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Anti-mycobacterial and Anti-inflammatory Activity of Punica granatum peel Extract Against Mycobacterium Tuberculosis

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ABSTRACT: Tuberculosis (TB) is one of the most prevalent infections in the world causing high mortality in developing countries. There is an urgent need for improved TB drugs due to development of drug resistant TB strains. Since herbal medicines play an important role in combating bacterial infections, the present study was performed to evaluate anti-tubercular activity of pomegranate fruit peels in Golestan province, north of Iran where the highest prevalence of TB was reported. Hydro-alcoholic extract of pomegranate fruit peel was screened for anti-tubercular activity by disc diffusion (DD) method. The anti-inflammatory effect of the extract was evaluated by cytokines measurement using ELISA in a model of phagocytized intracellular Mycobacterium in dU937cells. Phytochemical properties of the extract were assessed by free radical-scavenging activity test and also evaluation of the total phenolic, flavonoids and gallic acid concentrations. Our results showed the inhibitory effect of Punica granatum peel extract on all strains of Mycobacterium tuberculosis even on drug resistant TB strains. Cytokines production in culture media and Phytochemical analysis of the extract showed the anti inflammatory and antioxidant activity of the extract respectively. These findings revealed the potential ability of the Punica granatum peel against mycobacterium tuberculosis *in vitro*.

Keywords: Herbal plants; Mycobacterium Tuberculosis; Punica granatum; Anti-inflammatory; Antioxidant activity.

INTRODUCTION

Natural products isolated from herbal plants have played an important role in discovery of drugs against infectious diseases in the last decades so that Almost 75% of the approved anti-infective drugs are derived from medicinal plants[1]. Mycobacterium tuberculosis (Mtb), the etiological agent of tuberculosis (TB) is one of the most prevalent infections in the world causing high mortality in developing countries[2]. The emergence of multiple drug resistant (MDR) and extensive drug-resistant (XDR) strains of mycobacterium continually is increased. Therefore improving the current TB control drugs is urgently needed[3-4]. Recently, several studies showed that pomegranate (Punica granatum) possesses several pharmacological activities such as antimicrobial, antioxidant, and anti-inflammatory activity, furthermore, pomegranate is an amazing source of phenolic acids, gallic acid, quercetin (flavones), and other secondary metabolites in its juice, peels and seed oil[5-6]. Pomegranate is native to the Mediterranean region from northern India to Iran and has been extensively used in the folk medicine of many countries[7-9]. The water-methanol extract of pomegranate peel showed antimicrobial activity against eleven microorganisms, such as Bacillus subtilis, Staphylococcus aureus, Yersinia enterocolitica, Listeria monocytogenes, Saccharomyces cerevisiae, and Aspergillus niger[10-11]. Punica granatum also is a powerful suppressor of inflammation and has antioxidant activity[12] which is highly correlated with the contents of total phenolic and flavonoid compounds[13]. Golestan province in north of Iran where the highest prevalence of TB was reported[14], has appropriate climate and biodiversity of plants, however no information is available on the anti-mycobacterial and anti-inflammatory activity of local herbal plants

against Mycobacterium tuberculosis probably due to the high risk of TB transmission. This is the first study which was performed in the area to evaluate the antimycobacterial activity of pomegranate peel extract, as a well-known plant in folklore medicine, against Mycobacterium tuberculosis strains. The extract also was examined for anti-inflammatory activity in an in vitro model of Mycobacterium tuberculosis-infected monocytes. The antioxidant activity and phenol and flavonoid contents also were measured to evaluate the phytochemical properties of the extract.

MATERIAL AND METHODS

Preparation of pomegranate peel extract: Punica granatum (P. Granatum) was chosen to be studied because of the inhibitory effect of this plant on the nontuberculous mycobacteria revealed in the previous study[15]. Punica granatum fruit peels were collected from the southeast of Golestan province, Iran (Ramian) then it was identified in the herbarium of Faculty of Plant Science, Islamic Azad University of Gorgan branch. The P. granatum peel was air-dried in the dark at room temperature and then ground into the fine powder using electric blender. Plant hydro-alcoholic extract was prepared by maceration method, briefly 30 g of dried powder was soaked in 100 ml of 70% Ethanol for 72 hrs at room temperature. Then, the macerated plant material was extracted with 70% ethanol solvent by percolator apparatus at room temperature. The plant extract was removed from percolator and filtered twice through Whatmann filter paper No. 1 (Whatmann, UK). The filtrates were evaporated using rota evaporator. The final products were sticky-brown substances and stored in a laboratory refrigerator prior to test. The concentrated plant extracts were dissolved in dimethyl sulphoxide (DMSO) (SIGMA, USA) to get a stock solution of 200 mg/mL and further diluted to varying 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[16].

Antibacterial activity: Antibacterial activity was determined by disc diffusion method as described by Taylor et al[17]. A total of five strains of Mycobacterium Tuberculosis were used; four clinical isolates were identified by conventional methods[18] 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, was kindly provided by Dr. Hashemi (Ahvaz university of Medical Sciences, Ahvaz, Iran); All strains were maintained on Löwenstein-Jensen slants. Approximately 4-5 mg (a loopful) of Mycobacterium was taken from a fresh subculture then was suspended in sterile saline in screw-cap tubes containing glass beads. The tubes were vortexed for 2 min and allowed to stand for 20 min. Then the supernatants were adjusted to the opacity of a no. 0.5 McFarland standard. One hundred µl of Mycobacterial suspension was spread onto the surface of the Lowenstein Jenson Media. Sterile paper (6 mm diameter) were loaded with 40 µL of extract (varying concentrations: 200,100,50,25,12.5, 6.25, 3.12, 1.6 mg/ml) and then left to dry. The impregnated-disks were placed on the surface of plates in which the microorganisms were cultured. 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. A clear zone visible all around the impregnated-disk was indicative of the inhibitory effects. 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, purchased from Pasture Institute of Iran, was used as a model of in vitro infection to evaluate the anti-inflammatory activity of pomegranate peel extract by cytokines detection[19]. 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% CO_2. These monoblasts (U937) are undifferentiated, upon standard induction, rapidly differentiate into a macrophage-like, cytokine producing phenotype (dU937)[20]. For differentiation, cells were harvested at exponential growth phase, washed and suspended in complete medium containing 5 ng/ml of phorbol 12-myristate 13-acetate (PMA, Sigma, USA). The cells were seeded into culture plates at a cell Density of 2×10^5 cells/well and allowed to differentiate for 3 days before use. Cell viability was determined by Mtt assay and 0.2% trypan blue exclusion test.

Macrophage infection: 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±Neilsen (ZN) staining and light microscopy was used[21].

Measurement of cytokine production: M.tuberculosis- infected dU937 cells were treated with the extract (200 mg/ml) for 48 hours. Concentrations of the cytokines in the cell culture supernatants were determined by enzyme-linked immunosorbent assay (eBioscience). Sensitivities of the assays were 1pg/ml for IL-10, 5 Pg/ml for TNF-α and 0.92 Pg/ml for IL6.

Phytochemical tests: To evaluate the phytochemical properties of the Punica granatum peel extract, free radical-scavenging activity, and total phenolic and total flavonoids content were measured. The stable1, 1-diphenyl-2-picryl hydrazyl radical (DPPH), quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one),BHA(butylated hydroxyanisole) and BHT(butylated hydroxytoluene) were purchased from Sigma Chemical Co. (St., Louis, USA). Gallic acid, Folin-Ciocalteu reagent and methanol were purchased from Merck Co. (Germany).

Free radical scavenging activity determination: Diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extract [22]. Different concentrations of extract were added at an equal volume to methanolic solution of DPPH (100µM). 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[23]. Briefly, 0.5 ml of the sample or gallic 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[23]. 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 quercetin and gallic acid 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 "quercetin and gallic acid" in the extract was identified by comparison with pure standard.

Statistical analysis: Statistical analysis was determined by SPSS software and student's t-test and the differences were considered to be significant if the P value was <0.05.

RESULTS AND DISCUSSION

Antibacterial activity: Punica granatum peel extract exhibited significant in vitro antimycobacterial activity against all strains of Mycobacterium Tuberculosis; the mean of inhibitory zone was 19.5±3.5 mm at concentration of 200 mg/ml of extract (Table1). No significant differences were observed between MDR and non-MDR strains (P< 0.05).

Table 1: Antimycobacterial activity of punica granutum peel extract against M. tuberculosis strains tested based on disc-diffusion method. Inhibition zone diameter around test disc (mm). Data expressed as mean ± standard deviation (n= 3) (P<0.05)

Bacteria	Punica granutum peel extract (mg/ml)					Isoniazid	Rifampin
Concentration	200	100	50	25		5µg/ml	5µg/ml
MDR_1	22	18	15	10		R	R
MDR_2	17	13	10	8		R	R
Non-MDR_1	18	15	14	10		20	19
Non-MDR_2	20	16	13	8		19	18
$MDR_{1\&2}$	19.5±3.5	15.5±3.5	12.5±3.5	9±1.4		R	R
Non-$MDR_{1\&2}$	19±1.4	15.5±0.7	13.5±0.7	9±1.4		19.5	18.5
H37RV	20	17	15	13		S	S

Macrophage infection by Mycobacterium tuberculosis: Incubation of the human promonocytic cell line (U937) with phorbol ester (PMA) for 72 h induces the cells to differentiate to macrophage-like cells in vitro. Undifferentiated U937 cells were unable to bind M. tuberculosis H37Rv, but differentiation using PMA, significantly enhanced their ability to bind mycobacteria, particularly in the presence of serum opsonins. 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).

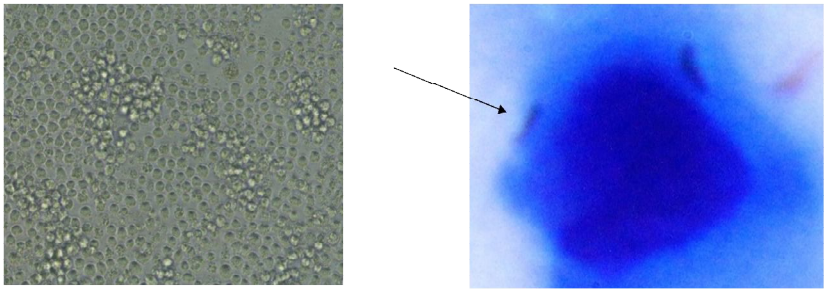

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Figure 1: (A) The cluster and adherent form of dU937 cells after treatment. (B) Intracellular Mycobacterium tuberculosis was showed by Zeihl ± Neilson (ZN) staining and light microscopy.

Cytotoxicity of the extract: Cytotoxicity of the extract on U937 cells was examined by Mtt assay and trypan blue exclusion test. According to our experiment, pomegranate peel extract did not show cytotoxic effect against U937 cells (IC50> 200mg/ml).

The effects of pomegranate peel extract on cytokines productions: Following identification of the promising anti-mycobacterial activity, the anti-inflammatory effect of the extract was evaluated using M.tuberculosis-infected dU937 cells. 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 ($p<0.05$). Secreted TNF-α was decreased significantly after treatment of infected- dU937 cells with Punica granatum peel extract ($p<0.05$).

Production of IL-6 and IL-10 were decreased to the undetectable level by M.tuberculosis-infected dU937cells and get back up to the same levels which existed before infection after treatment with the extract. The results in each panel represent pooled data from three independent experiments (Table 2).

Table 2: Cytokines secretion by dU937 cells infected with Mycobacterium tuberculosis. Cytokines were measured by ELISA method in the presence and absence of the extract (200 mg/ml). Values represent the means of culture supernatants collected from triplicate wells assayed by ELISA (P< 0.05).N; no detectable

Cytokines(pg/ml)	Cell	Cell & Bacteria	Cell & Bacteria & Extract
TNF-α	20	30	10
IL-6	1.4	N	1.5
IL-10	2	N	1

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 (Table3). The content of quercitin as a main flavonoid content and gallic acid, a type of phenolic acid in the extract was estimated by HPLC method. The content of quercetin and gallic acid in the extract was 37.75 and 31.90 mg/gr respectively according to the standard curve.

Table 3: IC50 (µg/ml) values of DPPH scavenging activity and total phenolic and flavonoid content of the extract

Sample	IC50 (µg/ml)	Total phenol(mgGAEg-1)	Total flavonoid (mgQUEg-1)
Pomegranate peel extract	2.17± 0.1	189 ± 0.7	123.8 ± 0.3
BHA	3.25 ± 0.351(µg/ml)	-	-
BHT	2.93 ± 0.404(µg/ml	-	1

Although Different parts of the fruit-peel and seeds of Pomegranate have been reported to have many biological and antimicrobial activities against a range of both Gram positive and negative bacteria[24], a few studies have done on the antibacterial and anti-inflammatory activity of Pomegranate peel extract against Mycobacterium tuberculosis. The present study was conducted to investigate the activity of pomegranate fruit peel against Mycobacterium tuberculosis. In the first stage of the study antibacterial activity of the hydro-alcoholic extract of Punica granatum peel was tested by the agar-plate disk diffusion method. The results of this assay showed the strong activity of the extract against all strains of Mtb at the concentration of 200 mg/ml. There were no significant differences in the zones of growth inhibition between the MDR and sensitive strains (P<.05). However, the effectiveness of this inhibition was strongly related to the concentration of extract. The antibacterial activity of the pomegranate peel extracts might be due to the presence of various phytochemicals such as, gallic acids and quercetin[25] which have been discussed later. In the second stage of the current study, the anti-inflammatory effect of the extract was evaluated by cytokines producing measurement in an invitro model of M.tuberculosis-infected dU937cells.

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 and the extract decreased it in the culture media supernatant of infected cells. In contrast production of IL-6 and IL-10 were decreased after infection with M.tuberculosis and increased after treatment with the extract. TNF-α is essential for the initiation of immune response against Mtb infection, and for an effective granuloma formation. In spite of its essential roles in the host's immunity to Mtb infection, overproduction of TNF-α in pulmonary TB may cause fevers, weakness, night sweats, necrosis, and progressive weight loss[26]. Paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) is a common and potentially serious complication occurring in patients with severe tuberculosis and also occurring in HIV-infected patients

being treated for tuberculosis (TB) using combined antiretroviral treatment. The initiation of antituberculosis treatment in these patients may be accompanied by clinical deterioration and even death before any improvement occurs[27-28].Experiments with recombinant BCG-expressing TNF-α demonstrated that high levels of TNF-α could cause destructive inflammation; the relative amount of TNF-α at the site of infection determined whether the cytokine acts as a protective or a destructive mediator. These observations suggest that increases in plasma TNF-α levels may be associated with clinical deterioration observed early in the treatment of severe tuberculosis[29]. The restoration of macrophage competence after ART (Standard antiretroviral therapy) or antibiotic initiation results from a complex mechanism, probably involving a sudden and violent immune reaction with a cytokine storm, such as TNF-α and IFN-γ). This overreaction might be controlled using corticosteroids and thalidomide. While TNF-α level in the plasma increased following initiation of therapy, other monocyte proteins including IL-6 decreased. IL-6 is one of the most important mediators of the acute phase response and acts as both a pro-inflammatory and anti-inflammatory cytokine. The mechanism underlying this dissociation between production of TNF-α and IL-6 in tuberculosis patients is unknown[30]. IL-10 is an anti-inflammatory cytokine. During infection it inhibits the activity of Th1 cells, NK cells, and macrophages, all of which are required for optimal pathogen clearance and tissue damage[31-32]. In our study the production of IL-6 and IL-10 was decreased after infection of cells with M.tuberculosis and increased after treatment with the extract. In summary, the protective immune response to Mtb infection, which prevents progression to active disease, may be attributed to the finite balance between proinflammatory and immunoregulatory mechanisms. IL-10 represents one such regulatory mechanism that Mtb could be exploiting in order to establish a chronic infection. Therefore Modulation of cytokine secretion may offer novel approaches in the treatment of tuberculosis. One strategy in the modulation of cytokine expression may be through the use of herbal medicines. A class of herbal medicines, known as immunomodulators alters the activity of immune function through the dynamic regulation of informational molecules such as cytokines[33]. Effective anti-microbial agents possess three essential capabilities of antibacterial, antioxidant and anti-inflammatory activities. The experimental data gathered herein shows significant antibacterial, antioxidant and anti-inflammatory activities of the Hydro-alcoholic extract of Punica granatum peel which might be due to the synergistic action of compounds present in it. According to our investigation, Punica granatum peel extract showed high antioxidant activity and high contents of the flavonoid and phenolic phytochemicals. Phenolic acids and flavonoids have repeatedly been implicated as natural antioxidants in fruit, vegetable especially pomegranate fruit[34-35]. Additionally, the plant extract was not found to be toxic to human cells.

CONCLUSION

Pomegranate peel extract appeared to have strong anti-tuberculosis, anti-inflammatory and antioxidant activity. Therefore, Punica granatum 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.

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