In vitro Macrophage Nitric Oxide and Interleukin-1 Beta Suppression by *Moringa peregrina* Seed

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ABSTRACT

Objectives: *Moringa peregrina* have long been used in folk medicine to treat diseases including fever, headache, burns, constipation, gut pains and inflammatory. Nitric oxide (NO) and interleukin-1 beta (IL-1β) play an important role in the pathophysiology of inflammation. The objectives of this study were to determine the effect of *Moringa peregrina* seed ethanolic extract (MPSE) on the viability of and NO and IL-1β production by lipopolysaccharide (LPS)-activated macrophage (J774A.1) cell line.

Materials and Methods: The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used to determine the cytotoxic effect of MPSE treatment at concentrations ranging from 31.15 to 1000 µg/ml. The NO concentration was determined by Griess assay and IL-1β proinflammatory cytokine concentration by ELISA in the supernatant of MPSE-treated LPS-activated J774A.1 cell culture.

Results: The results show that the MPSE was not cytotoxic at 1000 µg/ml while significantly ($p<0.001$) inhibited NO and IL-1β production by the LPS-activated macrophage J774A.1 cells.

Conclusion: These findings suggest that the *Moringa peregrina* seed extract can be used to treat and prevent inflammatory diseases through the inhibition of inflammatory mediators.

Key words: *Moringa peregrina*, nitric oxide, interleukin-1β, inflammation.

***Moringa peregrina* Tohumlarıyla *in vitro* Makrofaj Nitrik Oksit ve İnterlökin-1 Beta Baskılanması**

ÖZ

Amaç:: *Moringa peregrina* geleneksel tıpta uzun yıllardan beri ateş, baş ağrısı, yanık, kabızlık, gut ağrıları ve inflamasyonların tedavisinde kullanılmaktadır. Nitrik oksit ve interlökin-1 beta inflamasyonun patofizyolojisinde önemli rol oynamaktadır. **Gereç ve Yöntemler:** Bu çalışmada, *Moringa peregrina* tohumları etanol ekstresinin sitotoksik ve lipopolisakkarit ile aktive edilmiş makrofaj hücre hattının (J774A1) nitrik oksit ve interlökin-1 beta üretimini baskılayıcı etkileri araştırılmıştır. Ekstrenin sitotoksik etkilerini tayin etmek için 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür yöntemi kullanılmıştır. İndüklenmiş makrofaj kültür süpernatantında nitrik oksit düzeyleri Griess yöntemi ile, interlökin-1 beta proinflamatuvar sitokin düzeyleri enzim aracılı immünosorbent yöntemi ile tayin edilmiştir.

Bulgular: Sonuçlar, MPSEnin J774A1 hücrelerine toksik olmadığını göstermiştir. Ayrıca, ekstre lipopolisakkarit ile aktive edilmiş J774A1 hücre makrofajlarında nitrik oksit ve interlökin-1 beta üretimini önemli ölçüde baskılamıştır.

Sonuç: Bu bulgular, *Moringa peregrina* tohum ekstrelerinin, inflamatuvar mediyatörlerin aşırı üretiminin eşlik ettiği inflamatuvar hastalıklardan korunma ve bu hastalıkların tedavisinde yararlı olabileceğini göstermektedir.

Anahtar Kelimeler: *Moringa peregrina*, nitrik oksit, interlökin-1 beta, inflamasyon

INTRODUCTION

Inflammatory process plays a key role in the development of various cases such as gastritis, diabetes, atherosclerosis, and cancer[1]. Macrophages have critical roles in inflammatory response by phagocytosis or producing inflammatory mediators such as nitric oxide (NO) and pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α). These inflammatory molecules can be induced by certain stimulants such as lipopolysaccharide (LPS), which can stimulate and activate macrophages[2,3]. NO is a signalling protein synthesised by nitric oxide synthase (NOS) from L-arginine. It is a short-lived intercellular biomolecule, which performs key roles in the regulation of a variety of inflammatory diseases. It has also important antitumor and antiviral properties.[4-6] IL-1β is considered as a key inflammatory cytokine responsible for the induction inflammatory reactions and the production of reactive oxygen species (ROS).[6,7] Although cytokines and nitric oxide are having special roles in mediate immune function, the same molecules have been concerned in enhanced expression which might cause chronic inflammatory diseases and tissue injury.[8,9]

Moringa peregrina (Forssk.) Fiori can be found in Africa and countries that bordering the Red Sea.[10] In folk medicine, all parts of this plant are used for the treatment of abdominal pains, diabetes, headache, fever and burns. It is also administered to pregnant women to facilitate the fetus delivery.[11] The pharmacological studies have been reported the validation of this plant to be used as anti-inflammatory, anti-microbial, antiulcer, and antioxidant.[10,12] *M. peregrina* seeds oil contains high amounts of oleic acid, linoleic acid, tocopherols and phenolic compounds, which help to reduce inflammation.[13,14] Thus, this study was undertaken to investigate the effect of *M. peregrina* seed extract, which might provide natural drug for treatment of inflammatory-releated disease, on nitric oxide and pro-inflammatory cytokine IL-1β production in lipopolysaccharide (LPS) - induced macrophage cell line J774A.1.

MATERIALS AND METHODS

Plant material and extraction

The *M. peregrina* seeds were authenticated by Dr Maha Kordofani (Resident Botanist) at the Botany Department, Faculty of Science, and University of Khartoum. Fresh seeds were dried in room temperature, powdered and macerated in 1:5 dried plant weights to solvent (ethanol) volume ratio for 3 days. The filtrate was collected

and the residues were subjected to further macerated with ethanol. The filtrates were combined and concentrated to dryness under reduced pressure using rotary evaporator at 45°C to 50°C in order to obtain the crude extracts.[15]

5(3-(4, 5-Dimethylthiazol-2-yl)-2.5-diphenyl Tetrazolium Bromide) MTT Assay

Extract used in all cell culture assays was diluted in the growth media of the J774A.1 cell line. The vehicle for initial stock of drug was 0.1% DMSO.

Effects of MPSE on the viability of Macrophage were detected using the MTT assay. The J774A.1 cells were seeded at a density of 5.0×10^3 cells/ml in a 96-well plate, treated with MPSE at concentrations ranging from 31.25 to 1000 µg/ml, or left untreated as a control and incubated for 24h under 5% CO_2 at 37°C. 20 µL of MTT solution was added to each well and incubating the plate for 3 hours, after which the purple formazan was dissolved with dimethyl sulfoxide (DMSO). Absorbance was determined at 570 nm with reference at 630 nm using a microplate reader (Tecan, Austria). Each experiment was repeated three times with triplicate wells for each concentration[16].

Nitric oxide (NO) assay

Nitrite concentration was detected using the Griess reaction. Pretreatment of macrophage cells with MPSE at the concentrations ranging from 31.25 to 200µg/ml, or 0.5 µg/ml dexamethasone (DXM) as a positive control, and then incubated for 1 hour. To trigger the inflammatory response, lipopolysaccharide (LPS) was added to the treatment wells of the 96-well plate at a concentration of 1 µg/mL/well. Nitrite in cell culture supernatants were quantified according to methods described previously.[17]

Interleukin-1β (IL-1β) Cytokine determination via ELISA

The Macrophage cell suspension with concentrations adjusted to 3×10^5 cells/ml were seeded into 24-well plates and cultured for 24 h. Pre-treatment of cells with MPSE at the concentrations ranging from 31.25 to 200µg/ml, or 0.5 µg/ml dexamethasone (DXM) as a positive control, and then incubated for 1 hour under the same culture conditions. 1µL of 1mg/ml LPS was then added to the treatment cells to activate the macrophages. Enzyme-linked immunosorbent assay (ELISA) kits (Cusabio Biotech Co. Ltd, USA) were used for interleukin IL-1β determinations in the

supernatants, using a spectrophotometric measurement depending on the manufacturer's instructions. The cytokine concentrations were calculated as percentage to LPS-induced control, which was set to 100% IL-1β production.

Statistical analysis

All Data were expressed as mean ± standard error, and statistical significance was determined by one-way analysis of variance (ANOVA) with Turkey post-hoc test using Graph Pad prism 6.0 statistical software with significant differences set at $p < 0.01$ and $p<0.001$.

RESULTS

Cytotoxicity assay

Detection of suitable concentration ranges, which are not toxic, can be used for further *in vitro* anti-inflammatory screening assays of the MPSE. The colorimetric assay results showed that increasing concentrations of MPSE have caused reduction of macrophage cells viability. On the other hand, MPSE was not toxic to macrophages at concentrations ranging from 31.25 to 125µg/mL when compared to culture media without seed extract which act as control (Figure 1).

Inhibition effects of M. peregrina on NO production

To assess the potential of MPSE to modulate NO releasing in macrophage, nitrite concentrations were detected in the culture supernatants of LPS-induced macrophages in the absence or presence of MPSE. Results shown in figure 2 demonstrated that the treated LPS group activated nitrite production by the macrophage cells. On the other hand, treatment with different concentrations of MPSE well as DXM significantly ($p<0.001$) inhibited nitrite generation from the LPS-induced macrophage. The MPSE suppressed nitrite production to 64.2%, 43.1%, 34.9%, and 30.1% of the LPS-stimulated control at the concentrations of 25, 50, 100 and 200 µg/ml., respectively.

Effects of M. peregrina on LPS-induced IL-1β expression in J774A.1 macrophages

IL-1β is one of the potent activator that may stimulate NO production in macrophages. The activation of macrophages with LPS trigged the expression of proinflammatory cytokines IL-1β in a concentration-dependent manner as shown in figure 3. The MPSE significantly suppressed LPS-induced IL-1β expression in a concentration-dependent manner with the values of 54.4%, 49.7%, 24.6%, and 21.9% of the LPS-stimulated control at the concentrations of 25, 50, 100 and 200 µg/ml, respectively. Besides, pretreatment of stimulated cells with MPSE significantly ($p<0.001$) decreased the expression of IL-1β in comparison to untreated control cells with MPSE.

DISCUSSION

Many traditional plants have been shown to possess excellent medicinal properties against various diseases. However, *M. peregrina* seeds have been reported to be widely used in traditional medicine, only a few scientific studies exist on its therapeutic efficacy and mechanism of action[11,14,18,19]. As a follow-up to that studies, we intend to investigate the effects of MPSE that may be considered as a potential anti-inflammatory drug on NO and IL-1β in LPS-induced J774A.1 macrophage cells. Macrophages are the predominant cells in the immunologic responses[21]. In the laboratory, J774A.1 macrophage cell line is one of the most common cells used for screening the anti-inflammatory drugs *in vitro*, because these cells share phenotypic and functional features with normal macrophages[20,22]. In this study, the cytotoxicity assay of MPSE on J774A.1 cells showed that MPSE did not possess toxic effect on macrophage cells since the cell viability is more than 80%. The concentrations ranging from 31.25 to 200 µg/ml have been chosen for anti-inflammatory screening of MPSE on J774A.1 cells.

The secretion of NO and IL-1β can be stimulated by a variety of compounds including LPS, a macrophage activator. Thus, one of the phenomenons in inflammation is massive production of these molecules by the activated macrophages causing intense inflammatory reactions[23].

In this study, it has been explored that MPSE caused a dose-dependent suppression of nitrite levels in LPS -induced macrophages. Nevertheless, the generation of pro inflammatory cytokines, such as IL-1β, and tumour necrosis factor (TNF-α) is important for the induction of NO production in LPS- induced macrophages through

NF-αB activation[7,24,25]. This study has also explained that MPSE can modulate the IL-1β expression in inflammatory cells. The inhibited level of NO synthesis observed in macrophages culture, might be related to antioxidant capacity and suppression of pro-inflammatory cytokines release provided by MPSE. The MPSE have been reported to contain high amounts of oleic acid, linoleic acid, tocopherols and phenolic compounds, which are attributed to the NO radical scavenging and anti-inflammatory properties of extract[1,3,14,25,26]. Our outcome is in agreement with findings reported by Fard *et al* who proved that *M. oleifera* has a significant inhibitory effect against the secretion of NO and IL-1β.

CONCLUSION

In conclusion, *M. peregrina* seeds, which act as inhibitors of NO and IL-1β production in LPS-activated macrophage cells, may be suggested as good anti-inflammatory agents that could normalize the conditions created by inflammation. This study has supported the traditional use of seeds of *M. peregrina* in the treatment of inflammatory- related conditions.

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Conflict of interest statement

The authors declared no conflict of interest.

Acknowledgments

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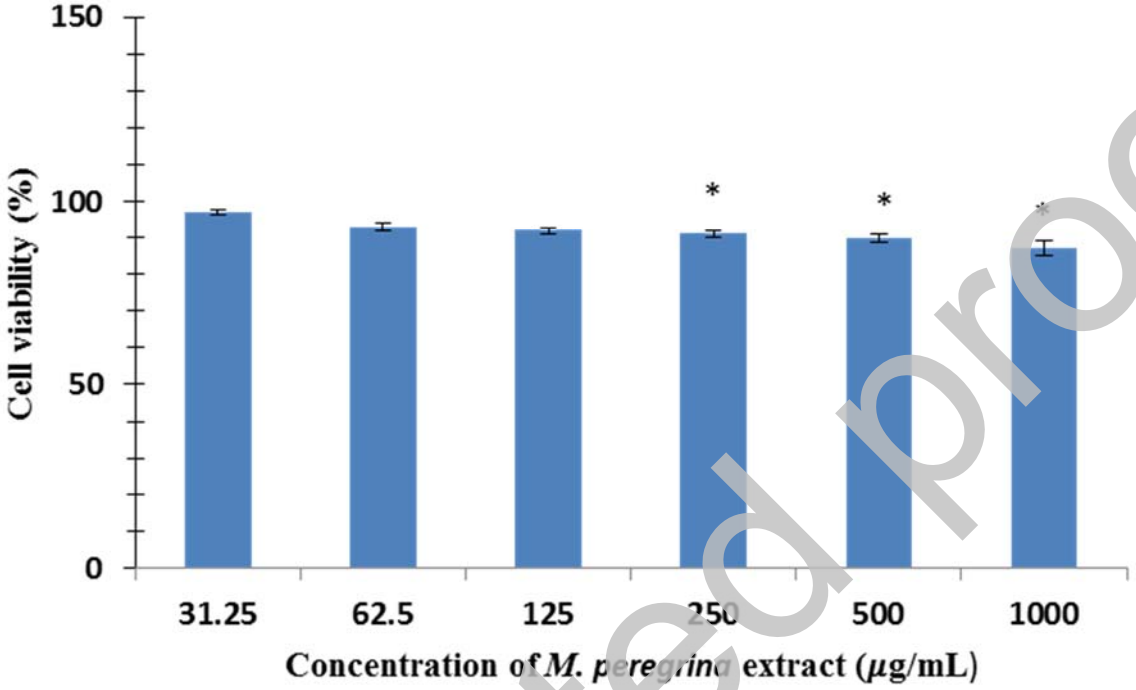

Figure 1: Viability of J774A.1 macrophage via MTT assay after treatment with MPSE. Values are mean ± standard deviation. $p < 0.01$ versus control.

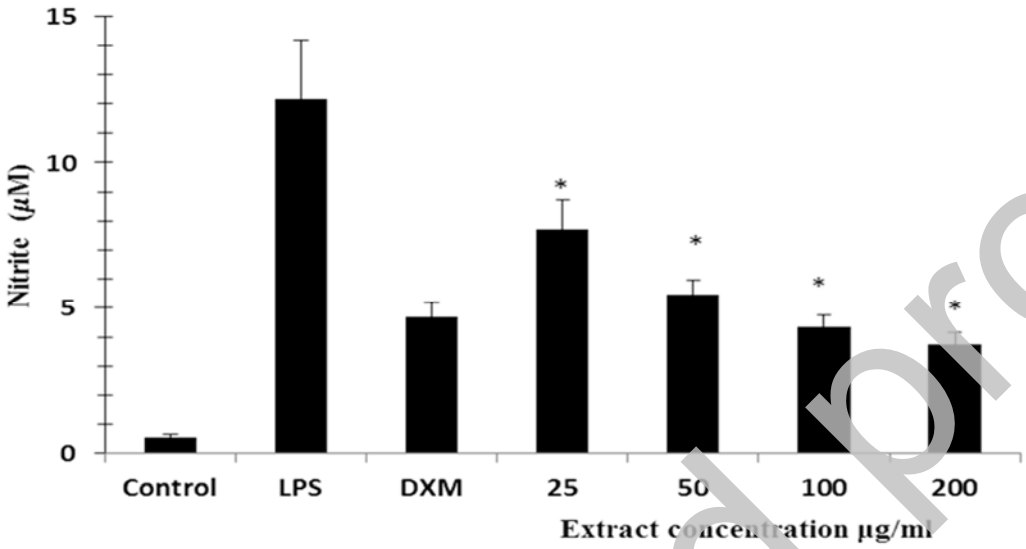

Figure 2: Effects of MPSE, and DXM on nitric oxide production by LPS- induced macrophage J774A.1 cells. Values are mean ± SD. * indicate significantly different from those of untreated lipopolysaccharide-activated J774A.1 cells (LPS) at $p < 0.001$.control.

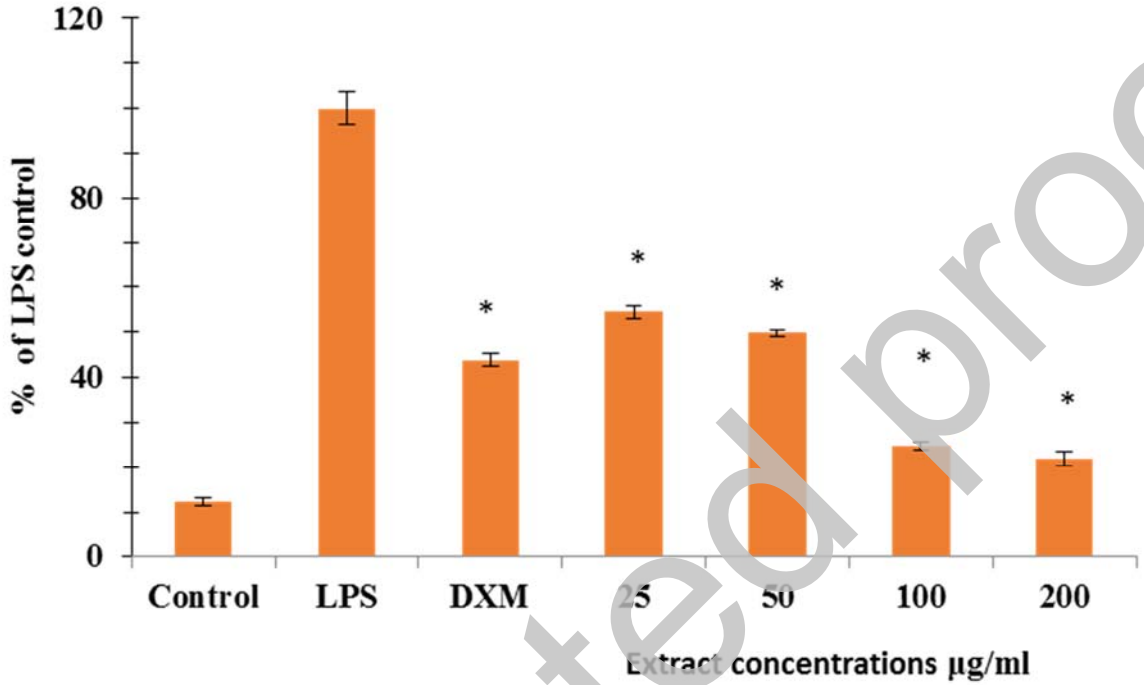

Figure 3: Effects of MPSE, and DXM on IL-1β generation by LPS- induced J774A.1 macrophage cells. Values are mean ± SD. * indicate significantly different from those of untreated lipopolysaccharide-activated J774A.1 cells (LPS) at $p < 0.001$.