**In vitro Enhancement of Polyclonal IgM Production by Ethanolic Extract of Nigella sativa L. Seeds in whole Spleen Cells of Female BALB/c Mice**

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**Abstract**

*Nigella sativa* L. has long been used in the traditional medicine for treatment of many diseases including infections, immunomodulations and cancers. In this study, the immunostimulating activity of ethanolic extract of *N. sativa* was investigated in light of the production of immunoglobulin M (IgM) and the proliferation of murine splenic cells *in vitro*. The production of IgM in cultural supernatants was determined by an Enzyme-linked Immunosorbent Assay (ELISA) and the proliferation of mice spleen cells was measured by 3-(4,5-dimethylthiazol-2-y)-2,5-diphenylterazolium bromide (MTT) assay. Our investigations resulted that 70% ethanolic extract of *N. sativa* at a dose of 0.1 mg/ml significantly augmented the production of polyclonal IgM compared to control. The extract could not significantly increase the proliferation of splenocytes. Thus, our results demonstrate that ethanolic extract of *N. sativa* exhibited immunostimulating activities mainly by the differentiation of B cells to plasma cells rather than proliferation; also suggest that *N. sativa* prevents infection through the enhancement of humoral immunity along with other mechanisms.

**Keywords:** *Nigella sativa*, IgM, proliferation, differentiation, immunostimulant, splenocytes, BALB/c mice.

**Introduction**

*Nigella sativa* L. (black cumin) is one of the most revered medicinal seeds in history. The Prophet Hazrat Mohammad (P.B.U.H.) is quoted as saying that the black seed can heal every disease except death. The popularity of the plant is highly enhanced by the ideological belief in the herb as a cure for multiple diseases. Therefore, scientists all over the world have great interest on this holy seed, and black cumin seed has been extensively studied, particularly for the justification of its broad traditional therapeutic value. Traditionally, black seed is used for treating skin conditions (such as, psoriasis, eczema, pimples, burns, skin infections, moisturizers, etc.), as joint pain reliever, diuretic, diaphoretic, stomachic, liver tonic and digestive, dyspepsia, diarrhea, aromatic and carminative (Ramadan, 2007; Atta-ur-Rahman et al., 1992; Al-Gaby, 1998). The seeds are used to cure obstinate hiccups, vomiting, dropsy, obesity, intermittent fevers, hepatic disorders, chronic headache and migraine, as anthelmintic and to prevent antibacterial and other parasitic infections (Nadkarni, 1976; Kapoor, 1990; Evans, 1996; Al-Gaby, 1998; Ramadan and Moersel, 2002a). Scientific research reported that *Nigella sativa* L. and its constituents have anticancer (Islam et al., 2004; Salomi et al., 1992; Rooney and Ryan, 2005; Worthen et al., 1998; Banerjee et al., 2009) antidiabetic, antiradical, analgesic, anti-inflammatory, spasmolytic, brochodilator, hepatoprotective, renal protective, antihypertensive, antioxidant, antimicrobial, and immunomodulatory properties (Ramadan, 2007). Previous studies reported that *Nigella sativa* and its ingredients enhanced cell mediated immunity but suppressed humoral immunity (Islam et al., 2004; Swamy and Tan, 2000; Salem, 2005). Thus, the present study was designed to evaluate the activity of...

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crude ethanolic extract of N. sativa on the humoral immune response and found that the ethanolic extract potentially augmented the production of polyclonal IgM in vitro. The proliferation of murine splenocytes was also considered in this study.

Materials and Methods

Collection of sample and preparation of extract: N. sativa seeds were purchased from commercial shop situated at Dhaka, Bangladesh. The botanical identification was authenticated in the Department of Botany, Jahangirnagar University, Savar, Dhaka, Bangladesh and where a voucher specimen was preserved. The collected seeds were crushed to powder. The powder was exhaustively extracted with 70% ethanol at room temperature with occasional shaking for 7 days. The extract was decanted and the solvent was removed by evaporation followed by freeze-drying under reduced pressure at lower temperature to obtain the crude extract.

Chemicals and reagents: RPMI-1640 and Eagle’s minimum essential medium (MEM) were purchased from ICN Biomedicals (Irvine, CA, USA) and Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Lipopolysaccharide (LPS) from Escherichia coli 055:B5, bovine serum albumin (BSA) (Fraction V), Tween 20, and fetal calf serum (FCS) were purchased from Sigma-Aldrich (Japan).

Purified mouse IgM, goat anti-mouse IgM antibody (Ab), and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM Ab were obtained from Zymed Laboratories Inc (San Francisco, CA, USA), Organon Teknika Corporation (Durham, NC, USA), and Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA), respectively. MTT [3-(4,5-dimethylthiazol-2-y)-2,5-diphenylterazolium bromide] reagent was purchased from Sigma Chemical Co. (Japan).

Mice: Female BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained under specific pathogen-free conditions in the animal facility of Okayama University and were approved by the Animal Research Control Committee of Okayama University, Japan.

Preparation of murine spleen cells: Spleen cells from BALB/c female mice, depleted of erythrocytes, were prepared by lysis of erythrocytes with ammonium chloride as described previously (Aoyama et al., 2005; Goto et al., 2010).

Mice were killed and the spleens were collected aseptically. The spleens were mashed with spatula through the strainer to pass the cells into MEM (pH adjusted to around 7.0 with 1 N NaOH) in a petridish (Iwaki, Japan). Cells were suspended by pasteur pipette and screened by passing through a mesh into the centrifuge tube. The cell suspensions were then centrifuged for 5 min at 4°C and 2000 rpm. The supernatants were removed and ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA; pH 7.2) was added to the cells into the centrifuge tube for lysing the erythrocytes for 5 min at room temperature. MEM was added to the centrifuge tube, suspended and centrifuged for 5 min at 4°C and 2000 rpm. The supernatants were removed and the cell pellets were washed twice with MEM. The cells were re-suspended in MEM and passed through a mesh into another centrifuge tube to collect the spleen cells. The viability of the prepared splenocytes was determined by the Trypan-blue exclusion technique and cells having viability higher than 70% were used for the experiments.

Cell culture: Freshly prepared spleen cells were suspended in basal culture medium (RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml of penicillin G and 100 µg/ml of streptomycin). The cells (2.5 × 10^5 cells/100 µl/well) were plated in 96-well U-bottom plates (Nunc, Roskilde, Denmark) and incubated for 30 min at 37°C in a fully humidified atmosphere containing 5% CO2. 50 µl of 2-mercaptoethanol (2-ME) (0.2 mM), diluted with the basal culture medium, was added into each well and plates were incubated for 5 days with or without the addition of N. sativa extract and LPS, diluted with the basal medium, at 37°C in the CO2 incubator. The cultural supernatants were then collected and frozen at −30°C for IgM-ELISA (Enzyme-linked Immunosorbent Assays) and the cells pellets were used for proliferation study.

Cell proliferation study by MTT method: The growth of cultured cells was determined by MTT method as
described by Hansen et al., (1989). Briefly, at the end of incubation of cells for 120 hrs, 160 µl supernatants were removed. 60 µl of fresh medium and 25 µl of MTT solution were added in each well and the plate was incubated for 2 hrs. After addition of 100µl stock solution in each well, the plate was incubated overnight in dark at 37ºC and the absorbance was measured at 570 nm by using a plate reader.

**ELISA for the determination of IgM production:** The IgM production levels were measured by a sandwich ELISA as described previously (Goto et al., 2010). Briefly, each well of 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 50 µl/well of goat anti-mouse IgM antibody (10 µg/ml), diluted with phosphate buffer saline (PBS), and incubated the plates overnight in the dark at 4 ºC. The plates were washed three times by PBS containing 0.05% Tween 20 (wash-buffer) (200 µl/well). The wells were blocked with 200 µl of 1% bovine serum albumin (BSA) in PBS for 2 hours at room temperature. After washing the plates 100 µl/well of cultural supernatants (diluted with 1% BSA-PBS-Tween 20, accordingly) or standard mouse-IgM were added into each well, and the plates were incubated for 2 hours at room temperature. The plates were again washed three times by wash buffer (200 µl/well). Fifty µl per well of horseradish peroxidase-conjugated goat anti-mouse IgM antibody (0.2 µg/ml) was added into each well and the plates were incubated for 1 h at room temperature. After washing the plates 100 µl/well of 0.1 M citrate buffer (pH 4.0) containing 2.5 mM 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.17% H2O2 were added. The plates were incubated for 10 min at room temperature and the optical densities at 405 nm were measured using an automatic plate reader (Bio-Rad Laboratories, USA).

**Statistical analysis:** The experimental results are expressed as means ± S.E.M. of three independent experiments. The differences between the control and treated groups were analyzed by one-way analysis of variance (ANOVA), followed by Dunnett’s T3 test. P values less than 5% were regarded as significant.

**Results and Discussion**

**Enhancement of polyclonal IgM production by the extract of N. sativa L.:** BALB/c female mice spleen cells were sub-cultured with or without *N. sativa* extract for 5 days and the amount of IgM production in the cultural supernatants were determined by an IgM-ELISA as mentioned previously. LPS was used as a positive control. As shown in the Figure 1, the extract at a dose of 0.1 mg/ml significantly enhanced the production of polyclonal IgM in cultural supernatants of murine splenocytes as compared to control. The extract at a dose of 0.01 mg/ml insignificantly augmented the IgM level but the higher doses (1 mg/ml & 2 mg/ml) potentially suppressed IgM production.

![Figure 1](image_url)  
**Figure 1** Evaluation of ethanolic extract of *Nigella sativa* (L.) extract for the production of polyclonal IgM in cultural supernatants of murine splenocytes. BALB/c mice whole spleen cells (2.5×10⁵ cells/well) were incubated with the indicated concentrations of *Nigella sativa* extract at 37ºC in the 5% CO₂ incubator for 5 days. The IgM levels in the supernatants were determined by an IgM-ELISA. The data are means ± S.E.M. of three independent experiments. *P<0.05, as compared with the control (Dunnett’s T3 test).

**Effect of N. sativa extract on the proliferation of murine spleen cells:** BALB/c female mice whole spleen cells were sub-cultured with or without the indicated doses of *N. sativa* extract for 5 days and the proliferation of cells were measured by MTT assay as described in the materials and methods section. Our investigation resulted that the extract could not significantly induce the proliferation of splenocytes at any dose starting from 0.01 mg/ml to 2 mg/ml as compared to control (Figure 2). Likewise the IgM production, the extract at the doses of 1 mg/ml and 2 mg/ml remarkably suppressed cells proliferations.

Our data presented here indicate that the crude ethanolic extract of *N. sativa* L. potentially increased (3-
fold higher than control) the production of polyclonal IgM in murine spleen cells in vitro (Figure 1). The enhanced antibody production is an indication of the differentiation of B cells to antibody secreting plasma cells (Yoshihara et al., 2007; Goto et al., 2010; Ichiyama et al., 2009). Thus, the result demonstrated that N. sativa extract promoted the differentiation of B cells. Although the production of IgM was increased at lower concentrations (0.01 mg/ml and 0.2 mg/ml) of extract, the higher doses (1 mg/ml and 2 mg/ml) were found to drastically suppress IgM production, suggesting that the extract contains an inhibitory substance(s) which is effective at higher concentrations.

We also observed that N. sativa extract could not significantly promote the proliferation of murine spleen cells (Fig. 2). Previous study reported the enhanced proliferative response of murine splenocytes by N. sativa in the presence of ConA, but not LPS (Swamy and Tan, 2000). Thus, the previous study supports the present finding for activity of N. sativa for the proliferation of splenocytes. However, further research is recommended in this regard.

**Conclusion**

N. sativa extract significantly promoted the production of IgM in vitro, suggesting that N. sativa seeds can be used for the up-regulation of humoral immunity. Thus, its use in traditional medicine for the prevention of infection and enhancement of immunity is justified. In contrast to the previous reports on the effect of N. sativa for humoral immune response, our report suggests that *Nigella* enhances the humoral immunity rather than suppression. Thus we can conclude that immunostimulating activity of N. sativa is not only due to its upregulation of cell-mediated immunity but also due to the promotion of humoral immunity. However, further investigations are recommended.

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