The effect of dietary supplementation of Nigella sativa (black seeds) on cell mediated immunological function of male Wistar rats

Hany Mahmoud
Center of Scientific Foundation for Experimental Studies and Research, Ismailia, Egypt, 41511.
Amani Almallah
Anatomy and Embryology Department, Faculty of Medicine, Suez Canal University, Egypt.
Heba Gad EL-Hak
Zoology Department, Faculty of Sciences, Suez Canal University, Egypt
Tahany Aldayel (TSALdayel@pnu.edu.sa)
Nutrition and food science, Department of Physical Sport Sciences, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia.
Heba Abdelrazek
Department of Physiology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.
Howayda Khaled
Zoology Department, Faculty of Sciences, Suez University, Egypt.

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Abstract

This experiment aimed to investigate the effect of dietary *Nigella sativa* on cell mediated immune response. A total of eighteen male Wistar rats were divided equally into control and black seeds at 30 and 50 g/kg/diet (Sa30 and Sa50), respectively for 30 days. Weight gain, feed intake, feed conversion ratio (FCR), cell mediated immune response was monitored after injection of 0.1 mL of 10% phytohemagglutinin (PHA). Intumesce Index, serum total antioxidant capacity (TAC), Catalase (CAT), Interleukin-12 (IL-12), gamma interferon (γ-IF) and tumor necrosis factor alpha (TNF-α) were determined. Histopathological examination and immunohistochemistry of splenic caspase-3 and CD 8 were done. *Nigella sativa* significantly (p<0.05) improved weight gain and FCR in Sa30 and Sa50 groups. Significant increase for Intumesce Index in Sa50 group was observed. Total TAC, CAT, IL-12, γ-IF and TNF-αincreased significantly (P<0.05) in Sa30 and Sa50 groups. Histological examination of PHA stimulated foot pads showed more leukocytes infiltration and edema in a dose dependent pattern. Splenic caspase-3 and CD 8 showed significant (P<0.05) decrease and increase, respectively in Sa30 and Sa50 groups. *Nigella sativa* seeds had immunostimulatory ability through their antioxidant potential, cytokines induction, CD 8 promotion and reducing splenic apoptosis.

Introduction

The immunostimulants enhance cell-mediated immune response by the activation of antigen-specific cytotoxic T-lymphocytes, phagocytes and the discharge of several cytokines toward antigen [1] to achieve therapeutics [2]. Immunostimulant ropes to overwhelm the immunosuppressive effects of infectious agents and stress that interface and/or harm the immune cells function [3]. Diverse substances have been displayed immunostimulatory effects which are; plants or animal derivatives, microbial products, hormones, synthetic chemical and vitamins [4]. Herb, plant extracts and animal originated products are widely used because they are not expensive, can be easily obtained and act versus a wide-ranging spectrum of pathogens [5]. Oral administration of plant or herbal extracts as immunostimulants is considered the most superlative method of immunostimulation [6].

Herbal medicines extracted from plants or plant extracts itself have historically been used to enhance health. Recently, scientists have been keen on recognizing their key ingredients and comprehending their mechanisms of action [7-10]. One of them is black seed, or *Nigella sativa* belonging to the family Ranunculacea that has a rich religious and historical backgrounds [11]. It has been grown and used in various parts of the world as a food additive, spice and remedy for a large variety of diseases, like headache, bronchial asthma, nasal congestion, toothache, allergies, back pain, hypertension, obesity, gastrointestinal troubles and numerous types of cancer [12]. Also *Nigella sativa* seeds can minimize fatigue [13] and depression [14] beside increasing the strength of the body [15]. Moreover, *Nigella sativa* was found to possess immunostimulatory effects, due to its documented components, in various inflammatory and immunologic diseases [16] such as experimental allergic encephalomyelitis, colitis, arthritis [17], sensitized animals [18], asthma sufferers patients [19] and chemical war victims [20].

Ahmed and El-Sayed [21] indicated that dietary black seeds supplementation successfully improved body gain percentage, feed intake, serum biochemical and immunological parameters in rats. *Nigella sativa* has been given numerous beneficial properties including its ability for promoting antioxidants [22]. Newly, clinical and experimental studies have demonstrated that *Nigella sativa* extracts have many therapeutic effects including; antidiabetic effect [23-25], immunomodulating properties [12,26-29], analgesic and anti-inflammatory activity [30-34], antitumor against various cancer diseases [35] and antiulcerogenic effects [36-38]. *Nigella sativa* oil showed anti-inflammatory, anti-arthritic and anti- nociceptive activities in arthritic rats [39]. In addition, modern toxicological studies have shown that crude seed extracts and some of their active ingredients (volatile oil, thymoquinon) may have protective effects against hepatotoxicity and nephrotoxicity caused by either chemical substances or diseases [40-44]. Moreover, *Nigella sativa*activates bone marrow and immune cells [45] and increases the production of interferon [46], defending normal cells against cell death by viruses killing [47], destroys tumor cells [48] and increases the amount of antibodies generating B cells [17].

The current study aimed to explore the effect of dietary *Nigella sativa* seeds supplementation on growth performance parameters, FCR, Intumesce Index as an indicator for immune function in Wistar rats. This was achieved by investigating the parameters of total antioxidant and inflammatory cytokines, such as total antioxidant capacity (TAC), catalase activity, interferon gamma (IF- γ), interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) in rat model. Also, histopathologic changes in the popliteal lymph node and spleen were also examined; in addition to the immunohistochemical expression of caspase 3 and CD8 in the spleen.

Materials And Methods

Herbal plants

Black seeds (100% organic *Nigella sativa* seeds) were bought from a local market, kept with a voucher number 54782: 228/056/572 in Prophetic Medicine Foundation, Ismailia, Egypt. The whole seeds were daily shriveled in a blender and mingled well with basal diet just before administration to rats. Quantitative phytochemical constituent's analysis of *Nigella sativa* seeds were done using previously reported protocols as follow: preparation of seeds extract for quantitative phytochemical constituent's analysis as previously mentioned by Silahtaroglu, et al. [49].

Phytochemical analysis of *Nigella sativa* seeds and antioxidant activity

Phytochemical screening of phenolic compounds was done according to Oladeji, et al. [50]. Total phenolic content was estimated in *Nigella sativa* seeds according to Khattak and Simpson [51]. The total flavonoid content was estimated in *Nigella sativa* seeds extract based on the method of Nergiz and Ötleş [52]. The antioxidant activity of the seeds was assessed by the method described by Mariod, et al. [53].

Experimental animals

Eighteen adult male Wistar rats (105-115 g) were got from the Animal House in Suez Canal University, Faculty of Sciences, Ismailia, Egypt. The rats were acclimated for 2 weeks in clean cages under standard conditions and got free access to water and feed. The animals' room was subjected to natural daylight rhythm and adequate ventilation during the whole experimental period (30 days).

Ethical approval.

This animal experiment was carried out in accordance with EU Directive 2010/63/EU that strictly followed to minimize suffering of animal experiments, The experimental work was approved by the Institutional Animal Ethics Committee of Faculty of Veterinary Medicine Animal Ethics Committee, Suez Canal University with Number: 2020041.

Experimental design and diets

Experimental rats were randomly splitted into 3 groups (6 rats in each group); control and two treatment groups. Rats of the control untreated rat fed on a basal formulated diet (1% vitamin mixture, 2% choline chloride, 3.5 % salt mixture, 4.7% corn oil, 5% fibers, 10% sucrose, 20 % protein, and the remainder was corn starch up to100 %) which encountered the nutritional requirements of rats, according to Nutrient Requirements of Laboratory Animals [54]. Rats of the second (Sa30) and third (Sa50) treatments fed on the previous basal diet plus 30 g/kg and 50 g/kg black seedpowder, respectively for 30 days. The selected *Nigella sativa* seeds doses were according to Bashir et al. and Mohamed et al. [55,56].

Growth performance parameters

The feed intake and weights of each experimental animal/group were recorded at the commencement and the end of the experimental to monitor the growth output parameters using the following formulae:

Weight gain (g rat^{-1}) = W_f-W_0.

Feed conversion Ratio (FCR) = FI/ (W_f-W_0).

Where, W_0 and W_f were the initial and final weights of the rats per group, respectively and FI was feed intake.

Phytohemagglutinin injection and Intumesce Index

The left foot of each experimental rat/ group was injected 0.1 mL of 10% (v/v) phytohemagglutinin (PHA) (L 9017, Sigma Aldrich, USA) in PBS. The right foot pad of the same rat was kept as control through inoculation with 0.1 mL of PBS [57,58]. The rats were kept for 24 h, then the thickness of the lateral and dorso-ventral aspects of the left foot pad at the injection point was assessed via manual micrometer. The same person made the injections and steps to reduce the mistake. The Intumesce Index was determined as the measured ankle size-primary ankle size/primary ankle size.

Blood and tissue collection

The rats were anesthetized with tetrahydrofuran inhalation anesthesia after overnight fasting. Blood samples were drawn, at the end of experiment, from orbital venous plexus under the effect of the latter anesthesia in plain tubes. Sera were alienated and kept at -70 °C until analysis of antioxidants (catalase and TAC), IF-γ, IL-12 and TNF-α.

Rats were scarified and the spleen was excised, microscopically examined and weighed in relation to body weight to obtain the relative weight. Also, popliteal lymph node of PHA injected paw and PBS injected one were excised. Both spleen and popliteal lymph nodes were fixed in a 10% neutral formalin solution for histopathological examination and immunohistochemical detection.

Catalase activity and TAC

Catalase activity and TAC were measured in the sera by colorimetric method and following kits (K773 and K274) instruction that were purchased from BioVision Inc., Milpitas, CA, USA.

IF-γ, IL-12 and TNF-α levels

IF-γ, IL-12 and TNF-α levels were assayed via rat enzyme linked immunosorbent assay sandwich ELISA kit (Thermo Fisher Scientific, USA) according to producing company directions. IF-γ (BMS629) with detection limit 11.0 pg/mL; Serum IL-12 (KRC0121) with detection limit <2.5 pg/mL and TNF-α levels (BMS621) with detection limit 9.9 pg/mL. They were carefully checked for their sensitivity, specify and reliability. Spectrophotometer and a microplate reader (Biotech, USA) were used to measure the absorbance.

Histopathology and immunohistochemistry examination

Formalin fixed spleen and foot paws were progressively dehydrated, cleared, then submerged in paraffin wax. Numerous 5μm sections were obtained and stained with Haematoxylin and Eosin (H&E) according to Bancroft and Gamble [59] for histopathological examination.

Immunohistochemistry of splenic caspase 3 was done using a primary antibody for caspase 3 (#PAI29157, Thermo Scientific Co., USA) and CD8 (Cat. No. 6A242, Santa Cruz, CA, USA) at dilution rates 1:1000 and 1:200, respectively. The procedures were performed secondary polyvalent Biotinylated antibody according to the methodology of Zhao et al. and Elgawish et al. [60,61], respectively. The percentages of the IHC-stained area (IHC area %) were acquired via ImageJ software as described by Elgawish and Abdelrazek [61]. Tissues were examined blindly to which group the samples belonged.

Statistical analysis

Data were tested for normality and they found to follow normal distribution. The results were presented as the mean ± standard error of mean (SEM). The differences between groups were calculated using one-

way analysis of variance (ANOVA) followed by Duncan post hoc multiple comparison tests (SPSS software, version 16.0; SPSS Inc., Chicago, IL, USA) with significance at $P < 0.05$.

Results

Phytochemical constituent analysis of *Nigella sativa* seeds

The standard laboratory procedures for phytochemical screening demonstrated the presence of phenolic compounds (p-hydroxybenzoic; Catechin; Chlorogenic; Ferulic; Sinapic; p-coumaric; Kaempferol) Table (1). The results indicated that the highest amount of phenolics was p-hydroxybenzoic (69.685 µg/g) while the lowest amount was Kaempferol (1.277 µg/g). As shown in Table (2), the contents of *Nigella sativa* seeds from total phenol compound were 2.077 mg equivalents to Gallic acid , total flavonoid contents is 0.565 mg equivalents to catechin and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities of black seeds was 1.367 g equivalents to Trolox.

Table (1): Phenolic profile (µg/g) of black seeds *(Nigella sativa)*

Compound	Contents (µg/g)
p-hydroxybenzoic	69.685
Cateachin	32.824
Chlorogenic acid	2.554
Ferulic acid	6.628
Sinapic	21.979
p-coumaric	45.147
Kaempferol	1.277

Table (2): Total phenols content, flavonoid content and DPPH activity in black seeds extract *(Nigella sativa)*

	Total Phenols (mg GAE/g)	Total Flavonoids (mg CE/g)	DPPH (mg TE/g)
Black seeds	2.077	0.565	1.367

Values of Total Phenols content were manifested as Gallic acid equivalents (GAE). Total flavonoid contents were expressed as catechin equivalents (CE). The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging activities of black seeds were equivalent to Trolox (TE).

Growth performance parameters:

There was no mortality or change in the behavior during the study. *Nigella sativa* positively affected the growth performance of rats. Rats given dietary *Nigella sativa* 30 and 50 mg/kg, respectively, demonstrated significant (P<0.05) promotion in weight gain than control rats. Analysis of data revealed that FCR was significantly affected by the *Nigella sativa*. Rats received *Nigella sativa* 30 and 50 mg/kg basal diet showed significant (P<0.05) reduction in FCR than control group (Table 3).

Table (3): Growth performance of rats, relative spleen weight and Intumesce Index of *Nigella sativa* seeds fed groups (30 and 50 g/kg) and control

Treatment Groups	Weight gain (g)	Average Food intake per week (g)	Feed conversion ratio (FCR)	Relative Spleen weight (%)	Intumesce Index
Control	23.00±4.3 b	367.50±34.5	17.39±1.9^{b}	0.46±0.02^{b}	0.02±0.01^{b}
Sa30	37.10±3.9 a	390.81±32.4	10.86±1.9^{a}	0.49±0.01^{b}	0.04±0.02^{ab}
Sa50	39.00±2.5 a	460.60±26.0	12.67±1.1^{a}	0.60±0.03^{a}	0.06±0.01^{a}

Data are presented as mean ± SE, n=6 per group. Means within the same Colum carrying different superscript letters are significantly different performed by one-way ANOVA, followed by Duncan post hoc analysis P < 0.05 (Sa30: *Nigella sativa* fed group 30 g/kg diet & Sa50: *Nigella sativa* fed group 30 g/kg diet).

Relative weight of the spleen

There was no noteworthy finding in the spleen external appearance. The relative weight of the spleen in the sa50 rats significantly (P< 0.05) improved than those of the control group. Relative weight of spleen did not differ in 30 g/ kg black seeds fed group than control rats (Table 3).

Intumesce Index

There was a significant (P < 0.05) increase in Intumesce index of ankle of rats fed 50 g/kg *Nigella sativa* for 30 days compared to control groups. However, 30 g/kg black seeds fed group was non-significantly differed than control and 50 g/kg black seeds fed groups (Table 3).

Catalase and TAC

Serum catalase activity revealed significant (P <0.05) increase in Sa30 and Sa50 groups than control. Also, serum TAC was significantly increased (P<0.05) in Sa30 and Sa50 groups than control (Table 4).

IF-γ, IL-12 and TNF-α levels

The serum levels of IF-γ, IL-12 and TNF-α in in Sa30 and Sa50 groups exhibited significant (P<0.05) promotion than control one (Table 4).

Table (4): Interferon gamma (IF-γ), interleukin- 12 (IL-12), tumor necrosis factor alpha (TNF-α), , total antioxidant capacity (TAC) levels and catalase activity of *Nigella sativa* seeds fed groups (30 g/kg & 50 g/kg) and control

Treatment	Cytokines parameters			Antioxidant parameter	
	IF-γ (pg/mL)	IL-12 (pg/mL)	TNF-α (pg/mL)	TAC (mmol/l)	CAT (mmol/l)
Control	461.8±3.9^{c}	12.89±0.1^{c}	6.83±0.03^{c}	0.864±0.003^{c}	3.85±0.01^{c}
Sa30	566.5±6.2^{b}	15.72±0.1^{b}	8.804±0.1^{b}	1.070±0.03^{b}	4.27±0.03^{b}
Sa50	627.5±1.9^{a}	20.89±0.1^{a}	11.17±0.1^{a}	1.31±0.02^{a}	4.87±0.01^{a}

Data are presented as mean ± SE, n=6 per group. Means within the same Colum carrying different superscript letters are significantly different performed by one-way ANOVA, followed by Duncan post hoc analysis P < 0.05 (Sa30: *Nigella sativa* fed group 30 g/kg diet & Sa50: *Nigella sativa* fed group 30 g/kg diet).

Spleen histopathology and histomorphometry

Microscopical examination of control and *Nigella Sativa* treated spleens revealed no histopathological lesions in normal white and red pulps separated by marginal zones. The white pulp consists of follicle with pale germinal center and peripherally located central arterioles (Fig. 1). Histomorphometric analysis of 30 and 50 g/kg *Nigella Sativa* spleens showed that area of the white pulp, periarterial lymphoid sheath and the germinal center showed significant increase compared to the spleen of control rats (Fig. 1) that appeared to be dose dependent.

The CD8 protein expression appeared as brownish color in splenocytes. Spleen of control group showed very weak expression of CD8 expression. In contrast to spleen of Sa30 and Sa50 groups showed more expression pattern compared to control group (Fig. 2). The percent area of positive Immunohistochemical expression of splenocytes containing CD8 significantly (P<0.05) increased in Sa30 and Sa50 groups compared to control groups.

On the other hand, spleen of control group showed a strong expression of caspase 3 expression that was represented by brown coloration. In contrast to spleen of Sa30 and Sa50 groups showed more decrease to the expression pattern of caspase 3 protein compared to control group (Fig. 3). The percent area of positive Immunohistochemical expression of splenocytes containing caspase 3 protein significantly ($P < 0.05$) decreased in rats fed (30 and 50) g/kg *Nigella sativa* seeds daily for 30 days compared to control groups.

The popliteal lymph node of control group injected with PHA Fig. 4 (A&D) showed a reduction in the width of the cortex. The lymphoid follicle with a pale staining area (germinal center) was poorly demarcated. Reactive inflammatory hyperplasia of the lymph node showed a slight increase in the number of lymphocytes seen extending to the medulla and dispersed throughout the whole section. Severe degeneration with mild necrotic area and lymphatic sinus ectasia were also observed. The histological inspection of the popliteal lymph nodes of Sa30 Fig. 4 (B&E) and Sa50 Fig. 4 (C&F) groups showed medullary lymphoid hyperplasia with a slight increase in the number of lymphocytes that were seen extending to the medulla and distributed throughout the whole section. Sa50 lymph nodes showed parafollicular hyperplasia.

The footpad 24 hours after PHA injection Fig. 5 showed the control footpad with marked cellular diffuse infiltration in the connective tissue with edema in the dermis. In the other hand, the footpad of male rats fed *Nigella sativa* seeds showed an increased in the inflammatory response with an increase of lymphocyte infiltration and edema in the dermis when matched with control group.

Discussion

Nigella sativa seeds are one of the most frequently used plants in traditional medicine. They play a significant role as anti-inflammatory [62], antioxidant [63,64] agents and immunological activator [65]. Some researchers have demonstrated that *Nigella sativa* has influences on the immune system; it could increase the antibody response [66] and ameliorate inflammation [67,68] as well as immunological attacks [69]. Therefore, the current study investigated the cellular mediated immunomodulatory action of *Nigella sativa* seeds on male adult Wister rats with their influence on performance. To the best of our knowledge, current study is the first study presuming experimental evidence that dietary *Nigella sativa* has immunomodulatory properties against 10% PHA in rats. Additionally, no studies have described the immune response to the PHA skin test in rat fed *Nigella sativa* seeds.

Analyses of *Nigella sativa* seeds showed a strong antioxidant free radical DPPH scavenging action which may be attributed to the high levels of phenolic and flavonoid constituents. Similarly, Adetuyi and Ibrahim [70], Hameed et al. [71], Hirose et al. [72] and Zhang et al. [73] found the DPPH function associated with the phenolic and flavanoid components.

Nigella sativa seeds fed groups showed a significant improvement in final body weight and FCR relative to control group. *Nigella sativa* seeds greatly improved growth performance. This growth performance

promotion could be due to the nutritional value of *Nigella sativa* key components which contain high fatty acid percentages and essential amino acids [74-76]. Moreover, *Nigella sativa* contributes an enhancing effect to digestive enzymes [77,78] and gastrointestinal motility [79] thus, improving feed utilization and FCR. The previous results were in harmony with Dollah et al. [80] and Ahmed and El-Sayed [21].

Spleen represent enlarged lymphatic tissue which responsible for clearance of the damaged old particles of the body and foreign particles from the blood [81]. In the present study, dietary *Nigella sativa* seeds were found to increase relative splenic weight at dose 50 g/kg. Moreover, the splenic histomorphometry of both Sa30 and Sa50 groups was significantly higher than control. These results harmonized with Ghonime et al. [45] as *Nigella sativa* was proven to have lympho- regenerating effect in lymphoid organs [82]. This illustrates its role in increasing splenic weight.

According to our results, feeding *Nigella sativa* seeds had resulted in a significant increase of catalase activity and TAC rates compared with the control group. These results proposed that feeding of *Nigella sativa* seeds participated in cellular protection as a source of antioxidant molecules and indirectly as a stimulator to the activity of these enzymes [83,84]. The active ingredients of *Nigella sativa* seeds such as p-hydroxybenzoic acid [85], Chlorogenic acid [86], Catechin [87], sinapic acid [88]. ferulic acid [89], p-coumaric acid [90] and kaempferol [91] were established to have antioxidant influences beside ROS scavenging potential thus, promoting higher antioxidant enzymes level. The abolishing of oxidative stress has close association with promotion of body weight [92]. Moreover, the later antioxidant potency of *Nigella sativa* seeds ingredients could be attributed to their immunostimulant effect [17,93-96]. This was manifested by increased TNF-α and IF-γ toward PHA stimulation as well as increased splenic lymphoproliferation denoted by the increased histomorphometric parameters in *Nigella sativa* treated groups.

Phytohaemagglutinin (PHA) is a mitogen derived plant that provokes leucocytes recruitment in both innate and adaptive immune responses at the place of inoculation resulting in a quantifiable tissue swelling that could quantify such immune response [97-99]. Current results demonstrated significant increase in Intumesce index of Sa50 group. These results were parallel to the observed upgrading of cellular infiltration, edema and lymphocytes infiltration at PHA injected paws in Sa30 and Sa50 groups. The increments of IF-γ, IL-12 and TNF-α in Sa30 and Sa50 groups as cellular immune promoting cytokines were confirmative to *Nigella sativa* immunostimulatory effect.

Interleukin 12 (IL-12) is a substantial immunomodulatory cytokine which is manufactured by macrophages, dendritic cells and antigen presenting cells. The production of this cytokine during infection adjusts innate immune responses and determines the adaptive immune responses sequence to be elicited. Also, IL-12 can evoke the assembly of IF-γ from T helper type 1 (Th1), activated CD8 T cells and natural killer cells that in turn, aggravates macrophages to destroy intracellular organisms [100]. IF-γ prompts the differentiation of CD4+ T cells into Th1 cells that further produces IF-γ again [101]. Moreover, IL-12 provokes TNF-α production that possesses a pivotal role in scenario of immune regulation through monitoring lymphocyte proliferation, survival and apoptosis via paracrine/autocrine signals [102-104]. The

later function of TNF-α is concerned with maintenance of immune homeostasis and self-tolerance [105,106]. The crosstalk between innate and adaptive immune system that is arbitrated by IL-12 and IF-γ, contributes a substantial role in infectious agent control. The present study revealed significant promotion in the levels of IF- γ, IL-12 and TNF-α in Sa30 and Sa50 groups that seemed to be dose dependent. Present data were in harmony with Gholamnezhad et al. [107], Aljabre et al. [108], Gholamnezhad et al. [109] and Titiek et al. [110]. These results explained the cell mediated immunostimulatory effect of *Nigella sativa* where IL-12 enhanced IF-γ that acted in autocrine and paracrine manner to increase CD8 cells activity. Both γ-IF and TNF-α could stimulate macrophages activity to eliminate infectious agent [111,112].

Nigella sativa seeds treated rats showed a significant increase in the CD8 immunoreactivity in splenocytes. Salem et al. [113] had shown similar results where they found that addition of thymoquinone stimulated CD8 and markedly increased IF-γ production. Our findings indicate that the expression of CD8 may be linked to the activation state of the T cell when injected with PHA. CD8 cells are crucial constituent of the cellular immune response where their frequency is increased during conditions where the immune system is activated by infection [114] or autoimmune disease or after transplantation [115]. $CD8^+$ T cells produce cytokines like IF-γ and TNF-*α*. The latter is a proinflammatory cytokine that initiates apoptotic gesturing and inhibits viral replication as well as gene expression [116]. Also, CD8+ T cells could directly attack and induce cytolysis to the infected targets [117,118].

Nigella sativa seeds treated rats showed a significant decrease in the expression of caspase-3 splenic immunoreactivity in a dose dependent pattern. on the same trend, Salem [12] found that spleen of treated with *Nigella sativa* associated with decreasing rates of apoptosis. Numerous lines of evidence had indicated that *Nigella sativa* seeds are able to modulate pro-inflammatory cytokines as multiple cell signaling molecules [27], apoptotic proteins [119] and antioxidants [12]. Reduction of caspase 3 protein expression in *Nigella sativa* seeds administered groups indicated their anti-apoptotic potential that could be attributed to the antioxidant ingredients in them. This was manifested by the elevated TAC and catalase activities in *Nigella sativa* treated groups. Moreover, the reduced splenic caspase 3 denoted active dynamic status in such organ toward PHA injection that was augmented by the increased splenic CD8 expression as well as serum IF-γ, IL-12 and TNF-α. This scenario was reflected on the increased edema and infiltrations of inflammatory cells in the PHA stimulated foot pads with increment in Intumesce Index. Moreover, white pulp, periarterial lymphoid sheath and the germinal center were significantly increased in Sa30 and Sa50 groups as a reflection of active splenic performance with lower apoptosis.

Histological assessment of lymph nodes is crucial to comprehend the immunologic effects of chemicals [120]. PHA stimulates a characteristic pattern of reaction in lymph nodes. The control group injected with PHA has shown lymphoid follicle size reduction with medullary lymphoid hyperplasia, severe degeneration with mild necrotic area and lymphatic sinus ectasia. The appeared histopathological alteration of lymph node is due to its inflammatory response to the PHA injection which trigger an

immunologic response according to O'Dowd et al. [121].The groups Sa30 and Sa50 lymph nodes showed medullary lymphoid hyperplasia and lymphoid follicle size reduction with less notable distortion in architecture seen in the control sections. Hyperplasia occurs in lymph node is an acute immune response to antigens [122]. Therefore, PHA acts as antigen stimulator. Sa50 lymph nodes showed parafollicular hyperplasia where the follicle pushed to the periphery of the node beneath of the capsule that may be a response to the reactive hyperplasia of the lymph node [123]. *Nigella sativa* immunologic response is explicit dose dependent trend where the higher dose showed the least damage and more lymphocyte to the popliteal lymph nodes tissues of the experimental rats.

With consideration to all the previous data, it seems that *Nigella sativa* possesses a favorable cell mediated immune response toward PHA injection through its antioxidant active ingredients that positively influenced weight gain and FCR. The effect of *Nigella sativa* seeds was represented by increased IL-12 that promoted CD8 IF-γ production and TNF-α that could effectively face infectious agent. This active immunomodulatory cell mediated immune response was accompanied with active splenic state of increased CD8 expression and reduced caspase 3 as apoptotic marker. These were manifested by increased lymphoid histomorphometry in spleen with increased chemotaxis and inflammatory reaction at the site of PHA injection. Also, popliteal lymph node of PHA injected leg showed lymphoid hyperplasia.

Conclusion

The existing study established that *Nigella sativa* seeds are useful to be introduced in foods where the positive effect of these seeds is to boost the overall growth output parameters, FCR and immunological response. Later effects are due to their antioxidant constituents that promoted cell mediated cytokines production, splenic CD8 and reduced splenic caspase-3 expression. From this study *Nigella sativa* seeds could be useful as a dietary supplement that has a positive modulatory effect to cell mediated immunoresponse to disease. Thus, *Nigella sativa* dietary supplementation could be beneficial in viral or bacterial infections where cellular mediated immune response plays a pivotal role.

Declarations

Author Contributions: H.S.M., A.A.A., H.N.G.E., T.S.A., H.M.A.A. and H.E.K. conceptualization; H.N.G.E., H.E.K. and H.M.A.A. methodology; H.S.M. and A.A.A. analysis; H.S.M. and H.M.A.A. investigation; A.A.A., H.N.G.E. and H.E.K; writing – original draft; H.S.M., A.A.A., H.N.G.E., T.S.A., H.M.A.A. and H.E.K. writing – review & editing; T.S.A. visualization; H.S.M., H.M.A.A. and T.S.A. Supervision. All authors have read and agreed to the published version of the manuscript.

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Figures

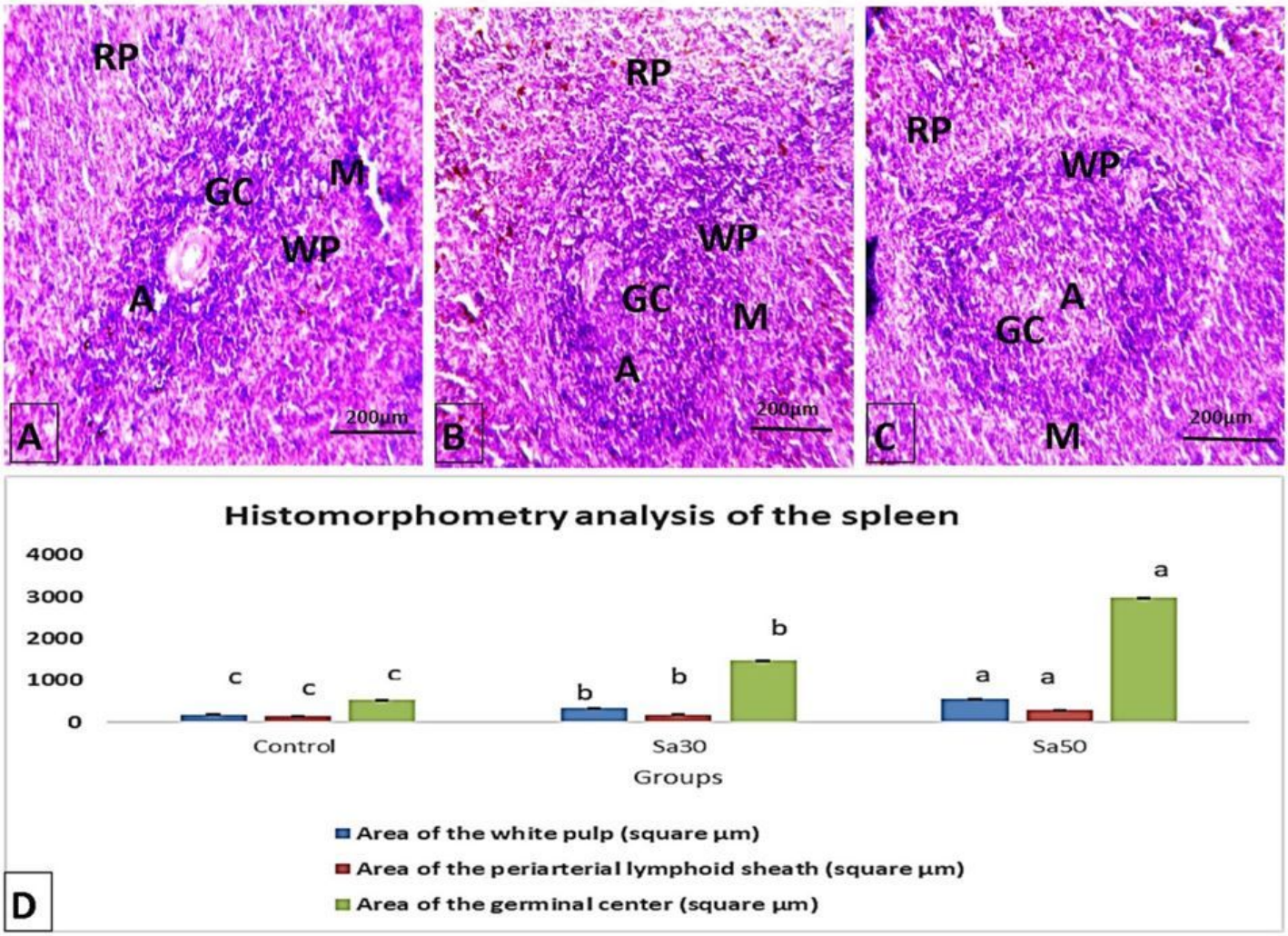

Figure 1

Photomicrograph of (A) Control rat Spleen, (B) Sa30 treated rat spleen, (C) Sa50 treated rat spleen showed remarkable histopathological changes of the spleen architecture with distinct red pulp (RP), white pulp (WP) with central arteriole (A) and germinal center (GC) surrounded with marginal zone (M). (Hx&E stain) X 200. (D) Histomorphometry analysis of the spleen of rats of Nigella sativa seeds fed groups (30 g/kg & 50 g/kg) and control. (n=6, data were represented as mean ± SE). Different superscript letters are significantly different performed by one-way ANOVA, followed by Duncan post hoc analysis $P < 0.05$ (Sa30: Nigella sativa fed group 30 g/kg diet & Sa50: Nigella sativa fed group 50 g/kg diet).

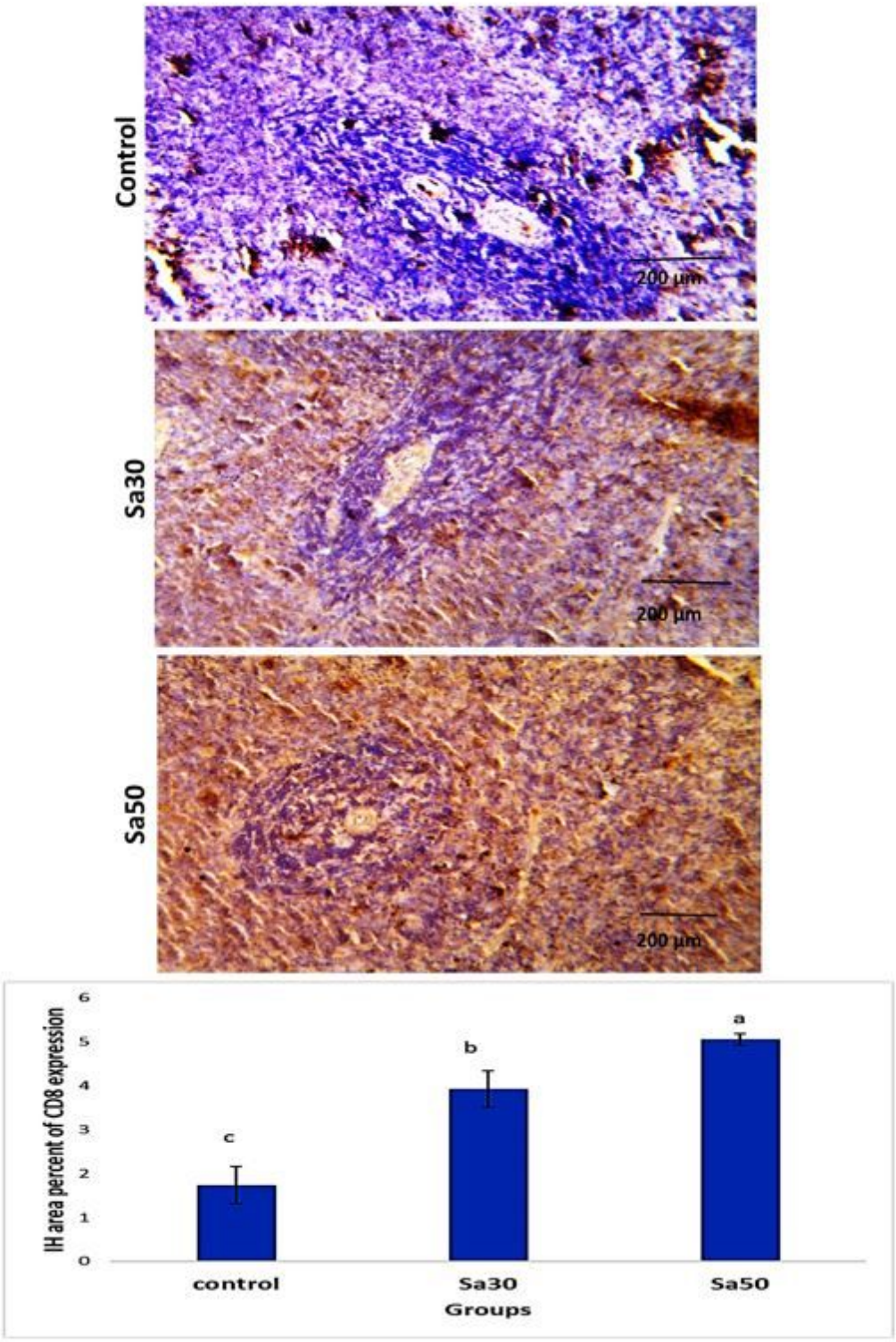

Figure 2

Immunohistochemistry stained sections in the control and treated spleen of rat showing, the area percent of positive splenocytes containing CD8 expression (×200) and a histogram showing a mean percentage of positive CD8 reactivity. Different superscript letters are significantly different performed by one-way ANOVA, followed by Duncan post hoc analysis $P < 0.05$.

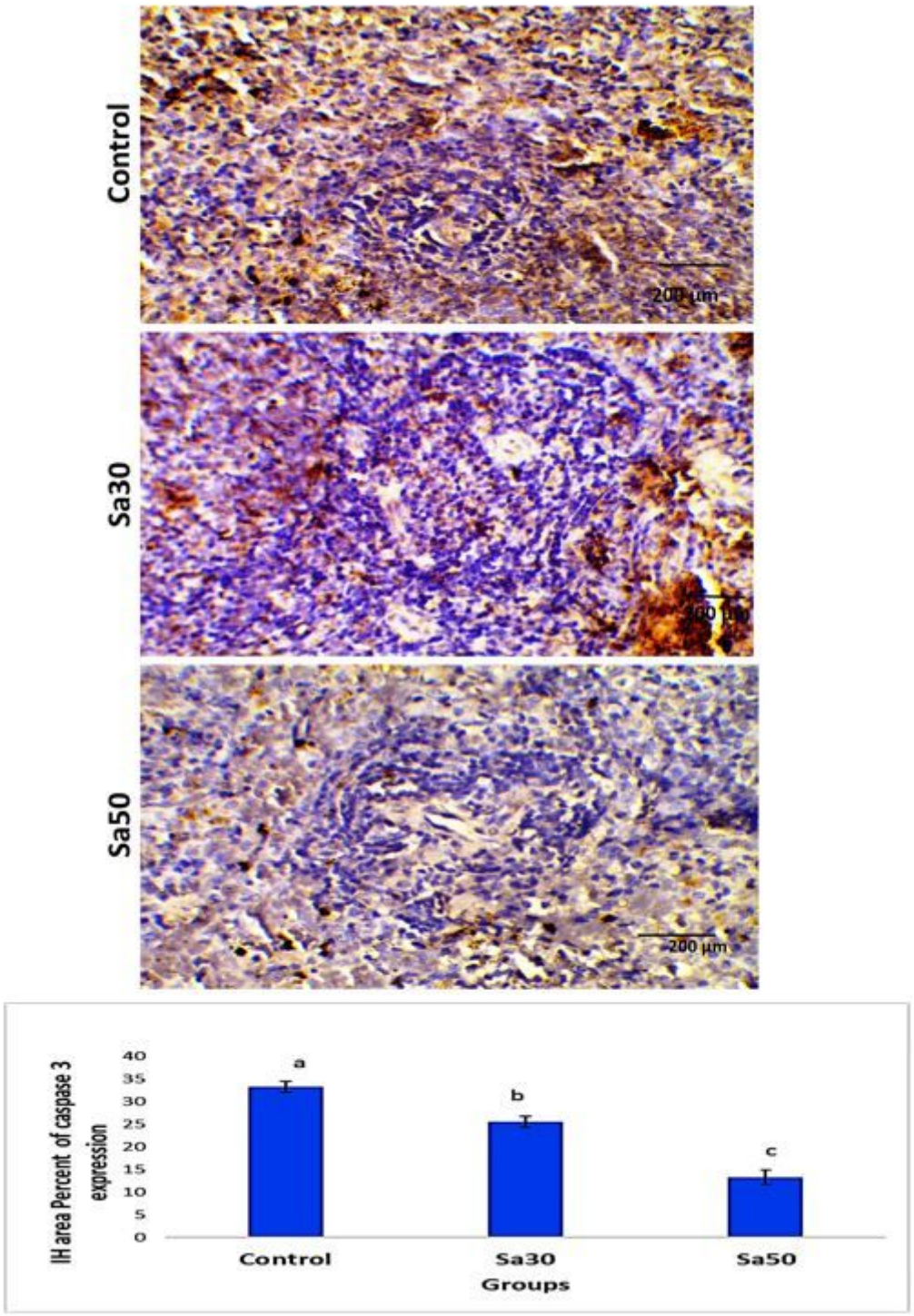

Figure 3

Immunohistochemistry stained sections of control and Nigella sativa seeds fed groups (30 g/kg & 50 g/kg) treated rats' spleen showed, the area percent of positive splenocytes containing Caspase-3 protein expression (×200) and a histogram showing a mean percentage of positive Caspase 3 reactivity. Different superscript letters are significantly different performed by one-way ANOVA, followed by Duncan post hoc analysis $P < 0.05$.

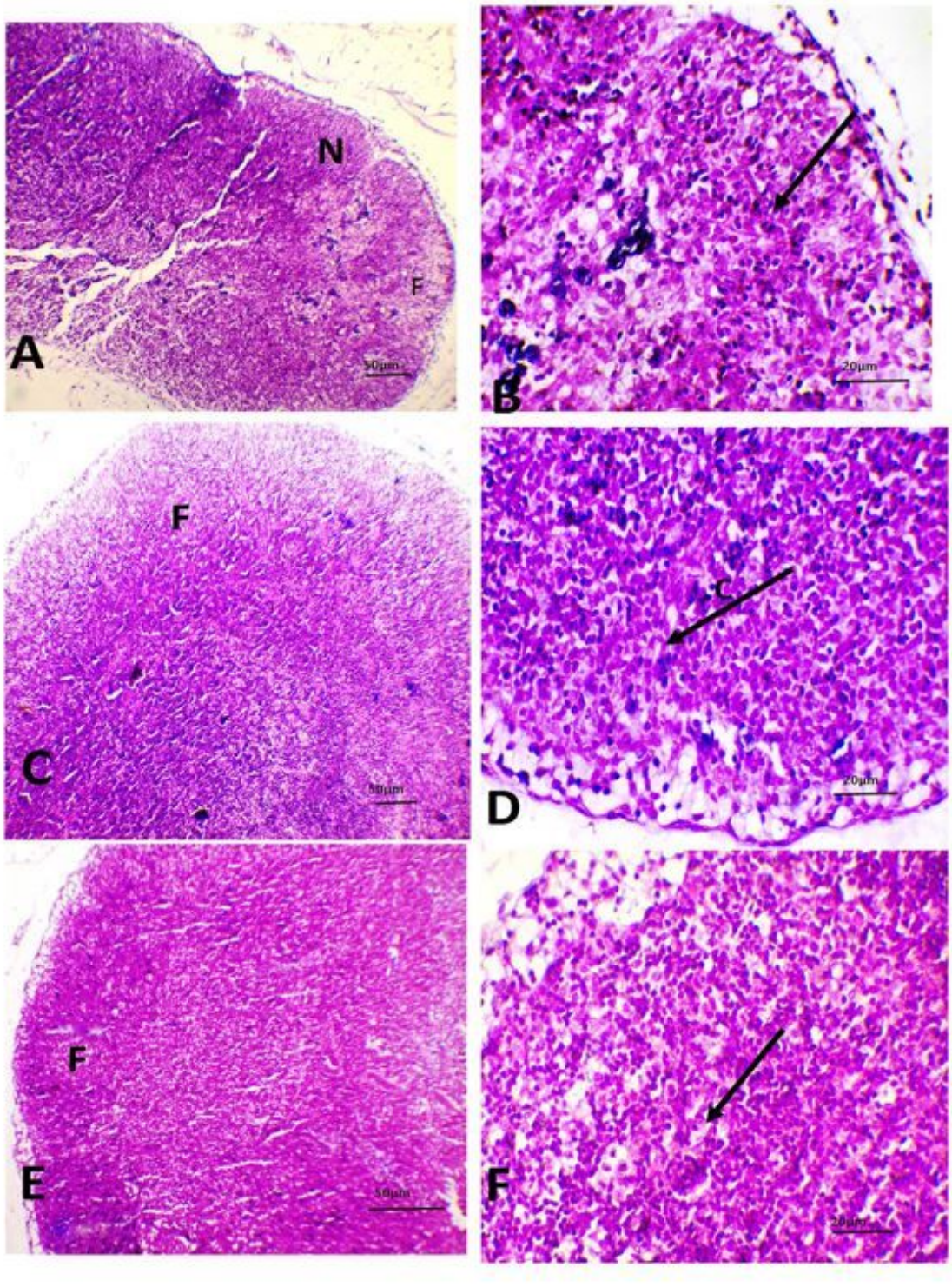

Figure 4

Photomicrograph of (A&B) of control rat popliteal lymph node showed severe degeneration with mild necrotic area (N) and a slight increase in the number of lymphocytes (arrow). (C &D) Sa30 treated rat popliteal lymph node showed lymphoid follicle (F) with its germinal center and a slight increase in the number of lymphocytes (arrow). E&F Sa50 treated rat popliteal lymph node showing a parafollicular hyperplasia (F) and a slight increase in the number of lymphocytes (arrow) (Hx&E stain, X5 & X 10).

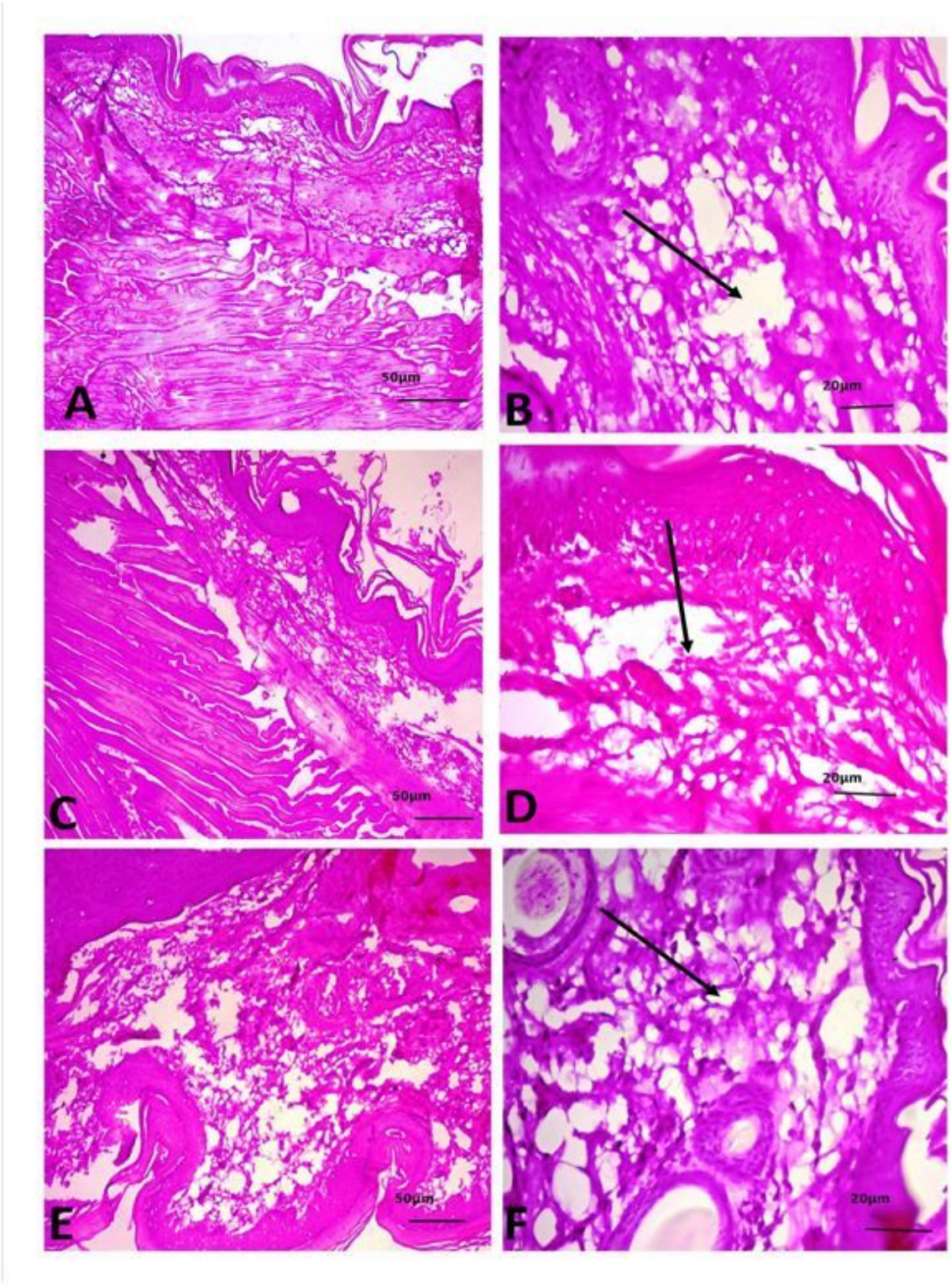

Figure 5

Representative photographs from foot-pad biopsies of PHA model animals showing the effect of Nigella sativa seeds against PHA-induced inflammation in rats. Controls (A&B), Rats fed 30 mg Nigella sativa seeds in the diet for 30 days (B&C), Rats fed 50 mg N. sativa seeds in the diet for 30 days (C&D). →: lymphocytic infiltration and edema in the dermis (Hx&E stain X100, &X 200).

Supplementary Files

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- Supplementaryofblackseed.pdf