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Pistacia atlantica gum aqueous extract modulates humoral and cellular immune responses in BALB/c mice

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Abstract: Natural herbal products contain different compounds that can modulate immune and inflammatory diseases. This investigation aimed to examine the influence of *Pistacia atlantica* aqueous extract on cellular and humoral immunity in mice following the exposure to sheep red blood cells (SRBCs). For this purpose, male BALB/c mice (n = 40) were randomly assigned into 4 equivalent groups. The animals received 1×10^9 SRBCs in complete Freund's adjuvant (CFA) intraperitoneally 3 times with two-week intervals. Five days after the final injection, blood samples were taken from the animals. Additionally, 1×10^9 SRBCs were also injected into the mice's left hind footpad 48 h before the blood collection. Three groups of mice received various oral doses of the extract (100, 200, and 400 mg/kg) for 52 consecutive days after the study onset. Measurements were performed on the levels of humoral and cellular immune responses, splenocytes' ability to uptake neutral red, respiratory burst, and cell viability. The mice receiving *Pistacia atlantica* in all concentrations displayed significantly low delayed type hypersensitivity (DTH) reactions in a dose-dependent manner compared to the control animals. The treated mice also presented a dose-dependent, significantly lower average anti-SRBC titer than that in the control animals. IL-17 and IL-10 cytokine levels respectively decreased and increased significantly in the mice that received *Pistacia atlantica* compared to the control group. Splenocyte proliferation and respiratory burst declined significantly in mice treated with *Pistacia atlantica* as opposed to the normal control group but phagocytosis ability was significantly decreased. The immunomodulatory impacts of *Pistacia atlantica* might be partially caused by immunity diversion from proinflammatory cytokine IL-17 into antiinflammatory cytokine IL-10.

Key words: *Pistacia atlantica*, humoral and cellular immunity, immunomodulation, BALB/c mice

1. Introduction

Modern strategies for treating autoimmune diseases have significantly improved the consequences of these diseases and raised a novel promise for better recovery [1]. Presently, immunosuppression is the backbone of autoimmune disease treatment. Traditional drugs used for treating autoimmune inflammatory diseases include disease modifying antirheumatic drugs (DMARDs), glucocorticoids, and nonsteroidal antiinflammatory drugs (NSAIDs) [2,3].

Natural products such as medicinal plants were used for the treatment of human diseases many years ago [4]. However, traditional medicine along with modern therapies continues to be an important basis for future medication and remedies [5]. Additionally, natural products, consisting of intrinsically diverse structures (compared with synthetic compounds), are the main sources of bioactive agents for developing novel medicines [6,7]. Production of drugs from medicinal plants has mostly been dependent upon isolation

of materials which are the source of biological activities of the plants [8].

Pistacia atlantica (from the family Anacardiaceae) as an indigenous fruit of Iran has been traditionally used in traditional medicine [9]. This plant has constituents such as carotenes, tocopherols, and alcohols. Tocopherols and tocotrienols have antioxidant activity similar to vitamin E, which is advantageous for human health [10]. *Pistacia atlantica* has a variety of beneficial features for treating diseases such as hepatic, digestive, and neurological disorders [11,12]. According to Shakarami et al. [13], the aqueous extract of *Pistacia atlantica* gum has potential as a natural medicine for treating asthma. Tanideh et al. [14] presented evidence that oral and rectal administration of oil extract from *Pistacia atlantica* at high doses could lead to some positive physiological and pathological changes of colitis in a rat model; thus, it might be effective for treating ulcerative colitis.

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This study aimed to examine the influence of *Pistacia atlantica* gum aqueous extract on cellular and humoral immunity in mice following immunization by sheep red blood cells (SRBCs).

2. Materials and methods

2.1. Reagents

Complete Freund's adjuvant (CFA), natural red, nitro blue tetrazolium, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum and RPMI 1640 were purchased from GIBCO/Life Technologies Inc. (Gaithersburg, MD, USA). The ELISA kits for assaying cytokines were purchased from Abcam (Germany).

2.2. Animals

Male BALB/c mice aged from 6 to 8 weeks old were provided by the Pasteur Institute, Tehran, Iran. For one week before the experiments, the animals were kept under fixed settings of 12 h light/dark photoperiod and under temperatures of 22–24 °C. The mice were kept under conditions compatible with the protocols of the Ministry of Health and Medical Education of Iran. The study was performed under a license issued by the Medical Ethics Committee of the Khorramabad University of Medical Science for Animal Studies (IR.LUMS.REC.1397.165).

2.3. *Pistacia atlantica* gum extraction

Fresh *Pistacia atlantica* gum was obtained from Khorramabad grocery stores (Lorestan, Iran). The gum was identified and characterized by a herbarium collection manager at the University of Khorramabad (Herbarium No. HLu27061397). It was then washed, dried, ground, and vacuum-dried using an electric mill. The resultant powder was soaked in distilled water for 48 h before filtration. The extract was incubated at 50 °C, followed by exposure to redistillation [15].

2.4. Gas chromatography–mass spectrometry (GC-MS) analysis

GC-MS examination was done with a Shimadzu GC-17A (Kyoto, Japan) gas chromatograph connected to a Shimadzu Quadruple-MS model QP5050 mass spectrometer. The mixtures were partitioned on a fused silica capillary column with dimensions of 30 m × 0.22 mm i.d. covered with a 0.25 µm layer of BP-5 (Shimadzu) and a split/splitless injector with an internal glass liner of 1 mm. Ultrapure helium as a carrier gas was utilized at an ionization voltage of 70 eV. The temperatures of the injector and interface were 280 °C and 260 °C, respectively. The mass ranged between 35 and 450 amu. The oven temperature limit was similar to the one mentioned earlier for the GC. The extract components were identified in a

DB5 column under similar chromatographic conditions through calculating the retention indexes under the settings based on the temperature for n-alkanes (C8–C20). The extract components were determined by comparing their mass spectra with those of the internal reference mass spectra library (NIST08 and Wiley 9.0) [15].

2.5. The experimental scheme, immunization protocol, and assessment

The mice were randomly assigned into 4 groups, namely a control and 3 treatment groups, with 10 animals in each group. The treatment groups were administered various amounts of the extract (100, 200, and 400 mg/kg) via the oral route from the start of the study (beginning of immunization), maintained continuously during the survey while taking blood from the animals. The dose was selected according to the earlier studies with murine models. Normal control mice were treated with an equivalent volume of PBS with the same treatment plan of the test groups. Antigen (1×10^9 SRBCs) was emulsified in CFA and injected to mice intraperitoneally 3 times with 2-week intervals. Five days after the final injection, blood samples of animals were taken from their hearts, after determining the amounts of the anti-SRBC antibody in their blood with a microhemagglutination experiment based on previous studies.

Additionally, to assess cellular immunity, mice were exposed to subcutaneous injection of 1×10^9 SRBCs in 50 µL of PBS into the left hind footpad 48 h before bleeding time. At the same time, a similar amount of PBS was administered into the right footpad of the control group.

Before the bleeding, the thickness of the footpad was measured using a dial caliper and the average percentage increase in the thickness of the footpad was established based on the equation below [16]:

$$\left[\frac{(\text{left footpad thickness}) - (\text{right footpad thickness})}{(\text{right footpad thickness})} \right] \times 100$$

2.6. Measuring cytokines

Aseptic isolation of spleen cells from mice was performed at bleeding time. Briefly, single-cell suspensions of splenocytes were prepared in RPMI 1640 medium complemented with red blood cells (RBCs) and 10% fetal bovine serum, eliminated by RBC lysis buffer. Then cell suspensions (2×10^6 cells/mL) underwent incubation in plates with 24 wells and were pulsed by PHA solution of 50 µL (1 mg/mL). The culture supernatants were gathered after 72 h. IL-10 and IL-17 cytokines were assayed by ELISA test according to the instructions of the provider of the kits [17].

2.7. Splenocyte proliferation

The proliferation potency of the splenocytes was assessed by the MTT test. The splenocytes were cultured in 96-well, flat-bottomed plates (1×10^5 cells/100 µL/well) in

RPMI 1640 medium supplemented with 10% fetal bovine serum, and activated by a PHA solution of 50 μ L (1 mg/mL), or culture medium alone. Following incubation for 72 h, the cultures were pulsed with MTT solution of 20 μ L (5 mg/mL) at 37 °C for 4 h. Afterward, DMSO (150 μ L) was added to dissolve the formazan crystal and agitated vigorously. The optical density (OD) was read at 550 nm by a microplate reader (Dynatech, Denkendorf, Germany). The assays were run in triplicates and the findings were expressed as the index of proliferation based on the ratio of the OD550 of the stimulated cells with MOG35-55 to the OD550 of nonstimulated cells [18].

2.8. Measuring respiratory burst in splenocytes

In splenocyte populations, the phagocytic cells' respiratory burst was examined by NBT dye reduction based on the partially modified previous description of the test [20–22]. Briefly, a mixture was prepared with splenocyte suspension (100 μ L), *Staphylococcus aureus* suspension (10^8 cell/mL) of 0.1 mL, and 0.1% NBT of 0.1 mL in PBS (pH 7.4). This mixture underwent incubation for 15 min at ambient temperature and was then held for a further 15 min at 37 °C. The reduced dye was extracted in dioxane and quantified at 520 nm [19].

2.9. Phagocytic assay of splenocytes population by neutral red

The primary phagocytic ability of the splenocyte population (opsonin-independent phagocytosis) was measured using neutral red uptake through the addition of neutral red solution (dissolved in 10 mmol/L PBS at a 0.075% concentration) of 200 μ L to the splenocyte population and incubation of cells for 1 h. After discarding the supernatant, cells were rinsed 2 times in PBS to eliminate

the neutral red not undergoing phagocytosis by cells. This was followed by the addition of cell lysate solution (0.01% acetic acid and ethanol at the ratio of 1:1, 200 μ L/well) to the cells and then incubation at 4 °C overnight. The optical density was read at 490 nm by a microplate reader [20].

2.10. Statistical analysis

The data were analyzed with one-way ANOVA and presented as mean \pm standard deviation (SD) to compare the experimental groups. Once significant differences were detected by ANOVA, mean values between different groups were compared by Tukey's multiple post hoc test. Differences between the groups were considered significant at $P < 0.05$.

3. Results

As represented in Table 1, GC-MS analysis of *Pistacia atlantica* gum aqueous extract demonstrated the highest levels of active ingredients including α -pinene and β -pinene at 77.9% and 3.66%, respectively. As shown in Table 2, mice that received different concentrations of *Pistacia atlantica* displayed significantly lower DTH reactions dose-dependently compared with the control groups. The treated mice also presented significantly lower average antibody titers dose-dependently than that in control animals (Table 2). Footpad diameter (indicative of DTH) and antibody titer were significantly reduced by the concentrations of 100, 200 ($P < 0.05$), and 400 mg/kg ($P < 0.01$) of the extract, and IL-17 cytokine levels decreased significantly while IL-10 increased significantly in splenocytes of mice subjected to *Pistacia atlantica* in comparison with the cells from the control group (Table 3). Changes in IL-17 and IL-10 levels were all dose-dependent.

Table 1. The concentrations (%) of different compounds in aqueous extract of *Pistacia atlantica* gum.

Compound	RI	Amount (%)	Compound	RI	Amount (%)
Alpha-Pinene	939	77.9	Linalool	1103	0.13
Camphene	953	0.88	Chrysanthenone	1146	0.08
Verbenene	967	0.43	Rans-Limonene-Oxide	1149	0.09
Beta-Pinene	980	3.66	Pinocarveol	1152	0.59
Beta-Myrcene	991	6.26	Cis-Sabinol	1149	1.7
Delta-3-carene	1011	0.24	Isopinocamphone	1181	1.93
Alpha-Terpinene	1018	0.03	Pinocarvone	1162	0.14
P-Cymene	1026	0.3	P-Mentha-1,5-dien-8-ol	1185	0.05
L-Limonene	1031	0.76	Terpinene-4-ol	1190	1.02
1,8-Cineole	1033	0.14	P-Cymen-8-ol	1199	0.25
Trans-beta-Ocimene	1050	0.18	Myrtenol	1190	0.55
Gamma-Terpinene	1062	0.07	Cis-Carveol	1241	0.15
Terpinolene	1088	0.21	Isobornyl acetate	1302	0.2

Table 2. The effects of the aqueous extract of *Pistacia atlantica* gum on humoral (antibody titer) and cellular (percentage of footpad thickness) DTH reactions (* P < 0.05 and # P < 0.01, different treated groups in comparison with the control group).

Group	Antibody titer	Percentage of footpad thickness
Control	448 ± 128	1.975 ± 0.170
100 mg/kg	80 ± 32*	1.5 ± 0.081*
200 mg/kg	40 ± 16*	1.375 ± 0.095*
400 mg/kg	12 ± 4.61#	1.225 ± 0.170#

The viability of splenocytes was assessed by MTT assay, which measured cell metabolic activity. Splenocyte proliferation (Figure 1) and respiratory burst (Figure 2) dropped significantly in mice treated with *Pistacia atlantica* compared to the normal control group.

Viable phagocytic cells engulf neutral red (NR) as a vital stain and keep it in their lysosomes. The data of the present study (Figure 3) indicated that treatment of mice with *Pistacia atlantica* could significantly potentiate the opsonin-independent phagocytic ability of splenocytes dose-dependently.

4. Discussion

Immunity is defined as an ability displayed by animals against damage induced by microorganisms and their products [21]. Immunity is generally associated with the response of the body towards all foreign antigens. Although immunity is advantageous to host, it may occasionally be injurious, resulting in tissue injuries and diseases (hypersensitivity and autoimmune diseases) [22].

Phytochemicals are natural products originating from plants. A considerable number of phytochemicals derived from various plants show promise in modulating immune reactions [23]. Flavonoids, flavanol, quinones, phloroglucinols, polysaccharides, glycosides, terpenoids, essential oils, alkaloids, glycolipids, biopolymers, phenolics, macrocyclic lactones, saponins, cannabinoids, polyacetylenes, gallic acid, coumarins, lignans, artemisinin, alpha-amyrin, kaempferol, hexacosanol, and vitamin C are among the most common phytochemicals. These phytochemicals lead to a variety of immunomodulating effects [24]. The present research aimed at examining the impacts of *Pistacia atlantica* as a natural product on BALB/c mice immunized with SRBCs.

Previous studies showed that a high dose of *Pistacia atlantica* extract used orally and rectally ameliorated the physiological and pathological status of colitis, which is an immune system disorder, in a rat model [14]. Recently, the use of *Pistacia atlantica* extract has shown significant

Table 3. Cytokine production assay following treatment with the extract of *Pistacia atlantica* gum. Splenocytes isolated from mice after immunization with SRBCs were cultured with PHA of 50 µL (1 mg/mL) for 72 h. The amounts of IL-17 and IL-10 in culture supernatants were measured by ELISA after 72 h (* P < 0.05 and # P < 0.01, various treated groups in comparison with the control group).

Group	IL-17 level	IL-10 level
Control	38.75 ± 5.18	34.5 ± 3.87
100 mg/kg	33 ± 2.16*	42 ± 3.16*
200 mg/kg	31.5 ± 3.41*	44.25 ± 2.98*
400 mg/kg	25.75 ± 3.59#	47.5 ± 3.41#

increases in antioxidant defense, vascular endothelial growth factor (VEGF), and hydroxyproline but reduced amounts of malondialdehyde (MDA) in rats that underwent experimental skin burns [25]. Moreover, *Pistacia atlantica* could markedly decrease wound size in comparison with burns in the control animals along with greater advantageous impacts than sulfadiazine as the positive control [25].

Shakarami et al. [13] presented evidence of significant decreases in the number of bronchoalveolar lavage fluid (BALF) eosinophilic cells and the amounts of anti-ovalbumin IgE, IL-4, IL-5, and IL-17 cytokines, as well as pathologic injury to the lung tissue induced by aqueous extract of *Pistacia atlantica* gum. The same authors further demonstrated significant elevations in the levels of antiinflammatory IL-10, TGF-β, and INF-γ in the extract-treated groups compared to the asthmatic groups, which received dexamethasone. As stated above, the extract of *Pistacia atlantica* possesses antiinflammatory activities which act as natural sources in treating immune system disorders.

Recently published literature has reported that natural products originating from plants have a major effect on differentiating T cell subsets and migrating the T cells into tissues. T cell-mediated immunity has considerable impact in organ-specific autoimmune diseases [26]. Delayed type hypersensitivity (DTH) is among the usual reaction patterns of immunity mediated by T cell. The primary requisite for DTH response is to prime a particular effector group of antigen-specific T cells [27].

The initial demonstration of Th17 cells' contribution to autoimmunity was in mice with a defective p19 chain of IL-23, in which T cells generating the IL-17 were statistically less than those in wild type mice, underlining the significance of the IL-23/Th17 axis in the pathogenicity of these autoimmune diseases [28].

IL-17 (known as IL-17A as well) has strong proinflammatory activity, making it a critical agent in

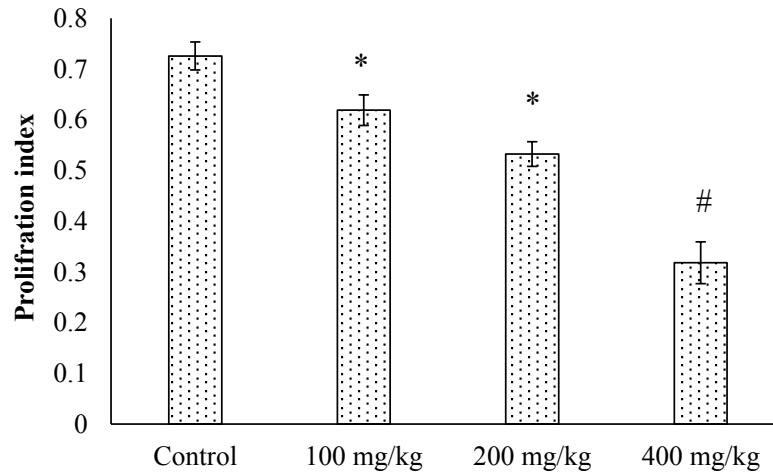


Figure 1. Assessment of mice splenocyte proliferation following challenging of the immune system with SRBCs and treatment by *Pistacia atlantica* gum extract (* $P < 0.05$ and # $P < 0.01$, various treated groups in comparison with the control group).

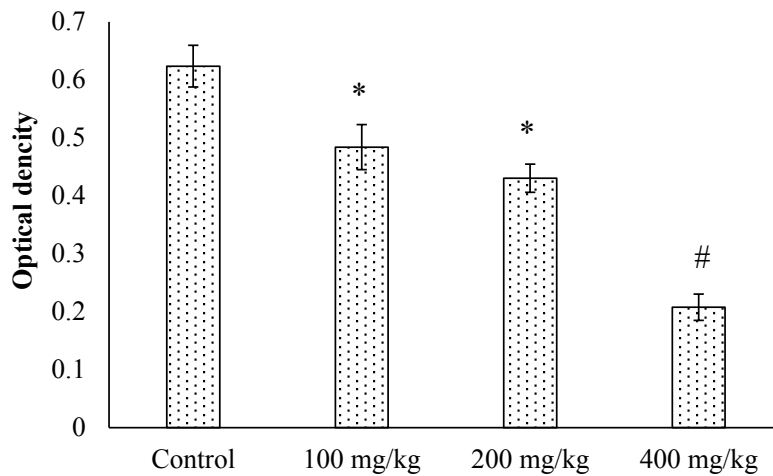


Figure 2. Assessment of splenocytes respiratory burst in mice following the exposure of the immune system to SRBCs and treatment by *Pistacia atlantica* gum extract (* $P < 0.05$ and # $P < 0.01$, various treated groups in comparison with the control group).

regulating DTH reactions. Apparently, Th17 cells mimic the inflammatory reaction and regulate the tissue injury. It generally appears that Th17 cells have higher pathogenicity than other cells in immunopathological settings [29].

According to the findings of this study, IL-17 production was significantly decreased by treatment with *Pistacia atlantica* following immunization of mice with SRBCs. DTH response was also reduced by this extract. Our results present a new understanding of the possible mechanisms that underlie the immunomodulatory impacts of *Pistacia atlantica*.

As an immunoregulatory cytokine, IL-10 has a critical contribution to inflammatory and immune reactions,

termination of macrophage activity in DTH responses, and subsequently the prevention of tissue damage [30]. This cytokine shows strong immunosuppressive and antiinflammatory activities on myeloid cell function, providing a concrete foundation to be applied in acute and chronic inflammatory illnesses. Suppression of the immunostimulatory activities of IL-10 can furnish new strategies in treating humoral autoimmune diseases, infectious diseases, and cancer [30]. The results showed a decrease in IL-17 production and an increase in IL-10 production by *Pistacia atlantica* extract, which might justify the respiratory burst reduction in splenocytes of mice under *Pistacia atlantica* treatment.

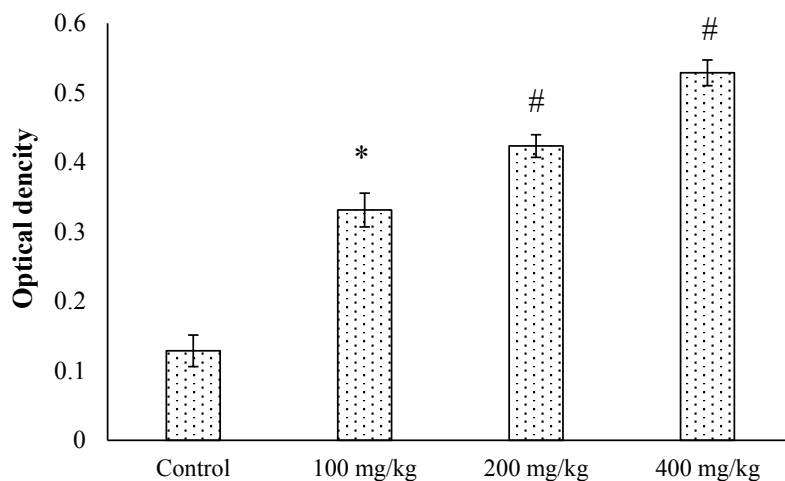


Figure 3. Assessment of neutral red uptake by mice splenocytes following the exposure of the immune system to SRBCs and treatment by the extract of *Pistacia atlantica* gum (* $P < 0.05$ and # $P < 0.01$, various treated groups in comparison with the control group).

Reactive oxygen species (ROS) have a crucial contribution to the elimination of invading microorganisms by phagocytes. Nonetheless, upon increased or unsuitable production, ROS are engaged in acute host tissue damage and other immunopathological situations [14].

Notably, in the investigation by the authors, the mice showed increased phagocytic activity and uptake of neutral red under *Pistacia atlantica* gum extract treatment, with significant drops in respiratory burst against opsonized heat-killed baker's yeast and splenocyte proliferation.

The findings of respiratory burst, neutral red, and MTT assays demonstrated that despite an improvement in phagocytosis ability of spleen cells in the mice treated with *Pistacia atlantica* gum, there were reductions in their killing activity and proliferation, suggesting an induction of bodily antiinflammatory ability as a result of this treatment. In conclusion, our results revealed that *Pistacia atlantica* gum has immunomodulatory effects in

the mouse model and it can be used in the treatment of autoimmune diseases as a natural product.

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Conflict of interest:

The authors report no conflict of interest.

Informed Consent:

This manuscript reports the results of experimental investigations conducted with mice and an approval code was received (IR.LUMS.REC.1397.165).

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