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In vitro Evaluations of Cytotoxicity and Anti-inflammatory Effects of *Peganum harmala* Seed Extracts in THP-1-derived Macrophages

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Based on knowledge from traditional Greco-Arab herbal medicine, this *in vitro* study aims to evaluate the cytotoxicity and the anti-inflammatory mechanism of *Peganum harmala* (*P. harmala*) seeds by measuring the expression and release of both anti-inflammatory (IL-10) and pro-inflammatory (IL-1, IL-2, IL-6 and TNF-α) cytokines in human THP-1-derived macrophages.
Methodology: Cells were treated with 1µg lipopolyssacharide/ml (LPS) in the absence and presence of increasing concentrations of extracts from the *P. harmala* seeds (PHS-extract). During the entire experimental period, we used extract concentrations (up to 250 µg/ml) that had no cytotoxic effects as measured with MTT and LDH assays. The anti-inflammatory effects were assessed by measuring the levels of secretory proteins and mRNA of IL-1, IL-2, IL-6, IL-10 and TNF-α in LPS-activated (1µg/ml) human THP-1-derived macrophages.

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Results: PHS-extract remarkably increased IL-10 release and mRNA expression at a concentration of 64μg/ml and suppressed the expression and release of IL-1, IL-6 and TNF-α in dose-dependent manner. No significant effects on the expression levels of IL-2 were observed after treatment with PHS-extract.
Conclusions: These results indicate that PHS-extract probably exert anti-inflammatory properties throughout increasing the release and expression of IL-10 mRNA as well as by suppressing IL-1, IL-6 and TNF-α mRNA expression in THP-1 cells.

Keywords: Interleukins; TNF-α, pro-inflammatory; anti-inflammatory; Peganum harmala; Greco-Arab herbal medicine.

1. INTRODUCTION

Recently, there have been many studies concerning herbal-derived products with anti-inflammatory activity including but not limited to *Hypericum triquetrifolium, Punica granatum, Urtica dioica* and *Vitis vinifera* [1-3]. *P. harmala L.* (Wild rue, Syrian rue, Hirmel) is a wild-growing flowering plant which belongs to the Zygophylaceae family and is found abundantly in the Middle East and North Africa. From ancient times, it has been claimed to be an essential medicinal plant. The seeds of *P. harmala* have been used in the Greco-Arab Herbal medicine for treatment of various diseases. They have been used traditionally as an emmenagogue and an abortifacient agent in the Middle East and North Africa. There are several reports in the literature indicating a great variety of pharmacological activities for *P. harmala* such as anti-bacterial, antifungal anti-cancer and anti-inflammatory properties [4-7]. Although *P. harmala* has been used as an anti-inflammatory agent in Greco-Arab and Islamic medicine, the mechanisms of its anti-inflammatory activity await further studies.

Inflammation is the first response of the immune system to infection or irritation. It is regulated by cytokines. There are two types of cytokines, the pro-inflammatory cytokines, which promote inflammation and anti-inflammatory cytokines, which suppress the activity of pro-inflammatory cytokines. For instance, interleukin-4 (IL-4), IL-10, and IL-13 are potent activators of B-lymphocytes. However, IL-4, IL-10, and IL-13 are anti-inflammatory agents. They are anti-inflammatory cytokines by virtue of their ability to suppress genes for pro-inflammatory cytokines such as IL-1, tumor necrosis factor-α (TNF-α), and the chemokines. Thus, inhibitors of the pro-inflammatory cytokines have been considered as a candidate of anti-inflammatory drugs [8-11]. Lipopolysaccharide (LPS)-activated macrophages are usually used for evaluating the anti-inflammatory effects of various substances. LPS is a principle component of the outer membrane of Gram-negative bacteria, it is an endotoxin that induces septic shock syndrome and stimulates the production of pro-inflammatory mediators such as nitric oxide (NO), IL-1, IL-6, TNF-α, interleukins, prostanoids, and leukotrienes [12-14]. Therefore, LPS plays a key role not only in eliciting an inflammatory response but also in causing septic shock during a Gram-negative bacterial infection. Inflammatory responses are advantageous for eradicating bacteria, as long as they are under control. When out of control, however, dysregulated inflammation leads to the massive production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 by macrophages [15-16], which can cause tissue injury and multiple organ failure [11]. For example, the resident macrophages of the liver, the Kupffer cells, are among the first to respond to foreign antigens. Activated hepatic Kupffer cells play an essential role in LPS-induced liver injury [17]. Following contact with the CD14 protein, the complex triggers a signal cascade involving the nuclear factor kappa B. This factor enhances the expression of inflammation-related genes. The acute-phase response is regulated by cytokines released by activated Kupffer cells, notably IL-1, IL-6, and TNF–α [15-17]. Among these cytokines, IL-6, is a major inducer of the acute phase response. TNF-α production is not restricted to Kupffer cells in the liver. The acute-phase response was found to be affected trough hepatocyte-derived IL-6 and TNF-α in an autocrine loop and the NO production of parenchymal liver cells [18]. TNF-α stimulates the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and thus induces cell damage [19]. ROS have been implicated in the pathogenesis of many forms of liver disease. When liver cells are exposed to excesses of ROS, oxidative stress occurs and alters several cellular functions [18,19].

Anti-inflammatory cytokines block inflammatory process or at least suppress the intensity of the cascade. Cytokines such as IL-4, IL-10, IL-13, and TGF-β suppress the production of IL-1, IL-8, TNF-α, and vascular adhesion molecules. Therefore, a "balance" between the effects of the types of cytokines is thought to determine the outcome of an inflammatory disease. Macrophage-derived IL-10 affects the growth and differentiation of various cell types of the immune system *in vitro*. It inhibits the production of inflammatory cytokines such as IL-1, IL-6, and TNF–α by LPS-activated macrophages [20,21].

Results obtained here suggest that PHS-extract probably exerts anti-inflammatory effects through differential modulation of anti-inflammatory (IL-10) as well as proinflammatory cytokines IL-1, IL-6, and TNF-α mRNA expression.

2. MATERIALS AND METHODS

2.1 Preparation of *P. harmala* Extracts (PHS-extract)

A hundred grams of air-dried plant material were added to one liter of distilled water and boiled for ten minutes. The boiled water extract was filtered through filter paper and the filtrate was freeze-dried in a lyophilizer (48-72 hours). The freeze-dried crude extracts were stored at -70℃. These crude extracts were used for the following experiments.

2.2 Cell Culture

The human monocytic cell line THP-1 (ATCC® TIB-202) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). These cells express various receptors that are found in normal monocytes and have been used as a model system for macrophage biology and leukemia since 1980. Cells were grown in RPMI-1640 medium (Roswell Park Memorial Institute) with a high glucose content (4.5 g/l), supplemented with 10% vol/vol inactivated fetal calf serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin, and 10µg/ml streptomycin.

2.3 MTT Assay

The tetrazolium dye, MTT, is a widely used *in vitro* test to measure the cell viability and/or the metabolic state of the cells. This assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. For the MTT assay, 20,000 cells from THP-1cell line were seeded per well of 96-microtiter plates. Cells were then differentiated to macrophages by the addition of Vitamin D3 (0.1 µM) and 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24h. Twenty-four hours after cell seeding, cells were incubated with increasing concentrations of water PHS-extract (0-500 µg/ml) for 24 hours at 37℃. Following the removal of the culture media, cells were washed in phosphate buffered saline. The cells were then incubated in serum-free RPMI to which MTT (500µg/ml) was added to each well (100µl), and incubated for a further four hours. Then the medium was removed and the cells were incubated for 15 minutes with 100µL of acidic isopropanol (0.08 N HCl) to dissolve the formazan crystals. The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated control cells.

2.4 Lactate Dehydrogenase

In the Lactate dehydrogenase (LDH) assay the leakage of the cytoplasm located enzyme LDH into the extracellular medium is measured. The presence of the exclusively cytosolic enzyme, LDH, in the cell culture medium was indicative of cell membrane damage.

For the LDH assay, 20,000 cells from THP-1cell line were seeded per well of 96-microtiter plates. Cells were then differentiated to macrophages by the addition of Vitamin D3 (0.1 µM) and PMA (100 ng/ml) for 24h. Twenty-four hour after cell seeding, cells were exposed to varying concentrations of PHS-extract (0-500 µg/ml) for 24h, the supernatants were then collected from each well. Cells were then lysed with a cell lysis solution for 30 minutes at room temperature. The cell lysates were collected. LDH activity was measured in the supernatants and the cell lysates with the help of CytoTox 96 assay kit (Promega, WI, USA) in accordance with the manufacturer's instruction. The absorbance was determined at 490 nm with 96-well plate ELISA reader. The percent of LDH released was determined using the formula: LDH release = (Absorbance of the supernatant)/(absorbance of the supernatant and cell lysate)*100.

2.5 Immunoassay for Cytokines

THP-1 Cells were seeded in 6-well plates at a density of 2.5 X 10^5 cell/ml. THP-1 cells were differentiated to macrophages with PMA (100 ng/ml) and Vitamin D3 (0.1 µM). Twenty-four hours after cell differentiation, cells were exposed to increasing concentrations of PHS-extract in a fresh serum-free medium in both, the absence and presence of LPS (1 µg/ml). Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify the secretion of TNF-α, IL-6 and IL-10. The absorbance at 450nm was read by a microplate reader (model 680; Bio-Rad Laboratories, Mississauga, ON, Canada) with the wavelength correction set at 550 nm. To calculate the concentration of TNF-α, IL-6 and IL-10, a standard curve was constructed using serial dilutions of cytokine standards provided with the kit.

2.6 Total RNA Isolation and cDNA Synthesis

Total RNA was isolated from cells using the Rneasy Plus Mini Kit (QIAGEN) according to manufacturer's instructions, and immediately frozen at –80°C until use. DNase-treated RNAs were used to synthesize cDNA with the Transcriptor First Strand cDNA Synthesis Kit using random hexamers as specified by the manufacturer (Maxima First Strand cDNA Synthesis Kit for RT-qPCR by Thermo). All RNA samples from a single experimental setup were reverse transcribed simultaneously and in duplicate, to minimize variation in the reverse transcription reaction.

2.7 RT-PCR Analysis

PCR amplifications and Advanced relative quantification analysis were achieved using a Light Cycler 480 instrument (Roche Applied Science) with software version LCS480 1.5.0.39. All reactions were performed in duplicates with the Light Cycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science) in a final 20 µl volume with 2.5 mM $MgCl_2$, 0.2 µM of each primer and 2 µL cDNA. Total RNA (0.1 µg) were used for a single reaction. Nucleotide sequences of oligonucleotide primers for the housekeeping glyceraldehyde-3-phosphate dehydrogenase (G3PDH) plus the IL-1, IL-2, IL-6, IL-10, or TNF-α primers pairs were used for RT-PCR which described elsewhere respectively [22]. Amplification conditions consisted of an initial pre-incubation at 95°C for 10 min (polymerase activation) followed by amplification of the target cDNA for 45 cycles (95°C for 15 s, 60°C for 20 s and extension time at 72°C 30 s).

2.8 Statistical Analysis

Error limits cited and error bars plotted represent simple standard deviations of the mean. When comparing different samples, results were considered to be statistically significant when $P < 0.05$ (Student's t test for unpaired samples).

3. RESULTS

3.1 Cytotoxicity Measurements

LDH and MTT assays were carried out in order to evaluate non-toxic concentrations of PHS-extract in human THP-1-derived macrophages.

3.2 LDH-release Test

Lactate dehydrogenase, an enzyme located in the cytoplasm, catalyzes the oxidation of L-lactate to pyruvate. There are two possible causes for the presence of lactate dehydrogenase in the cell culture media,: The first is cellular death and the second is a 'leak' in a cell membrane. When cells membranes are damaged, the lactate dehydrogenase activity in the media is remarkably elevated. Fig. 1 shows that in consistent with the MTT results, no significant changes of the LDH levels in the culture medium after exposure to extracts from PHS-extract at concentration up to 250 mg/ml in the presence or absence of LPS. A slight, but significant increase, was observed after treatment with concentration higher than 250 µg/ml.

Based on the MTT and LDH results, we decided to exclude concentrations of 500 µg/ml and to use concentrations below 250 µg/ml in the following experiments.

3.3 MTT Test

The metabolic activity can be evaluated by measuring the activity of the mitochondrial enzyme succinate dehydrogenase using the MTT test. This test is widely used in the *in vitro* evaluation of the toxicity of plant extracts. We applied the MTT test to evaluate the safety of extracts from PHS-extract in THP-1-derived

macrophages. Cells were exposed to increasing concentrations (0-500µg/mL of culture medium) of PHS-extract for 24h. No sign of any negative effects were observed after treatment with concentrations up to 250 µg/ml (Fig.1A). Concentrations higher than 250 µg/ml caused a significant reduction in the cell viability similar results obtained when the cells treated with PHS-extract in the presence of LPS (Fig. 1B).

Fig. 1A

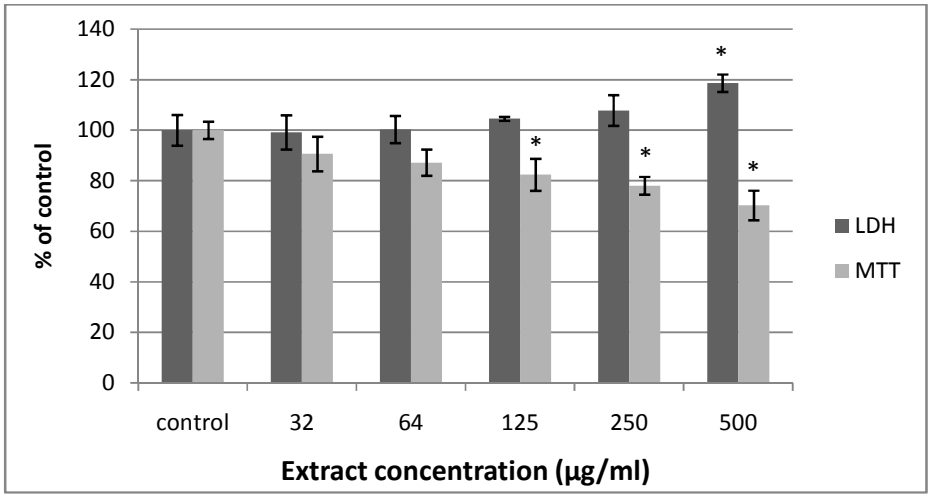

Fig. 1B

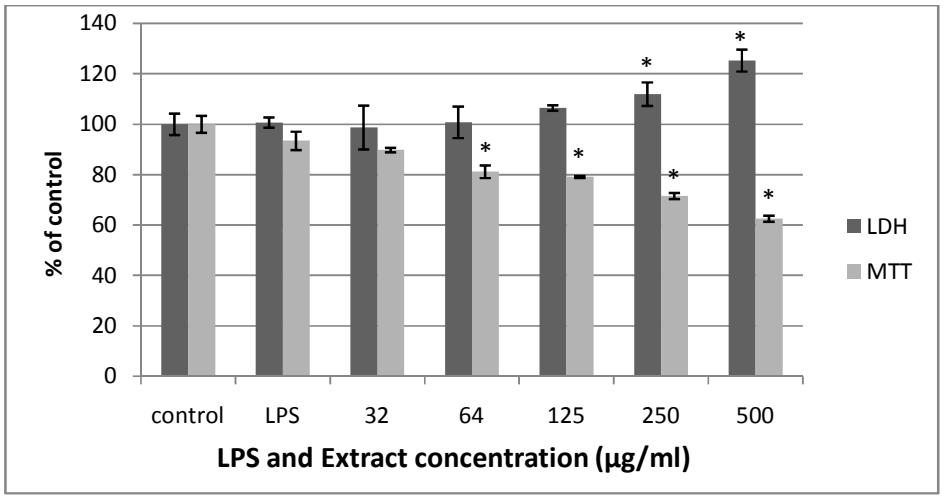

Fig. 1. MTT and LDH Assay in THP-1-derived macrophages after an overnight treatment with various concentrations of PHS-extract in the absence (A) or presence of 1µg/ml LPS (B). The absorbance of the MTT formazan was determined at 570 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage)of PHS-extract treated cells relative to untreated cells. Values represent means ± SD of three independent experiments carried out in triplicates

3.4 Effects on Gene Expression

We further studied the effect of PHS-extract on IL-1, IL-2, IL-6, IL-10, and TNF-α mRNA levels in LPS-activated THP-1-derived macrophages. As expected, mRNA expression of the pro-inflammatory effectors TNF-α and IL-1, IL-6, was dramatically elevated in THP-1-derived macrophages treated with LPS (1 μg/ml). Concomitant with its effect on TNF-α and IL-6 protein levels, PHS-extract decreased in a dose-dependent manner the mRNA levels of TNF−α and IL-6. A maximum reduction of 55% and 100% (compared to LPS stimulated cells) was reached for TNF-α and IL-6 respectively at dose of 125 μg of PHS-extract/ml (Fig. 2). In contrast, IL-10 mRNA expression was elevated to 500% of the control cells (none stimulated cells) levels at of 62 μg/ml of PHS-extract (Fig. 3B). No significant effects on the expression levels of IL-2 were observed after treatment with PHS-extract (data not shown).

Fig. 2 A

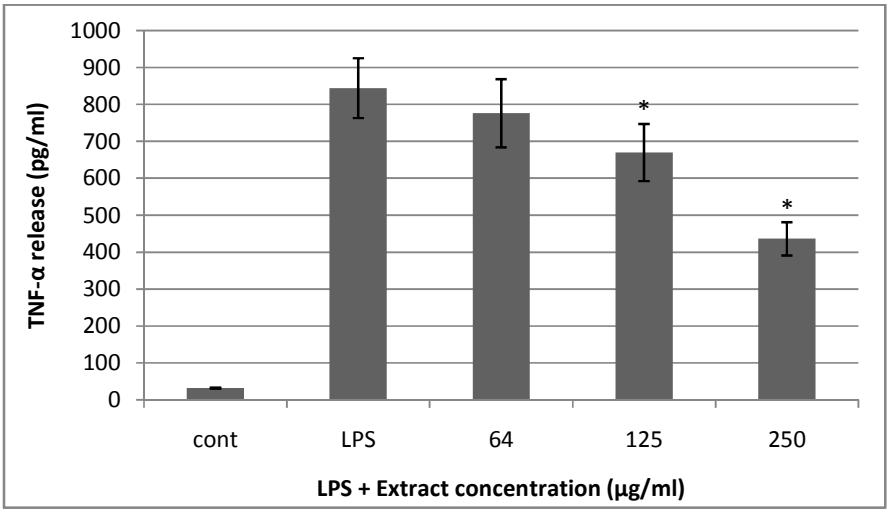

Fig. 2 B

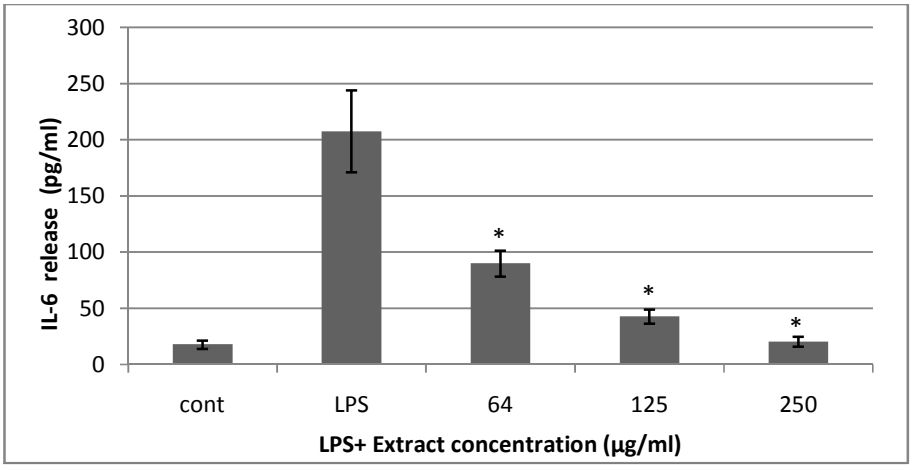

Fig. 2. Dose-dependent inhibition of LPS mediated production of IL-6 and TNF-α by PHS-extract. For each concentration treatment, the level of IL-6 and TNF- α release was measured after 4h and 6h after treatment, respectively. The bar heights represent the values, as a percentage of the control, of means ± SD of three independent ELISA experiments carried out in triplicates

3.5 IL-6, IL-10 and TNF-α

Production of the cytokines by THP-1-derived macrophages was tested in the culture supernatants using commercial ELISA kits. THP-1-derived macrophages produce detectable amounts of IL-6 and TNF-α after stimulation with LPS. Maximal TNF-α, IL-6 and IL-10 concentrations were detectable in the culture supernatants 4h, 6h, 24h after LPS stimulation, respectively (Data not shown). Therefore, these time points were used to characterize the effect of PHS-extract on TNF-α, IL-6, and IL-10 production in cultured THP-1 (Fig. 2 and Fig 4). Treatment of macrophages with PHS-extract alone did not affect the levels of any of the studied cytokines. Fig. 2 shows the TNF-α and IL-6 secretion into the culture supernatant of untreated and LPS treated THP-1 cells. PHS-extract inhibited the secretion of the two pro-inflammatory cytokines in a dose dependent manner. TNF-α and IL-6 secretion from LPS stimulated THP-1 cells was about 844pg/ml and 207 pg/ml, respectively. However, when those stimulated cells were treated with 250 µg/ml PHS-extract, TNF-α and IL-6 secretion was decreased to 463 pg/ml (~ 50% reduction) and 26 pg/ml (non-stimulated cells level), respectively. No significant cytotoxic effects were evident at these treatments (Fig. 1). These results were confirmed by assessing the mRNA expression at the same treatment conditions. TNF-α, IL-6 and IL-1 mRNA levels were elevated upon LPS treatment by 4, 4.5 and 9 folds, respectively. When the THP-1 cells were treated with 62µg/ml PHS-extract, theses cytokines expression was dramatically reduced by about 2, 4 and 5 folds respectively (Fig. 3A).

PHS-extract increased the secretion of the anti-inflammatory cytokine, IL-10 reaching 21 pg/ml of control levels at a concentration of 64µg/ml (Fig. 4). However, either IL-10 section, nor its gene expression were elevated at 125 or 250 µg/ml PHS-extract (Fig. 3B and Fig. 4). No significant cytotoxic effects were seen in these treatments (Fig. 1).

4. DISCUSSION

Herbal medicines containing *P. harmala* have been used in traditional Arab herbal medicine to treat various inflammatory diseases. However, only few studies have been conducted to evaluate the effects of *P. harmala* on inflammation; the first response of the immune system to infection or irritation. It is caused by cytokines (e.g. TNF-α, IL-1 and IL-6) and by eicosanoid (e.g. PGE2) production and secretion [10,21]. Macrophages are the major players of the innate immune response that promote inflammation via production of various key biomediators, including cytokines such as TNF-α and IL-6 and chemokines such as IL-8 [23-24]. However, these same cells can adapt an anti-inflammatory behavior in order to stop the acute inflammation and retrieve a steady state through the secretion of immuno-modulating agents such as IL-10. The human THP-1 cell line and THP-1-derived macrophages have been reported to be useful cellular models for both host/pathogen interaction studies and anti-inflammatory drug screening [2]. Here, we used THP-1-derived macrophages to assess the effect of PHS-extract on *in vitro* LPS-induced inflammation.

Pro-inflammatory cytokines, TNF-α and IL-6 are prominent contributors to chronic inflammatory disorders [10,21,24]. In recent years, various medicinal plant-derived products have been reported to modulate the mRNA and protein production of pro-inflammatory cytokines as well as anti-inflammatory cytokines. Flavonids, such as a moradicin, genistein, and silybin were found to reduce TNF-α production from LPS-treated RAW 264.7 cells [23]. Baicalin inhibited the induction of IL-1, IL-6, TNF-α, IFN-γ, monocyte chemotacticprotein-1 and macrophage inflammatory protein (MIP)-1at protein as well as at RNA levels from human blood monocytes treated with staphylococcal entero toxin [24]. In this study, we show that PHS-extract could modulate the regulatory mechanism of pro-inflammatory cytokines (TNF-α and IL-6) as well as anti-inflammatory cytokine (IL-10) in the LPS-activated THP-1-derived macrophages. PHS-extract remarkably inhibited in a dose-dependent manner the protein and mRNA expression of IL-6 and TNF-α. It is worth noting that treatment of macrophages with PHS alone did not affect the levels of any of the studied cytokines (Data not shown). These results suggest that PHS probably exerts anti-inflammatory effects through the suppression of IL-6 and TNF-α mRNA expression. PHS-extract was shown to decrease TNF-α activity in monocytes [25]. Similar results were found using different medicinal plants. For example, *Hypericum triquetrifolium* inhibited the production of NO and TNF-α, and the expression of iNOS and TNF-α, but not of IL-6 [2].

Fig. 3A

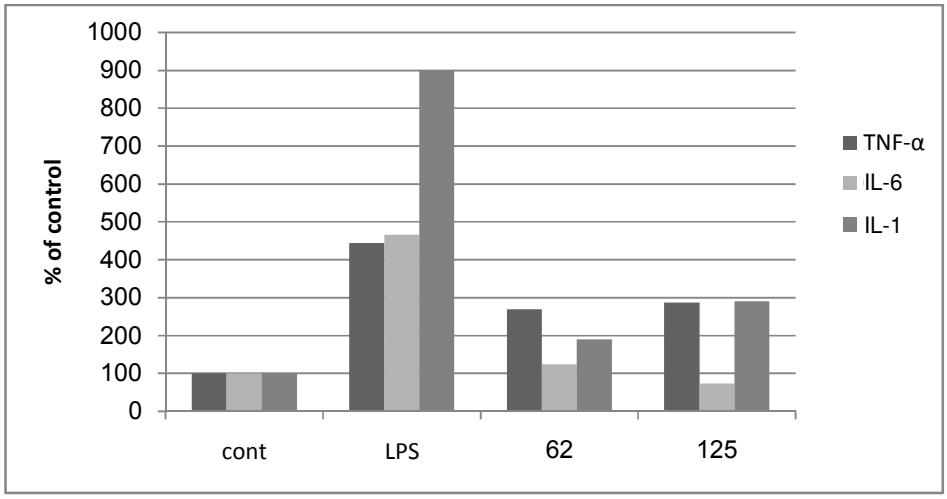

Fig. 3B

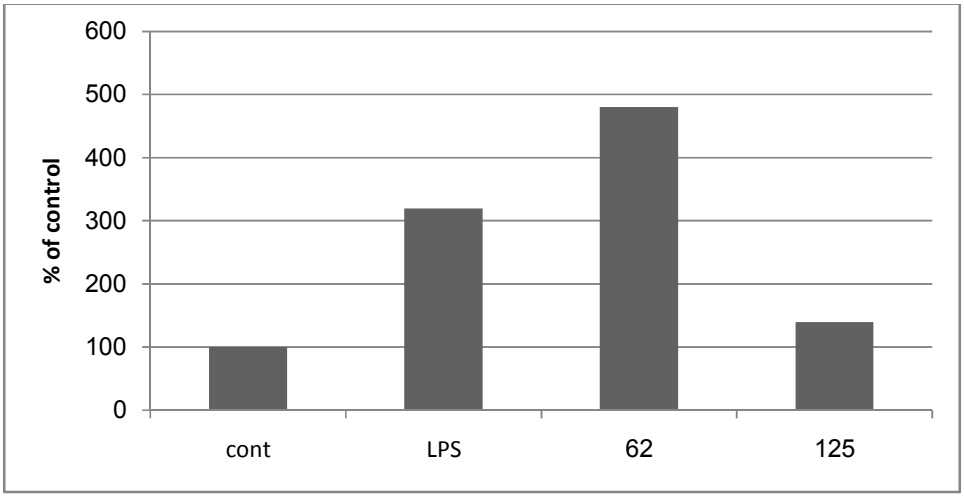

Fig. 3. PHS-extract effects on LPS-induced IL-1, IL-6, TNF-α (A) and IL-10 (B) gene expression in human THP-1-derived macrophages. Following 24h of THP-1 cells seeding, cells were treated with LPS in the absence and presence of PHS-extract for 2h, 4h, 6h, 12h, and 24h. Cells were then pooled, mRNA extracted and the mRNA levels were recorded as describe in materials and methods and is expressed as a percent of non-treated cells

Feverfew extracts were found to effectively reduce LPS-mediated TNF-α and CCL2 (MCP-1) releases by THP-1 cells [22-24]. Our results however empathize that the reduction in the pro-inflammatory cytokines and the elevation of the anti-inflammatory ones is due to inhibiting their mRNA transcription.

Treatment of THP-1-derived macrophages with PHS-extract remarkably increased levels of IL-10 protein and mRNA expression. These effects were evident at a significantly lower extract concentrations compared to maximal inhibitory concentrations of IL-6 and TNF-α. IL-10 is a potent immunoregulatory cytokine, the primary biological function of which seems to be the limitation and termination of inflammatory responses [26]. LPS treatment of THP-1-derived macrophages cells induced the secretion of a low level of the immunomodulator cytokine IL-10.

However, when PHS-extract was added to the cells, IL-10 level increased with the highest concentration at 64 µg/ml of PHS-extract.

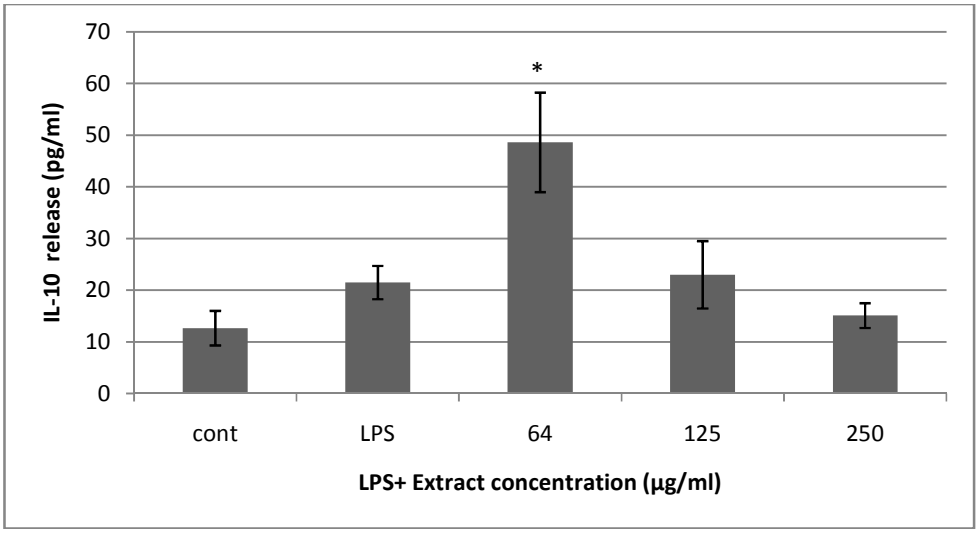

Fig. 4. PHS-extract effects on LPS mediated IL-10 production. For each concentration treatment, the level of IL-10 release is represented as a percentage of the control set at 100%. The bar heights represent the values of means ± SD of three independent ELISA experiments carried out in triplicates

Previous reports have clearly shown the antagonist effect of IL-10 on the secretion of pro-inflammatory cytokines [27,28], suggesting that PHS-extract -mediated inhibition of the LPS-induced secretion and mRNA expression of IL-6 and TNF-α may pass through the induction of IL-10 secretion. Several inflammatory diseases share the dual characteristic of a very low blood level of IL-10 and a high blood level of TNF- α. Furthermore, injection of the exogenous recombinant form of IL-10 caused a decrease in the blood level of TNF-α that has proven beneficial for such diseases [29,30]. The ability of PHS-extract extract to modulate both, the proinflammatory and anti-inflammatory cytokines in by LPS-activated THP-1-derived macrophages represents an additional argument for the suggestion that it is an alternative or a complement that may help in the treatment and/or prevention of inflammatory diseases. However, taking into consideration that PHS-extract affects the expressions of pro-inflammatory and the anti-inflammatory cytokines at different concentrations, a more detailed study regarding the effects of PHS-extract on these cytokines is necessary. However, since injection of exogenous recombinant form of IL-10 caused a decrease in the blood level of TNF-α [29,30] in addition to the reported antagonist effect of IL-10 on the secretion of pro-inflammatory cytokines [27,28] could explain the obtained results here.

5. CONCLUSION

P. harmala has been used in traditional Greco-Arab and Islamic herbal medicine to treat various inflammatory diseases [6,31,32]. Results obtained here suggest that PHS-extract probably exerts anti-inflammatory effects through differential modulation of IL-1, IL-6, IL-10 and TNF-αprotein and mRNA expression levels. Based on the current study, the increase in IL-10 protein levels and mRNA expression and the increase in expression of IL-6 and TNF-*α* mRNAs support the use of this plant for purposes of modulating the immune system. These results represent an additional argument for the suggestion that PHS-extract is an alternative or a complement that may help in the treatment and/or prevention of inflammatory diseases.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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