

Anti-Inflammatory Effects of Herbal-Derived Factors are Mediated By Down Regulation of Pro-Inflammatory Cytokines

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Abstract

The aim of the present *in vitro* study is to evaluate the role of pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6) in the observed anti-inflammatory effects of *Hypericum triquetrifolium* and *Peganum harmale*. Therefore, water extracts from the aerial parts of the two plants were tested for their anti-inflammatory effects in vitro using cells from the human monocyte cell line THP-1. Anti-inflammatory effects were assessed by measuring the levels of secretory proteins and mRNA of TNF- α and IL-6 production of LPS-activated THP-1 cells. Cells were treated with 5 μ g lipopolysaccharide/ml (LPS) in the absence and presence of increasing concentrations of plant extracts. During the entire experimental period, we used plant extracts concentrations (up to 250 μ g/ml) that had no cytotoxicity as measured in MTT assay. *Hypericum triquetrifolium* extracts totally reduced the LPS induced TNF- α expression and secretion. *Peganum harmale* extracts inhibited the IL-6 production in a dose dependent manner reaching the control levels of untreated cells at a concentration of 125 μ g/ml. These results demonstrate that the anti-inflammatory effects of the two plants are mediated through the inhibition of the pro-inflammatory cytokines TNF- α and IL-6.

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Introduction

Traditional therapies have been utilized by people in Mediterranean region who have faith in spiritual healers, homeopaths or even many herbalists. These are the first choice for people with problems such as infertility, impotence, diabetes, obesity, epilepsy, psychosomatic troubles, and many other diseases. Arabic medicine had shown remarkable achievements in curing inflammatory diseases in general, and clearly distinguishing between several subtypes of inflammatory diseases and has offered tens of plants for treating various types of inflammations (Said et al., 2002, Saad et al., 2005, Said et al., 2006, Said et al., 2008, and Azaizeh et al., 2006).

Inflammation is the first response of the immune system to infection or irritation. It is caused by cytokines. There are two types of cytokines; pro-inflammatory and anti-inflammatory. Thus, inhibitors of the pro-inflammatory cytokines have been considered as a candidate of anti-inflammatory drugs. Lipopolysaccharide (LPS)-activated macrophages are usually used for evaluating the anti-inflammatory effects of various materials. LPS is a principle component of the outer membrane of Gram-negative bacteria, is an endotoxin that induces septic shock syndrome and stimulates the production of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor- α (TNF- α), interleukins, prostanoids, and leukotrienes (Hewett and Roth, 1993; Watson et al., 1999, Kubes and McCafferty, 2000). Therefore, LPS plays a key role in not only 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, deregulated inflammation leads to the massive production of pro-inflammatory cytokines such as TNF- α , interleukin-1 (IL-1) and interleukin-6 (IL-6) by macrophages, which can cause tissue injury and multiple organ failure (Delgado et al., 2003). The inflammatory

process is controlled by immune-suppression cytokines such as IL-10 and IL-4. Macrophage-derived

Herbal medicines containing *Hypericum triquetrifolium* and *Peganum harmale* have been used in traditional Arab herbal medicine to treat various inflammatory diseases. The classic Arabic name for *Hypericum triquetrifolium* is *Dathi* or *Nabtat Yohanna* (Said et al., 2002, Said et al., 2005, Said et al., 2006, Said et al., 2008, and Azaizeh et al., 2006). Our previous studies show that *Hypericum triquetrifolium* is not used any more within the practitioner communities in the Galilee and in the West Bank. This fact reflects an extinction process of important elements of the Arab herbal medicine heritage (Said et al., 2002, Said et al., 2005, Said et al., 2006, Said et al., 2008, and Azaizeh et al., 2006). Utilizing the knowledge derived from traditional Arab herbal medicine and a recent in vivo report in which *Hypericum triquetrifolium* extract exhibited anti-inflammatory activity in rats (Ozturk et al., 2002), this study explored the anti-inflammatory mechanism of *Hypericum triquetrifolium* and *Peganum harmale*. Therefore, the expression and release of pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6) in human monocytic (THP-1) cells were measured here.

Materials and methods

Preparation of Plant Extracts: 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 frozen at -70°C until use. The frozen filtrate was freeze-dried in a lyophilizer (48-72 hours). The freeze-dried extracts were stored at -70°C for further evaluation. These crude extracts were used for the following experiments.

Cell culture: The human monocytic cell line THP-1 (ATCC 202-TIB) (Nathan et al., 1992) was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle's medium

(DMEM) with a high glucose content (4.5 g/l), supplemented with 10% vol/vol inactivated fetal calf serum, 1% nonessential amino acids, 1% glutamine, 100 U/mL penicillin, and 10µg/ml streptomycin. Cell lines were maintained in a humidified atmosphere of 95% O₂ – 5% CO₂ at 37°C. Cells were activated with PMA (100 ng/ml) and Vitamin D₃ (0.1 µM). Twenty-four hours after cell activation, cells were exposed to various concentrations of *Hypericum triquetrifolium* and *Peganum harmale* extracts in a fresh serum-free medium in both the absence, and presence, of LPS (5 µg/ml).

MTT assay: Twenty-four hours after cell seeding, cells were incubated with varying concentrations of water extracts of *Hypericum triquetrifolium* and *Peganum harmale* for 24 hours at 37°C. The MTT assay was carried out as previously described Saad et al., 2006).

Immunoassay for Cytokines: Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) were used to quantify TNF-α and IL-6. The absorbance at 450 nm 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 and IL-6, a standard curve was constructed using serial dilutions of cytokine standards provided with the kit.

Results

Toxicity measurements: the anti-inflammatory effects of *hypericum triquetrifolium* were evaluated here in cells from the human monocyte cell line. MTT and LDH assays were carried out in order to evaluate non-toxic concentrations of *Hypericum triquetrifolium* and *Peganum harmale*. We applied the MTT test to evaluate the safety of extracts from *Hypericum triquetrifolium* and

Peganum harmale in cells from the humane monocyte cell line THP-1. Cells were exposed to increasing concentrations (1-500 $\mu\text{g/mL}$ of culture medium) of *Hypericum triquetrifolium* and *Peganum harmale* extracts for 24h. No sign of any negative effects were observed after treatment with concentrations up to 250 $\mu\text{g/ml}$ (Fig.1). Concentrations higher than 250 $\mu\text{g/ml}$ caused a significant reduction in the cell viability.

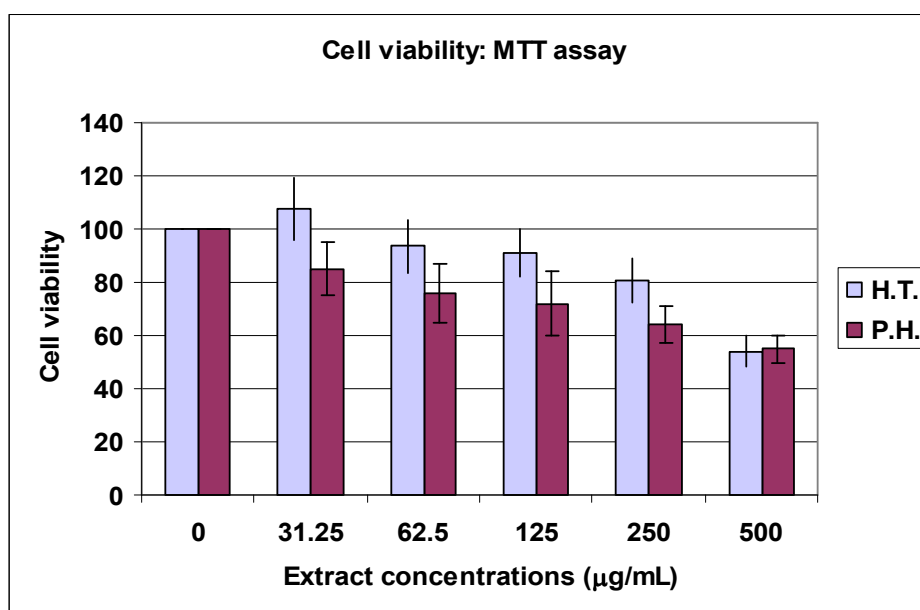


Figure 1: MTT Assay in THP-1 cells after an overnight treatment with various concentrations of extract from *Hypericum triquetrifolium* (H.T.) and *Peganum harmale* (P.H.). Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells. Values represent means \pm SD of three independent experiments carried out in triplicates.

Pro-inflammatory cytokines IL-6 and TNF- α : It was found that THP-1 produce detectable amounts of IL-6 and TNF- α after stimulation with LPS. Maximal TNF- α and IL-6 concentrations were detectable in the culture supernatants 4h and 6h

after LPS stimulation, respectively. Therefore, the 4h and 6h time points were used to characterize the effects of *Hypericum triquetrifolium* and *Peganum harmale* extracts on TNF- α and IL-6 production by cultured THP-1, respectively.

Figure 2 and Figure 3 show the TNF- α and IL-6 secretion into the culture supernatant of untreated and LPS treated THP-1. *Hypericum triquetrifolium* extracts inhibited the TNF- α production in a dose dependent manner reaching a 60% reduction compared to untreated cells at a concentration of 125 $\mu\text{g/ml}$ (Fig. 2). *Peganum harmale* extracts inhibited the TNF- α production reaching a 28% reduction compared to untreated cells at a concentration of 125 $\mu\text{g/ml}$ (Fig. 2). *Peganum harmale* extracts inhibited the IL-6 production in a dose dependent manner reaching the control levels of untreated cells at a concentration of 125 $\mu\text{g/ml}$ (Fig.3). No effects on the production levels of IL-6 were seen after LPS treatment in the presence of *Hypericum triquetrifolium* (Fig. 3).

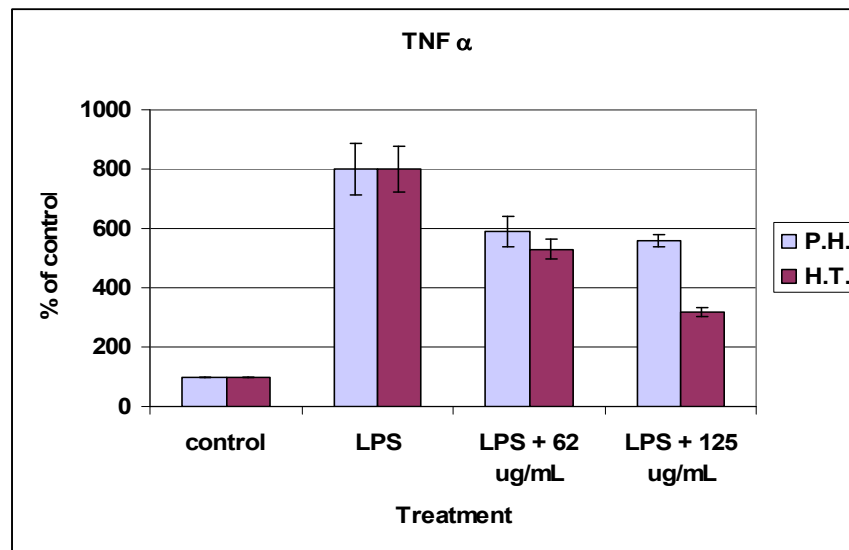


Figure 2: Dose-dependent inhibition of LPS mediated production of TNF- α by *Hypericum triquetrifolium* (H.T.) and *Peganum harmale* (P.H.) extracts. For each concentration treatment, the level of TNF- α release is represented as a percentage of the control set at 100%. The bar heights represent the values of means \pm SD of three independent ELISA experiments carried out in triplicates.

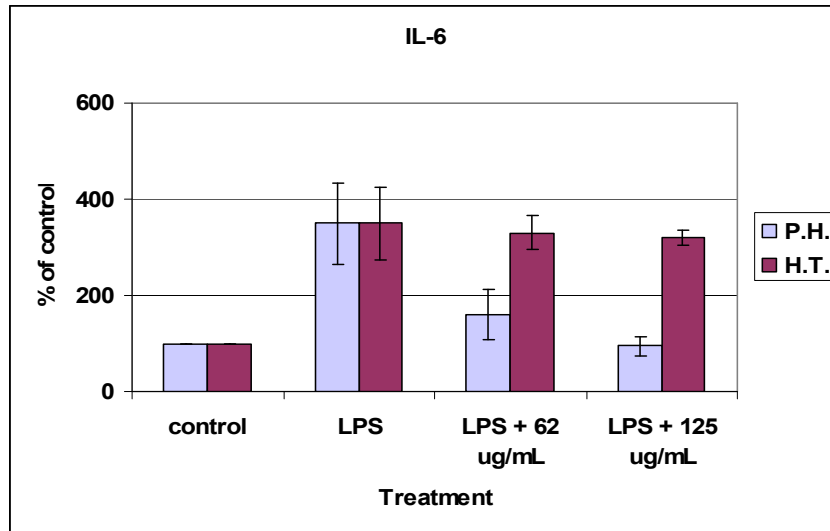


Figure 3: Dose-dependent inhibition of LPS mediated production of IL-6 by *Hypericum triquetrifolium* (H.T.) and *Peganum harmale* (P.H.) extracts. For each concentration treatment, the level of TNF- α release is represented as a percentage of the control set at 100%. The bar heights represent the values of means \pm SD of three independent ELISA experiments carried out in triplicates.

Discussion

Inflammation is the first response of the immune system to infection or irritation. It is caused by cytokines such as TNF- α , IL-1 and IL-6, and by eicosanoid such as PGE₂. Thus, inhibitors of these cytokines have been considered as a candidate for anti-inflammatory drugs. Monocytes/macrophages are key mediators of

inflammation and widely distributed in the body (Gilroy et al., 2004). Therefore, monocytic cell line THP-1, which represents an appropriate model system to study immune responses, was utilized to investigate the anti-inflammatory effects of *Hypericum triquetrifolium* and *Peganum harmale* extracts. Herbal medicines containing *Hypericum triquetrifolium* and *Peganum harmale* extracts 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 these two plants on inflammation. In this study, we show that both extracts could modulate the regulatory mechanism of pro-inflammatory cytokines (TNF- α and IL-6) in the LPS-activated THP-1 cells. *Hypericum triquetrifolium* inhibited the production and expression of TNF- α but not of IL-6. *Peganum harmale* inhibited the production of both, IL-6 and TNF- α .

Several cytokines are deeply associated with inflammatory diseases. In particular, TNF- α and IL-1 are prominent contributors to chronic inflammatory disorders (Gilroy et al., 2004). TNF- α and IL-1 receptor antagonists have been clinically successful to improve the symptoms of RA patients. SAIDs, such as prednisolone and dexamethasone, are known to reduce the production of these cytokines. In recent years, various medicinal plant-derived factors have been reported to regulate the production of pro-inflammatory cytokines. Flavonoids, such as amoradin, genistein, and silybin were proved to inhibit TNF- α production from LPS-treated RAW 264.7 cells (Cho et al., 2000). Baicalin inhibited the induction of IL-1, IL-6, TNF- α , IFN- γ , monocyte chemoattractant protein-1, macrophage inflammatory protein (MIP)-1, and MIP-1 at protein as well as at RNA levels from human blood monocytes treated with staphylococcal enterotoxin (Krakauer and Young 2001). Here, we demonstrate that *Hypericum triquetrifolium* and *Peganum harmale* inhibits the production of LPS-induced TNF- α production by down-regulating the transcription of the TNF gene. Similar results were found using different medicinal

plants. For example, feverfew extracts were found to effectively reduce Lipopolysaccharides (LPS)-mediated TNF- α and CCL2 (MCP-1) releases by THP-1 cells (Chen and Cheng 2007). Moutan Cortex extracts were reported to exhibit anti-inflammatory effects through the inhibition of iNOS and COX-2 expression by suppressing the phosphorylation of I-kBa and the activation of NF-kB. *Uncaria tomentosa* extracts inhibited the MAP kinase-signaling pathway and altered cytokine expression in THP-1 monocyte-like cells with *Uncaria tomentosa* (Chun et al., 2007). *Hypericum triquetrifolium* inhibits the production of LPS-induced TNF- α production by down regulating the transcription of the TNF gene but has no significant effects of the production levels of IL-6. These results suggest that the anti-inflammatory effects of *Hypericum triquetrifolium* and *Peganum harmale* is mediated via the down regulation of the transcription of the TNF gene.

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تتحقق فعالية النباتات المضادة للالتهابات بواسطة تأثيرها على السيتوكينات (Cytokines) الالتهابية والمضادة للالتهاب

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تلخيص:

تهدف الدراسة المعروضة إلى بحث تأثير مستخلصات مختلفة لنبتتي الداذي والحرمل في مقاومة الالتهابات. وهما نبتتان معروفتان، وفقاً للطب الشعبي المحلي الفلسطيني، بقدرتهما على مقاومة الالتهابات.

تأثير المستخلصات النباتية على انقسام الخلايا وعلى مستوى إنتاج السيتوكينات ($IL-6$ ، $TNF\alpha$). سيتم تقييمه مخبرياً (in vitro) بواسطة خلايا دم البلعمة أحادية الخلية THP-1، وذلك من خلال استخدام أنظمة فحصية محددة بوجود أو غياب محرّض الالتهاب من نوع LPS (Lipopolysacchride).

عليه فإنّ دراستنا تركّزت على فحص تأثير تلك المستخلصات على معدلات إنتاج طليعة السيتوكينات الالتهابية ($TNF\alpha$ ، $IL-6$). بالإضافة لإنتاج الـ RNA الرسول (mRNA) الخاص بـ $TNF-\alpha$ و $IL-6$.

خلال مجمل التجربة، استعملت تراكيز غير سامة (حتى $250\mu g/ml$) وفق ما أظهرت نتائج فحص الـ MTT. مستخلصات نبتة الداذي قلّلت بشكل كامل التعبير والإفراز عن السيتوكين $TNF-\alpha$ المستحث بواسطة LPS. بالإضافة لذلك، فإنّ مستخلص نبتة الحرمل بتركيز $125\mu g/ml$ أعاق عملية إنتاج السيتوكين $IL-6$ حتى أصبح بمستوى الخلايا غير المعالجة. هذه النتائج تؤكد أن التأثيرات المضادة للالتهابات لهاتين النبتتين تمر عبر كبت السيتوكينات $TNF-\alpha$ و $IL-6$ الموالية للالتهابات.