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RESEARCH ARTICLE

Nigella sativa Seed Extract Affects Granulocyte Phagocytosis and Lymphocytes Proliferation in Goats

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A B S T R A C T

Herbal medication is becoming very popular in today's world as people seek for natural remedies and avoid synthetic ones. These are widely used as immuno-modulatory agents. *Nigella sativa* is one of them and its extract contains a major component thymoquinone (TQ), which has immuno-modulatory activities. The objectives of current study were to evaluate the immunomodulatory effects of *Nigella sativa* extract in ethanol on the polymorphonuclear (PMN)/leukocyte phagocytosis and lymphocyte proliferation in goats. For this purpose, whole blood was analyzed with Phagotest® by flow cytometry and lymphocyte proliferation using WST-1® test kit. All doses of D4 and D8 dilutions of *Nigella sativa* exerted an inhibitory effect on phagocytosis activity, maximal effect was observed with 10 µl (highest dose). In contrast, all doses of the D6 dilution of *Nigella sativa* enhanced the phagocytosis activity in a dose-dependent manner. The maximum stimulating effect (median: 8%) was observed at the highest dose (l0 µl), which was significantly ($P<0.05$) different from other doses. Various doses of all tested dilutions (D4, D6, D8) of *Nigella sativa* stimulated lymphocyte proliferation. The maximum stimulating effect (median: 8%) was observed by the lowest dose (0.5 µl) of D4 dilution of *Nigella sativa*, which was significantly ($P<0.05$) different from other doses tested. Thus, ethanolic dilutions of *Nigella sativa* seed extract may be considered as an immuno- modulatory agent to cure immune mediated disorders.

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INTRODUCTION

The medicinal use of naturally occurring herbs has a rich historical background and many spices and herbs have been integrated into the traditional medicine of all human cultures. About 65-80% of world's population in developing countries is dependent principally on plants and herbs for their primary health care (Calixto, 2005). Ekor (2013) has reported that more than 80% of world population use herbal remedies annually suggesting that the use of herbal medicine is widespread and rapidly growing. This is because the gentle, synergistic and nourishing actions of herbal remedies make them an excellent choice of treatment. There are many such herbal plants available like *Allium sativum, Corcus sativus, Zataria multiflora, Thymus vulgaris* and *Nigella sativa* (Black seed).

Nigella sativa, also known as black cumin or black seed, is a dicotyledonous flowering plant which belongs to the botanical family *Ranunculacea.* Extensive studies on *Nigella sativa* seed extracts (ethanolic, aqueous, and methanolic) have shown valuable therapeutic effects in various disorders with a wide range of safe doses (Al-Baghir, 2010). TQ is a major phytochemical bioactive ingredient in *Nigella sativa* oil and extracts. Some of the medical benefits of *Nigella sativa* and its major active ingredient TQ are attributed to their anti-hypertensive, anti-histaminic, anti-cancer, anti-inflammatory, hypo-glycemic, and immunity-enhancing effects (Butt *et al.*, 2010; Khan *et al*., 2011; Randhawa and Alghamdi, 2011; Ahmad *et al.*, 2013; Shabana *et al*., 2013; Rahmani *et al*., 2014; Umar *et al*., 2017).

Although scarce experimental findings suggest that *Nigella sativa* can alter humoral and cellular immune responses. But, contemporary literature pointing to the immunomodulatory activity of *Nigella sativa* and its major active ingredient TQ cannot be undermined. The main objective of this study was to elucidate the effects of ethanolic preparations of *Nigella sativa* seed extracts on

phagocytosis of peripheral leucocytes and the proliferation in the mitogen-induced peripheral blood mononuclear cell (PMBC) of goats *in vitro*.

MATERIALS AND METHODS

Sixteen healthy goats, aged 2-10 years, reared in the stalls of Bonn University, Germany, were used in this study. Study was conducted after getting permission from institutional ethics committee.

Preparation: The *Nigella sativa* seeds were purchased from Faisalabad and got duly authenticated by the experts of Botany. All raw extracts and individual decimal dilutions were prepared according to the Homeopathic Arzneibuch (HAB-1) (1985).

Phagocytosis activity: The phagocytosing activity of PMN cells in the heparinized whole blood was determined with Phagotest® using a modified protocol. 100 µl of heparinized whole blood was mixed with 100 µl NaCl containing test doses of the *Nigella sativa* seed extract (10 µl, 5 µl and 1 µl) based in ethanol (43%). The controls received an equal quantity of ethanol (43%) in 100 µl NaCl for each dose of the drug. Phagocytosis was halted by placing all samples on ice simultaneously and by addition of quenching solution (15% trypan blue) for signal's suppression from non-phagocytized, adherent bacteria. Washed twice with washing solution (250g, 8 min, 4°C). Erythrolysis was done by using diethylene glycol/ formaldehyde solution during 20 min incubation at room temperature. After washing the samples, cells were stained by addition of DNA-staining solution containing Propidium Iodide (PI). Each sample was investigated in duplicate by flow cytometry. Calibrite® beads were used for instrument settings adjustment and to set fluorescence compensation.

Data were acquired with CellQuest® software on a Fluorescence-activated cell sorting (FACS) flow cytometer. The event number was set to 15000. The PMN cluster was gated in the scatter diagram (lin FSC vs lin SSC, its green fluorescence and red fluorescence were plotted (log FITC (Fluorescein isothiocyanate) vs log PI (Propidium Iodide). Phagocytosis activity was determined as a percentage of phagocytosing PMN.

Lymphocyte proliferation assay: All procedures involving cell culture were performed in sterilized conditions. The blood was diluted 1:1 with phosphate buffer saline (PBS) and 21 ml of blood/PBS mixture was layered over 12 ml of Histopaque 1077 (Sigma Labs., USA) in 50 ml conical bottom tubes. Following centrifugation (550 x g for 30 minutes), the interface cells were collected and washed twice with serum-free RPMI (Roswell Park Memorial Institute) 1640 medium. The cell pellet was re-suspended in complete medium (RPMI 1640 medium + 20% autologous serum + 100 IU Penicillin/ ml, 100 µg Streptomycin/ml, and 2% Glutamine). Cells were counted on a hemocytometer, and cell suspensions were diluted with RPMI medium to 5 x 10^5 cells/ml. The viability of cells (>95%) was determined by trypan blue dye exclusion.

Lymphocyte cultures were carried out in triplicate in 96 well flat-bottomed microtiter plates (Nunc, Denmark). All experimental wells received 5 x 10^4 cells (100 µl) and 100 µl of test substance solution containing 90 µl RPMI medium and respective volume of drug dissolved in 0.9% NaCl. All control wells received 90 µl RPMI medium and 10 µl NaCl. Total volume in all wells was 200 µl. Parallel a reference test was carried out with 2.5µg PHA along each culture. All cultures were carried out simultaneously using the same cell suspensions and the cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2 for 72 hours.

Following incubation, 20 µl WST-1 solution (Boehringer Mannheim, Germany) was added to each well and plates were incubated for an additional 4 hours. Measurements were done with the help of a multi-well spectrophotometer using a test filter of 450 nm and reference filter of 690 nm. Data were presented in Stimulation Index (SI) (%), calculated by using following formula:

$$SI = \frac{\text{(Mean CPM of mitogen stimulated wells)}}{\text{(Mean CPM of media stimulated wells)}} \times 100$$

Statistical analysis: The results are presented in the median and 25 and 75 percentile. Wilcoxon t-test was applied to compare the values for paired differences. All computations were carried out with SPSS 9.0 (SPSS Inc., Chicago, IL, USA) at 5% level of significance.

RESULTS

The results revealed that D4 and D8 dilutions of *Nigella sativa* seed extract exerted an inhibitory effect on PMN phagocytosis. This inhibitory effect was more pronounced for D4 dilutions than D8. Maximal effect was observed with 10 µl (highest dose) (Fig. 1). In contrast, all doses of D6 dilution of *Nigella sativa* seed extract in ethanol enhanced the leucocyte phagocytosis in a dose-dependent manner. The maximum stimulating effect (median: 8%) was observed at the highest dose (l0 µl), which was significantly ($P<0.05$) different from other doses.

Various doses (10, 2, 1 and 0.5 µl) of all dilutions (D4, D6, D8) of *Nigella sativa* under trial stimulated lymphocyte proliferation with different patterns. (Fig. 2). The maximum stimulating effect (median: 8%) was observed at the lowest dose (0.5 µl) of D4 dilution of *Nigella sativa*, which was found significantly ($P<0.05$) different from other doses tested. Stimulation index for D4 dilution increased with decreasing dose in a consistent pattern, being lowest with highest dose (10 µl). For D6 dilution, stimulation index remained same for different tested doses, showing minimum variation. For D8 dilution, stimulation index was lowest with highest dose (10 µl) and highest with 2 µl dose.

DISCUSSION

To our knowledge, this is the first study to investigate the effects of ethanol based seed extract of *Nigella sativa* on PMN cells and lymphocytes in goats. This in-vitro study was undertaken to demonstrate the effects of *Nigella sativa* seed extract in ethanol prepared according to the homeopathic principles, on the cultures of

peripheral blood mononuclear cells (PBMC) of goats, to determine whether the extract has immunomodulatory properties. All tested doses of D4 and D8 dilutions of *Nigella sativa* exerted an inhibitory effect on Leucocyte Phagocytosis, maximal effect was observed with 10 µl (highest dose). In contrast, all doses of the D6 dilution of *Nigella sativa* enhanced the leucocyte phagocytosis in a dose-dependent manner. No literature could be found regarding effect of *Nigella sativa* preparations in ethanol. However, literature does mention effect of *Nigella* sativa on leucocytes, which has been discussed. Al-Saaidi *et al*. (2012) reported that total leucocytes count and percentages of lymphocytes, monocytes and eosinophils were significantly decreased in rabbits administered with *Nigella sativa* seed extract @ 1.5 g/kg. Similar findings stating that both activity and capacity of intraperitoneal macrophages decrease by increasing dosages of *Nigella sativa* oil in laboratory rats have also been reported by Sriningsih (2008). Haq *et al*. (1995) observed *Nigella sativa*'s suppressive effect on chemiluminescence of phagocytic cells but no effect of *Nigella sativa* on killing rate, phagocytic rate or phagocytosis without *Nigella sativa* or with different concentrations of *Nigella sativa* fraction.

One of the active components of *Nigella sativa* is TQ. The immunotherapeutic efficacy of TQ is attributed to its anti-histaminic, antitoxic, and anti-inflammatory properties (Salem, 2005; Forouzanfar *et al*. 2012; Amin and Fayyad, 2015). Stimulation of PMN Leucocytes with TQ showed protective action against superoxide anion radical generated biochemically, photochemically or derived from calcium ionophore, suggesting that it has a potent superoxide radical scavenger role (Ramadan and Moersel, 2002). Besides, TQ has been reported to induce apoptosis in adult T-cell leukemia/lymphoma by decreasing glutathione and increasing reactive oxygen species (ROS) and levels of ROS underlie the differential cellular response to TQ (Manasse, 2013). Furthermore, TQ exhibits anti-tumor effect against lungs, breast, prostrate, colon, liver and pancreatic cancer (Akram and Afzal, 2016).

The lymphocyte proliferation was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). The maximum stimulating effect (median: 8%) was observed at the highest dose (l0 µl), which was significantly ($P<0.05$) different from other doses. Various doses of all tested dilutions (D4, D6, D8) of *Nigella sativa* stimulated lymphocyte proliferation with decreasing doses. Using mouse splenocytes to evaluate the immuno-modulating activity of *Nigella sativa* seeds extract in ethanol, Swamy and Tan (2000) demonstrated that the extract itself does not have any immunomodulatory activity, but presence of optimal doses of mitogen gives significant potentiation of the immune response. However, Haq *et al*. (1995) reported that *Nigella sativa* at lower concentrations (0.5 µg/ml) stimulate the lymphocyte response to allogenic cells. Of considerable interest is that the low molecular weight fraction (<10 kDa) of *Nigella sativa* enhanced the proliferative activity of lymphocytes, however, killing rate of bacteria and phagocytosis was independent of higher (50 µg/ml) or lower (0.5 µg/ml) concentration of *Nigella sativa.*

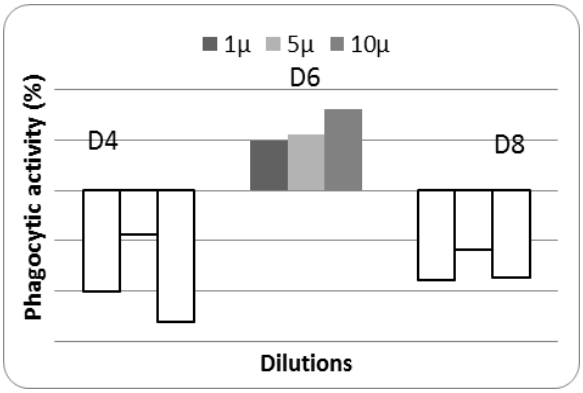

Fig. 1: Effect of different doses (1-10 µl) of various ethanolic dilutions (D4, D6, D8) of *Nigella sativa* extract on PMN phagocytosis activity in goats. Results are expressed as relative index.

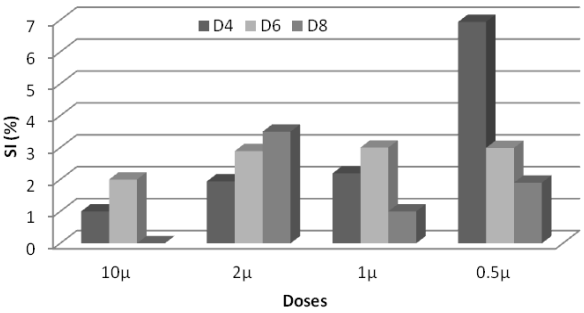

Fig. 2: Relative change in the caprine lymphocyte proliferation after addition of various doses (0.5-10 µl) of various ethanolic dilutions (D4, D6, D8) of *Nigella sativa* seed extract.

El-Kadi *et al*. (1989) have further reported that treatment of human volunteers with whole seeds powder of *Nigella sativa* at doses of 1 g twice daily (orally) for 4 weeks enhanced the ratio of T-lymphocytes helper cells to T-suppressor cells by 72%. It also enhanced the number and function of T-killer cells. Previous studies reported that *Nigella sativa* increased both IL-1B and IL-3 (Haq, 1995; Ali and Blunden, 2003) and suggested that *Nigella sativa* had a prominent effect on a subset of CD4 positive T-cells (T- lymphocytes). The enhanced IL-1B production indicates a stimulatory effect on macrophages. *Nigella sativa* oil is reported to exhibit immune-potentiating, immune-modulating and interferon-like activities and increase interferon gamma, macrophages and CD4+ T cells (Mady *et al*., 2013).

Conclusions: It is conceivable from these findings that ethanolic dilutions of *Nigella sativa* extract may be considered as a strong candidate for immunotherapeutic applications which require an increase in PMN phagocytic activity or enhanced lymphocyte proliferation. These conditions include cancer, AIDS, autoimmune and parasitic diseases as well as in other disorders associated with immune deficiency states. However, further studies are needed to investigate mechanisms underlying these immuno-modulatory properties of ethanol bases *Nigella sativa* seed extract.

Authors contribution: ASQ and HE conceived the idea and supervised the project, ASQ designed the project, conducted laboratory work, and HE, ASQ and SR prepared the manuscript.

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