IFN-γ [13].

Macrophage activation by bacterial LPS promotes the secretion of cytokines and secondary mediators, such as leukotrienes and prostaglandins (PG) [30]. The rate-limiting step in PG synthesis is that catalyzed by COX, an enzyme that exists in two isoforms each encoded by a different gene: COX-1 is constitutively expressed in most cell types [31], while COX-2 is expressed at very low levels and is strongly influenced by growth factors and proinflammatory stimuli, including LPS [32]. Previous studies have shown that NO is necessary for maintaining prolonged COX-2 gene expression [33], and both NOS-2 and COX-2 expression by macrophages generate massive production of NO and PGE$_2$, both of which have cytotoxic and pro-inflammatory activities [34]. In the present study, Vimang at 4 μg/ml significantly reduced COX-2 mRNA levels; however, significant reduction of COX-1 mRNA levels was obtained only with Vimang at 400 μg/ml. To achieve significant reductions of both COX-2 protein levels and PGE$_2$ protein levels, Vimang at 40 μg/ml was required. The observed effect of Vimang on COX-2 expression may be at least partially attributable to its principal polyphenolic component MA. It has been reported that various polyphenols (notably those present in green tea) are capable of inhibiting both COX-2 expression and PGE$_2$ expression in diverse cell types [29,35].

Cytokines are local protein mediators, now known to be involved in almost all important biological processes, including cell growth and activation, inflammation, immunity and differentiation [36]. Macrophage activation by bacterial LPS induces the secretion of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6, TGF-β, and GM-CSF [30]. In the present study, Vimang and DX significantly reduced mRNA levels of TNF-α, IL-1β and GM-CSF, three cytokines with proinflammatory effects [37]. Evidence of various types demonstrates that plant polyphenols, including MA, have an inhibitory effect on mRNA transcription of LPS-induced proinflammatory cytokines [25,38].

The cumulative evidence suggests that TNF-α is not only an inflammatory mediator but is also the key regulator of the production of other cytokines involved in inflammation, such as IL-1, GM-CSF, IL-6, and IL-8 [39,40]. In the present study, the observed inhibition by Vimang of TNF-α mRNA levels in inflammatory macrophages stimulated with LPS and IFN-γ was accompanied by a reduction in TNF-α levels in the culture supernatant, indicating good correlation between the two methods (RT-PCR for determination of mRNA levels, and ELISA for determination of protein levels). The inhibition of TNF-α expression and release by Vimang appears to involve this product's polyphenols, notably MA [15]. Other polyphenols apart from MA are known to be able to inhibit the expression and release of TNF-α [41,42]. Glucocorticoids are likewise effective inhibitors of TNF-α gene transcription [43], so that the steroids present in Vimang may also act additively together with the polyphenols in the blockage of TNF-α gene transcription and TNF-α release.

Transcriptional regulation of proinflammatory cytokine genes is a multifaceted process that requires a combination of nuclear factors and co-activators for maximal gene transcription to occur [10]. NF-κB is activated by a wide variety of different stimuli including proinflammatory cytokines and bacterial LPS [44], and is retained in the cytoplasm by binding to the inhibitory IκB proteins (IκB, IκBβ, IκBε, p105 and p100), which block the nuclear localization sequences of NF-κB [45]. The transcriptional activation of NOS-2 and COX-2 is mediated by the binding of inducible transcription factors including NF-κB [46], and previous studies have shown that NF-κB is dispensable for the induction of COX-2 by LPS

[47]. As shown in the present study, both Vimang and DX block the transcription of NF-κB in inflammatory macrophages stimulated with LPS and IFN-γ, and do not affect transcription of the mRNA coding for IκB proteins. The inhibition of NF-κB mRNA transcription by Vimang may likewise be attributable to its constituent polyphenols. It has been shown that various plant polyphenols may inhibit the production of proinflammatory cytokines by inhibiting NF-κB expression [48] and/or by suppressing NF-κB activation through degradation of IκB [49] or prevention of NF-κB translocation to the nucleus [50]. For example, green tea extracts that contain polyphenolic free radical scavengers such as MA [25] block NF-κB and TNF-α mRNA and protein expression [51].

The present study has focused on the possible contribution of the polyphenol MA to the observed effects of Vimang on proinflammatory cytokines and enzymes involved in inflammation; however, it should be noted that Vimang also contains steroids and terpenoids (10–40%) [11,12]. There have not been many studies of the effects of terpenoids on inflammatory processes, but these molecules are known to inhibit cell surface expression of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VAM-1), both of which are crucial in the regulation of immune response and inflammation [52].

The results of the present study indicate that, in macrophages, Vimang has similar effects to the glucocorticoid DX on molecular signaling events controlling the expression of genes for primary inflammation mediators such as cytokines and secondary mediators such as NOS-2 and COX-2 enzymes. As a result, and in view of the anti-inflammatory effects of Vimang in vivo and its lack of toxicity after long-term oral administration [13], we suggest that Vimang may be useful in the treatment of acute or chronic inflammatory processes. Vimang may be of particular value as a replacement or partial replacement treatment in subjects receiving glucocorticoids, with the aim of reducing the glucocorticoid dose and thus reducing secondary effects.

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