

Study of the Immunomodulatory Properties of *Pistacia khinjuk* Extract on Rats

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ABSTRACT

Medicinal herbs are widely used as immunostimulants and immunomodulators in treating various ailments around the world. Many herbs used regularly have not been thoroughly studied for their immune-modifying properties. The immunostimulatory effect of the methanol extract of *Pistacia Shinjuku* was tested in male rats. The experiment consisted of seven groups of animals, each with eight healthy adult mice. Control group (G1): normal saline (0.5 mL) was taken for 14 days. Group 2 (G2) received intraperitoneal cyclosporine (CSA) (5 mg/kg/BW) for 14 days Group 3 (G3): interferon alfa-2a subcutaneous injection 34.5 mg/kg/BW) in two doses week for 14 days. Group 4 (G4) (methanol extract of *P. khinjuk*, 3 g/kg/body weight) for 14 days, Group 5 (G5): methanol extract of *P. khinjuk*, 1.5 g/kg/body weight) for 14 days. Group 6 (G6): methanol extract of *P. khinjuk* 3 g/kg/body weight) for 14 days after cyclosporine injection. Group 7 (G7): methanol extract of *P. khinjuk* 1.5 g/kg/body weight) for 14 days after cyclosporine injection. On day 15, whole blood was collected in clean ethylenediaminetetraacetic acid (EDTA)-vacutainer. Total white blood cells (WBCs) count, differential count, In addition to measuring the level of (IgM, IgG) in the blood serum. According to the results, the group treated with CsA showed an immunosuppressive effect with a significant decrease ($P > 0.05$) in the total number of WBC, differential count, antibody concentration, and cytokine level (TNF- α , CCL5 NF- κ B), but increased the level of TGF- β as compared with other groups. At the same time, there were immunostimulatory effects in groups treated with extract Group 4, 5, 6, and 7, with a significant increase ($p > 0.05$) in the total number of WBC, Differential count and antibody concentration, and Cytokine level (TNF- α , CCL5, NF- κ B). The decreased level of TGF- β , as compared to the CsA-treated Group. In conclusion, *P. khinjuk* possesses immunomodulatory and antioxidant properties against the adverse effects of cyclosporine in rats.

Keywords: Antibody, Cytokine, *Pistacia khinjuk*, WBC.

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INTRODUCTION

Medicinal plants always can play a role in the development of various medications. Various plant species have yielded several medicines with recognized medicinal benefits. There were over 800 species in the Anacardiaceae family, divided into nearly 82 genera. In folklore tales, *P. shinjuku*, a possible plant of the genus *Pistacia*, demonstrated amazing medical ability. Mastic, Ushgai, Gulgnoor, , Baneh, Guan, Kelkhong, and Gazan are some of the names used to describe the plant's variability in different areas.¹ Egypt, Syria, Turkey, Iraq, Iran, Afghanistan, and Pakistan are its native habitats. Fruit clusters are longer and wider, measuring up to 14 to 22 cm in length. They grow on a lower branch, giving the impression of additional width. Blue and black color tones were visible on the fruits. Between August and September, the seeds are yellowish and mature. At altitudes of around 2000 m, however, the season lasts until

October. Indigestion and toothache have been treated using the plant's resin. Bakhtiari's folkloric tales corroborated the usage of *P. khinjuk* as a tonic and astringent.² The indigenous utilize the resin from the plant known as Gulgul to treat eye infections because it has anti-inflammatory properties. Galls have been found to have a variety of biological functions. Folklore stated that it had antibacterial, anti-inflammatory, and antioxidant properties. The antioxidant and antibacterial activity of the *Pistacia* genus was shown to be comparable to that of antibiotics. In vivo tests demonstrated that the methanolic extract of *P. shinjuku* has the potential to be employed as a wound healer.³ Furthermore, the fruits of this uncontrolled plant are nutritious.⁴ The hydroalcoholic extract of *P. khinjuk*'s leaves and fruits has recently been found to have vermifugal activity.⁵ The oil of wild pistachio plants can control hypothyroidism and its impact on the serum lipid profile and leptin levels,

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according to *S. Nazifi, et al.*⁶ The essential oil of *P. khinjuk* has been demonstrated to have antihelminthic properties against *Echinococcus granulosus* protozoa.⁷ *P. khinjuk's* antibacterial and antifungal properties, as well as its positive effects on wound healing, have been reported in a variety of scientific domains.⁸ Three myricetin glycosides were identified to be the primary components of the extract of *P. khinjuk* in prior research.⁹ Spathulenol, germacrene B, aromadendrone, eudesmol, zizanol, caryophyllene, myrcene, pinene, cymene, and limonene were found to be the major constituents in the essential oil from the aerial parts of *P. khinjuk*, in addition, some studies identified other compounds in the essential oils of other *Pistacia*.¹⁰ Many of these compounds are recognized to be natural antioxidants. Locals eat the *P. khinjuk* fruit as a snack after grinding it and mixing it with other ingredients, and the fruit oil is used as frying oil. Natives have employed various sections of the *P. khinjuk* fruit as helpful treatments for various illnesses. The fruit of *P. khinjuk*, for example, is used to cure stomach, heart, and pulmonary problems. *P. khinjuk's* gum resin is also useful for wound healing and the treatment of stomach and gastrointestinal diseases.¹¹ Accordingly, the present study was proposed to evaluate the immunomodulatory effects of methanol extracts of *P. khinjuk* on rats.

MATERIALS AND METHODS

The Plants

P. khinjuk fruits were obtained from Sulaymaniyah city and classified at the National Herbarium of Iraq. The fruits were ground using a coffee grinder.

Preparation of Plant Extract

Plants were extracted as 50 grams of the plants' powder with 80% methanol 250 mL at 65°C for 3 hours using the soxhlet apparatus. The extracts solution were concentrated by dryness under reduced pressure in a rotary evaporator to yield dried crude extract, which was frozen at -20°C until use to prepare the required concentrations.

Blood Sample Collection and Serum Preparation

After the end of the injection period, the animals were sacrificed by being exposed to a small amount of Diethyl Ether

C₂H₅O₂ to draw blood from the heart by heart puncture. Then, the obtained blood samples were left at laboratory temperature for half an hour to obtain clotting. Then the blood was discarded by means of a centrifuge at a speed of 2500 revolutions per minute for a quarter of an hour and at a temperature of 4°C. The serum was withdrawn and kept in eppendorf tube in the freezer (-20°C) until use.

Measurement of IgG, IgM, WBC Count, and Differential Count

To measure the concentration of IgG, IgM, the single in gel radial immunodiffusion method was used, the kit prepared from LTA, Italy according to the manufacturer's instructions was used, while the WBC number and differential number were measured using a sphygmomanometer, While the cytokine activity was measured using the count prepared from MyBioSource, USA according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean ± standard error (SE), and differences between means were assessed by SPSS version 23. The significant differences between the rates were compared using the least significant difference test (LSD test), in which ($p > 0.05$) was considered significant.

RESULTS AND DISCUSSION

WBC Count and Differential Count

The results showed that cyclosporine intraperitoneal injection for 14 days led to its effects by significant reduction ($p > 0.05$) of WBC count and differential count in G2 compared with G1, G3. At the same time, there was a significant increase ($p > 0.05$) of these data in G4, G5, G6, and G7 as compared with G2 (Table 1). The results agreed with 12, which recorded a decrease in the number of lymphocytes and their differential number when injecting rabbits with CsA drug at the two doses of 25 and 1.5 mg/kg/body weight, CsA inhibits calcineurin by binding to the immune-modulator (cyclophilin) and prevent the de-phosphorylation of nuclear factor activating T lymphocytes (NFATs)¹² and its subsequent translocation from the cytoplasm to the nucleus in an IL-2-mediated process, and thus total

Table 1: Effect of methanol extract of *Pistacia khinjuk* on WBC count and deferential count level in the serum of male rats treated with cyclosporine.

<i>The percentage WBC count and differential count in the serum (Mean ± S.E)</i>						
Groups	Basophil	Eosinophil	Monocyte	Neutrophil	Lymphocytes	Total count
Control (normal saline)	0.35 ± 0.01 ^f	1.80 ± 0.01 ^f	3.20 ± 0.01 ^f	35.00 ± 0.73 ^e	56.74 ± 0.57 ^f	96.50 ± 0.02 ^f
Cyclosporine (5 mg/kg)	0.16 ± 0.02 ^g	1.05 ± 0.01 ^g	1.13 ± 0.01 ^g	20.79 ± 0.74 ^f	40.27 ± 0.48 ^e	63.94 ± 0.02 ^g
Peginterferon alfa-2a (34.5 mg/kg)	0.39 ± 0.03 ^e	1.88 ± 0.08 ^e	3.25 ± 0.06 ^e	37.73 ± 0.06 ^d	60.25 ± 0.66 ^a	104.07 ± 0.01 ^e
<i>P. khinjuk</i> (3 g)	0.41 ± 0.08 ^a	1.93 ± 0.011 ^a	4.82 ± 0.036 ^a	43.68 ± 0.17 ^a	69.02 ± 0.75 ^b	119.01 ± 0.05 ^a
<i>P. khinjuk</i> (1.5 g)	0.38 ± 0.05 ^b	1.84 ± 0.02 ^b	4.32 ± 0.04 ^b	42.43 ± 0.19 ^b	62.21 ± 0.74 ^g	112.00 ± 0.05 ^b
<i>P. khinjuk</i> (3 g)+ Cyclosporine(5 mg/kg)	0.34 ± 0.05 ^c	1.76 ± 0.02 ^c	3.81 ± 0.04 ^c	31.48 ± 0.20 ^g	50.88 ± 0.47 ^c	89.01 ± 0.03 ^c
<i>P. khinjuk</i> (1.5 g)+ Cyclosporine (5 mg/kg)	0.31 ± 0.06 ^d	1.61 ± 0.02 ^d	3.51 ± 0.02 ^d	28.57 ± 0.10 ^c	48.60 ± 0.04 ^d	82.91 ± 0.03 ^d

*Different letters represent a significant difference ($P > 0.05$) between means in columns, while similar letters represent a non-significant difference ($P > 0.05$) between these means.

WBC count decreased after treatment with CsA, that leads to significant decrease in IL-2 level. On the other hand, oral doses of cyclosporine caused a reduction in neutrophils.^{13,14} Immunosuppressive medications are important in the treatment of anemia. In rats given CsA at a daily oral dose of 7.5 mg/kg, there was a decrease in white blood cells (WBCs), red blood cell counts, and hemoglobin (Hb) concentration but an increase in platelet counts.¹⁵ Furthermore, when compared to the control animal, the administration of CsA (30 mg/kg) and ketoconazole (10 mg/kg) medicines resulted in a substantial drop in total WBC and lymphocyte counts.¹⁶ Another study found that daily cyclosporine doses (low, 2 mg/kg; medium, 5 mg/kg; and high, 10 mg/kg) decreased white cells and lymphocytes and impaired cell-mediated immunity in a dose-dependent manner when compared to an untreated group for 31 days.¹⁷ The current decrease in total WBCs and differential lymphocytic counts were all consistent with Lekhooa *et al.* and study.¹⁸

Meanwhile, giving *P.khinjuk* extract to CsA injected rats resulted in a considerable improvement in all hematological parameters, where was a significant increase ($p > 0.05$) in WBC count and differential count in G4, G5, G6, and G7 as compared with G2, with a significant difference between the low and high dose groups.¹⁹ As noted in a previous study, there are some effective compounds in *P. khinjuk* that act to activate bone marrow and hematopoietic progenitor cells.²⁰ The suppression or stimulation of body immune responses is capable of sustaining a disease-free state in a living species. Substances capable of triggering the host's defensive mechanisms through the immune system have been utilized to control diseases in humans and animals all over the world. When compared to the G1 and G2, all of the different types of blood cell count was raised for all of the doses of total crude extracts of *P. khinjuk* that were given to the animals. The increase in blood cells could be attributed to alkaloids, saponins, cardenolides, deoxy sugar, tannins, cardiac glycosides, flavonoids, anthraquinones, phenolics, steroids, glycosides, ascorbic acid, and other vitamins contained in the plant extract stimulating the bone marrow and lymphoid organs, The results are in agreement with what was reached by Mungole, *et al.*,²¹ which recorded an increase in the total number of white blood cells with an increase in the rates of mononuclear cells, lymphocytes and

neutrophils when using the alcoholic extract of black seed, and this indicates that the *P.khinjuk* plant has a significant role in immunity by increasing the number of white blood cells.

The Estimation of IgM and IgG Activity

Serum levels of IgM and IgG was recorded a significant decreasing ($p > 0.05$) in G2 compared with the G1, G3, while there was a significant increase ($p > 0.05$) of these data in in G4, G5, G6 and G7 as compared with G2 (Table 2). Most protective roles of immune cells are dependent on cell membrane fluidity, and CsA-induced cell membrane fluidity depression leads to thymus atrophy and B cell malfunction.²² After CsA exposure, serum IgG concentrations dropped significantly, indicating a toxic effect of CsA on humoral immune function.²³ In some cases, the immunosuppressive impact of cyclosporine has been demonstrated.²⁴ In a rat model and a human model,²⁵ cyclosporine therapy totally or partially inhibited IgG production. CsA and prednisolone treatment of hamsters resulted in reduced levels of circulating IgG against worm crude antigen.²⁶

The significant increase ($p > 0.05$) of these data in in G4, G5, G6, and G7 as compared with G2 due to that *P.khinjuk* contains substances such as flavonoids, tannins, pectins, hydroquinone, ascorbic acid, carotenoids, and polyphenols, polyphenols, several phenolic acids, caffeic acid, flavonol glycosides, and others that may interfere with various processes in the hematopoietic system's white blood cell formation stages.²⁷ The extract of numerous constituents could stimulate or inhibit natural substances that promote or repress the proliferation or suppression of certain blood cell components, including granulocytes colony-stimulating factors (G-CSF).²⁸ In innate immunity, neutrophils are a multifunctional cell type that helps to bacterial clearance by recognizing, phagocytosing, and destroying foreign materials.²⁹ whereas T and B lymphocytes are involved in and accountable for antibody formation, resulting in enhanced immunity,³⁰ mentioned that the use of herbs prevented the occurrence of loss of body weight and the thymus caused by cyclophosphamide (CP), with an increase in the numbers of IgG and IgM with little effect on CD4 and CD8 cells, and the reason for the increase in the numbers of antibodies may be due to containing The plant

Table 2: Serum level of IgM, IgG for male rats treated with cyclosporine and methanol extract of *Pistacia khinjuk*.

Groups	The percentage of IgM , IgG level in the serum(unit/liter)	
	IgM	IgG
Control (normal saline)	5.07 ± 0.14 ^g	3.88 ± 0.03 ^f
Cyclosporine (5 mg/kg)	3.96 ± 0.35 ^e	2.31 ± 0.05 ^g
Peginterferon alfa-2a (34.5 mg/kg)	6.00 ± 0.01 ^d	4.85 ± 0.02 ^e
<i>P. khinjuk</i> (3 g)	8.88 ± 0.05 ^a	5.95 ± 0.03 ^a
<i>P. khinjuk</i> (1.5 g)	7.92 ± 0.02 ^f	5.72 ± 0.03 ^b
<i>P. khinjuk</i> (3 g)+ Cyclosporine (5 mg/kg)	6.94 ± 0.07 ^b	5.16 ± 0.09 ^c
<i>P. khinjuk</i> (1.5 g)+ Cyclosporine (5 mg/kg)	6.09 ± 0.01 ^c	4.96 ± 0.01 ^d

*Different letters represent a significant difference ($P > 0.05$) between means in columns, while similar letters represent a non-significant difference ($P > 0.05$) between these means.

extract contains many compounds such as flavonoids, ascorbic acid, carotenoids, polyphenols, phenols, and flavonols. These compounds may interact with B cells and act as antigens, thus activating the subsequent proliferation and differentiation of B cells into antibody-producing plasma cells, which are essential in humoral immune responses. Therefore, the plant may enhance the immune response of the immune system's T and B lymphocyte subtypes and thus enhance antibody synthesis.³¹ This is consistent with the results of A Babaei, *et al*,³² as it obtained a significant increase in both the concentration of IgG and the number of white blood cells when treating rats with extract of saffron petals at a dose of 75 mg/kg, because of its effect on stimulating the immune system, and that some of these effects are through stimulating the secretion of cellular cytokines such as IFN which is the main cytokine to stimulate B lymphocytes to produce IgG. As a result, consuming dietary antioxidants boosts immunity. It protects the body from the negative effects of CsA and oxidative stress³³ that CsA can cause. *P.khinjuk* extract's active components to boost humoral and cellular immune responses by influencing neutrophil, macrophage, B, and T lymphocytes. These active chemicals work together or alone to improve the responsiveness of these cells, either directly or indirectly.³⁴

The estimation of cytokine levels in serum

The results showed that cyclosporine intraperitoneal injection for 14 days led to the appearance of immunosuppressive effects by significant reduction ($p > 0.05$) of TNF- α , NF- κ B, and CCL5. But increased level of TGF- β in G2 as compared with G1, G3, while there was a significant increase ($p > 0.05$) of these data in in G4, G5, G6, and G7 as compared with G2 (Table-3).

Producing cytokines is one of the first steps of the immune response and can provide important information about the nature of any immunotoxic responses. Since immune cells only produce a tiny amount of cytokines to meet their basic cellular requirements. The mechanism of immunosuppressive action of CsA is dependent on the inhibition of the nuclear factor of activated T-cells (NFAT), a CSA-sensitive transcription factor involved in the formation of interleukin-2 (IL-2), and the increased maturation of T lymphocytes required for the immune response.³⁵ Inhibition of this pathway by CsA leads to toxicity beyond immunosuppression and also prevents the expression of genes encoding cytokines and other proteins

required for the immune response. CSA has the ability to inhibit the transcription of cytokine genes by inhibiting the activity of the phosphatase enzyme present in calcineurin, which subsequently leads to the activation of the transcription factor NFAT and also regulates nuclear translocation, also inhibits the JNK and NF- κ B signaling pathway, and stimulates the transcription of TGF- β genes.³⁶ CsA stimulates TGF- β secretion, which then leads to fibrosis-mediated nephropathy and apoptosis of renal cells.³⁷ While 38 concluded. The ability of cyclosporine to inhibit NF- κ B activation, which in turn turned off the TNF- α gene and thus reduced acute vascular failure. The results of the current study also agreed with that of H Nam, *et al*.³⁹ A significant increase in the level of TGF- β was recorded when CsA was injected subcutaneously in rats for 4 weeks at a dose of 15 mg/kg/day. It also agreed with the findings of A Wenchao, *et al*,⁴⁰ which recorded a decrease in the level of CCL5 and TNF- α when rats were dosed with CsA at a dose of 5, and 20 mg/kg for a week. It was also mentioned that the effect of immunosuppressive on the amount of cytokine secretion is proportional to The dose given for this drug.

The administration of the plant extract led to a significant improvement in the level of cytokines in the blood serum, which indicates the active role of the plant as an anti-inflammatory, which reduced the hepatotoxicity caused by CSA. Since the plant contains polyphenolic compounds that have a significant role in defending against reactive oxygen species (ROS), which are inevitably produced when environmental stresses impair aerobic or photosynthetic metabolism, it has been shown that the amount of antioxidant and antimicrobial components increase under adverse environmental conditions in plant tissues.⁴¹ This is consistent with what was indicated by G D'Argenio, *et al*.⁴² It was concluded that garlic extract inhibits TGF- β growth factor and alleviates liver fibrosis in mice induced by CCL4 injection. G Yana, *et al*,⁴³ reported significant activation of TLR4 and NF- κ B in the inflammatory response induced by Lipopolysaccharide (LPS), where high expression of these cytokines correlates with expression of TNF- α and IL-1 β . It also showed that NF- κ B could act as an anti-apoptotic molecule. The change in the level of cytokines is also due to the plant's role in stimulating the proliferation of lymphocytes, which in turn leads to the production of cytokines that activate other immune cells such as B cells, antigen-presenting cells, and other T cells.

Table 3: Effect of methanol extract of *P. khinjuk* on cytokine level in the serum of male rats treated with cyclosporine.

<i>The percentage of cytokine level in the serum mean \pm SE (pg/mL)</i>				
<i>Groups</i>	<i>NF-κB</i>	<i>CCL5</i>	<i>TGF-β</i>	<i>TNF-α</i>
Control (normal saline)	0.24 \pm 0.18 ^a	0.30 \pm 0.06 ^b	0.20 \pm 0.04 ^f	0.26 \pm 0.04 ^a
Cyclosporine (5 mg/kg)	0.06 \pm 0.08 ^c	0.16 \pm 0.11 ^f	0.46 \pm 0.20 ^a	0.11 \pm 0.06 ^f
Peginterferon alfa-2a (34.5 mg/kg)	0.21 \pm 0.04 ^c	0.31 \pm 0.06 ^c	0.31 \pm 0.04 ^b	0.29 \pm 0.03 ^e
<i>P. khinjuk</i> (3 g)	0.24 \pm 0.06 ^a	0.34 \pm 0.11 ^a	0.23 \pm 0.02 ^e	0.26 \pm 0.08 ^a
<i>P. khinjuk</i> (1.5 g)	0.26 \pm 0.09 ^b	0.35 \pm 0.04 ^g	0.25 \pm 0.02 ^d	0.27 \pm 0.06 ^b
<i>P. khinjuk</i> (3 g)+ Cyclosporine(5 mg/kg)	0.21 \pm 0.04 ^c	0.27 \pm 0.06 ^d	0.27 \pm 0.02 ^g	0.24 \pm 0.02 ^c
<i>P. khinjuk</i> (1.5 g)+ Cyclosporine(5 mg/kg)	0.20 \pm 0.07 ^d	0.24 \pm 0.02 ^e	0.29 \pm 0.02 ^c	0.22 \pm 0.04 ^d

*Different letters represent a significant difference ($p > 0.05$) between means in columns, while similar letters represent a non-significant difference ($p > 0.05$) between these means.

CONCLUSION

The methanol extract of *P.khinjuk* fruit contains chemicals that have immunomodulatory action on both cell-mediated and humoral immune responses. It stimulated the synthesis of white blood cells and raised the activity IgM, and IgG. It enhanced hemagglutination titers, indicating that humoral immunity was being boosted. The findings support the widespread use of *P.khinjuk* as an immune booster by both local areas and traditional healthcare practitioners in treating various illness conditions.

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