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Induction of Humoral Immunity Through the Enhancement of IgM Production in Murine Splenic Cells by Ethanolic Extract of Seed of *Piper nigrum* L.

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Abstract

The immunostimulating activity of 70% ethanololic extract of *P. nigrum* L. was investigated by measuring the production of immunoglobulin M (IgM) and the proliferation of murine splenic cells *in vitro*. The production of IgM in cultured supernatants was determined by an enzyme-linked immunosorbent assay (ELISA) and proliferation of cells was measured by 3-(4,5-dimethylthiazol-2-y)-2,5-diphenylterazolium bromide (MTT) assay. The extract at the doses of 0.01 and 0.1 mg/ml significantly augmented the production of polyclonal IgM production (0.3206 and 0.5014 µg/ml, respectively) compared to control (0.1465 µg/ml). No dose of the extract could remarkably increase the proliferation of splenocytes. Thus, the present study clearly demonstrates the immunostimulating activity of ethanololic extract of *Piper nigrum;* exhibited mainly by the differentiation of B cells to plasma cells rather than proliferation of splenocytes.

Keywords: *Piper nigrum* L.; IgM; Proliferation; Differentiation; Immunostimulant; Splenocytes.

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1. Introduction

A good number of modern medicines have been obtained from natural sources, especially from natural plants having its relevant traditional medical usage [1]. Immunostimulating agents from medicinal plants are badly important to help the impaired immune systems, as an alternative to conventional chemotherapy, in conditions like receiving chemotherapy for the treatment of cancer, AIDS, burn, or in case of reduced defensive capacity of human body [2]. Although the immunostimulating activities of many herbs have been extensively studied in the recent years, investigations on various spices for immunoenhancing activity are relatively negligible. *Piper nigrum* L. (black pepper) seed,

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one of the oldest and well-known spice (Piperaceae), has long been used in the traditional medicine for the treatment of many diseases, such as, sore throat, skin diseases, arthritis, gastric ailments, cholera, malaria, bacterial infection [3, 4], gonorrhea, tuberculosis, respiratory tract infections [5] and for the enhancement of immunity [6]. Scientific investigations reported that black pepper and its main alkaloid piperin exhibited antioxidant, anti-inflammatory, anticancer activities [7, 8], promoted melanocyte proliferation and dendrite formation [9], antimicrobial [10, 11], anti-apoptotic [5, 12] and immunomodulatory activities [6, 13]. The present study was aimed to evaluate the immune-enhancing ability of crude ethanolic extract of black pepper on humoral immune response and splenocytes proliferation *in vitro* and found that the extract potentially promoted polyclonal IgM production.

2. Materials and Methods

Piper nigrum L. seed was purchased from commercial shop at Dhaka, Bangladesh. The botanical identification was authenticated at the Department of Botany, Jahangirnagar University, Savar, Dhaka, Bangladesh where a voucher specimen was preserved. The collected seeds were crushed to powder and exhaustively extracted with 70% ethanol at room temp with occasional shaking for 7 days. The extract was decanted and the solvent was removed by distillation followed by freeze-drying under reduced pressure at lower temp to obtain the crude extract.

Spleen cells from BALB/c female mice, depleted of erythrocytes, were prepared by lysis of erythrocytes with ammonium chloride as described previously [14]. Freshly prepared splenocytes (viability >70%) were suspended in basal culture medium (Roswell Park Memorial Institute Medium or RPMI 1640 medium, supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 U/ml of penicillin G and 100 µg/ml of streptomycin). The cells (2.5×10^5cells/100 µl/well) were plated in 96-well U-bottom plates (Nunc, Roskilde, Denmark) and incubated at 37ºC for 30 min in a fully humidified atmosphere containing 5% CO_2. Fifty 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 incorporation of *P. nigrum* extract and lipopolysaccharide (LPS). The cultured supernatants were then collected and frozen at -30ºC for IgM-ELISA and the cells pellets were used for proliferation study.

The proliferation of cultured cells was determined by MTT method as described by Hansen *et al.* [15], with some modifications. Briefly, at the end of incubation of cells for 120 hrs, 160 µl supernatants were removed. Sixty 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 stop solution in each well, the plate was incubated overnight in dark at 37ºC and the absorbance was measured at 570 nm by using an automatic plate reader (Bio-Rad Laboratories, USA).

The IgM production levels were measured by a sandwich ELISA as described previously [14]. 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 4ºC. 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 h at room temperature. After washing the plates 100 µl/well of cultured 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 h at room temperature. The plates were again washed three times by wash buffer (200µl/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% H_2O_2 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).

The experimental results were expressed as mean±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.

3. Results and Discussion

As shown in Fig.1, the extract at the doses of 0.01 and 0.1 mg/ml enhanced the production of polyclonal IgM in cultured supernatants of splenocytes by 2.5 and 3.5-folds respectively, compared to control. LPS was used as a positive control. The extract at a dose of 1 mg/ml could not alter IgM level rather the higher dose (2 mg/ml) potentially suppressed IgM production.

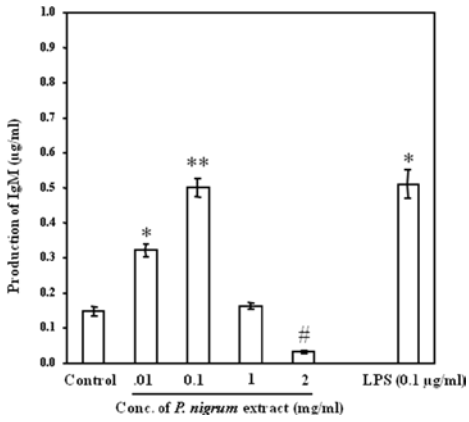

Fig. 1. Evaluation of the activity of ethanolic extract of *Piper nigrum* (L.) extract for the production of polyclonal IgM in cultural supernatants of murine splenocytes. BALB/c mice whole spleen cells

(2.5×10^5 cells/well) were incubated with the indicated concentrations of *P. nigrum* L. at 37ºC in the 5% CO_2 incubator for 5 days. The IgM levels in the cultural supernatants were determined by an IgM-ELISA. The data are means ± S.E.M. of three independent experiments. **P*<0.05, #*P*<0.05, and ***P*<0.01, as compared with the control (Dunnett's T3 test).

The investigation found that the extract could not significantly promote the proliferation of splenocytes at any dose starting from 0.01 to 2 mg/ml compared to control (Fig. 2). Likewise the IgM production, the extract at a dose of 2 mg/ml remarkably suppressed the proliferation of cells.

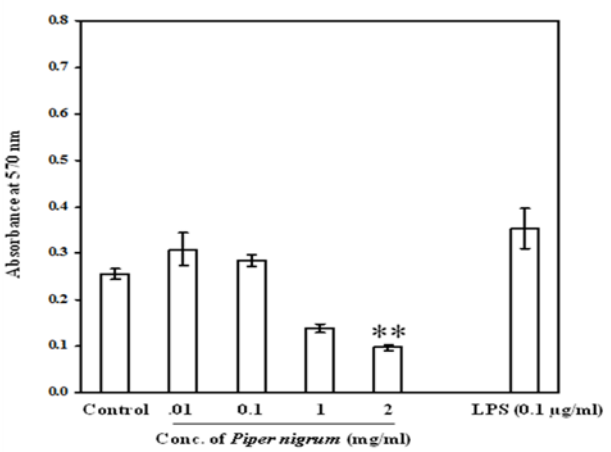

Fig. 2. Effect of ethanolic extract of *Piper nigrum* L. extract on the proliferation of murine spleen cells in culture. BALB/c mice whole spleen cells (2.5×10^5 cells/well) were incubated with the indicated concentrations of *P. nigrum* at 37ºC in the 5% CO_2 incubator for 5 days. The proliferations of cells were measured by MTT method. The data are mean±S.E.M. of three independent experiments. ***P*<0.01, as compared with the control (Dunnett's T3 test).

The above data resulted the enhancement of polyclonal IgM production 3.5-folds higher than that of control by the crude ethanolic extract of *P. nigrum* (L.) in vitro (Fig. 1). IgM is the first antibody produced in humoral immune response to fight against infectious agents or antigens, such as, bacteria, viruses, parasites, etc. [16]. Therefore, the induction of B cells to produce enhanced IgM antibody ultimately results to stronger humoral immunity to defend the body against antigens. The enhanced antibody production is an indication of the differentiation of B cells to antibody secreting plasma cells [14]. Thus, the present result demonstrated that black pepper seed extract promoted the differentiation of B cells to plasma cells. Although the production of IgM was increased at lower concentrations (0.01 and 0.1 mg/ml) of extract, the higher dose used in this experiment (2 mg/ml) was found to drastically suppress IgM production, suggesting that the extract may contain an inhibitory substance(s) which is effective at higher concentrations.

Previous studies reported that *P. nigrum* L. extract and piperine possess antitumor [17], anti-inflammatory [7] and immunomodulating potential [7, 17, 18]. Pathak and Khandelwal [6] reported anti-oxidative, anti-apoptotic and chemo-protective ability of piperine in blastogenesis, cytokine resease and restoration of spleen cells population, and they recommended the therapeutic usefulness of piperine in immuno-compromised situations. Another study reported piperine to protect cells against cisplatin-induced apoptosis [12]. All the above reports clearly indicate the immunomodulatory role of black pepper through cellular immune responses. The immune system has its two arms to defense cell-mediated immune response and humoral immune response. As the activities of black pepper on the cell-mediated immune responses were extensively studied by many scientists, the action of black pepper on humoral immune response was targeted in this study. Only one report is available which resulted the enhancement of circulating antibody titre and antibody forming cells by piperine [13]. This report supports the finding of the present investigation in which black pepper potentially augmented humoral immune response through the production of antibody (IgM) (Fig. 1).

Piperine is the main alkaloid of black pepper [19] and is considered to be its therapeutic active constituent [20]. Therefore, the enhancement of IgM production in the present study was most probably be due to the presence of piperine in the black pepper. Chun *et al.* [21] prepared purified polysaccharides from black pepper and reported that purified anti-complementary polysaccharides from *Piper nigrum* can act as a supplement for immune-enhancement. Therefore, the polysaccharides present in the black pepper may also contribute to the enhancement of humoral responses as well.

The effect of black pepper on the proliferation of spleen cells *in vitro* was also studied and it was found that black pepper extract could not induce the proliferation of cells (Fig. 2). Thus, the overall result suggests that black pepper enhanced humoral immunity through the differentiation of B cells rather than proliferation, and its use in traditional medicine for the prevention of infection and enhancement of immunity is justified. However, further investigations are recommended.

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