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Immunomodulatory and Anti-Inflammatory Effect of Thymoquinone on Rat Liver and Kidneys

Timokinonun Rat Karaciğer ve Böbrekleri Üzerindeki İmmunmodülatör ve Antiinflamatuvar Etkisi

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ABSTRACT

The *Nigella sativa* plant has a very long medical history. It has been used by ancient Egyptians for many purposes. Studies have reported that the plant has antibacterial, antifungal, anticancer, antidiabetic, antioxidant, and immunomodulation effects. The main active ingredient of the plant is a substance called thymoquinone. The aim of the present study was to investigate the immunomodulatory and anti-inflammatory effects of thymoquinone on rat liver and kidneys. Rats were shared into 5 groups the first group (1 mg/kg thymoquinone, intraperitoneally), the second group (2 mg/kg thymoquinone, intraperitoneally), the third group (10 mg/kg thymoquinone, gavage), the fourth group (20 mg/kg thymoquinone, gavage), and control group. The groups contained 7 rats and the experiment continued for 42 days. The localization and expression of interleukin 2, interleukin 4, interferon gamma, and tumor necrosis factor alphain the liver and kidneys are shown in vivo, and the immunomodulation effects of thymoquinone in the system have been shown. The effects of thymoquinone differ according to the cytokine type, administration methods, and dose. As a result, the present findings demonstrated that the immunomodulatory effect of thymoquinone on the liver and kidneys varies according to the organ, application method, and application dose. For interleukin 2, the most effective form of administration in the liver is intraperitoneal and low dose; for kidneys, it is a low-dose gavage administration. For interleukin 4, low-dose gavage administration has been observed to be more effective in the liver and kidneys. Similarly, it is concluded that especially 10 mg/kg gavage applications are more effective for liver and kidneys in interferon gamma and tumor necrosis factor alpha expressions.

Keywords: Immunomodulation, immunohistochemistry, inflamation, rat, thymoquinone

ÖZ

Nigella sativa bitkisinin çok uzun bir tıbbi geçmişi vardır. Antik Mısırlılar tarafından birçok amaç için kullanılmıştır. Çalışmalar bitkinin antibakteriyel, antifungal, antikanser, antidiyabetik, antioksidan ve immünomodülasyon etkilerine sahip olduğunu rapor etmiştir. Bitkinin ana etken maddesi timokinon adlı bir madde içerir. Bu çalışmanın amacı, timokinonun sıçan karaciğer ve böbreklerindeki immünomodülatör ve anti-inflamatuar etkilerini incelemekti. Sıçanlar, birinci grup (1 mg/kg timokinon, intraperitoneal), ikinci grup (2 mg/kg timokinon, intraperitoneal), üçüncü grup (10 mg/kg timokinon, gastrik gavaj), dördüncü grup (20 mg/kg timokinon, gastrik gavaj) ve kontrol grubuna ayrıldı. Gruplar yedi sıçandan oluşturuldu ve deney 42 gün boyunca devam etti. Karaciğer ve böbreklerdeki interlökin-2, interlökin-4, interferon gama ve tümör nekroz faktörü alfanın lokalizasyonu ve ekspresyonu in vivo olarak gösterildi ve sistemde timokinonun immünomodülasyon etkileri gösterildi. Sonuç olarak, mevcut bulgular, timokinonun karaciğer ve böbrekler üzerindeki immünomodülatör etkisinin organa, uygulama yöntemine ve uygulama dozuna bağlı olarak değiştiğini göstermektedir. Interlökin-2 için karaciğerde intraperitoneal ve düşük dozun en etkili uygulama şekli olduğu; böbrekler için ise düşük doz gastrik gavaj uygulamasının daha etkili olduğu görülmüştür. İnterlökin-4 için ise karaciğer ve böbreklerde düşük doz gastrik gavaj uygulamasının daha etkili olduğu gözlemlenmiştir. Benzer şekilde, özellikle 10 mg/kg gastrik gavaj uygulamalarının interferon gama ve tümör nekroz faktörü alfa ekspresyonu için karaciğer ve böbreklerde daha etkili olduğu sonucuna varılmıştır.

Anahtar Kelimeler: İmmunomodülatör, immunohistokimya, antiinflamatuar, sıçan, timokinon

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INTRODUCTION

Herbs are invaluable sources of new drugs and interest in medicinal herbs was advanced due to the increased efficiency of herbs-derived new medicines.[1] Recently, the daily use of food products of plant origin, which are not at risk for health and do not have excessive side effects, for therapeutic purposes has increased. Since ancient times, various plants have been used to treat various ailments. Plants are treated the same way today, and the industry associated with them is growing rapidly from year to year.[2] In developing countries as well as in developed countries, herbal remedies are used to treat different basic medical problems.[3] The most important reason for this is that herbal treatments are more effective, safe, less toxic, easily available, and affordable than chemical drugs.[4] Therefore, serious research is being carried out on the therapeutic potential and medicinal uses of plants.

Nigella sativa of the Ranunculaceae family is part of the black seed used as food. *Nigella* seeds are known to be used to support a healthy life, promote healthy aging, improve the quality of life, and most importantly, prevent diseases, that is, to help preventive medicine. *Nigella sativa*, commonly grown in the Middle East and West Asian countries, is used for treatment in different parts of the world. Nowadays, herbal medicines are widely utilized to address a diverse range of conditions, including bronchial asthma, cough, headache, toothache, and various kinds of cancer.[5-9] The main active ingredient of the plant is a substance called thymoquinone.[10] In scientific research, *Nigella* seed is one of the products that can be useful to consume due to the vitamins, active ingredients, and important fatty acids found in its structure. *Nigella*'s pharmaceutical effectiveness has been scientifically proven with clinical findings and it has also been a preferred plant for the treatment of medical conditions. One of the important properties of the black seed is the immunomodulatory effect of the substances in its composition.[11-15] Substances that alter the immune response by increasing or decreasing the strength of the immune system are called immunomodulatory substances. Immunomodulatory substances activate the immune system by stimulating cytokines such as interleukin (IL) 1, IL-2, IL-6, tumor necrosis factor (TNF), and interferon gamma (IFN-γ). The cytokines that play an important role in acquired immunity are IL-2, IL-4, IL-5, TGF-β, IL-10, and IFN-γ. Many studies have shown that black seed has immunomodulatory effects. As a result of studies, it has been reported that *Nigella* oil increases T-cell proliferation, triggering cellular immunity and suppresses B cells, that is, humoral immunity.[11] These findings obtained by in vitro studies were also supported by in vivo studies.[16-18] Based on the recent in vitro and in vivo data, it can be said that black seed can increase cellular immunity while suppressing humoral immunity. However, further experimental studies are needed to confirm this hypothesis. Thanks to such studies, the immunomodulatory effects of black seed can be measured based on natural immune reaction mediators in diseases.

The liver is an organ found only in vertebrates with many functions, including the production of enzymes necessary for detoxification, protein synthesis, and digestion. It is one of the most important organs of digestive system with its secretions and is also a unique immunological organ and has an important place among immune system-specific tissues. The liver contains immunologically active cells, especially in the fetus; T cells are first produced by the fetal liver.[19] They play a significant role in forming immune memory and maintaining and establishing immune tolerance. Like the liver, the kidneys are highly functional organs. Erythropoietin secreted in the kidneys is involved in the production of new red blood cells. The kidneys are responsible for gastrin metabolism. It is seen that the risk of a peptic ulcer due to disorders in gastrin metabolism is increased in patients with acute and chronic kidney disease. At the same time, both the liver and kidneys are organs that are included in the mononuclear phagocytic system. Kupffer cells in the liver and mesangial macrophages in the kidney are immune-related cells involved in phagocytosis.[19,20]

Studies have shown that black cumin extracts and thymoquinone increase T-cell population, cluster of differentiation 3 (CD3), CD4, and CD8 surface antigens and the number of immune system cells. As a cause of this increase, it was found that thymoquinone stimulates hematopoiesis, and thus the increase is formed as a result of the influence of immune system-related cells.[21-23] In in vitro and in vivo studies where *Nigella sativa* and its thymoquinone proteins are used, these proteins stimulated IL-1β and TNF-α productions.[24,25]

In the studies conducted, the effects of *Nigella sativa* were generally examined by applying either orogastric (oral) or intraperitoneal applications. But detailed information has not been given about the effectiveness of implementation paths. The aim our study was to compare the immunomodulatory effects of thymoquinone in different routes (oral vs intraperitoneal) as well as different dosages on the liver and kidneys of the rats. In summary, our goal was to determine the effective application route and dosage of thymoquinone with regard to the rat liver and kidneys.

MATERIALS AND METHODS

All experimental protocols were approved by Ondokuz Mayıs University Animal Experiments Ethics Committee (Date: 14.07.2015, Number: 2015/51).

Animals and Experimental Protocol

Thirty-five female adult Sprague–Dawley rats with an average weight of 250-300 g were used in the study. The rats were randomly assigned to 5 groups (n = 7) and the study continued for 42 days. The first group received 1 mg/kg dose of thymoquinone (Sigma 03416-100MG, St. Louis, Missouri, USA) intraperitoneally (ip). The second group received 2 mg/kg dose of thymoquinone ip. A dose of 10 mg/kg thymoquinone was administered to the third group via gavage. The fourth group received 20 mg/kg dose of thymoquinone with gavage. The fifth group was the control group with no intervention.

Histological Analysis

The rats in all groups were sacrificed and their kidney and liver tissues were collected. Tissues were fixed in a 10% formalin solution, and then tissue samples were embedded in paraffin blocks following routine histological procedures. After cutting sections of 5 μm thickness from paraffin blocks, Crossman's trichrome staining method was used to examine the histological structures.[26] Slides were examined under light microscope (Nikon E-80İ). Photography was done using Nikon digital sight imaging system (Nikon DS-Fi1).

Immunohistochemical Staining

The liver and kidney samples, prepared as 5 μm thick sections from paraffin blocks, underwent immunohistochemical staining. Interleukin 2 (1/750) (Biont, YID5405), IL-4 (1/750) (Biont, YID2904), TNF-α (1/200) (Biont, 5026), and IFN-γ rabbit polyclonal antibodies (1/500) (Biont, 2791) were utilized as primer antibodies for this

staining process.[27] After deparaffinization, the sections were subjected to proteolysis by heating in a citrate buffer solution (pH 6) for antigen retrieval, using a 700 W microwave oven. The tissues were treated with a 3% hydrogen peroxide solution, which blocks the peroxidase enzymes present in the tissues. After the washing step with phosphate buffer saline (PBS), the immunehistochemistry (IHC) kit's (Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC Kit, Abcam, ab64264,Cambridge, UK) serum was applied to the slides to prevent nonspecific protein binding. The primary antibodies were incubated at +4°C for 24 hours. As a negative control group, tissue samples were treated with PBS solution alone. Following the washing step, the samples were treated with a biotinylated secondary antibody, and subsequently incubated with a streptavidin–horseradish peroxidase complex. In the final step, 3,3′-diaminobenzidine was employed as the chromogen. The tissue preparations were counterstained with Mayer's hematoxylin and coverslipped using a mounting medium. In the immunohistochemical evaluations, staining intensity was graded on a scale of 0 to 3 as follows: no staining (–), weak staining (+), moderate staining (++), and intense staining (+++).[28]

Enzyme-Linked Immunosorbent Assay Analysis

Serum levels of IL-2 (Rat IL-2 Elisa Kit), IL-4 (Rat IL-4 Elisa Kit), TNF-α (Rat TNF-α Elisa Kit), and IFN-γ (Rat IFN-γ Elisa Kit) were measured using specific kits according to the procedures specified by the manufacturers. The levels of IL-2, IL-4, TNF-α, and IFN-γ were quantified in nanograms per milliliter (ng/mL).

Statistical Analysis

The statistical analysis of parameters was conducted using the Statistical Package for the Social Sciences, version 21.0 software (IBM Corp.; Armonk, NY, USA). The assays are presented in the median values. For parameters exhibiting a normal dispersion, an analysis of variance was conducted, while the Kruskal–Wallis test was employed for parameters demonstrating an abnormal dispersion. The level of statistical meaning was set at $P < .05$.[29]

RESULTS

Histological Results

The liver and kidney sections of all groups were examined by staining with the Crossman's trichrome staining method. In the liver, hepatocytes, vessels, and duct system had a normal histological structure. No important distinction was observed among the groups in terms of the histological structure (Figure 1A-E). Microscopic examinations of the kidney tissues revealed a normal histological structure. However, when all groups were compared with the control groups, it was detected a different degree of degeneration on renal tubule cells in all experimental groups (Figure 2A-E).

Immunohistochemical Results

Interleukin 2

In the liver, immune positive reaction intensities in the control group, the second group (2 mg/kg, ip) and the third group (10 mg/kg, gavage) were similar to each other with weak intensity. When

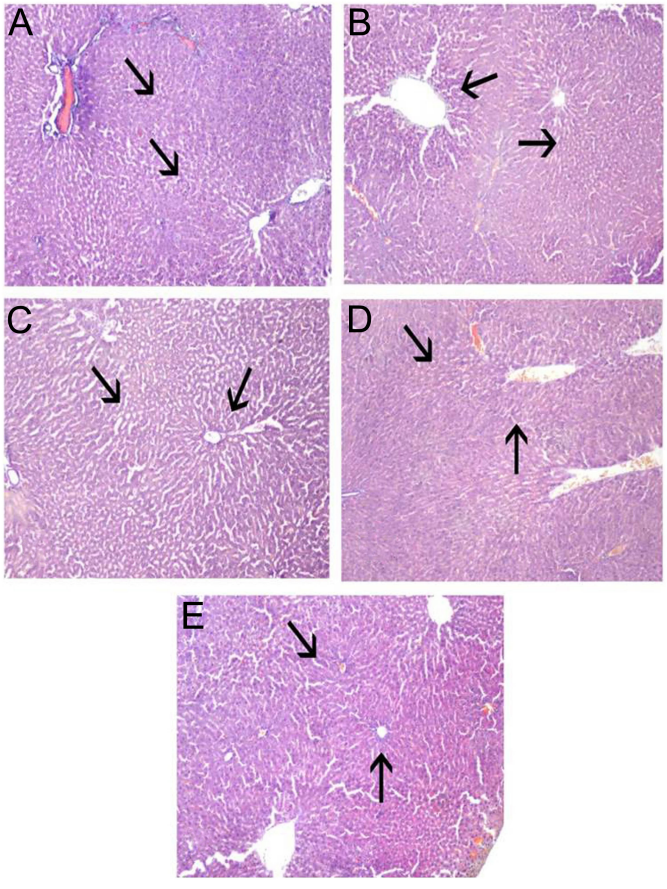

Figure 1. 1 mg/kg ip group (A), 2 mg/kg ip group (B), 10 mg/kg gavage group (C), 20 mg/kg gavage group (D), control group (E) liver. Arrows: hepatocyte 10×. ip, intraperitoneally.

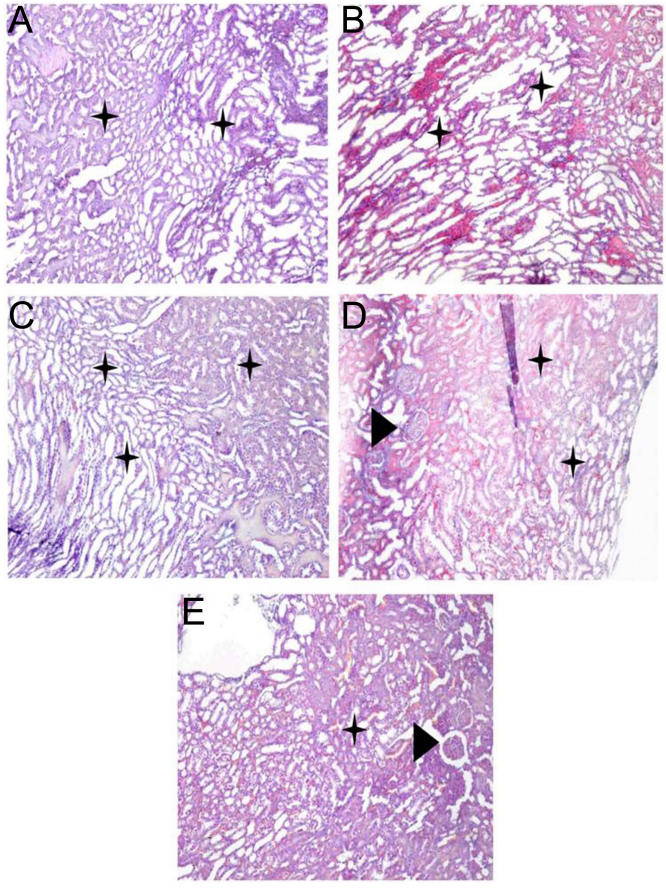

Figure 2. 1 mg/kg ip group (A), 2 mg/kg ip group (B), 10 mg/kg gavage group (C), 20 mg/kg gavage group (D), control group (E) kidney. +: kidney tubules, arrowheads: kidney glomeruli 10×. , intraperitoneally.

compared to other groups, the intensity of the immune positive reaction was quite weak in the first group (1 mg/kg, ip). The most intense reactions were observed in the fourth group (20 mg/kg, gavage) (Figure 3A1-E1, Table 1). In the kidneys, different intensities of immune positive reactions were observed in the cortex and medulla across all groups. The most intense reaction in the cortex was detected in the fourth group (20 mg/kg, gavage); immune positive reactions were similar and mild in the first group (1 mg/kg, ip), the second group (2 mg/kg, ip), and third group (10 mg/kg, gavage). In the control group, a weak immune positive reaction was detected. The most intense reaction in the cortex was detected in the control group. The moderate immune positive reactions were observed in the first group (1 mg/kg, ip) and second group (2 mg/kg, ip). The reactions were weaker in the third group (10 mg/kg, gavage) and fourth group (20 mg/kg, gavage) compared to the other groups (Figure 4A1-E1, Table 2).

Interleukin 4

In the liver, the most intense reaction was in the second group (2 mg/kg, ip) and the control group, while the weakest immune positive reaction was seen in the third group (10 mg/kg, gavage) (Figure 3A2-E2, Table 1). In the kidneys, weak immune positive reactions were observed in the cortex across all groups. In the medulla the most intense immune positive reaction was in the first group (1 mg/kg, ip), while the reaction intensities were similar and moderate in the second group (2 mg/kg, ip) and control groups. The weakest immune reactions were in the gavage-applied groups (Figure 4A2-E2, Table 2).

Tumor Necrosis Factor Alpha

In the liver, the most intense immune positive stainings were in the first group (1 mg/kg, ip), second group (2 mg/kg, ip), and fourth group (20 mg/kg, gavage). The intensity of immune positive reaction was weak in the third group (10 mg/kg, gavage) and control groups (Figure 3A3-E3, Table 1). In the renal cortex, the reaction intensity increased in entirely groups compared to the control group. The immune positive reactions in the first group (1 mg/kg, ip) and the second group (2 mg/kg, ip) were more intense than those of the third group (10 mg/kg, gavage) and fourth group (20 mg/kg, gavage). In the kidney medulla, the immune positive reactions were stronger in the first group (1 mg/kg, ip) and fourth group (20 mg/kg, gavage), compared to the other groups. In the second group (2 mg/kg, ip), weak immune-positive reactions were observed similar to the control group, while mild reactions were observed in the third group (10 mg/kg, gavage) (Figure 4A3-E3, Table 2).

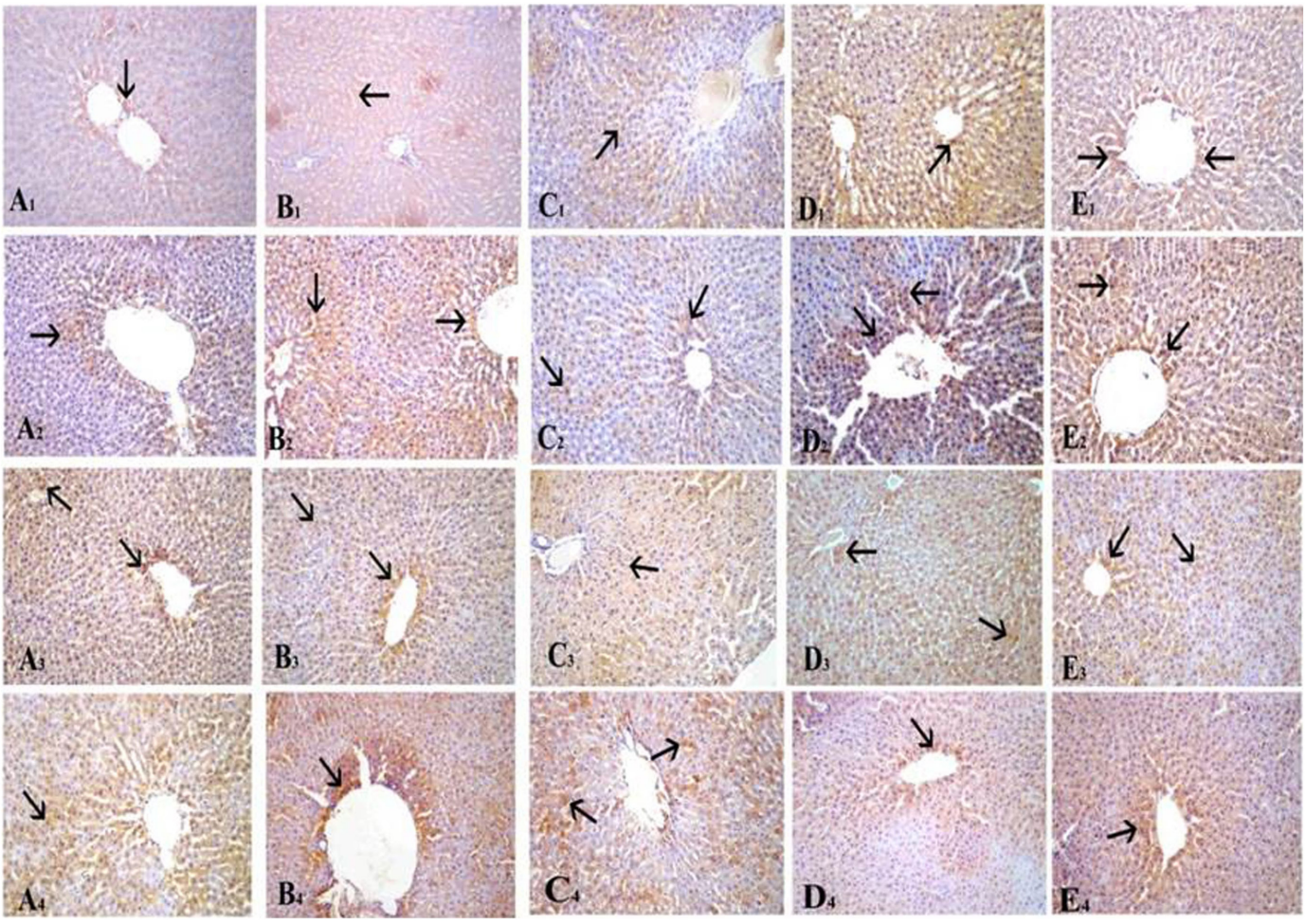

Figure 3. 1 mg/kg ip group (A_1), 2 mg/kg ip group (B_1), 10 mg/kg gavage group (C_1), 20 mg/kg gavage group (D_1), control group (E_1) IL-2 expression in liver. 1 mg/kg ip group (A_2), 2 mg/kg ip group (B_2), 10 mg/kg gavage group (C_2), 20 mg/kg gavage group (D_2), control group (E_2) IL-4 expression in liver. 1 mg/kg ip group (A_3), 2 mg/kg ip group (B_3), 10 mg/kg gavage group (C_3), 20 mg/kg gavage group (D_3), control group (E_3) tumor necrosis factor alpha expression in liver. 1 mg/kg ip group (A_4), 2 mg/kg ip group (B_4), 10 mg/kg gavage group (C_4), 20mg/kg gavage group (D_4), control group (E_4) interferon gamma expression in liver. Arrow: hepatocyte 20×. IL-2, interleukin 2; IL-4, interleukin 4.

Table 1. Comparison of Semiquantative Immunohistochemical Staining Scores of IL-2, IL-4, IFN-γ, and TNF-α in Study Groups (Liver)

Parameters	1 mg/kg TQ ip	2 mg/kg TQ ip	10mg/kg TQ gavage	20 mg/kg TQ gavage	Control
IL-2	±	+	+	++	+
IL-4	+	+++	±	++	+++
IFN-γ	++	+++	+++	++	+++
TNF-α	++	++	+	++	+

Negative to weak (±), eak (+), moderate (++), and strong (+++).
IFN-γ, interferon gamma; IL-2, interleukin 2; IL-4, interleukin 4; TNF-α, tumor necrosis factor alpha; TQ, thymoquinone.

Interferon Gamma

In the liver, the most intense immune positive reactions were in the second group (2 mg/kg, ip) and the third group (10 mg/kg, gavage), while moderate immune positive reactions were observed in the other groups (Figure 3A4-E4, Table 1). In the kidneys, the reaction intensities in the cortex region were weak in all groups. In the medulla, the intensity of immune reactions was similar and intense across all groups (Figure 4A4-E4, Table 2).

Biochemical Results

Interleukin 2, IL-4, TNF-α, and IFN-γ levels were measured by the enzyme-linked immunosorbent assay method with serum obtained from the blood (Table 3).

Considering the IL-2 serum biochemistry levels, significant decreases were detected across all experimental groups compared to the control group (Table 3). Interleukin 4 levels were decreased in the other 3 experimental groups except for the second group (2 mg/kg, ip) compared to the control group, but the difference was (Table 3). Interferon gamma levels were decreased in the first group (1 mg/kg, ip) and the third group (10 mg/kg, gavage) compared to the control group. While the second group (2 mg/kg, ip) was at similar levels to the control group, there was an increase in the fourth group (20 mg/kg, gavage) compared to the control group with no statistically significant differences between the groups (Table 3). Interferon gamma levels were decreased in the second group (2 mg/kg, ip) and 10 mg/kg gavage groups compared to the control group, while an increase was observed in the first group (1 mg/kg, ip) and the third group (10 mg/kg, gavage). The differences between the groups were not statistically significant (Table 3).

DISCUSSION

Many medicinal plants and their products have been documented to have immunomodulatory and therapeutic properties; *Nigella sativa* is one of these plants.[11,30] In the literature, no study was found in which the method of administration and doses of

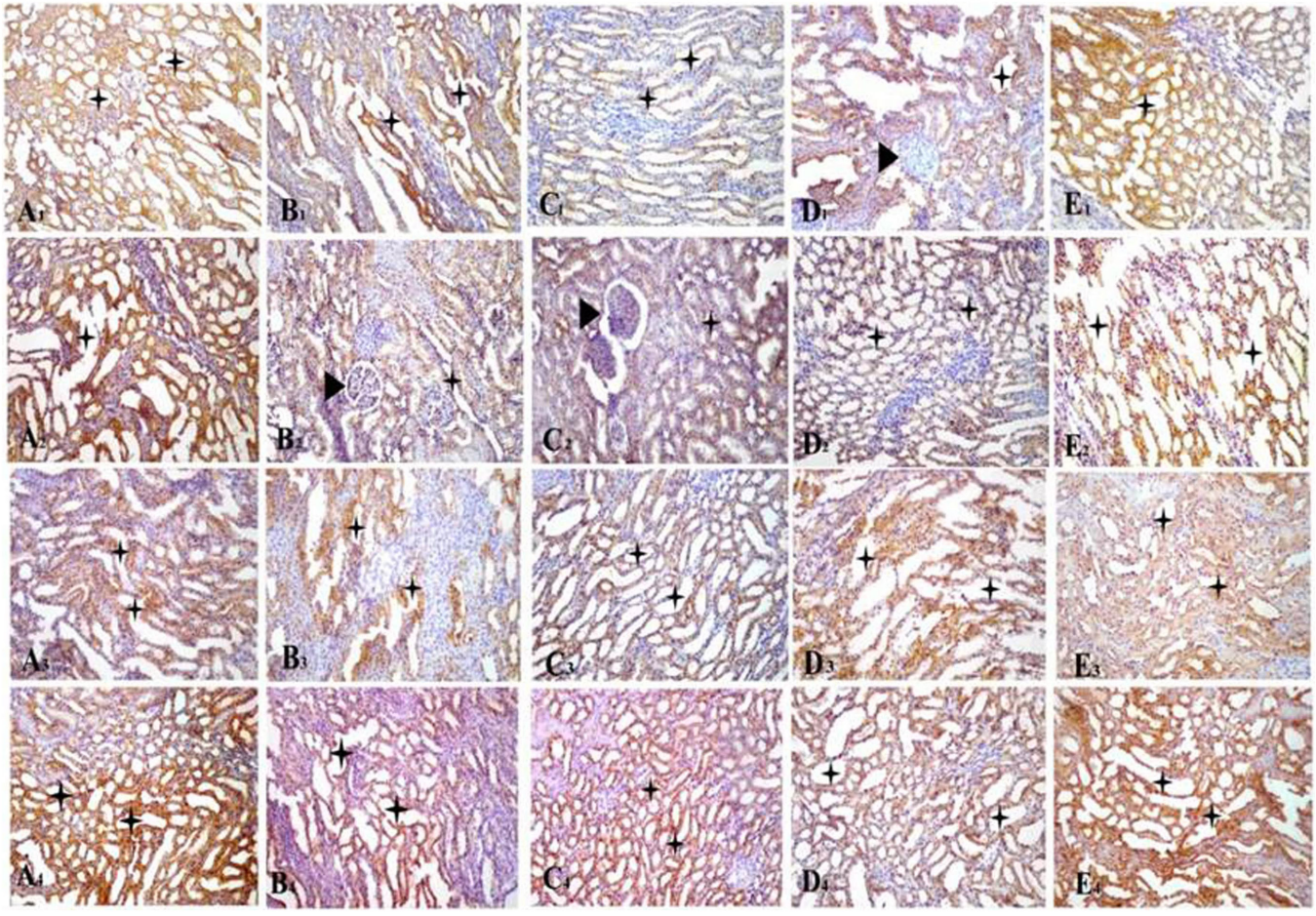

Figure 4. 1 mg/kg ip group (A_1), 2 mg/kg ip group (B_1), 10 mg/kg gavage group (C_1), 20 mg/kg gavage group (D_1), control group (E_1) IL-2 expression in kidney. 1 mg/kg ip group (A_2), 2 mg/kg ip group (B_2), 10 mg/kg gavage group (C_2), 20 mg/kg gavage group (D_2), control group (E_2) IL-4 expression in kidney. 1 mg/kg ip group (A_3), 2 mg/kg ip group (B_3), 10 mg/kg gavage group (C_3), 20 mg/kg gavage group (D_3), control group (E_3) tumor necrosis factor alpha expression in kidney; 1 mg/kg ip group (A_4), 2 mg/kg ip group (B_4), 10mg/kg gavage group (C_4), 20mg/kg gavage group (D_4), control group (E_4) interferon gamma expression in kidney. +: kidney tubules, arrowhead: kidney glomeruli, 20×. IL-2, interleukin 2; IL-4, interleukin 4.

Table 2. Comparison of Semiquantative Immunohistochemical Staining Scores of IL-2, IL-4, IFN-γ, and TNF-α in Study Groups (Kidney)

Parameters	1 mg/kg ip, TQ C/M	2 mg/kg ip TQ, C/M	10 mg/kg gavage, TQ C/M	20 mg/kg gavage, TQ C/M	Control C/M
IL-2	±/++	±/++	±/+	++/+	+/+++
IL-4	+/+++	+/++	+/+	±/+	+/++
IFN-γ	+/+++	±/+++	±/+++	++/++	+/+++
TNF-α	++/+++	++/++	+/+	+/+++	±/++

Negative to weak (±), weak (+), moderate (++), strong (+++).
C, kidney cortex; IFN-γ, interferon gamma; IL-2, interleukin 2; IL-4, interleukin 4; M, kidney medulla; TNF-α, tumor necrosis factor alpha; TQ, thymoquinone.

Table 3. ELISA Results of IL-2, IL-4, IFN-γ, and TNF-α Levels in Experimental Groups

TQ	n	IL-2	IL-4	IFN-γ	TNF-α
Control	7	2.34 ± 0.16[b]	62.45 ± 3.44	62.08 ± 3.45	184.93 ± 11.61
1 mg/kg ip	7	1.94 ± 0.06[a]	58.79 ± 4.50	56.46 ± 4.42	189.14 ± 10.18
2 mg/kg ip	7	1.74 ± 0.11[a]	70.48 ± 4.33	62.33 ± 1.53	182.70 ± 6.54
10 mg/kg gavage	7	1.84 ± 0.10[a]	60.78 ± 5.02	58.19 ± 4.45	177.90 ± 12.24
20 mg/kg gavage	7	1.80 ± 0.09[a]	56.41 ± 4.60	64.96 ± 2.61	268.06 ± 45.61
*P**			–	–	–

Dashes refer to nonsignificant *P*-values ($P < .05$).
IFN-γ, interferon gamma; IL-2, interleukin 2; IL-4, interleukin 4; TNF-α, tumor necrosis factor alpha; TQ, thymoquinone.
*$P < .05$.[a, b]Difference between values with different letters in the same column is significant ($P < .05$).

thymoquinone were compared. Therefore, this study was conducted to evaluate which administration method and dose are more effective by comparing the dose and administration methods.

In our study, all liver and kidney tissues were evaluated in detail. When the histological effects of the administration of thymoquinone at different doses by intraperitoneal and oral gavage routes on liver and kidney tissues were evaluated, no significant structural difference was detected between the groups. The results of our study are similar to the results of Yuncu et al's[31] studies on rat livers.

As a result of immunohistochemical staining in our study, IL-2 expression decreased in the liver and kidneys compared to control groups. Cobourne-Duval et al[32] reported in their study that thymoquinone plays an important role in the regulation of proinflammatory cytokines; they said that it can achieve this effect by inhibiting the expression of IL-2, IL-4, and IL-6. In our study, the decrease of IL-2 in thymoquinone-administered groups is similar to the results of study of Cobourne-Duval et al.[32] Likewise, it was determined that there was a decrease in the expression of IL-4 in the groups administered low-dose thymoquinone in the liver. Similarly, in the study of Gunel et al[33] which created allergic rhinitis, it was reported that there was a dose-dependent decrease in IL-4 expression after thymoquinone applications.

Interferon gamma and TNF-α expressions differed as a result of the administration of thymoquinone at different doses and in different forms. Gholamnezland et al reported that the expressions of TNF-α and IFN-γ vary depending on the dose of thymoquinone. They stated that high-dose thymoquinone administrations are also effective in immunomodulation by causing a decrease in cytokine release. The fact that the thymoquinone used in our study caused different intensities of reductions in cytokine release in both the liver and kidneys is consistent with the other studies.[33-35]

In this study, the possible immunomodulating effects of thymoquinone administered orally and intraperitoneally at different doses on the liver and kidneys were investigated comparatively. Expression of IL-2, IL-4, IFN-γ, and TNF-α were demonstrated in the liver hepatocytes and renal tubules across all groups. Observation of different immune reactions in all groups showed that various administration forms of thymoquinone did not inactivate cytokines. However, the effects of thymoquinone differ according to the cytokine type, administration methods, and dose. For IL-2, the most effective form of administration in the liver is intraperitoneal and low dose; for kidneys, it is a low-dose gavage administration. For IL-4, low-dose gavage administration has been observed to be more effective in the liver and kidneys. Similarly, it is concluded that especially 10 mg/kg gavage applications are more effective for liver and kidneys in relation to IFN-γ and TNF-α expressions.

In conclusion, the present findings demonstrate that the immunomodulatory effect of thymoquinone on the liver and kidneys varies according to the organ, application method, and application dose. However, different molecular methods are needed to explain the reasons for the differences, the effective dose, and the method of administration in detail. It is hoped that future studies will confirm the immunomodulatory functions of thymoquinone and also confirm the importance of the form and dose of administration and therefore its possible therapeutic efficacy against various diseases and medical conditions.

Ethics Committee Approval: All experimental protocols were approved by the Ondokuz Mayıs University Animal Experiments Ethics Committee (Date: 14.07.2015, Number: 2015/51).

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – Ş.T.; Design – Ş.T.; Supervision – Ş.T.; Resources – Ş.T.; Materials – Ş.T., T.E., A.E.; Data Collection and Processing – Ş.T., N.D., E.A.; Analysis and Interpretation – Ş.T., T.E.; Literature Search – Ş.T., N.D., E.A.; Writing Manuscript – Ş.T.; Critical Review – Ş.T., T.E., A.E., N.D., E.A.

Declaration of Interests: The authors declare that they have no competing interest.

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