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ORIGINAL ARTICLE

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

Moringa peregrina Tohumlarıyla *İn Vitro* Makrofaj Nitrik Oksit ve İnterlökin-1 Beta Baskılanması

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ABSTRACT

Objectives: *Moringa peregrina* has long been used in folk medicine to treat diseases including fever, headache, burns, constipation, gut pains, and inflammation. Nitric oxide (NO) and interleukin-1β (IL-1β) play an important role in the pathophysiology of inflammation. The objectives of this study were to determine the effect of *M. 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 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 enzyme-linked immunosorbent assay 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 but significantly ($p<0.001$) inhibited NO and IL-1β production by the LPS-activated macrophage J774A.1 cells.

Conclusion: These findings suggest that *M. 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

ÖZ

Amaç: *Moringa peregrina* geleneksel tıpta uzun yıllardan beri ateş, baş ağrısı, yanık, kabızlık, gut ağrıları ve inflamasyonların tedavisinde kullanılmaktadır. Nitrik oksit (NO) ve interlökin-1β (IL-1β) inflamasyonun patofizyolojisinde önemli rol oynamaktadır. Bu çalışmada, *M. peregrina* tohumları etanol ekstresinin (MPSE) sitotoksik ve lipopolisakkarit (LPS) ile aktive edilmiş makrofaj hücre hattının (J774A1) NO ve IL-1β üretimini baskılayıcı etkileri araştırılmıştır.

Gereç ve Yöntemler: Ekstrenin sitotoksik etkilerini tayin etmek için 3-(4,5-dimetiltiyazol-2-il)-2,5-difeniltetrazolyum bromür yöntemi kullanılmıştır. İndüklenmiş makrofaj kültür süpernatantında NO düzeyleri Griess yöntemi ile, IL-1β proinflamatuvar sitokin düzeyleri enzim aracılı immünosorbent yöntemi ile tayin edilmiştir.

Bulgular: Sonuçlar, MPSE'nin J774A1 hücrelerine toksik olmadığını göstermiştir. Ayrıca, ekstre LPS ile aktive edilmiş J774A1 hücre makrofajlarında NO ve IL-1β üretimini önemli ölçüde baskılamıştır.

Sonuç: Bu bulgular, *M. 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-1β, inflamasyon

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INTRODUCTION

The inflammatory process plays a key role in the development of various conditions, 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 (IL)-1β, IL-6, and 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 signaling protein synthesized by NO synthase (NOS) from L-arginine. It is a short-lived intercellular biomolecule that 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 a key inflammatory cytokine responsible for the induction of inflammatory reactions and the production of reactive oxygen species.[6,7] Although cytokines and NO play special roles in mediating immune function, the same molecules have been involved 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 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 fetus delivery.[11] Pharmacological studies have reported the validation of this plant for anti-inflammatory, antimicrobial, antiulcer, and antioxidant use.[10,12] *M. peregrina* seed oil contains high amounts of oleic acid, linoleic acid, tocopherols, and phenolic compounds, which help to reduce inflammation.[13,14] Thus, the present study was undertaken to investigate the effect of *M. peregrina* seed extract, which might be used as a natural drug for treatment of inflammatory-related disease, on NO 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, University of Khartoum. Fresh seeds were dried at room temperature, powdered, and macerated in 1:5 dried plant weight to solvent (ethanol) volume ratio for 3 days. The filtrate was collected and the residues were subjected to further macerating with ethanol. The filtrates were combined and concentrated to dryness under reduced pressure using a 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

The 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 the drug was 0.1% dimethyl sulfoxide (DMSO).

Effects of MPSE on the viability of macrophages 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 24 h under 5% CO_2 at 37°C. Then 20 µL of MTT solution was added to each well and the plate was incubated for 3 h, after which the purple formazan was dissolved with DMSO. Absorbance was determined at 570 nm with the reference at 630 nm using a microplate reader (Tecan, Austria). Each experiment was repeated three times with triplicate wells for each concentration.[16]

NO assay

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

IL-1β cytokine determination via ELISA

The macrophage cell suspensions with concentrations adjusted to 3×10^5 cells/mL were seeded into 24-well plates and cultured for 24 h. The cells were pretreated with MPSE at concentrations ranging from 31.25 to 200 µg/mL, or 0.5 µg/mL DXM as a positive control, and then incubated for 1 h under the same culture conditions. Then 1 µL of 1 mg/mL LPS was added to the treatment cells to activate the macrophages. ELISA kits (Cusabio Biotech Co. Ltd, USA) were used for interleukin IL-1β determinations in the supernatants, using spectrophotometric measurement according to the manufacturer's instructions. The cytokine concentrations were calculated as percentage to the 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 ANOVA with Tukey's *post-hoc* test using GraphPad 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 MPSE. The colorimetric assay results showed that increasing concentrations of MPSE caused reduction in macrophage cell viability. On the other hand, MPSE was not toxic to macrophages at concentrations ranging from 31.25 to 125 µg/mL when compared to culture media without seed extract acting as the control (Figure 1).

Inhibition effects of M. peregrina on NO production

To assess the potential of MPSE to modulate NO release in macrophages, nitrite concentrations were detected in the

culture supernatants of LPS-induced macrophages in the absence or presence of MPSE. The 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 as well as DXM significantly ($p<0.001$) inhibited nitrite generation from the LPS-induced macrophages. The MPSE suppressed nitrite production to 64.2%, 43.1%, 34.9%, and 30.1% of the LPS-stimulated control at 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 a 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 values of 54.4%, 49.7%, 24.6%, and 21.9% of the LPS-stimulated control at concentrations of 25, 50, 100, and 200 µg/mL, respectively. Moreover, 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. Although *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 those studies, our aim was to investigate the effects of MPSE, which may be considered a potential anti-inflammatory drug, on NO and IL-1β in LPS-induced J774A.1 macrophage cells. Macrophages are the predominant cells in immunologic responses. In the laboratory, the J774A.1 macrophage cell line is one of the most common types of cells used for screening anti-inflammatory drugs *in vitro*, because these cells share phenotypic and functional features with normal macrophages.[20-22] In the present study, the cytotoxicity assay of MPSE on J774A.1 cells showed that MPSE did not have a toxic effect on macrophage cells since cell viability was more than 80%. Concentrations ranging from 31.25 to 200 µg/mL were 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 phenomena in inflammation is massive production of these molecules by activated macrophages, causing intense inflammatory reactions.[23]

In the present study, it was found that MPSE caused dose-dependent suppression of nitrite levels in LPS -induced macrophages. Nevertheless, the generation of pro inflammatory cytokines, such as IL-1β, and TNF-α is important for the induction of NO production in LPS-induced macrophages through NF-αB activation.[7,23,24] The present study also determined that MPSE can modulate IL-1β expression in inflammatory cells. The inhibited level of NO synthesis observed in the macrophage culture might be related to the antioxidant capacity and suppression of pro-inflammatory cytokine release provided by MPSE.

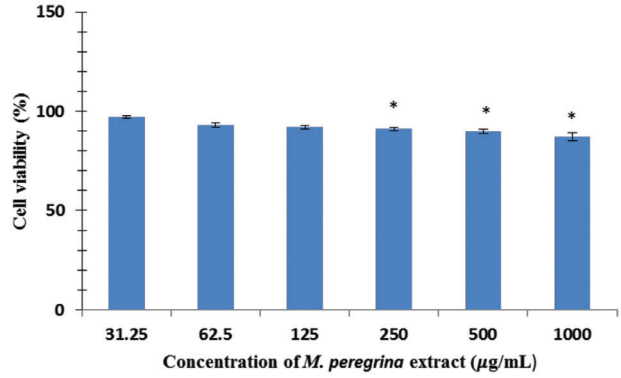

Figure 1. Viability of J774A.1 macrophage via MTT assay after treatment with MPSE. Values are mean ± standard deviation. $p<0.01$ versus control

MPSE: *Moringa peregrina* seed ethanolic extract

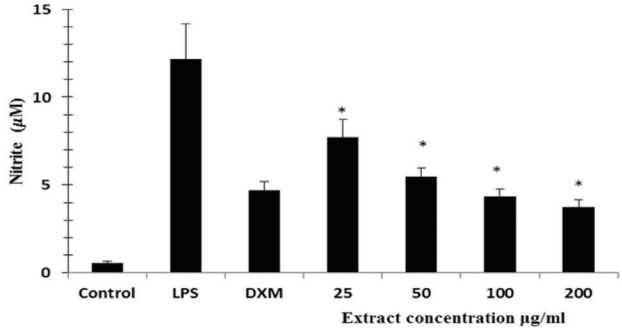

Figure 2. Effects of MPSE, and DXM on nitric oxide production by LPS-induced macrophage J774A.1 cells. Values are mean ± standard deviation. * Indicates significantly different from those of untreated lipopolysaccharide-activated J774A.1 cells (LPS) at $p<0.001$

MPSE: *Moringa peregrina* seed ethanolic extract, DXM: Dexamethasone, LPS: Lipopolysaccharide

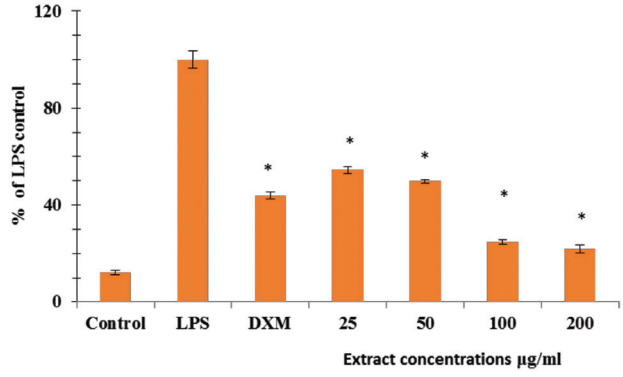

Figure 3. Effects of MPSE and DXM on IL-1β generation by LPS-induced J774A.1 macrophage cells. Values are mean ± standard deviation. * Indicates significantly different from those of untreated lipopolysaccharide-activated J774A.1 cells (LPS) at $p<0.001$

MPSE: *Moringa peregrina* seed ethanolic extract, DXM: Dexamethasone, LPS: Lipopolysaccharide, IL: Interleukin

MPSE has 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.[1,3,14,24-26] Our outcome is in agreement with findings reported by Fard et al.,[26] who stated that *M. oleifera* has a significant inhibitory effect on the secretion of NO and IL-1β.

CONCLUSIONS

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: No conflict of interest was declared by the authors.

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