

Bioactive Extract from *Moringa oleifera* Inhibits the Pro-inflammatory Mediators in Lipopolysaccharide Stimulated Macrophages

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ABSTRACT

Introduction: Inflammation is a well-known physiological response to protect the body against infection and restore tissue injury. Nevertheless, the chronic inflammation can trigger various inflammatory associated diseases/disorder. *Moringa oleifera* is a widely grown plant in most tropical countries and it has been recognized traditionally for several medicinal benefits. **Objectives:** The objective of this study was to investigate the anti-inflammatory properties of *M. oleifera* extract on lipopolysaccharide (LPS) - stimulated macrophages. **Materials and Methods:** The anti-inflammatory effect of *M. oleifera* hydroethanolic bioactive leaves extracts was evaluated by assessing the inhibition of nitric oxide (NO) production during Griess reaction and the expression of pro-inflammatory mediators in macrophages. **Results:** Interestingly, we found that *M. oleifera* hydroethanolic bioactive leaves extract significantly inhibited the secretion of NO production and other inflammatory markers such as prostaglandin E₂, tumor necrosis factor alpha, interleukin (IL)-6, and IL-1 β . Meanwhile, the bioactive extract has induced the production of IL-10 in a dose-dependent manner. In addition, *M. oleifera* hydroethanolic bioactive leaves extract effectively suppressed the protein expression of inflammatory markers inducible NO synthase, cyclooxygenase-2, and nuclear factor kappa-light-chain-enhancer of activated B-cells p65 in LPS-induced RAW264.7 macrophages in a dose-dependent manner. **Conclusion:** These findings support the traditional use of *M. oleifera* plant as an effective treatment for inflammation associated diseases/disorders.

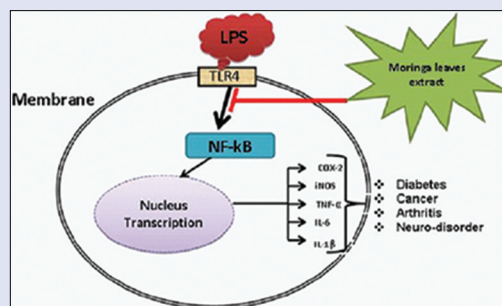
Key words: Anti-inflammatory, lipopolysaccharide, *Moringa oleifera*, pro-inflammatory, RAW264.7 murine macrophage

SUMMARY

- Hydroethanolic extracts of *Moringa oleifera* effectively inhibit the NO produc-

tion in LPS induced inflammatory model.

- M.oleifera* crude extracts successfully modulate the production of pro-inflammatory mediators in LPS stimulated macrophages.
- M.oleifera* extracts suppressed the expression of inflammatory mediators in LPS stimulated macrophages.



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INTRODUCTION

Inflammation is a protective mechanism of organisms to defense against harmful stimuli.^[1,2] It involves various molecular pathways with a wide variety of physiological processes.^[3] However, up-regulated inflammation can lead to many diseases such as cancer,^[4] asthma, allergic rhinitis, atopic dermatitis, and rheumatoid arthritis.^[1] In the United States, inflammatory diseases were reported with the high prevalence among their population.^[5] Around 46.4 million individuals were diagnosed with arthritis; 1.3 million adults and 294,000 children were reported for rheumatoid arthritis and juvenile arthritis, respectively.^[6] Inflammation process of cellular dysfunction can be induced through microbial stimulus; lipopolysaccharide (LPS) is a common prototypical endotoxin can directly activate macrophages. Monitoring evidence have been recommended that activated macrophage produce a higher amount of inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin (IL)-6, and other inflammatory mediators.^[1] Macrophages produced by bone marrow cells are directed to different body organs and tissues such as lung and liver are

closely connected with tissue homeostasis; moreover, it performed crucial role within the natural and acquired immune system. The macrophage is the foremost cell in the inflammatory process and exerts two opposite impacts, stimulatory and inhibitory effects on inflammation process, which include phagocytic effect based on the nature and intense of the stimulus. Macrophages, derived from blood (monocytes), are actively

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involved in tissue inflammation site against noxious insult; they are capable to perform a wide array of function. Inflammatory macrophages have the ability to kill through apoptosis, clearance of proliferating resident stromal, infiltrating leukocytes, and parenchymal cells.^[7]

The World Health Organization has estimated that 80% of the population in the developing countries use traditional medicine for their primary health care needs and the majority of this therapy requires the use of herbal extract and their active components.^[8] Almost all modern drugs are produced originally through traditional herbal resources. These have progressed to develop the conventional medicines that use both isolated natural compounds and synthetic drugs. Herbal supplements are popular and their formulations have led to the several novel generation of phytomedicines that are more effective than before.^[9] Development of efficient nonsteroidal anti-inflammatory drugs (NSAIDs) with minimal or no gastrointestinal (GI) side effects is an area of interest in drug discovery industry. The use of most commonly recommended drug from analgesics such as aspirin currently have been restricted because of their potential side effects such as severe gastric disorders. Lesser gastrointestinal side effects seen with Cyclooxygenase -2 inhibitors are resolved at a slow phase.^[10-12] Furthermore, an enhancement of cardiovascular and cerebrovascular events, specifically in patients with an increased risk of thrombosis has been evaluated in the chronic use of some COX-2-specific inhibitors. Anti-inflammatory drugs which include “biologicals” such as anti-cytokine therapies show a significant decrease in host defense toward the infection.^[3] As a result of the steroidal and NSAID side effects, the researchers started to focus on natural compounds as a keen source of alternative drugs. Numerous herbal medicines pave the way for leading novel therapeutic compounds against inflammation, despite they are inexpensive, safe, highly tolerated, and convenient for numerous patients.^[9,13]

Moringa oleifera Lam (syn. *Moringa pterygosperma* known as “The Miracle Tree,” “Horseradish-tree,” or “Ben oil tree”) is well-known and the most commonly spread species of Moringaceae family. It offers a remarkable range of medicinal application due to its possession of numerous nutritional/therapeutic candidates. *M. oleifera* grows through the diverse part of the world Asia, Africa, India, Pakistan, Cambodia, Philippines, and America.^[14] Different parts of the plant extracts and derived compounds have been reported with various medicinal properties. The root extract has been demonstrated to have anti-fertility and anti-inflammatory agents. Moreover, the seed possesses anti-inflammatory impact, anti-hypertensive effects, and ability to decrease lipid peroxidation. *Moringa* Leaves extract have been studied for its antioxidant effect through various *in vitro* and *in vivo* approaches and also it has various pharmacological properties such as hypolipidemic, anti-atherosclerotic,^[15] wound healing,^[16] hepatoprotective,^[17-19] antimicrobial, antinociceptive, and radioprotective properties.^[20] These extracts have eliminated the neuropathic pain^[21,22] and protection against oxidative stress in an *in vivo* model of Alzheimer’s disease.^[23] The pods, leaves, and flowers of *M. oleifera* commonly have been consumed as a vegetable in the south of Asia. Since, the whole plant provides nutrition and medicinal value against diabetes, cancer, rheumatoid arthritis, and other ailments; *M. oleifera* is also called as “miracle tree.”^[24] The reason behind the enormous pharmacological activities of *M. oleifera* might possibly due to various secondary plant metabolites present in *M. oleifera* such as flavonoids, phenolics, vitamins, carotenoids, minerals, sterols, amino acids, alkaloids, and glycosides.^[8] *M. oleifera* leaves contains phenolic acids such as quinic acid and chlorogenic acid that exhibit high antioxidant activities^[25,26] and recently β -sitosterol, was isolated from *Moringa* leaves fraction^[27] and it has reported to reduce the intestinal uptake of dietary cholesterol effects.^[28] *M. oleifera* seeds contains antitumor and anti-hypertensive effects of O-ethyl-4-(α -L-rhamnosyloxy) benzyl carbamate and its seven derivatives such as niazirin, niazimicin, and β -sitosterol.^[29] Therefore, the purpose of this study was to screen three different hydroethanolic solvent

gradients of *M. oleifera* bioactive leaves extract for cytotoxicity and NO production. Furthermore, the anti-inflammatory activity of *M. oleifera* hydroethanolic bioactive leaves extract in LPS-stimulated RAW264.7 murine macrophage cells was evaluated by quantifying the expression of various inflammatory markers in comparison with dexamethasone, a known anti-inflammatory compound.

MATERIALS AND METHODS

Chemicals and reagents

The murine macrophage RAW264.7 cell line (ATCC, TIB-71) was examined as an *in vitro* model to investigate the anti-inflammatory properties of *M. oleifera* hydroethanolic extract. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS), penicillin, streptomycin for cell culture were obtained from Nacalai (Kyoto, Japan). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPSs isolated from *Escherichia coli* 0111:B4 were purchased from Sigma–Aldrich Co., (St. Louis, MO, USA). Prostaglandin E₂ (PGE₂) parameter assay kit, TNF- α , IL-6, IL-1 β , and IL-10 mouse ELISA kits were purchased from R and D Systems (Minneapolis, MN, USA). The bicinchoninic acid (BCA) protein assay kit was obtained from Pierce (Thermo scientific, USA). Anti- β -actin–horseradish peroxidase (HRP) and secondary polyclonal antibody-conjugated HRP, were purchased from Santa Cruz (CA, USA). Primary antibodies against inducible nitric oxide synthase (iNOS), COX-2, nuclear factor kappa-light-chain-enhancer of activated B-cells p65 (NF κ B-p65), were purchased from Abcam (Cambridge, MA, USA).

Preparation of plant extract

Fresh and mature *M. oleifera* leaves were collected from Garden-2, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, washed and dried at room temperature for 18 h. Then, the leaves were oven dried at 42°C for 2 consecutive days and grinded them with electronic laboratory blender. Three different hydroethanolic solvent gradients were prepared (ethanol: Distilled water, 50:50 [50%], 70:30 [70%], and 90:10 [90%]), and they were macerated for 3 days at room temperature. The soaked material was stirred every day and filtered through filter paper. Then, the extract obtained from maceration process was concentrated under vacuum on a rotary evaporator at 40°C. The crude extract was freeze-dried, weighed, and stored at –80°C for future use. For each experiment prior to the treatment of cell culture, *M. oleifera* hydroethanolic extract was dissolved in DMEM and sonicated in an ultrasonic bath (Mettler Electronic Corporation, Anaheim, USA) at 25°C.

Cell culture

Murine RAW264.7 macrophage cell line was purchased from ATCC (VA, USA) and cultured in DMEM supplemented with 10% FBS, 1% streptomycin/penicillin at 37°C in a 5% CO₂ incubator. Dexamethasone, which is commonly known as a classic glucocorticosteroid drug and frequently used in clinical practice, was selected as a positive control with a concentration of 0.5 μ g/ml.^[30] RAW264.7 macrophage cells stimulated by LPS (1 μ g/ml) without any other intervention component were considered as a negative control^[31] while cells incubated by medium were considered as normal control.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for cell viability

The effect of *M. oleifera* hydroethanolic bioactive leaves extract on cell viability was measured by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT)

to violet formazan crystals (nonwater-soluble) inside the cell. The cells were cultured in 96-well plates at a density of 10^5 cells/ml (100 μ L/well) and incubated for 24 h. Cells were treated with three different hydroethanolic gradients (50%, 70%, and 90%) with different concentrations through serial dilution (1000–15.62 μ g/ml) and this was followed by 24 h incubation. Then, 20 μ L MTT solution at 37°C was added (5 mg/ml in phosphate-buffered saline) with 4 h incubation. The optical density of formazan in solution was measured by using microplate reader at 570 nm wavelength.

Determination of nitric oxide

The effects of *M. oleifera* hydroethanolic bioactive leaves extract on NO production in RAW264.7 cells were determined based on Griess reaction. In brief, RAW264.7 cells were cultured in 24-well plates at a density of 10^5 /ml and incubated for 24 h followed by 1-h pretreatment with *M. oleifera* hydroethanolic (50%, 70%, and 90%) extract and dexamethasone and co-incubated with LPS for 24 h. Griess reagent (0.1% N-[1-naphthyl] ethylenediamine-HCl and 1% sulfanilamide and 5% H_3PO_4) was added to the supernatant and after 10 min incubation reading by ELISA microplate reader at 540 nm wavelength.

Determination of inflammatory mediator's by ELISA

Cells (1×10^6 cells/ml) were plated into 6-well culture plate and treated with *M. oleifera* 90% hydroethanolic bioactive leaves extract or dexamethasone, then stimulated for 24 h with LPS. In brief, capture antibodies for TNF- α , IL-6, IL-1 β , and IL-10 were coated in 96-well plates by overnight incubation. After the incubation time and washing immune complex of capture antibody, samples/standard, and detection antibody. Finally, plates were detected by reacting with streptavidin horseradish – HRP – tetramethylbenzidine detection system. Reactions were stopped by addition of 2M H_2SO_4 , and absorbance was read at 450 nm by a microplate reader. In addition, PGE₂ production was determined using an ELISA kit (with coated capture antibody) according to the manufacturer's instructions.

Western blot analysis

The cells were cultured in 6-well plates and incubated with *M. oleifera* 90% hydroethanol bioactive leaves extract in the presence and the absence of LPS for 24 h. After washing with ice-cold phosphate buffer saline (PBS), the cells were lysed in a cell lysis buffer containing the protease and phosphatase inhibitor cocktail. Cells lysed dilution was centrifuged at 14,000 rpm for 25 min to discard cell debris. The protein concentration was measured by using BCA assay according to the manufacturer's instruction. Equal amounts of total cellular proteins (30 μ g) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (GE Healthcare, USA). Each membrane was incubated with blocking solution (5% nonfat skim milk) in PBS containing 0.5% Tween 20 (PBST, pH 7.4) at room temperature for 1-h. This was followed by an overnight incubation at 4°C with the appropriate primary antibodies iNOS, COX-2, NF κ B-p65 (1:1000 dilution), the membranes were washed with PBS containing Tween 20 for 1-h, then incubated with a 1:2000 dilution of HRP-conjugated secondary antibodies for 1-h at room temperature. The membranes were washed again with PBST for 1-h then the immune reacted proteins were detected using a Chemiluminescence System (ChemiDoc, BioRad, USA). The bands obtained were quantitated and analyzed using ImageJ Software (BioTechniques, New York, NY, USA).

Statistical analysis

The results reported are summarized from three independent experiments and expressed as the mean \pm standard deviation (SD).

Data were presented as means \pm SD and the $P < 0.05$ and $P < 0.001$ were considered as statistically significant. Significant differences were examined using an analysis of variance with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Effect of *Moringa oleifera* on cell viability

Three different hydroethanolic gradients of *M. oleifera* bioactive leaves extract were examined for cytotoxicity study with MTT assay. The 90% hydroethanolic extract showed the highest cell viability and more than 85% of the cells were viable at 125 μ g/ml and 250 μ g/ml concentrations ($*P < 0.05$) [Figure 1]. Accordingly, (90:10) 90% *M. oleifera* hydroethanolic bioactive leaves extract at 250, 125 μ g/ml concentrations was selected for future anti-inflammatory experiments.

Inhibition of nitric oxide production by *Moringa oleifera* in lipopolysaccharide-stimulated RAW264.7 cells

RAW264.7 murine macrophage cells were shown to produce NO through stimulation with LPS. Significant inhibition of NO production was observed after treatment with three different hydroethanolic gradients of *M. oleifera* bioactive leaves extract. In addition, NO production was significantly suppressed after treatment with dexamethasone ($*P < 0.001$) [Figure 2].

The effect of *Moringa oleifera* on lipopolysaccharide-induced inflammatory cytokines and prostaglandin E₂ production

The cytokine production levels of TNF- α , IL-1 β , IL-6, and PGE₂ in the cell-free culture supernatants were significantly enhanced in respond to LPS induction [Figure 3a-d], which has proven the successful establishment of *in vitro* inflammatory model. The 90% *M. oleifera* hydroethanolic bioactive leaves extract significantly suppressed the production level of TNF- α , IL-1 β , IL-6, and PGE₂ in a concentration-dependent manner ($*P < 0.001$) ($**P < 0.001$) ($#P < 0.05$)

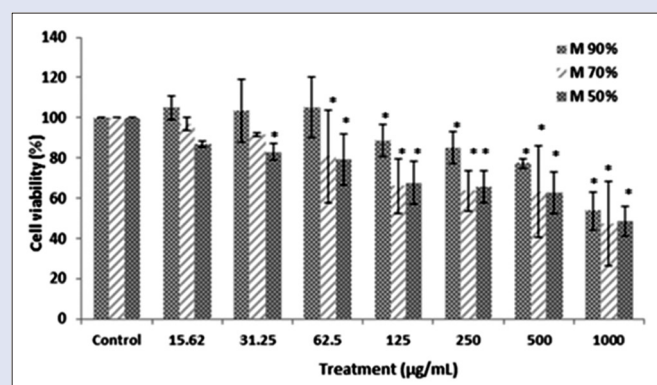


Figure 1: The effect of three different hydroethanolic gradients of *Moringa oleifera* leaves extract (90%, 70%, and 50%) on the viability of RAW 264.7 cells. Cytotoxicity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay after 24 h treatment with *Moringa oleifera* extract (15.62–1000) μ g/ml. Values are presented as the mean of three independent experiments in triplicate, and data are shown as mean \pm standard deviation. Statistical analysis using one-way analysis of variance with Tukey's *post-hoc* test ($*P < 0.05$) as compared with the untreated control group

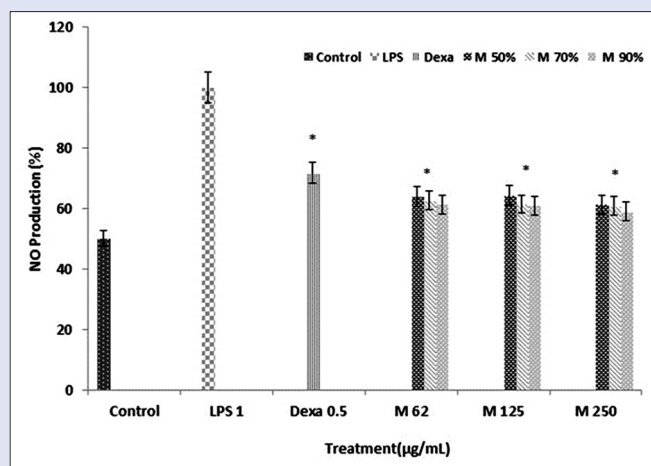


Figure 2: Effects of three different hydroethanolic gradients of *Moringa oleifera* bioactive leaves extract (90%, 70%, and 50%) on lipopolysaccharide-induced nitric oxide production in RAW264.7 macrophage. Cells were treated with different concentration of *Moringa oleifera* (62 µg/ml, 125 µg/ml, and 250 µg/ml) in the presence of 1 µg/ml lipopolysaccharide for 24 h. Dexamethasone (0.5 µg/ml) was used as a positive control. Control values were obtained in the absence of lipopolysaccharide or *Moringa oleifera*. Three independent assays were performed in triplicate, and data are shown the mean \pm standard deviation statistical analysis using one-way analysis of variance with Tukey's *post-hoc* test. (* $P < 0.001$) indicates significant differences from the lipopolysaccharide treated group and nontreated group

[Figure 3a-d] and meantime, it has increased the production of IL-10 in a dose-dependent manner (* $P < 0.001$) [Figure 3e]. Meanwhile, dexamethasone has also exhibited the significant inhibition in TNF- α , IL-1 β , IL-6, PGE₂ production, and enhancement in IL-10 level (* $P < 0.001$) (** $P < 0.001$) (# $P < 0.05$) [Figure 3a-e].

Effect of *Moringa oleifera* on expression of inflammatory markers inducible nitric oxide synthase, cyclooxygenase-2, and nuclear factor kappa-light-chain-enhancer of activated B-cells p65 on lipopolysaccharide-induced macrophages

Western blot analysis was performed to evaluate the inhibitory effects of *M. oleifera* on inflammatory mediators in LPS stimulated macrophages. RAW264.7 cells were treated with 90% *M. oleifera* hydroethanolic bioactive leaves extract in the presence and the absence of LPS for 24 h. The protein expression of iNOS and COX-2 was clearly enhanced through stimulation with LPS, and *M. oleifera* hydroethanolic bioactive leaves extract considerably suppressed iNOS and COX-2 pro-inflammatory mediators, and NF κ B-p65 protein expression in a dose-dependent fashion [Figure 4a]. In addition, to understand whether *M. oleifera* hydroethanolic bioactive leaves extract suppressed NF- κ B pathway activity, we measured the protein expression of NF κ B-p65 and it exhibited significant inhibition effect through LPS stimulated RAW264.7 cells. In the quantitative determination, β -actin expression level as an internal standard was comparable between each target protein. The density ratio of iNOS, COX-2, and NF κ B-p65 was significantly decreased after treatment with *M. oleifera* through LPS-induced RAW264.7 cells (* $P < 0.05$) [Figure 4b].

DISCUSSION

Macrophages perform a crucial function in host defenses toward noxious substances and the inflammation process.^[2-7] It has been well-established that LPS, a part of Gram-negative bacteria membranes is identified by toll-like receptor 4 (TLR4) on the cell membrane of the macrophages. It activates the release of pro-inflammatory mediators through TLR4-NF κ B signaling pathways, which mediate host harmful injury.^[31] Overproduction of pro-inflammatory cytokines by activated macrophages has been concerned in the pathophysiology of numerous chronic inflammatory illnesses. Therefore, LPS-stimulated macrophages are used as a model to investigate on inflammation and the mechanisms action of anti-inflammatory agents.^[2,7] Recently, it has been notable that more natural products are becoming utilized as a treatment to reduce several acute and chronic diseases.^[32,33]

In the field of natural products drug discovery, plant extractions process with the appropriate solvent system is very essential to identify and isolate the pharmacological compounds from medical plants. A previous investigation by Fakurazi *et al.*,^[34] has been reported that hepatoprotective properties of 80% hydroethanolic *M. oleifera* leaves extract in hepatotoxicin induced animal model. However, in the present study, we have evaluated three different hydroethanolic gradients of *M. oleifera* leaves extracts based on cell specific toxicity, anti-oxidant, and anti-inflammatory properties.^[35] Several anti-inflammatory drugs have recently been exhibited to carry a free radical scavenging mechanism as part of their pharmacological activities.^[36] Oxidative stress-induced activation of NF κ B has been identified in several chronic inflammatory diseases exhibiting a crucial link among modulation of NF κ B molecules and antioxidant properties.^[37] Furthermore, it could be suggested that antioxidant potential of *M. oleifera* leaves might be one of the cellular mechanisms of its anti-inflammatory potential in LPS stimulated macrophages.^[38-40]

Cell viability assay was performed for three different *M. oleifera* hydroethanolic leaves gradients 90% hydroethanolic bioactive leaves extract revealed the highest cell viability as compared with 70% and 50% concentration. It can be due to the availability of more nutrition and some certain compounds for cells. This result was in agreement with our previous study by Karthivashan *et al.*,^[35] which introduced 90% hydroethanolic solvent as the best solvent. It could possibly digest leaves tissue and may release antioxidant and nutritionally important active compounds of *M. oleifera* leaves. Consistent with the previous findings by Araújo *et al.*,^[41] indicated that *M. oleifera* aqueous seeds extract also exhibited higher cell viability on peritoneal macrophages cells. Previous investigations have shown that, the maximum duration of release of inflammatory mediators from LPS stimulated macrophages was 24 h with 1 µg/ml concentration and this LPS concentration is nontoxic to macrophages.^[42-45]

NO is an essential mediator and regulator of inflammatory responses. In the inflammatory processes overproduction of NO reacts with superoxide causes cytotoxicity and tissue damage in several organisms. NO-induced oxidative stress is associated with several illnesses for instance atherosclerosis, septic shock, diabetes, Alzheimer's disease, and Parkinson's disease. NO inhibitor compounds have been reported as an effective treatment for such diseases.^[46] In animal tissues synthases enzyme (NOS) oxidase L-arginine to L-citrulline and produce NO supplements. Three isoforms of NOS which include NOS I or (nNOS) - the neuronal form, NOS II or iNOS, contained in numerous cell types following inflammatory stimulation (e.g., macrophages), and NOS III (or eNOS) - constitutive enzyme mainly reveal in the endothelium. The three isoforms have the same molecular construction

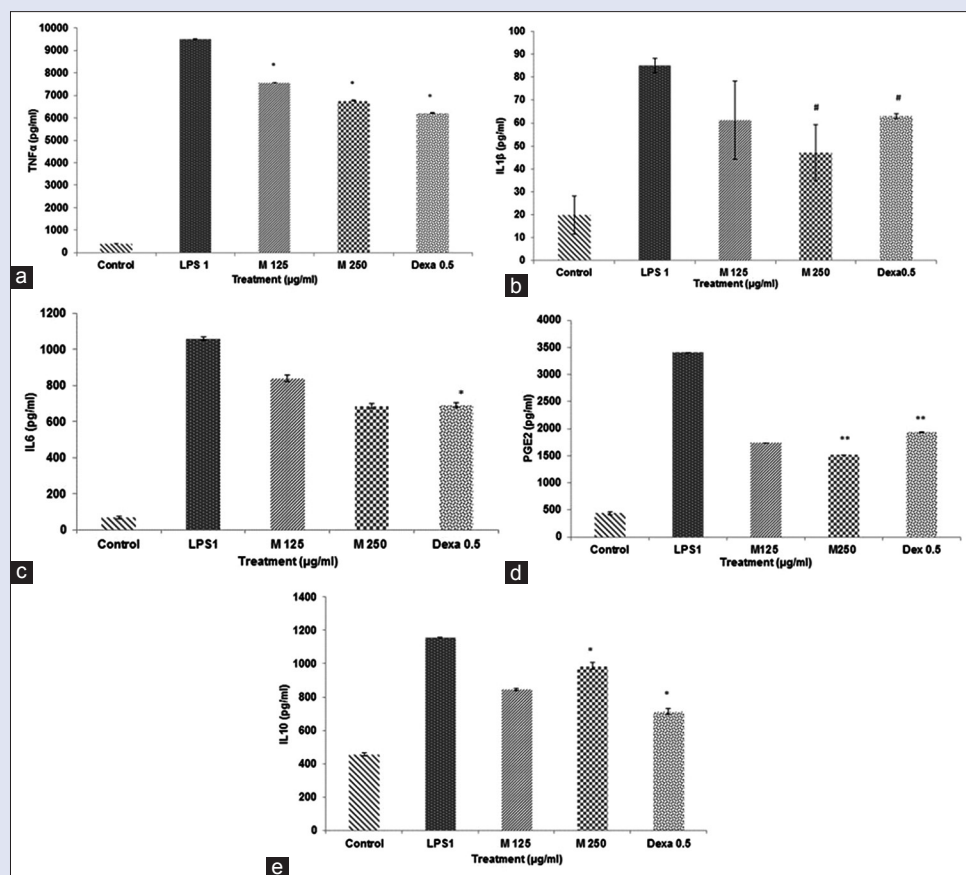


Figure 3: (a-e) Effect of *Moringa oleifera* 90% hydroethanolic bioactive leaves extract on the production of tumor necrosis factor alpha, interleukin-1 β , interleukin-6, prostaglandin E₂, and interleukin-10 cytokines in lipopolysaccharide-stimulated RAW 264.7 cells. Cells have been exposed with lipopolysaccharide (1 μ g/ml) alone or lipopolysaccharide plus different concentrations of *Moringa oleifera* (125 μ g/ml, 250 μ g/ml) and dexamethasone for 24 h. Three independent assays were performed in triplicate, and the data are shown the mean \pm standard deviation statistical analysis using one-way analysis of variance with Tukey's *post-hoc* test. It shows a significant difference with lipopolysaccharide treated and untreated groups (* P < 0.001). It exhibits significant difference with lipopolysaccharide treated group (** P < 0.001). It shows a significant difference with lipopolysaccharide treated group (* P < 0.05)

and involve numerous cofactors.^[47] Recent study by Lee, *et al.* has demonstrated that licochalcone E which is isolated and characterized from the roots of *Glycyrrhiza inflata*, revealed potent anti-inflammatory effect by suppressing NO production and protein expression of iNOS in a dose-dependent fashion.^[48] In this study, the effect of three different hydroethanolic *M. oleifera* leaves gradients (50:50 [50%], 70:30 [70%], and 90:10 [90%]) were found to significantly inhibit LPS-induced NO production. Moreover, the inhibitory effect of positive control (dexamethasone) was similar to *M. oleifera* leaves extract. In addition, protein expression of iNOS was inhibited after treatment with *M. oleifera* hydroethanolic bioactive leaves extract confirming the suppressive effect of *M. oleifera* on NO production. These findings might conclude that *M. oleifera* leaves acts as an effective anti-inflammatory agent against various ailments.

The mechanism of action of numerous anti-inflammatory drugs is through PG synthesis suppression which usually mediated by COX. Between two isoforms of COX (COX-1 and COX-2), COX-1 has been recommended to produce a physiological amount of PGs for typical platelet, kidney, and stomach function. COX-2 is a mediator with high activity at inflammatory sites in animals and human patients with the inflammatory disorder.^[49] PGE₂ is one of the most effective inflammatory mediators which acts in all inflammatory processes leading to typical signs of inflammation such as pain, swelling, and redness.^[50] PGE₂ has

been altered from arachidonic acid through the COX-2 catalytic activity. COX-2 also might be affected specifically at its enzymatic activity via NO and iNOS.^[49] A recent study by Kim *et al.*, has exhibited that composition of essential oil from fingered citron (*Citrus medica* L. var. *sarcodactylis*) caused dose-dependent suppression of LPS-stimulated PGE₂ production and inhibited COX-2 protein expression.^[2] In this study *M. oleifera* hydroethanolic bioactive leaves extracts significantly reduced PGE₂ production and COX-2 protein expression. It may suggest that the anti-inflammatory effect of *M. oleifera* hydroethanolic bioactive leaves extracts could possibly be due to its suppressive effect on PGE₂ production via blocking COX-2 protein expression. The cross-talk among the iNOS and COX-2 pathways has been identified through several researchers and few researches have concluded that NO seems to reduce COX-2 expression.^[51] Targeting iNOS and COX-2 has been known as a useful strategy to prevent acute and chronic inflammatory diseases. These inflammatory mediators regulate the inflammation process by producing NO and PGE₂, respectively.^[52] As a result active compounds with dual suppression effects on iNOS and COX-2 expression would have remarkable potential on healing the acute and chronic inflammation.

Pro-inflammatory cytokines, which includes TNF- α and IL-6 are classified as main inflammatory mediators which are produced by monocyte and macrophages in the inflammatory procedure.^[15] TNF- α

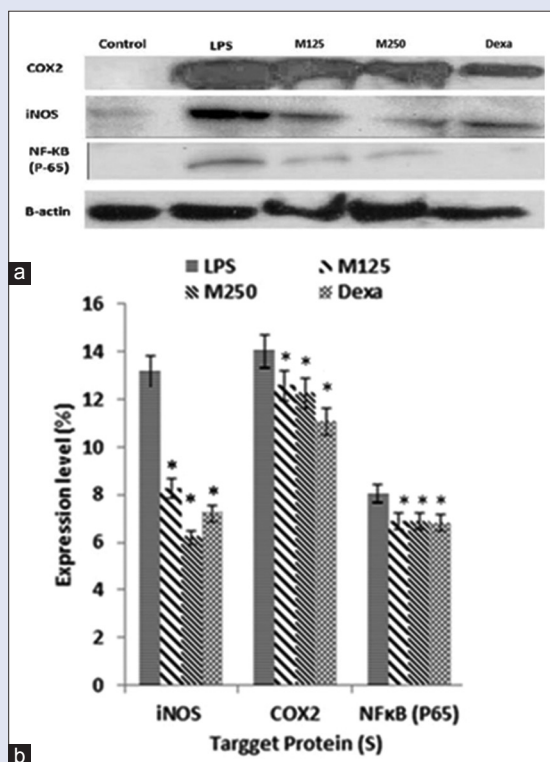


Figure 4: (a) Effect of *Moringa oleifera* hydroethanolic bioactive leaves extract on lipopolysaccharide-induced inducible nitric oxide synthase, cyclooxygenase-2, and nuclear factor kappa-light-chain-enhancer of activated B-cells p65 expression in RAW264.7 cells. Cells were stimulated with lipopolysaccharide (1 $\mu\text{g/ml}$) in the presence of *Moringa oleifera* hydroethanolic leaves extract (125 $\mu\text{g/ml}$, 250 $\mu\text{g/ml}$) and dexamethasone (0.5 $\mu\text{g/ml}$) for 24 h at 37°C. Cell lysates were extracted, and protein expression levels were analyzed by Western blot. β -actin was used as the loading control. (b) The level of each protein was measured and normalized to β -actin. The values are reported as the mean \pm standard deviation percentage of three independent experiments. Statistical analysis using one-way analysis of variance with Tukey's *post-hoc* test. It shows a significant difference with lipopolysaccharide treated group (* $P < 0.05$)

is a key mediator in inflammatory responses, as well as the initiation of apoptosis. It might stimulate the development or expression of IL-6, IL-1 β , PGE₂, collagenase, and adhesion molecules eliciting a variety of physiological functions such as septic shock, inflammation and cytotoxicity.^[46] In addition, it represents a function on the developmental pathogenesis of numerous inflammation-associated chronic illnesses such as cancer, obesity, and cardiovascular diseases. TNF- α and IL-6 are mainly produced by macrophages in response to LPS through NF κ B activation.^[15] The IL-1 family plays a crucial role in both host defense and inflammation. To date, up to 11 members of this family have been recognized. IL-1 α and IL-1 β both are synthesized as precursor molecules by several different cell types and both are pro-inflammatory cytokines. IL-1 β is a main mediator of inflammation which performs a basic role in tissue injury restore along with protection toward microbial pathogens. In these conditions, systemic (i.e. bone marrow) and/or local (i.e., endothelial) reactions to this cytokine are responsible for beneficial impacts, which include cellular infiltration and neutrophil mobilization, respectively. However, an extra amount of this cytokine may possibly have deleterious impacts on a diversity of cells and tissue.^[53] IL-6, which has been originally defined as a

B-cell differentiation factor is regarded as a multifunctional cytokine which regulates immune responses, hematopoiesis, acute phase response, and inflammation. Increased IL-6 amount in many cases is correlated with several diseases which include rheumatoid arthritis, systemic-onset juvenile chronic arthritis, osteoporosis, psoriasis, polyclonal plasmacytosis, malignant plasmacytoma, Crohn's disease, and encephalomyelitis. Therefore, inhibitors of IL-6 could possibly be beneficial in treating of inflammatory autoimmune illnesses.^[54] A recent study has been published on anti-inflammatory properties of plumbagin, derived from the plants of the *Plumbaginaceae* family which exhibited suppressing effect on the production of TNF- α , IL-1 β , and IL-6 from LPS-stimulated RAW264.7 cells in a dose-dependent fashion.^[55] In the present study, *M. oleifera* hydroethanolic bioactive leaves extract exhibited significant inhibition in LPS-induced TNF- α , IL-1 β , and IL-6 production in a dose-dependent manner. These results recommended that *M. oleifera* leaves may possess anti-inflammatory characteristic and help to reduce some inflammatory associated disorders. We believe that these anti-inflammatory properties of *M. oleifera* bioactive leaves extracts might be related to the presence of various active compounds which was identified by us through liquid chromatography-mass spectrometry (MS)/MS.^[35]

IL-10 is one of the pleiotropic cytokines, which regulated the function of numerous adaptive immune-related cells. Although, IL-10 is strongly known as an immunosuppressive and anti-inflammatory cytokine, it performs other crucial roles such as the ability to activate B-cells, T-cells, natural killer cells, and mast cells.^[56] A study by Zhao, *et al.* has reported that Corilagin (beta-1-O-galloyl-3,6-(R)-hexa hydroxyl diphenyl-D-glucose) a member of the tannin family significantly enhanced IL-10 production in a dose-dependent manner through LPS stimulated RAW264.7 cells.^[30] In the current study, *M. oleifera* hydroethanolic bioactive leaves extract revealed significant induction in IL-10 amount as an anti-inflammatory cytokine in a dose-dependent fashion which was in agreement with anti-inflammatory properties of *M. oleifera* bioactive extract.

NF κ B characteristics as a hetero- or homo-dimer, which is often classify in five NF κ B subunits NF κ B1 (p50 and its precursor p105), NF κ B2 (p52 and its precursor p100), RelA (p65), RelB, and c-Rel. Among all, p50:p65 is the most studied heterodimers, which are activated through the classical pathway and basically stimulated gene expression. The transcription factor NF κ B is often a crucial regulator of numerous cellular functions such as cell survival and inflammation. Interestingly, for this kind of functions the role of p50:p65 dimer is most highlighted. Moreover, cell survival and inflammation stimulation are related to the p50:p65 dimer function.^[57] In resting cells NF κ B is situated in the cytoplasm as a nonactive complex bound to I- κ B α , as an inhibitory protein. When cells become activated through inflammatory stimuli the inhibitory protein is phosphorylated and consequently degraded then, dissociated to provide free NF κ B. As a result, free NF κ B will translocate into the nucleus where it connected to κ B binding sites in the promoter part of target genes. Basically, transcription of pro-inflammatory markers increases through this binding connection. Therefore, suppression of NF κ B signaling pathway production might describe the potent activity of *M. oleifera* bioactive leaves extract as an inhibitor of inflammatory cytokines and mediators. Basically, NF κ B factor p65 after stimulation with LPS translocate from the cytoplasm to the nucleus.^[31] In the current study, we found that the translocation of NF κ B factor p65 was significantly inhibited by *M. oleifera* bioactive leaves extract in a dose-dependent fashion. It could be recommended that the anti-inflammatory impact of *M. oleifera* bioactive leaves extract is through NF κ B pathway inhibition.

CONCLUSION

Although a few immunotherapy studies on *M. oleifera* have been reported in the past the anti-inflammatory activity of *M. oleifera* hydroethanolic bioactive leaves extracts was first explored. The current study demonstrated that *M. oleifera* hydroethanolic bioactive leaves extract exhibited a remarkable anti-inflammatory effect on LPS induced inflammation in macrophages. Bioactive extract of *M. oleifera* effectively suppressed iNOS and COX-2 protein expression and also the production of NO and PGE₂ stimulated by LPS. Moreover, it decreased the pro-inflammatory cytokines production (TNF- α , IL-1 β , and IL-6) induced by LPS in macrophages, and the level of IL-10 was increased through suppression of signaling cascades leading to the activation of NF κ B-p65. In addition, this study suggested that the anti-inflammatory activity from bioactive compounds contain in *M. oleifera* hydroethanolic bioactive leaves extract can promote effective treatment to manage the inflammatory disorder.

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Conflicts of interest

There are no conflicts of interest.

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