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Aqueous extract of *Mangifera indica* inhibits LPS induced inflammatory effects in RAW 264.7 macrophage cell

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ABSTRACT

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are involved in various pathophysiological processes such as inflammation and carcinogenesis. As part of our search for anti-inflammatory activities of plant extracts, we examined the inhibitory effect of *Mangifera indica* (MI) on iNOS and cox-2 expression, in Lipopolysaccharide (LPS) (0.1 µg/ml) treated macrophage RAW 264.7 cells. The water extract of MI dose dependently inhibited NO production in LPS activated Raw 264.7 macrophage cell. MI reduced LPS induced mRNA expression of iNOS, COX-2, tumor necrosis factor (TNF-α), IL (interlukin)-1β, and GM-CSF (Granulocyte monocyte-colony stimulating factor) in concentration-dependent manner, as determined by RT-PCR. These data indicate that MI may have an anti-inflammatory property as evidenced from its inhibition of iNOS, COX-2, TNF-α, GM-CSF and IL-1β gene expression. However, more detailed studies using additional models are necessary to further characterize the effects of MI in inflammatory disorders.

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INTRODUCTION

Tropical country owing to its favorable climatic influences has been blessed with immense natural resources including explored and unexplored herbal medicinal plants. Recently, 546 species have been identified as having medicinal properties and therapeutic use (Ghani, 1998). Medicinal plants are the great importance to the health of individual and communities. The medicinal values of those plants possess some chemical active substances that produce a definite physiological action on the human body and animal health. The most important bioactive substances are alkaloid, tannin, flavonoid and phenolic compounds (Edeogra, 2005). Therefore, the present research has been designed to investigate anti-inflammatory effects of leaves extract of *Mangifera indica* (Mango) available in tropical countries.

In inflammatory processes, large amount of the pro-inflammatory mediators, such as, nitric oxide (NO) and prostaglandin E2 (PGE2) are generated by the inducible isoform NO synthase (iNOS) and cyclooxygenase-2 (COX-2) (Posadas et al., 2000). NO is a short lived messenger molecule synthesized from L-arginine by a family of enzyme known as nitric oxide synthases (NOS). There are three isoform of NOS: endothelial NOS, (eNOS), neural NOS (nNOS) and inducible NOS (iNOS). The inducible NOS is expressed in response to pro-inflammatory cytokines and lipopolysaccharides (Geller, 1993; Sunyer,1996). Prostaglandins produced from arachidonic acid by cyclooxygenases .There are two isoforms of cyclooxygenase: cyclooxygenase-1(cox-1) and cyclooxygenase-2 (cox-2). Constitutively present cox-1, which protects gastrointestinal mucosa and inducible isoform is cox-2, which mediates acute and chronic inflammation, pain, and cellular repair mechanism (Liang et al; 1999**)**. Tumor necrosis factor-α (TNF-α) is an inflammatory cytokines, which is produced by various cells including macrophages, lymphocytes, neutrophil and mast cells. TNF-α is responsible for the production of apoptosis and development of humoral immune response. Among that, the high concentration of TNF-α create some negative effect, such as tissue injury and potentiation of septic shock (D. Wallach, 1999; E. Shohami, 1999; R. Ettinger, 1998). Interleukin-6 is multifunctional cytokines that play important roles in host defense, acute phase reaction, immune responses, nerve cell functions and hematopoiesis (M. Hibi, 1996; T. Hirano, 1998; J. Van Snick, 1990; T. Taga , 1997; T. Hirano, 1994).

Macrophage generates inflammatory process by the release of chemokines (e.g. GM-CSF) and cytokines (e.g. TNF-α, IL-1β, and IL-6). There are several inflammatory stimuli such as lipopolysaccharides (LPS) and pro-inflammatory cytokines that stimulate immune cells to up-regulate such inflammatory states **(**Gallucci et al., 1998) so that , they are important in exploring molecular mechanism of action for the subsequent development of new potential anti-inflammatory drugs.
In this study, we attempted to determine the anti-inflammatory activity of MI in inflammation using an in vitro model system of mouse macrophage cell (RAW 264.7) line induced with LPS.

Materials and Methods

Reagents

Dimethylsulfoxide (DMSO), LPS, Griess's reagent, was obtained from the Sigma Co (St. Louis, MO). All other reagents were of the first grade.

Preparation of Extract

Mangifera indica (MI) stem bark was collected from market. The stem bark extract of *M. indica* was prepared by decoction for 1 h in accordance to the method of (Garrido et al. 2004) as described earlier (Dhananjaya et al., 2011).

Cell culture

RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin, and 5 % FBS. Cells were grown at 37°C and 5% CO_2 in humidified air. Cells were incubated with. *MI* at different concentrations (25, 50,100, 200 µg/ml) or positive chemical and stimulated with LPS 0.1µg/ml for 18hrs.

Cell Viability test

A cell proliferation assay was also performed to exclude the possibility that the observed NO inhibition was false positive due to the cytotoxic effects. Cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously (Hong et al., 2003**)**. All experiments for the measurement of nitric oxide inhibition were conducted three times, each time with three independent observations and the results were averaged.

Measurement of nitrite

In order to determine the concentration of NO, nitrite (NO_2^-) was measured using the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid), as described previously (Hong et al., 2003). After preincubation of the RAW 264.7 cells (1X10^{6} cells/ml) for 24 h, the cells were incubated with 25, 50, 100 or 200 µg/ml of *water* extracts of *MI* with LPS (0.1 µg/ml) for 18 h. One-hundred µl of supernatant from each well of the cell culture plates were transferred into 96-well micro plates, and the supernatant was then mixed with equal volume of Griess reagent at room temperature. The absorbance at 550 nm was determined in a Spectramax 250 micro plate reader. The concentrations of nitrite were calculated from regression analysis using serial dilutions of sodium nitrite as a standard. The percentage inhibition was calculated based on the ability of extracts to inhibit NO formation by cells compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as 0% inhibition.

Extraction of total RNA and Semiquantitative RT-PCR

Total RNA from the RAW264.7 cells were prepared by adding Easy blue Reagent (iNtRON Biotechnology Co. Korea), according to the manufacturer's instructions. The total RNA solution was stored at -70℃ until used.

Semi quantitative RT reactions were carried out using RT premix (Bioneer Co. Korea). Briefly, total RNA (2 µg) were incubated with oligo-dT_{18} at 70℃ for 5 minutes and cooled on ice for 3 minutes, and for 90 minutes after the addition of RT premix at 42.5℃. The reactions were terminated at 95℃ for 5 minutes for the inactivation of reverse transcriptase. The PCR reaction was further conducted using the PCR premix (Bioneer Co. Korea) with the appropriate sense and antisense primers for Glyseraldehyde-3-phosphate dehydrogenas (GAPDH, sense primer, 5'-CAC TCA CGG CAA ATT CAA CGG C-3'; antisense primer, 5'-CCT TGG CAG CAC CAG TGG ATG CAG G-3'), iNOS (sense primer, 5'- CCC TTC CGA AGT TTC TGG CAG CAG C-3'; antisense primer, 5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'), COX-2 (sense primer, 5'- CAC TAC ATC CTG ACC CAC TT -3', antisense primer, 5'- ATG CTC CTG CTT GAG TAT GT -3'), interleukin (IL)-1β (sense primer, 5'- CAG GAT GAG GAC ATG AGC ACC-3'; antisense primer, 5'- CTC TGC AGA CTC AAA CTC CAC-3'), interleukin (IL)-6 (sense primer, 5'-GTA CTC CAG AAG ACC AGA GG-3'; antisense primer, 5'-TGC TGG TGA CAA CCA CGG CC-3'), tumor necrosis factor (TNF)- (sense primer, 5'-TTG ACC TCA GCG CTG AGT TG -3'; antisense primer, 5'- CCT GTA GCC CAC GTC GTA GC-3'), and GM-CSF (sense primer, 5'- AGG ATG TGG CTG CAG AAT TTA CTT TTC -3'; antisense primer, 5'- TCA TTT TTG GAC TGG TTT TTT GCA TTC -3') under incubation conditions (a 45-second denaturation time at 94℃, an annealing time of 45 seconds at 55 to 60℃, an extension time of 45 seconds at 72℃, and final extension of 10 minutes at 72℃ at the end of the cycles. The PCR products were separated in 1% agarose using electrophoresis of BioRad Co. The relative intensities were calculated using Eagle eyes image analysis software (Stratagene Co. La Jolla). The resulting densities of the iNOS, COX-2, IL-1, TNF-α, and GM-CSF bands were expressed relative to the corresponding densities of the GAPDH bands from the same RNA sample. GAPDH, a housekeeping gene, was used as RNA internal standard.

Statistical analysis: Data were analyzed by a one-way analysis of variance, followed by a post-hoc Dunnett's test. All data are presented as means ± S.E.M.

Result

Effects of MI **on LPS-induced NO production and cell viability**

To analyze the potential anti-inflammatory properties of MI, we used Raw 264.7 macrophage cells, which can produced NO upon stimulation with LPS (0.1ug/ml). Cell were pre-incubated with MI for 1 hour and then stimulated with LPS (0.1ug/ml) for 18 hours. Both LPS and sample were not treated in Control group. After cell culture media were collected, nitrite levels were determined, and MI was found to reduced NO production in a dose dependent manner (Fig-1).Therefore ,these result suggest that inhibition of LPS induced NO production is due to iNOS gene .The potential cytotoxicity of MI was evaluated by MTT assay after incubating cells for 24 hrs in absence or presence of LPS, but cell viabilities were not affected at a concentrations used (with 25, 50, 100 or 200 µg/ml) to inhibit NO

production(Fig-2). Thus, the inhibitory effects were not attributable to cytotoxic effects.

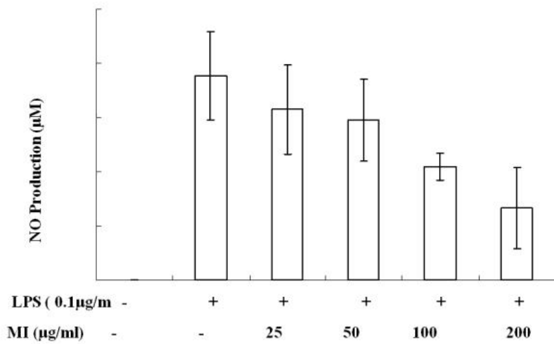


Figure: 1. Effect of MI on the NO production of RAW cells. RAW 264.7 cells were incubated with various concentration of MI and LPS (0.1 µg/ml) for 18 hours. NO production was determined with Griess's reagents. Each value is the means ± SEM of the three independent experiments performed in triplicate.

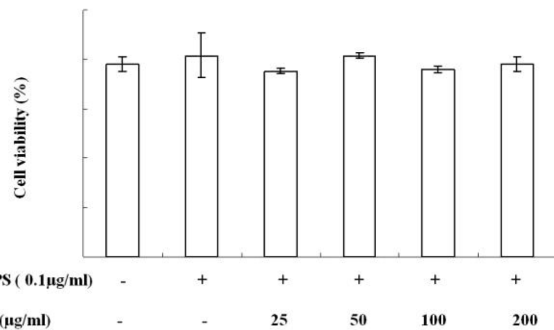
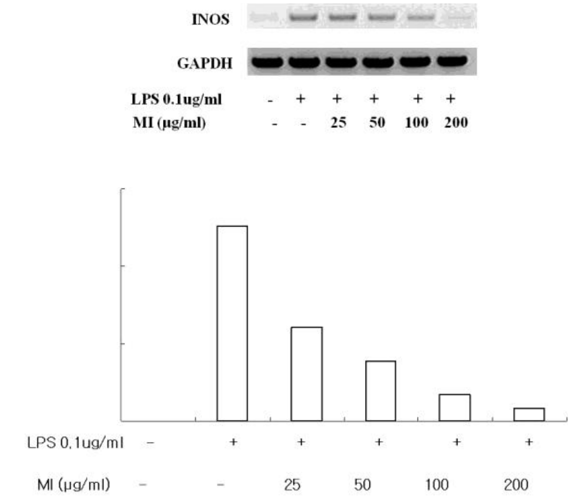


Figure-2 The effects of MI extract on LPS (0.1ug/ml)-induced iNOS , COX-2 mRNA expression. RAW 264.7 cells were treated with LPS (0.1ug/ml) in the presence of various concentration of MI extract for 18 hours. (A)Level of inos mRNA were determined by RT-PCR analysis. GAPDH mRNA was used as an internal control. (B) Ratio of GAPDH and iNOS expression. The figures present the representative results from three separate experiments, which give similar results. (2C) Level of COX-2 mRNA was determined by RT-PCR analysis. GAPDH mRNA was used as an internal control (2D) Ratio of GAPDH and COX-2 expression.

Effect of MI on LPS-induced iNOS and COX-2 mRNA expression

RT-PCR analyses were performed to determine whether the inhibitory effects of MI on the pro-inflammatory mediators (NO, PGE2) were related to modulation of the expression of iNOS and COX-2. In un stimulated Raw 264.7 cells, iNOS and Cox-2 mRNA expression were not detected, but LPS remarkably up regulated their mRNA gene expression levels and pretreatment with MI inhibited these up-regulation concentration dependent manner (Fig-3A, and 3B).The pro-inflammatory cytokines, prostaglandins and NO generated through stimulated macrophages play critical roles in inflammatory disease such as sepsis and arthritis (Szabo, 1998; Martel-Pelletier, 2003**)**. Therefore, inhibition of pro-inflammatory cytokines or iNOS and COX-2 expressed in inflammatory cells, offers us a new therapeutic strategy for the treatment of inflammation. In this study, it was found that MI inhibits iNOS and COX-2 gene expression in mouse macrophages cells and it probably act at transcriptional level, as evidenced by dose dependent reduction in their mRNA level. Inhibition of the LPS induced expression of these molecules in RAW 264.7 cells by MI was not due to MI cytotoxicity which was assayed by MTT assay. In general, these results indicate that the inhibitory effects of MI on LPS induced NO and PGE2 production are caused by iNOS and COX-2 suppression.



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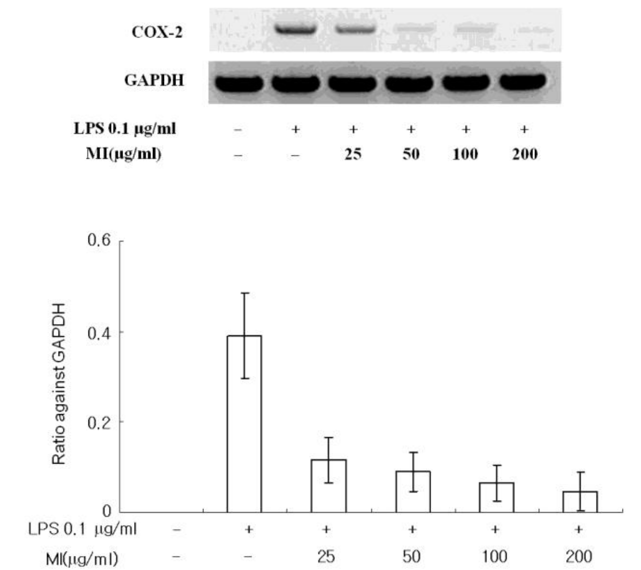

B

Figure-3 The effects of MI extract on the cytotoxicity of RAW 264.7 cells. RAW cells were incubated with either *MI* and LPS or LPS (0.1µg/ml) for 24 hrs. The determination of cell viability was performed using MTT assay, as described in Materials and Methods. Each value is the means ± SEM of the three independent experiments performed in triplicate.

Effects of MI on others pro-inflammatory cytokines

The present study demonstrated that water extract of MI has inhibitory effect on the expression of TNF-α, IL-1β, and GM-CSF, in LPS (0.1µg/ml) stimulated Raw 264.7 macrophage cell in dose dependent fashion. On the other hand MI was not inhibited IL-6 mRNA in LPS induced Raw 264.7 macrophages cell.

Discussion

At adequate concentrations, NO can generate or modify intracellular signals, there by affecting the function of immune cells, as well as tumor cells and resident cells of different tissue and organs. However, its uncontrolled release can cause inflammatory destruction of target tissue during an infection (MacMicking,1997; Bogdan,2001; Miljkovic, 2004) . The modulation of iNOS-mediated NO release is one of the major contributing factors during the inflammatory process (Kim et at., 2004). Therefore, agents with the ability to inhibit iNOS expression are potentially beneficial in the treatment of conditions associated with an overproduction of NO, including septic shock and inflammation (Hobbs et al., 1999). In our study, MI decreased NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner, and reduced mRNA expression levels in RAW264.7 cells, which indicates that the inhibition of NO production by MI was a result of the inhibition of iNOS gene expression.

PGE2 is another proinflammatory mediator involved in inflammatory responses and it is generated by the sequential metabolism of arachidonic acid by cyclooxygenase (COX) (Tannenbaum., 1996). COX exists in two isoforms: COX-1 and COX-2. COX-1 is a constitutively expressed enzyme with general housekeeping functions. COX-2 is an inducible isoform of cyclooxygenase, and its most important role is in inflammation (Vane, 1998; Lim, 2004), and it is responsible for proinflammatory PGE2 production. Similar to iNOS inhibition, we found that MI also suppressed LPS-induced mRNA expression levels in RAW264.7 cells.
It has been reported that, the pro-inflammatory cytokines, such as GM-CSF, TNF-α, IL-1β, and IL-6 have an significant role in inflammatory processes (Franzen, 2004; Blanco, 2008; Chen, 2008). The IL-6 family, IL-6, IL-11 and oncostain M, shows pro-inflammatory and anti-inflammatory effects. Also IL-6 family plays a vital role in innate and adaptive immune response (Blanco et al., 2008). IL-1β is crucial for chronic inflammatory disease (Dayer ,2004; Blanco, 2008). Hemopoietic pro-inflammatory cytokine, GM-CSF, was used in various hematological disorders for proliferation and differentiation of neutrophilic, eosiniphilic, and monocytic lineages.

TNF-α is a cytokine possessing both growth stimulating as well as inhibitory properties and is mainly secreted by the macrophages, though others, like mast cells, adipose tissue, and fibroblasts also secrete TNF-α. It is involved in a wide variety of cellular reactions like proliferation, differentiation, differentiation, apoptosis and inflammation (Ackermann et al., 2007). During inflammation, it triggers a cascade of cytokines responsible for increased mobilization of macrophages to the site of inflammation and also increases vascular permeability. In our study, addition of LPS increased the production of TNF-α but the addition of MI caused a modest attenuation of LPS induced TNF-α production (Fig-4).

In conclusion, we found that water extract of MI inhibits the NO production and the mRNA expression of iNOS, COX-2, TNF-α, IL-1β, and GMCSF cytokines in LPS stimulated RAW 264.7 cells. From these result it can be suggested that water extract of MI may be useful for the treatment of inflammatory disease

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