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Assessment of Cytotoxic and Immunomodulatory Properties of *Piper nigrum* Linn. (White Pepper) Seed Extract

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ABSTRACT

Macrophages are tissue-based phagocytes that play the central role in initiating defence mechanism of host immunity. Macrophage expresses inducible nitric oxide synthase (iNOS) that inhibit pathogen replication by releasing a variety of effector molecules which includes nitric oxide (NO). In the present study ethanol extract of *Piper nigrum* Linn. (white pepper) seed was investigated for its cytotoxicity and *in vitro* immunomodulatory properties using cell proliferation and NO determination assay with J774a-1, macrophage cell line. The ethanol seed extract was found to exhibit toxicity at higher concentrations of 50µg/ml and 100µg/ml. The seed preparation was observed to enhance both, the proliferation of macrophage cell with higher percentage proliferation of 29.24 at 12.5µg/ml and the production of NO with significant stimulation level of 47.74% at 12.5µg/ml compared to the control. Since the results show the modulator effect of *Piper nigrum* Linn. seed on macrophage cells, it could be considered to possess immunomodulatory potential.

Keywords: White pepper, nitric oxide, immunomodulatory, J774a-1 cells.

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INTRODUCTION

Inflammatory diseases of the skin, gut, respiratory tract, joints and central organs, as well as infectious diseases, are some primarily considered immunological disorders. [1] Stimulation and suppression of immune response are the two ways of immunomodulation. [2] Immune modulation helps to maintain disease free-state. Searching of substances with immunostimulative or immunorestorative effects could contribute to the maintenance of immune system. Alternative System of Medicine like Ayurveda, Siddha, Unani and Traditional

Chinese Medicine has gain its importance in the recent few years for its high potential in curing various disease with lesser side effects as compared with the synthetic drugs. [3] Immunomodulation using medicinal plants can provide an alternative approach to conventional chemotherapy for a variety of diseases. Many plants have been evaluated for immunostimulant and immunosuppressive properties using various techniques. [4]

White pepper is a value-added form of Black pepper (*Piper nigrum*) is widely used in different traditional systems of medicine like Ayurvedic and Unani System of medicines. [5] *P. nigrum* exhibits diverse pharmacological activities as they help in pain relief, rheumatism, chills, flu, colds, muscular aches and fever. Externally it is used for its rubefacient and as a local application for relaxed sore, throat and some skin

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disorder. [6] It has antimicrobial [7], antimutagenic [8], antioxidant, radical scavenging property [9], Immunomodulatory and antitumor activity. [10]

Nitric oxide (NO) is involved in a variety of important physiological processes such as vasodilatation, neurotransmission, and host defense against invading pathogens. [11] Macrophages play major roles in immunity and the inflammatory responses involved in host defense. NO produced by activated macrophages has been shown to mediate host defenses, such as antimicrobial and antitumor activities, but excess production can cause tissue damage associated with acute and chronic inflammation. [12] Therefore, the degree of NO production induced by iNOS may reflect the degree of inflammation and provides an indicator for assessing inflammatory processes.

Though different therapeutic potentials of *P. nigrum* have been published, there are very few reports on the immunomodulatory activity of the plant. Therefore in the present study, effect of *P. nigrum* seed on certain immune parameters closely related to inflammatory processes *i.e.* complement activity and macrophage proliferation was studied. This study can also help in understanding the mechanism of action of this drug and its possible use as an immunomodulator.

MATERIALS AND METHODS

Plant material

Seed of *Piper nigrum* Linn. was procured from an ayurvedha store in Chennai. The Plant seed was authenticated by the Department of Medicinal Botany, National institute of Siddha, Chennai and a voucher specimen [voucher number - NISMB2052015] was submitted.

Preparation of extract

The washed and air dried seed of *P. nigrum* was homogenized in a grinder to powder form. The powdered seed was extracted with ethanol (250 ml, 8h) at temperature 70°C by using soxhlet extractor. The extract was concentrated and dried using rotary evaporator to obtain the crude extract in a viscous semi solid form. The extract was filter sterilized and stored at 4°C until further use. [13]

Cell culture and *in vitro* cytotoxic activity

For cytotoxicity assay [14], HEp-2 cell obtained from the National Centre for Cell Science (NCCS; Job no.-989), Pune, India was cultivated in minimum essential medium provided with 5% FBS and L-glutamine (Himedia Labs, Mumbai). After cultivation, 1×10^4 cells were dispensed into each well of 96 well micro plate containing 150 ml fresh medium for 24 h before treatment with the extract to allow attachment of cells to the wall of the plate. The extract at different concentrations (3.12, 6.25, 12.5, 25, 50 and 100 µg/ml) was added to the cell monolayer in triplicate and incubated for 48 h at 37°C in a 5% CO₂ atmosphere. A total of 21 wells out of 96 wells were used for the toxicity analysis. After incubation, each cells were added with 10µl/well of 3-(4, 5-dimethylthiazol-2-yl)-2,

5-diphenyltetrazolium bromide [MTT (5 mg/ml)] (Himedia Labs, Mumbai) into each wells and cell viability was studied after 4 h of incubation at 37°C. The reaction was stopped by adding 100µl DMSO (Dimethyl sulfoxide) and the color intensity was measured at 540 nm with a Microplate reader (Biotek, USA). [15]

Cell proliferation assay

Macrophage culture and *in vitro* treatment

J774a-1, a macrophage cell line obtained from NCCS (Job no.-2121) were cultured in 25 cm³ flasks in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100µg/ml streptomycin, in a humidified incubator, at 37°C and 5% CO₂ atmosphere. Cells were added (1×10^6 cells/ml) to 96-well microtiter plates (100µl/well). Increasing amounts of seed extracts (3.12, 6.25, 12.5, 25, 50 and 100µg/ml) were added simultaneously to the culture medium. 21 microplate wells out of 96 were used for the assay, of which 18 were sample treated and 3 were left untreated for comparing and evaluating. After 48 h incubation, the cells were used to assess the proliferation and their supernatants were harvested for NO determination.

Determination of NO production

The production of NO was estimated from the accumulation of NO₃ and NO₂ in J774a-1 supernatants using the Griess reagent. [16] Briefly, equal volumes (100µl) of nitrite samples for standard curve and Griess reagent [1% sulphanilamide plus 0.1% a-naphtylamine (v/v) in 2.5% phosphoric acid (Himedia Labs, Mumbai)] were mixed and incubated at room temperature for 10 min. The absorbance values were measured at 540 nm using a microplate reader and the results were expressed in percentage of the NO production in comparison with control which is the supernatant from wells with untreated wells.

MTT assay for cellular viability and proliferation

After collecting supernatants for NO measurement, Cell proliferation of seed extract treated J774a-1 cell was evaluated by the MTT assay. [15] Briefly, cells were incubated in 96-well plates for 4 h with 10µl of 5 mg/ml MTT in 90µl of 5% DMEM. The insoluble purple particles were solubilized in DMSO and the absorbance was measured at 540 nm. The OD values obtained from cells cultured with seed extract were considered to be experimental conditions, whereas samples cultured with 5% DMEM only were considered as controls. The cell proliferation results were also expressed in percentage of proliferation in comparison with blank control.

Statistical analyses

The results were expressed as the mean ± SD. The significant difference was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Cytotoxicity

Cytotoxicity screening models provide important preliminary data of the plant extracts with potential immunomodulatory properties for further work. The cytotoxicity evaluation of *P. nigrum* ethanol seed extract at various concentrations (3.12, 6.25, 12.5, 25, 50 and 100 µg/ml) was shown in Table 1. It was observed that seed extract inhibited cell viability and growth of HEp-2 cells with the increase of concentration. 100 µg/ml and 50 µg/ml of extract showed more significant effect against HEp-2 cell lines, whereas 12.5, 6.25 and 3.12 µg/ml showed less effect on cells.

Table 1: Cytotoxicity effects of ethanol *P. nigrum* seed extract on the HEp-2 cells.

S. No.	Concentration	% Inhibition
1.	100	31.54 ± 1.365
2.	50	28.84 ± 1.076
3.	25	24.52 ± 0.596
4.	12.5	20.00 ± 1.746
5.	6.25	12.63 ± 2.813
6.	3.12	03.10 ± 0.525

The data plotted represent Mean ± SD of triplicate experiments (n-3).

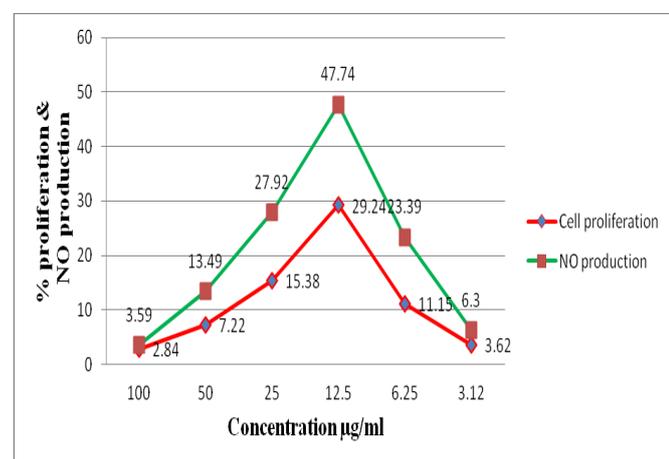


Fig. 1: Effect of ethanol extract of *P. nigrum* on macrophage cell proliferation and NO production.

Proliferation

The immunomodulatory effect in the cell proliferation model has been a target of study in the search for new therapeutic agents of natural origin. [17-18] The activation of the immune response in this model promotes cell proliferation with an increase in the number of cells present in the culture within a defined period [19] and can be identified through MTT reduction by mitochondrial dehydrogenase of living cells. [15]

The ethanol extract of *P. nigrum* assessed in this study induced an increase in cell proliferation depending on the varying dose with an average percentage of cell proliferation ranging from 2.84% to 29.24% (Fig. 1). The extract at 25 µg/ml and 12.5 µg/ml, under both cell culture conditions showed significantly higher proliferation values (15.38% and 29.24% respectively) than the control. Prior studies also reported the use of proliferation response of immune cells to compounds of natural origin as a parameter to analyze immunomodulatory potential. Various doses of aqueous extract of cardamom [20] resulted with an

increase in Splenocytes proliferation from lower to higher concentrations. The immunomodulatory response of *Piper nigrum* (Black pepper) and *Elettaria cardamom* methanol extract was assessed using macrophage proliferation assay. The extracts resulted with increase in proliferation from higher to lower concentrations. [21] Whereas in the present study, cell proliferation rate increased with decrease in the extract concentration and observed to reduce with rise in the concentration. The observed form of cell response to various doses in our study might be due to the presence of combination of various components at different proportion in the crude extract. The reduction in proliferation rate at higher concentration could be related to the toxic nature of the extract expressed at higher concentration, estimated via cytotoxic assay.

NO production

Macrophages play important roles in inflammation through the production of several pro-inflammatory molecules, including NO. NO has been recognized as a pleiotropic biological mediator, regulating diverse activities ranging from neuronal function to immune system regulation. [22] NO production by J774a-1 cells treated with varying concentration of seed extract was estimated (Fig 1.). Among the concentrations screened, the extract exhibited the significant stimulation of nitrite level say 47.74% at the concentration of 12.50 µg/ml followed by 27.92% and 23.39% at the concentration of 25 µg/ml and 6.25 µg/ml respectively. Diverse plant extracts have also been reported to affect NO production by murine macrophages. However, *Centella asiatica* was found to be an inhibitor of iNOS, while in an *in vitro* murine macrophage model *E. purpurea* was found to both increase and decrease iNOS activity as assessed by NO concentrations. [23-24] But in the present study the extract at its various concentrations showed induction of NO production in cells, indicating the presence of proinflammatory molecules. The stimulus in NO production in this report, compliments to macrophage proliferation noted at all concentrations used.

The present findings suggest that crude ethanol extract of *P. nigrum* had a significant impact on the proliferation rate and NO production by macrophage cells. In order to confirm whether the immunomodulatory activity is attributed to the presence of a single compound or compounds, the extract have to be further investigated for the isolation of bioactive compounds and assess their immunomodulation potency.

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