

Modulation Of Pro-Inflammatory Genes By α -Mangostin From *Garcinia mangostana*

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ABSTRACT : The role of α -Mangostin, a xanthone from the pericarp of mangosteen fruit, in the down regulation of proinflammatory genes was investigated by immune cytochemistry, RT-PCR and Western blot analysis using MDA-MB-231 breast cancer cells as in vitro model. The MDA-MB-231 cells were treated with different concentration of α -Mangostin (2.5 – 20 μ M) and stimulated with LPS (1 μ g/ml) for expression of COX-2 and iNOS. The results showed the induction of COX-2 and iNOS Protein in MDA-MB-231 cells and the expression was markedly attenuated in the cells pretreated with α -Mangostin in a dose dependent manner both at the transcriptional and translational level.

KEYWORDS: Proinflammatory genes, COX-2, iNOS, Inflammation, α -Mangostin

I. INTRODUCTION

Inflammation encompasses multiple processes by an organism in response to injury that is related to harmful stimuli such as infection by a pathogen, exposure to bacterial endotoxin or chemical exposure. Inflammatory responses are fundamental in host defense against external stimuli by activating immune cells, in an effort to maintain homeostasis [1]. In some disorders, the inflammatory process becomes continuous and subsequently develops chronic inflammatory diseases. Chronic inflammation is measured to be a momentous factor in many human diseases like type II diabetes, neurodegenerative diseases, several cancers and cardiovascular diseases. One of the significant anti-inflammatory mechanisms is the modulation of pro-inflammatory genes like COX-2 and iNOS [2, 3, and 4]. COX-2 and iNOS are inducible forms of cyclooxygenase and nitric oxide synthase enzymes that are over-expressed during inflammation and can lead to formation of tumor. In most of the normal tissues, the expression of pro-inflammatory genes (COX-2 and iNOS) is hardly detectable and induced in response to pro-inflammatory stimuli such as bacterial LPS [5]. COX-2 and iNOS are now recognized as markers for tumor progression documented for a variety of human cancers. The association between iNOS and COX-2 activation is acknowledged by a synergistic effect and they are often co-expressed during chronic inflammation in cancer tissues [6]. The synthesis of prostaglandin by COX-2 and NO production by iNOS are mainly regulated at the transcriptional level and their production can also be inhibited by down-regulating iNOS and COX-2 expression [7]. Therefore, the targeted regulation of iNOS and COX-2 is a promising approach for the inhibition of inflammation and carcinogenesis, as well as preventing cancer. Hence compounds inhibiting these inducible enzymes are important in the search for new anti-inflammatory and anti-cancer drugs.

The plant kingdom is a rich source of active components that lead to the discovery and development of numerous agents that can be used as medicine against several diseases [8]. α -Mangostin is a major xanthone derivative isolated from the pericarp of Mangosteen belongs to *Garcinia mangostana* Linn., of Southeast Asian Countries. The whole Mangosteen fruit, especially the xanthone packed pericarp, is being used traditionally to treat a variety of health disorders. Due to its easy isolation, α -Mangostin has been successfully used in traditional medicine [9]. The purpose of the present study was to investigate the role of α -Mangostin in the transcriptional and translational regulation of proinflammatory genes like COX-2 and iNOS.

II. MATERIALS

α -Mangostin (98-99.99% HPLC purified, ChromaDex, LGC Promochem India Pvt Ltd); MDA-MB-231 cell line (National Centre for Cell Science); DMEM, FBS, Trizol (GIBCO Laboratories); DMSO (HiMedia Laboratories); Griess reagent, LPS, COX-2, iNOS, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Primers (MWG Company); RT-PCR reagents (Promega); RT-PCR kit (Stratagene); anti-COX-1, anti-COX-2 and anti-iNOS (Caymen Chemicals); mouse anti-GAPDH (Santa Cruz Biotechnology); ECL kit (Millipore) and the other chemicals used in present study were of analytical grade.

III. METHODOLOGY

3.1 Cell culture and treatments

MDA-MB-231 human breast adenocarcinoma cell line was routinely maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2mM/L glutamine, 10% fetal bovine serum (FBS) and 10µg/ml of ciprofloxacin in a 5% CO₂ and 95% air incubator at 37°C [10]. The cultured MDA-MB-231 cells were treated with different concentrations of α-Mangostin (2.5, 5, 10, 15 and 20µM) and 0.1% DMSO (control) for 30min and then stimulated with LPS (1µg/ml) for 2h and 12h at 37°C for the expression of COX-2 and iNOS respectively.

3.2 Detection of COX-2 and iNOS expression in LPS stimulated MDA-MB-231 cells by Immunocytochemistry

The effect of α-Mangostin on the expression of COX-2 and iNOS protein in LPS (1µg/ml) stimulated MDA-MB-231 cells was detected by Immunocytochemistry [11]. In brief, 2x10⁵ cells / well were grown in a 6-well plate and incubated for 24h either with 10µM α-Mangostin or 0.1% DMSO (control). The treated cells were fixed, permeabilized and blocked with 0.5ml of 1% BSA for 30min. After rinsing with PBST, the cells were incubated with the primary antibody (1:500) anti-COX-2 and anti-iNOS and incubated with the secondary antibody (1:5000) for 30min and with peroxidase substrate 3, 3'-diaminobenzidine (DAB) for 5min. The cells were washed and counterstained with hematoxylin for 5min at room temperature. After counterstaining the cells were washed and covered with DPX mounting solution, cover slipped and observed under light microscopy.

3.3 Analyzing the role of α-Mangostin on the transcriptional regulation of COX-2 and iNOS genes

The effect of α-Mangostin on the mRNA profile of COX-2 and iNOS was determined by performing semi quantitative RT-PCR. The extracted RNA (1µg) was incubated for 50 minutes at 42°C with 2.5U/µL MMLV reverse transcriptase, 2.5µM random hexamers, 1mM dNTPs, 5mM MgCl₂, and 1U RNase inhibitor in a volume of 10µl for RT. The RT reaction was followed by PCR in a 25µl reaction containing 1µl RT product as template, 0.3mM dNTPs, 1.5mM MgCl₂, 40mM KCl, 50mM Tris-HCl (pH 8.3), 1U Taq DNA polymerase and 25pmol of each primers (iNOS, or COX-2 and GAPDH as an internal control). The mix was allowed to undergo PCR with temperature profile of initial denaturation at 94°C for 5min and 30 cycles of denaturation at 94°C for 30s, annealing for 45s, extension at 72°C for 1min and final elongation at 72°C for 5min. The annealing temperatures were varied depending on the gene, the primer sequences and their annealing temperatures were tabulated in Table 1 [12]. After amplification, PCR products (10µl of each sample) were subjected to electrophoresis on 1.5% agarose gels in 1x TAE buffer and visualized by ethidium bromide (0.5µg/ml) staining.

TABLE 1. THE GENE SPECIFIC PRIMER SEQUENCES AND THEIR ANNEALING TEMPERATURES

Gene	Primer sequences (5' to 3')		Annealing temperature
	Forward	Reverse	
COX-2	TTCAAATGAGATTGTGGGAAAATTGCT	AGATCATCTCTGCTGAGTATCTT	60°C
iNOS	ATGCCAGATGGCAGCATCAGA	ACTTCCTCCAGGATGTTGTA	57°C
GAPDH	GTGAAGGTCGGAGTCAAC	TGGAATTGCCATGGGTG	53°C

3.4 Quantification of nitric oxide production in LPS stimulated MDA-MB-231 cells

Nitric oxide production in culture media supernatant was assayed by measuring the stable degradation product of nitric oxide (NO), nitrite, using the Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) in α-Mangostin treated MDA-MB-231 cells [13, 14]. The treated cells were stimulated with LPS (1µg/ml) for 12h. After treatment with LPS, the supernatants from culture media were isolated and mixed with an equal volume of Griess reagent and incubated at room temperature for 15min. The absorbance was measured at 550nm and the nitrite concentration was determined using sodium nitrite (10-100µM) as a standard. 3.5 Impact of α-Mangostin on the expression of COX-2 and iNOS proteins The effect of α-Mangostin on the expression of COX-2 and iNOS proteins was determined by performing Western blot analysis [14]. The treated cells were washed with PBS (pH 7.4) and lysed in 100µl of cell lysis buffer (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1% sodium dodecyl sulfate (SDS), 10% glycerol, 5mM DTT, 1mM PMSF) on ice for 30min. Later, the lysates were subjected to centrifugation for 30min. at

10,000rpm. The supernatants were collected and the concentration of proteins was estimated [15]. The protein lysates (20µg) were separated on 10% SDS Polyacrylamide. The resolved protein bands were transblotted onto nitrocellulose membranes and probed with the primary antibodies (1:1000) anti-COX-1, anti-COX-2 and anti-iNOS overnight at 4°C. Blots were then washed with TBST and subsequently probed for 1h at room temperature with secondary antibody, anti-rabbit IgG (1:10,000). GAPDH was used as an internal standard for protein loading of cytoplasmic extracts. The bands were visualized by using an enhancer chemiluminescence (ECL) assay kit.

IV. RESULTS AND DISCUSSION

The effect of α -Mangostin on LPS induced expression of COX-2 and iNOS was detected in highly invasive breast cancer MDA-MB-231 cells. The cells exposed to α -Mangostin (10µM) were restimulated with LPS (1µg/ml) for 2h and 12h for the expression of COX-2 and iNOS respectively and analyzed by Immunocytochemistry. The induced expression of COX-2 and iNOS was observed in control (0.1% DMSO) cells (Fig. 1a) in contrast to α -Mangostin (10µM) treated cells (Fig. 1b). The over-expression of iNOS was seen at 12h of LPS stimulation in control cells (Fig. 2a) compared to α -Mangostin (10µM) treated cells (Fig. 2b). The molecular evidence for α -Mangostin's potential role as anti-inflammatory and anti-cancer agent was examined by performing RT-PCR and Western blotting analysis. The MDA-MB-231 cells were pre-treated with different concentrations (2.5 to 20µM) of α -Mangostin and with 0.1% DMSO (Control). Then, cells were stimulated with LPS (1µg/ml) for 2h and 12h correspondingly for the expression of COX-2 and iNOS mRNA. The total RNA was extracted and cDNA was synthesized by using relevant primers. The amplified PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. As shown in Fig. 3, the levels of COX-2 and iNOS cDNA significantly decreased in a dose-dependent manner compared to cDNA of GAPDH in α -Mangostin treated MDA-MB-231 cells at 12h of LPS stimulation. Moreover, α -Mangostin did not change the expression level of COX-1 in LPS stimulated MDA-MB-231 cells. COX-1 is expressed constitutively in most tissues and seems to be responsible for housekeeping role for normal physiological functions including maintenance of the integrity of the gastric mucosa and regulation of renal blood flow [16]. The γ -Mangostin potently inhibited LPS induced expression of COX-2 protein and mRNA but not the constitutive expression of COX-1 in C6 rat glioma cells [17]. The concentration of nitrite, a marker of nitric oxide production, was estimated by Griess assay in culture supernatants of MDA-MB-231 cells pre-treated with α -Mangostin. As shown in Fig. 4, the levels of nitrite concentration reduced dose-dependently by α -Mangostin in comparison with control.

The impact of α -Mangostin on the expression of COX-2 and iNOS proteins in LPS stimulated MDA-MB-231 cells was investigated by Western blot analysis. The results showed the induction of COX-2 and iNOS protein in MDA-MB-231 cells after LPS (1µg/ml) stimulation for 2h and 12h respectively. This expression was markedly attenuated in cells pre-treated with α -Mangostin (10-20µM). The concentration-dependent inhibition was observed in COX-2 expression but it was not noticed in the constitutive COX-1 protein expression in α -Mangostin treated cells (Fig. 5A & B). Similarly, the expression of iNOS protein was also inhibited with increasing concentrations of α -Mangostin (10-20µM) in contrast with GAPDH protein expression (Fig. 6A & B). These findings suggest that the inhibition of LPS induced pro-inflammatory genes (COX-2 and iNOS) expression in MDA-MB-231 cells by α -Mangostin was likely to be mediated at the level of transcription.

In another study, α - and γ -Mangostins, significantly inhibited NO and PGE₂ production from LPS stimulated RAW 264.7 cells and possessed anti-inflammatory effects [18]. Gambogic acid is a polyprenylated xanthone of *G. hanburyi*. The pre-treatment of LPS (1µg/ml) stimulated RAW 264.7 cells with Gambogic acid reduced the expression of COX-2 and iNOS after 2h and 12h respectively. The inhibition in the amount of nitrite production was observed after 12h of LPS stimulation in RAW 264.7 cells [14]. As the expression of both iNOS and COX-2 is induced by the same pro-inflammatory agents and is associated with inflammatory conditions, it has been proposed that inhibition of both iNOS and COX-2 would provide the most potent anti-inflammatory effect [19].

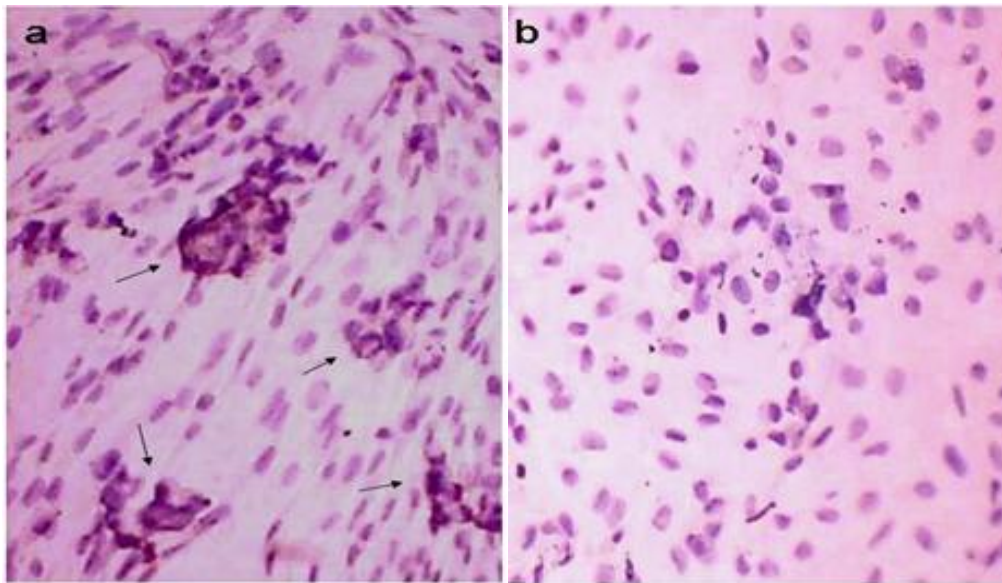


Figure1. Immunocytochemical analysis for detecting COX-2 expression in LPS stimulated MDA-MB-231 cells. a) Control cells and b) 10µMof α -Mangostin treated Pictures were taken with a 10x objective under light microscope.

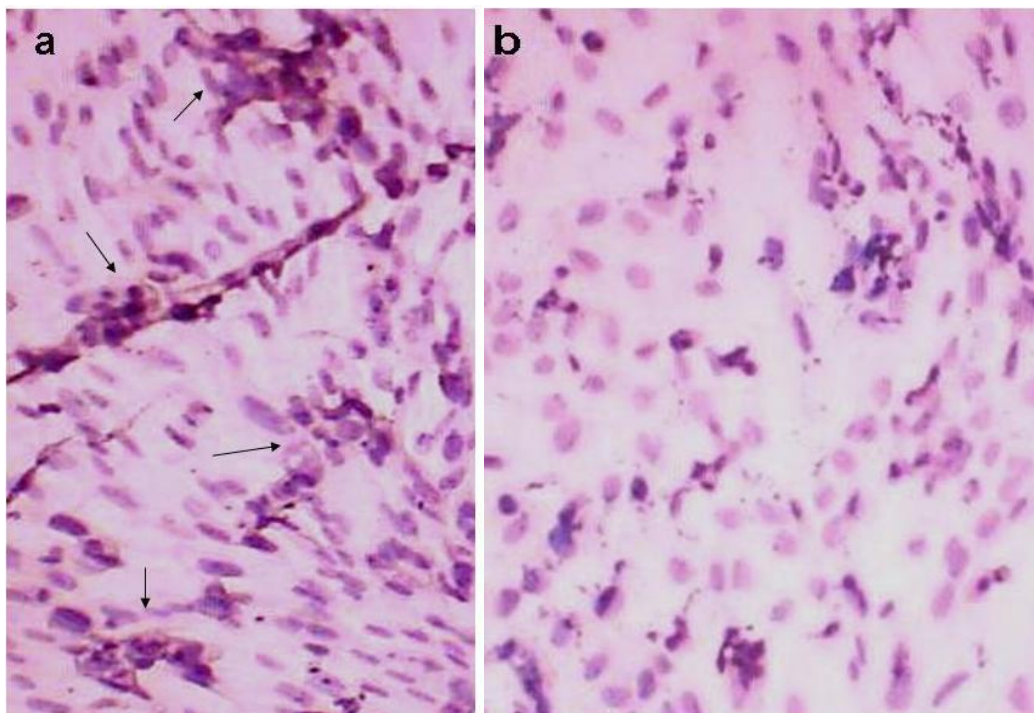


Figure2.Immunocytochemical analysis for detecting iNOS expression in LPS stimulated MDA-MB-231 cells. a) Control cells and b) 10µMof α -Mangostin treated cells. Pictures were taken with a 10x objective under light microscope

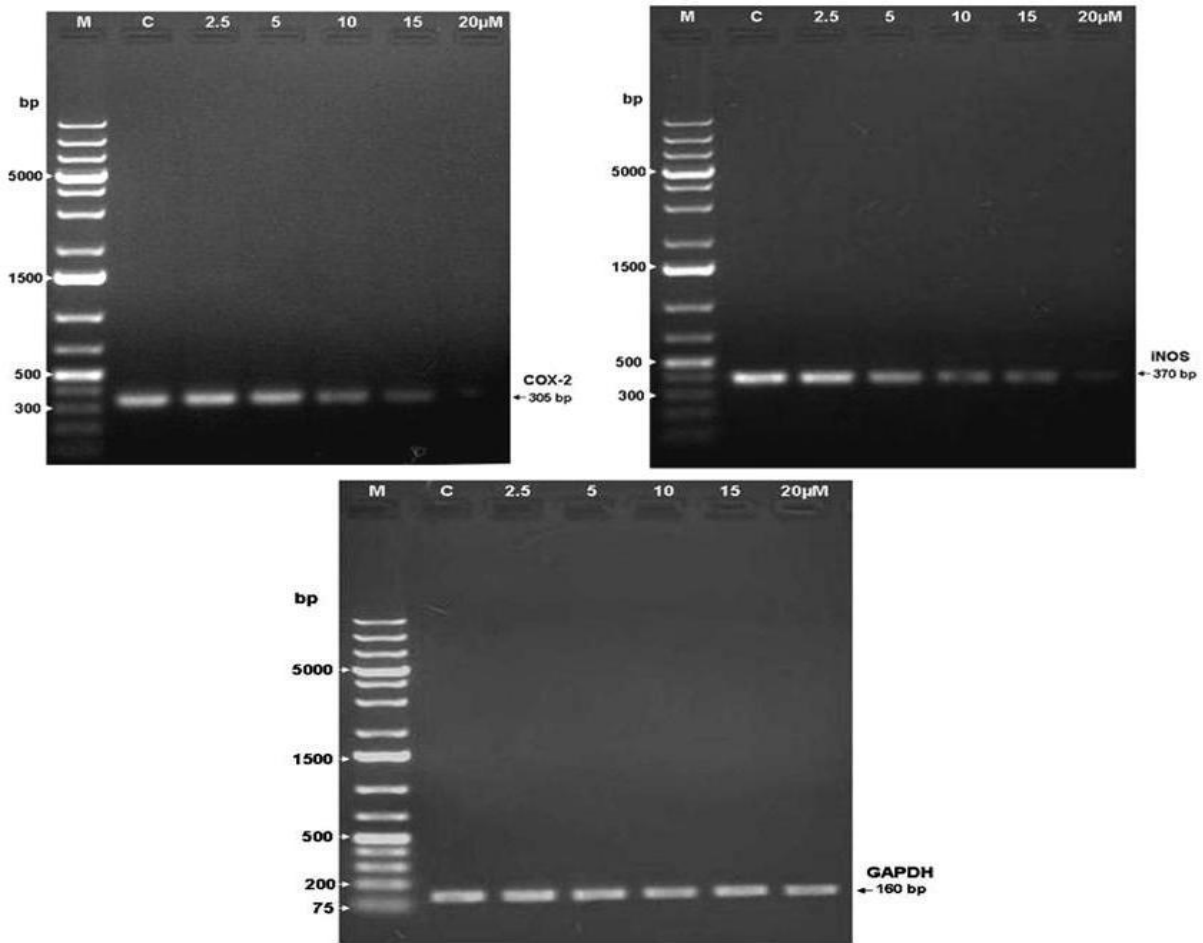


Figure3. The effect of α -Mangostin on the expression of COX-2 and iNOS genes in LPS-stimulated MDA-MB-231 cells determined by RT-PCR. Cells were pre-treated with the different concentrations (2.5 to 20 μ M) of α -Mangostin and with 0.1% DMSO (C). Then stimulated with LPS (1 μ g/ml) for 2h and 12h for the expression of COX-2 and iNOS respectively and total RNA from MDA-MB-231 cells was used as a template for cDNA synthesis and then subjected to PCR. The amplified PCR products were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. The cDNA of COX-2 (305bp) and iNOS (370bp) were detected and found that the expression levels were decreased with increasing concentrations of α -Mangostin. The GAPDH was used as the loading control. Data shown are the representative expression patterns from triplicate independent experiments.

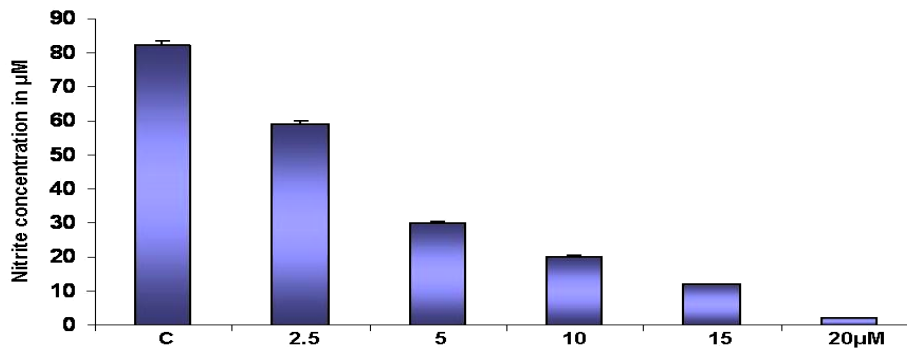


Figure4. The nitrite concentration in LPS-stimulated MDA-MB-231 cells was measured by Griess assay. The cells were pre-treated with different concentrations (2.5, 5, 10, 15 and 20 μ M) of α -Mangostin and with 0.1% DMSO (C), then stimulated with LPS (1 μ g/ml) for 12h. The nitrite levels were expressed as mean \pm SEM from triplicate determinants.

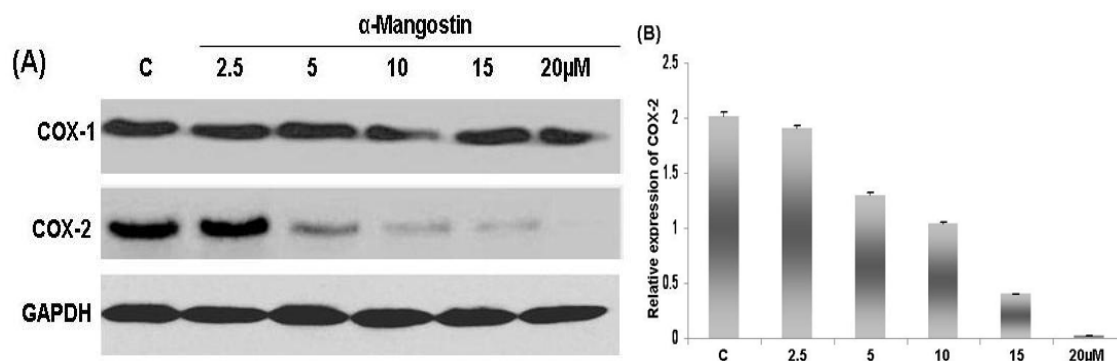


Figure 5. (A) The effect of α -Mangostin on the expression of COX-1 and COX-2 proteins in LPS stimulated MDA-MB-231 cells was assessed by Western blotting. After pre-treatment with the indicated concentrations of α -Mangostin and with 0.1% DMSO (C), cells were stimulated with LPS (1 μ g/ml) for 2h. Equal loading was confirmed by stripping the blot and reprobing it for GAPDH. Data shown are representative expression patterns from triplicate independent experiments.

(B) The densitometric data of COX-2 protein expression representing the relative density of the Western blot bands normalized to GAPDH.

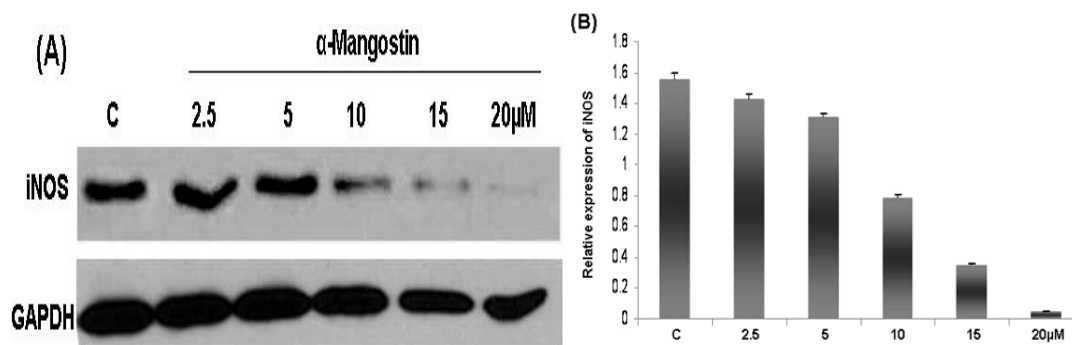


Figure 6. (A) The effect of α -Mangostin on the expression of iNOS in LPS stimulated MDA-MB-231 cells was assessed by Western blotting. After pre-treatment with the indicated concentrations of α -Mangostin and with 0.1% DMSO (C), cells were stimulated with LPS (1 μ g/ml) for 12h. Equal loading was confirmed by stripping the blot and reprobing it for GAPDH. Data shown are representative expression patterns from triplicate independent experiments.

(B) The densitometric data of iNOS protein expression representing the relative density of the Western blot bands normalized to GAPDH.

V. CONCLUSION

α -Mangostin (10-20 μ M) reduced the LPS (1 μ g/ml) induced expression of pro-inflammatory genes COX-2 and iNOS in MDA-MB-231 cells at the mRNA level as determined by RT-PCR. Similarly, the protein expression was decreased in concentration-dependent manner, as determined by Western blotting. α -Mangostin (10-20 μ M) down-regulated the LPS induced expression of COX-2 and iNOS genes in MDA-MB-231 cells, indicating the potential role of α -Mangostin both at transcriptional and translational level. In conclusion, our results implicate that α -Mangostin shows great potential as anti-inflammatory agent and may be utilized as an alternative herbal medicine to combat with chronic inflammatory diseases.

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