



Anti-mycobacterial and anti-inflammatory activity of *Peganum harmala*

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ABSTRACT

The aim of this study was to evaluate the antimycobacterial and anti-inflammatory activity of the methanolic extracts of *Peganum harmala* (Esfand) collected from Golestan province, north of Iran. Methods: Hydroalcoholic extract of seeds of *Peganum harmala* were obtained and screened for anti-mycobacterial activity by disc diffusion (DD) method. The anti-inflammatory activity of the extract was evaluated by cytokines measurement using ELISA in a model of phagocytized intracellular *Mycobacterium tuberculosis*, H37Rv strain, in dU937 cells. Free radical-scavenging activity, total phenolic, flavonoids and Harmalin concentrations were assessed to investigate phytochemical properties of the extract. Our data showed the inhibitory effect of the extract on growth of all strains of *Mycobacterium tuberculosis* even on drug resistant strains. Cytokines production in culture media showed the anti-inflammatory activity of the extract. The antioxidant (IC₅₀ (DPPH assay) was 53.6 ± 0.50 mg/L. The amount of total phenolic and flavonoids components was 61.5 ± 0.80 gGAE/kg and 42.20 ± 0.60 respectively. These findings revealed the potential ability of the *Peganum harmala*'s seed as a complementary medicine to treat tuberculosis.

Keywords: Herbal plants, *Mycobacterium Tuberculosis*, *Peganum harmala*, Anti-inflammatory, Cytokines

INTRODUCTION

Tuberculosis (TB) is second only to HIV/AIDS as the greatest killer worldwide due to a single infectious agent. In 2013, 9 million people fell ill with TB and 1.5 million died from the disease [1, 2]. This information is expected to increase in future due to the emerging of multidrug-resistant (MDR) and extensively-drug resistant strains (XDR) of TB organisms [3–5]. The emergence of MDR and XDR TB now makes urgent need for new strategy to cure TB worldwide [6-7]. About one-third of the world's population is infected with *M. tuberculosis* and that only around 10% break down with disease during their lifetime [8]. However in persons with compromised immune systems, such as people living with human immunodeficiency virus (HIV) or other conditions that impair the immune system risk of active TB is greater. Individuals with HIV infection who start antiretroviral therapy are susceptible to immune reconstitution disorders (IRIS). Tuberculosis-immune reconstitution inflammatory syndrome (TB-IRIS) is an exaggerated, dysregulated immune response against dead or viable antigens of *Mycobacterium tuberculosis* that frequently occurs after initiation of antiretroviral therapy despite an effective suppression of HIV viremia. These changes were temporally associated with a rise in the serum concentration of tumour necrosis factor- α and may have resulted from macrophage activation in response to release of mycobacterial cell wall antigens during treatment [9-11]. In patients with active tuberculosis, proinflammatory and immunosuppressive immune mechanisms are present concomitantly, the balance of which might be altered during the early stages of antituberculosis treatment leading to a worse stage of the disease [12]. Therefore using agents as complementary medicine to treat tuberculosis and the prevention of these complications is essential. Natural products isolated from plants have played an important role in discovery of drugs against infectious diseases and modulation of immune system either alone or in group [13]. *Peganum harmala* L commonly called as Esfand is famous for its antimicrobial activities, due to certain compound,

such as alkaloids that are found in its seeds and roots [14]. So far, several alkaloids with pharmaceutical activity including harmaline, vasicine, vasicinon and peganine have been obtained from the various parts of this plant. This plant is widely distributed in North Africa, Mediterranean, the Middle East, Pakistan, India and Iran [15]. *Peganum harmala* traditionally has been used in Iran as an antiseptic and disinfectant agent by burning its seeds [16]. The objective of this study was to assess the anti-mycobacterial and anti-inflammatory activity of *Peganum harmala* against *Mycobacterium tuberculosis* strains.

EXPERIMENTAL SECTION

Preparation of the extract

Peganum harmala (Esphand) was chosen to study because of the inhibitory effect of this plant on the *M. smegmatis* (PTCC 1307) and *M. bovis* BCG strains revealed in the previous study [17]. The seeds of *Peganum harmala* were collected from south east of Golestan province (2750 m – Tash Mountains) then was identified in the herbarium of Faculty of Plant Science, Islamic Azad University of Gorgan branch. The seeds were air-dried in the dark at room temperature and then ground into the fine powder using electric blender. Plant hydro-alcoholic extracts were prepared by maceration method [18]. Plant material was extracted with 70% ethanol solvent by percolator apparatus at room temperature then was removed from percolator and filtered twice through Whatman filter paper No. 1 (Whatman, UK). The concentrated plant extracts were dissolved in dimethyl sulphoxide (DMSO) (SIGMA, USA) to get a stock solution (200 mg/mL) and further diluted to these concentrations: 100, 50, 25, 12.5, 6.25, 3.12, 1.6 mg/mL. The final concentration of DMSO in all assays was 2% or less, which is nontoxic for mycobacteria [19].

Antibacterial activity

Antibacterial activity was determined by disc diffusion method as described by Taylor *et al* [20]. A total of five strains of *Mycobacterium Tuberculosis* were used; four clinical isolates were identified by conventional methods [21] including two MDR and two sensitive strains to rifampin and isoniazid from patients (local isolates) and a drug-susceptible reference strain of *M. tuberculosis*, H37Rv sensitive to rifampin and isoniazid, kindly provided by Dr. Hashemi (Ahvaz university of Medical Sciences, Ahvaz, Iran); All strains were maintained on Löwenstein-Jensen slants containing malachite green. Rifampin and Isoniazid was used as the positive control and discs treated with 2% DMSO were used as the negative control. The culture plates were sealed in plastic bags and incubated at 37°C for 3 weeks, after which the culture-growth was clearly visible on the agar. The diameter of the zone of inhibition around each of the discs was measured and recorded. Each experiment was performed in triplicate and each test was repeated at least two times.

Cell culture and differentiation

The human macrophage cell line U937 (from Pasture Institute of Iran) was used to evaluate the anti-inflammatory activity of the extract. Human U937 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, at 37 °C in an incubator containing 5% CO₂. These monoblasts (U937) are undifferentiated [22-23]. The cells were seeded into culture plates at a cell density of 2×10^5 cells/well. The cells were exposed to five ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma, USA) for 3 days to differentiate. Cell viability was determined by 0.2% trypan blue exclusion test.

Macrophage infection and cytokine production

The H37Rv strain of *M. Tuberculosis* was used to infect dU937 cells. Prior to infection, bacteria were opsonized as follows: 10^9 viable organisms were suspended in one ml of RPMI 1640 containing 50% AB⁺ serum and rocked for 30 min at 37°C. The bacteria were then resuspended in one ml of RPMI 1640 and clumps were disaggregated mechanically using glass beads. Immediately before infection, serum-coated *M. tuberculosis* was washed once in phosphate-buffered saline (PBS). The suspension of bacteria was added into the cell culture containing adherent macrophages at a multiplicity of infection (MOI) of 5 viable bacilli per cell in triplicate. After incubation at 37°C for 3 h, infected cells were washed three times with warm RPMI medium to eliminate free bacteria and were cultured in complete medium. For evaluation of mycobacterial binding, Zeihl-Neelsen (ZN) staining and light microscopy was used [24].

M. tuberculosis-infected dU937 cells were treated with different concentration of plant extract (200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml) for 48 hours. Cell viability was determined by 0.2% trypan blue exclusion test. Culture supernatants were collected from three wells of infected and uninfected control dU937 cells and then were frozen at -70 °C until assayed for human TNF- α , IL-10 and IL-6 concentration with commercial ELISA kits (eBioscience) according to the manufacturer's instructions. Sensitivities of the assays were 1 pg/ml for IL-10, 5 pg/ml for TNF- α and 0.92 pg/ml for IL-6.

Statistical analysis

Statistical significance was determined by SPSS software and student's t-test. The significance level was set at 0.05.

Phytochemical tests

To evaluate the phytochemical properties of the *Peganum harmala* extract, free radical-scavenging activity, and total phenolic and total flavonoids content were measured. The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH), BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) were purchased from Sigma Chemical Co. (St., Louis, USA).

Free radical scavenging activity determination

Diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract [25]. Different concentrations of extract were added at an equal volume to methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Scavenging of the DPPH free radical was measured using the following equation: % DPPH radical scavenging = [(absorbance of control – absorbance of test Sample) / (absorbance of control)] × 100. IC50 values denote the concentration of sample which is required to scavenge 50% of DPPH free radicals. BHA and BHT were used as references for radical scavengers.

Total phenols determination

Total phenolic contents were determined by FolinCiocalteu method [26]. Briefly, 0.5 ml of the sample organic acid (standard phenolic compound) was mixed with FolinCiocalteu reagent (5 ml) and aqueous Na₂CO₃ (4 ml, 1 M). The mixture was allowed to stand for 15 min and the total phenols were determined by colorimeters at 765 nm. Gallic acid was used as a standard for calibration curve. Total phenol values are expressed in terms of mg equal Gallic acid in 1 g powder dry plant. All measurements were performed in triplicate.

Total flavonoids determination

Total flavonoids content was estimated by the Aluminum chloride method [26]. Plant extract (0.5 ml) were mixed with 1.5 ml of solvent, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. They were kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. A standard calibration plot was generated at 415 nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of dry plant powder.

High-Performance Liquid Chromatography Analysis (HPLC)

HPLC quantification of harmalin was performed on a VWD Agilent 1200 series HPLC system with UV–VIS spectrophotometric detector (330 nm), column type; Agilent eclipse XDB C18, 5 μm, 150 × 4.6 mm. Solvent system consisting of 2-propanol, ACN (acetonitrile), Water and formic acid, was used as the mobile phase in isocratic mode. The flow rate was 0.75 ml/min and the column temperature was maintained at 25°C. The active compound "harmalin" in the extract was identified by comparison with pure standard

RESULTS**Antibacterial activity**

Peganum harmala extract exhibit antimycobacterial activity against all strains of Mycobacterium Tuberculosis, the mean of inhibitory zone was 18.7±3.5 mm at concentration of 200mg/ml of extract (Table1). No significant differences were observed between MDR and non-MDR strains (P< 0.05).

Table1: Antimycobacterial activity of *Peganum harmala* extract against *M. tuberculosis* tested based on disc-diffusion method

Bacteria	<i>Peganum harmala</i> extract (mg/ml)				Isoniazid 5 μg/ml	Rifampin 5 μg/ml
	200	100	50	25		
MDR ₁	20	16	12	10	R	R
MDR ₂	17	15	10	8	R	R
Non-MDR ₁	18	15	14	12	20	19
Non-MDR ₂	22	15	12	8	19	18
MDR _{1&2}	18.5±2.1	15.5±3.5	11.5±1.5	9±1.4	R	R
Non-MDR _{1&2}	19±1.4	14.5±0.7	12.5±0.7	9±1.4	19.5	18.5
H37RV	18	15	8	R	S	S

Inhibition zone diameter around test disc (mm). Data expressed as mean ± standard deviation (n= 3)

Macrophage infection and cytokine production

Approximately 50% of PMA treated U937 cells (dU937) binding one or more bacilli in the presence of serum. Mycobacterial binding and phagocytosis were evaluated using Zeihl & Neilsen (ZN) staining and light microscopy (Figure 1). Cytotoxicity of the extract was performed in dU937 cells by trypan blue exclusion test. The extract did not show cytotoxicity against dU937 cells at different concentrations ($IC_{50} > 200\text{mg/ml}$).

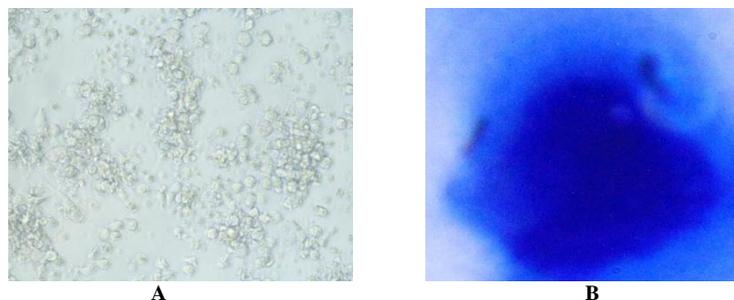


Fig.1 A; The cluster and adherent form of dU937 cells. B; Intracellular Mycobacterium tuberculosis was showed by Zeihl ± Neilsen (ZN) staining and light microscopy

The effects of *Peganum harmala* extract on cytokines productions

Culture supernatants of infected and uninfected control dU937 cells were investigated for TNF- α , IL-10 and IL-6 by ELISA method in the absence and presence of the extract. All of the above-mentioned cytokines were secreted by dU937 cells. Infection of dU937 cells by *M. tuberculosis* increased production of TNF- α significantly ($p < 0.05$) and it was decreased significantly after treatment with the extract ($p < 0.05$). Production of IL-6 and IL-10 were decreased to the undetectable level in *M. tuberculosis*-infected dU937 cells and also after treatment with the extract. The results in each panel represent pooled data from three independent experiments (Figure 2).

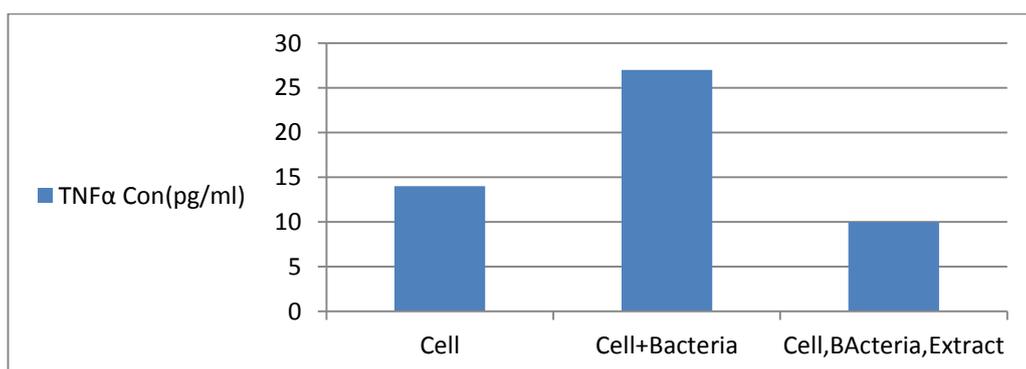


Figure 2. TNF- α were measured by ELISA method in the presence and absence of the extract. Values represent the means of culture supernatants collected from triplicate wells. Infection of dU937 cells by *M. tuberculosis* increased production of TNF- α significantly ($p < 0.05$) and it was decreased significantly after treatment with the extract ($p < 0.05$)

Phytochemical tests

Diphenyl-2-picryl hydrazyl radical (DPPH) method was used for determination of free radical-scavenging activity of the extract. The amounts of total phenolic content were determined by FolinCiocalteu method and Total flavonoids content were estimated by the Aluminum chloride method (Table 2). The content of Harmalin in the extract was estimated by HPLC method. According to the standard curve, Harmalin concentration in the extract was 21.25 mg/l.

Table 2: IC_{50} (Mg/L) values of DPPH scavenging activity and total phenolic (mgGAEg-1) and flavonoid (mgQUEg-1) content of the extract

Sample	IC_{50} (Mg/L)	Total phenol (mgGAEg-1)	Total flavonoid (mgQUEg-1)
<i>Peganum harmala</i>	53.6 ± 0.5	61.5 ± 0.8	42.2 ± 0.6
BHA	3.25 ± 0.351	-	-
BHT	2.93 ± 0.404	-	-

DISCUSSION AND CONCLUSION

Despite all efforts to control tuberculosis, the disease is still a public health problem in the world [27]. One of the issues considered in this regard is the complexity of the treatment of drug-resistant strains and also syndromes caused by the return of the ability of the immune system in patients receiving anti-TB drugs or AIDS patients treated

with antiviral drugs. Immune reconstitution inflammatory syndrome (IRIS) is a paradoxical inflammatory reaction against a foreign antigen in patients who have started antiretroviral therapy and who have undergone a reconstitution of their immune responses against this antigen [28]. Tuberculosis is the best-described presentation of IRIS [29-31]. The problems with current methods of tuberculosis treatment caused researchers to discover new drugs or alternatives to treat tuberculosis. The present study was conducted to assess the anti-mycobacterial and anti-inflammatory effects of the seeds of *Peganum harmala*, a medicinal plant reputed in folklore medicine for its anti-inflammatory and antimicrobial activities. *Peganum harmala L.* (Zygophyllaceae) commonly called as Esfand and Suryin Rue is widely distributed in North Africa, Mediterranean, the Middle East, Pakistan, India and Iran. *Peganum harmala* is a medicinal plant with antimicrobial, anti-inflammatory and analgesic properties [32]. The seeds of *P. harmala L.* contain about 2–6% pharmacologically active alkaloids, which are mostly carbolines such as harman, harmine, harmaline and harmalol [33]. Our data showed the strong antimycobacterial activity of hydro-alcoholic extracts of seeds of *Peganum harmala* against MDR and non-MDR isolates and a drug-susceptible reference strain of *M. tuberculosis* H37Rv. The Golestan province is a place in the north of Iran with high prevalence of tuberculosis [34] and a very proper climate for herbal plants to grow. It seems that there is a good opportunity to study on medicinal plants and their secondary metabolites to improve the treatment of infectious disease particularly tuberculosis.

All of the three cytokines namely TNF- α , IL-6 and IL-10 were secreted by dU937 cells. Infection of the cells with *M. tuberculosis* increased production of TNF- α . The production of TNF- α was decreased after treatment with the extract in the culture media supernatant of infected cells. In contrast, production of IL-6 and IL-10 were decreased after infection with *M. tuberculosis* to undetectable levels and it was also not detected after treatment with the extract. TNF- α is essential for the initiation of immune responses against *Mtb* infection [35, 36]. It is well established that treatment with TNF- α inhibitors, such as infliximab, adalimumab, and etanercept, can lead to the reactivation of *M. tuberculosis* [37]. Generally, there is a balance between the anti-inflammatory and inflammatory immune cells. With the use of TNF- α antagonist, however, anti-inflammatory Treg and Th2 cells dominate. When anti-TNF biologics are discontinued or reduced, skewing from Treg and Th2 cells towards the inflammatory responses of Th17 and Th1 cells may occur, thus leading clinically to IRIS. In recent years, there have been several case reports of patients who discontinued anti-TNF- α therapy in the setting of opportunistic infection with subsequent clinical worsening. This has been recognized to be a result of immune reconstitution, or “paradoxical reaction,” due to the removal of immune suppression [38-41]. Often times, cultures are negative during the clinical decline, suggesting that deterioration is secondary to an exuberant inflammatory response rather than the progression of the infection. Because the clinical presentation is often unclear, it is important to consider the differential diagnosis of IRIS, which includes resistance to antimicrobials, super infection, and drug-induced reactions. Since tuberculosis is the best-described presentation of IRIS following the discontinuation of TNF- α inhibition, using alternative drug in patients who received antimycobacterial treatments or antiretroviral drugs (ART) is important to the control of infection. The cases of life-threatening forms of TB-associated IRIS can be managed with corticosteroids or temporary discontinuation of ART. The use of systemic corticosteroids has been most widely employed in TB associated IRIS, perhaps because of the previously accepted role for immune modulation in some forms of TB [42-43]. According to our study the extract of *P. harmala* seed can be used as an anti-inflammatory compound with anti-TB drugs which currently used. Phytochemical screening of current study revealed the presence of alkaloids, polyphenolic compounds and flavonoids, in hydro-alcoholic extract of *P. harmala* and hence can be used as a potent antioxidant plant. Harmaline was first isolated from the seeds and roots of *P. harmala* and is the major alkaloid of this plant [44]. In conclusion, our data showed the anti tuberculosis and anti inflammatory activity of *peganiumharmala* via to the direct effect of the extract on the growth of bacteria and also the immunomodulatory effects of the extract by the effect on proinflammatory cytokines secretion.

Additionally, the plant extract was not found to be toxic to human cells. In summary pomegranate peel extract appeared to have strong antituberculosis, anti-inflammatory and antioxidant activity. Therefore, Punicagranatum peel could be an ideal complementary or alternative anti-tuberculosis agent. However further investigation need to be performed in animal models to confirm the data.

Acknowledgement

This work was financially supported by Infectious Diseases Research Center, Golestan University of Medical Sciences, which is greatly appreciated.

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