EFFECT OF *PLEUROTUS EOUS* ON NO PRODUCTION IN MURINE MACROPHGE AND SPLENOCYTE PROLIFERATION

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Summary

The mushroom *Pleurotus eous* were extracted with chloroform, ethyl acetate, methanol, water and hot water. All the extracts were screened for their splenocyte proliferation and nitric oxide (NO) production. The proliferation of splenocyte and macrophage were studied by MTT assay and Trypan blue dye exclusion method. The CTC_{50} value of all the extract was found to be in the range of 160 – 665 µgmL-1 against splenocytes and macrophages. All the extracts were enhanced the NO production in murine macrophage induced with LPS and the methanolic extract (100 µgmL-1) enhances 2-3 folds of NO production.

Key words: *Pleurotus eous*, Macrophage, Cytotoxicity, Nitric oxide, Mushroom

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Introduction

Edible mushrooms are nutritionally functional foods and a source of physiologically beneficial and nontoxic medicines. They have been used in folk medicine throughout the world since ancient times. Basidiomycetes mushroom is consumed as a food and for possible medicinal value. Infusion of the dried fruiting bodies has been used as a stimulant and as an auxiliary treatment of various diseases including cancer[1]. Pleurotus species are commonly called Oyster mushrooms. There are about species of this mushroom distributed worldwide in both temperate and tropical areas. Oyster mushrooms now rank as the second most important cultivated mushroom in the world[2].

Production of Nitric oxide (NO) molecule is an important cytotoxic function of macrophage use to resolve infection by several obligate intracellular protozoan and bacterial parasite. The inducible form of NO synthase enzyme (iNOS) produce large amount of reactive nitrogen molecule by oxidizing the terminal guanido nitrogen of L arginine[3]. It can be induced during macrophage activation and is an important messenger in diverse pathophysiological functions, including neuronal transmission, vascular relaxation, immune modulation, and cytotoxicity against tumor cells[4]. The ability of lymphocytes to respond to mitogen by undergoing mitotic proliferation is a distinctive characteristic and this reflects the immune potential of the organism[5].

Basic research on natural substances with immunomodulating properties has been performed by assays carried out by the stimulation of nonspecific immunity of innate responses, such as on the efficiency of granulocytes, macrophages, complement, and natural killer cells. In addition, researchers have investigated whether natural substances affect phagocytosis, lymphocyte proliferation, and T-lymphocyte migration[6]. In the present study the effects of various extract of *P. eous* on splenocyte proliferation and the release of NO in macrophage induced by LPS.

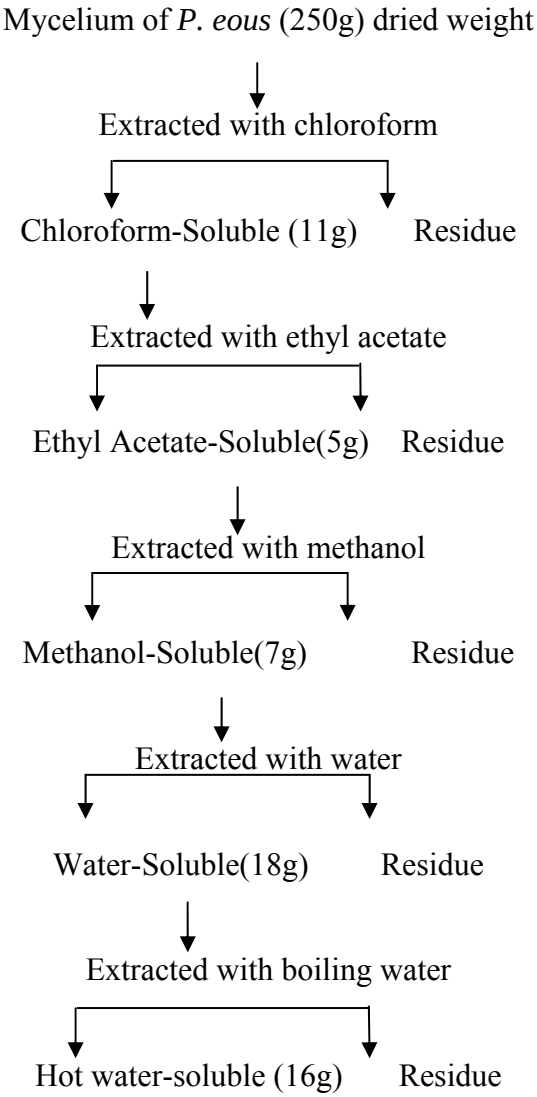
Materials and methods

Collection and Authentication of *P. eous*:

P. eous were collected in Tamil Nadu Agricultural University, in the month of November 2007. The plant was authenticated by Botanical Survey of India, Center for Plant Protection Studies, Tamil Nadu Agricultural University Coimbatore, India.

Extraction of *P. eous*

The fruiting bodies were dried in light shadow at room temperature. Dried mycelium of *P. eous* were extracted sequentially with chloroform, ethyl acetate,methanol, water and boiling water and the fractions were dried in rotary vacuum evaporator under reduced pressure[7], and various fraction obtained are summarized as below:



Isolation of Splenocytes and Macrophages

Male Balb/c mice (8–12 weeks old) were purchased from NIN, Hyderabad. All mice were kept at the animal facilities under specific pathogen-free condition until used. Mice were housed under a natural day–night cycle at a constant room temperature. Twelve animals were anesthetized and subjected to 5 ml ice cold PBS intraperitoneally[8]. Then, the peritoneal cells were extracted immediately and kept at 2-8^{0}C.To avoid non-specific attachment prior to washing for 3 times by centrifugation at 1500 rpm for 5 min using cold RPMI 1640 (Sigma Chemical Co).

Spleen cells of mice were obtained by gently teasing the organ[9]. Spleens were removed, homogenized in a glass homogenizer, suspended in RPMI-1640 and centrifuged at 3000rpm for 20min at room temperature. The cells were re-suspended in 5 ml RPMI-1640 medium and cell counts were done by tryphanblue dye exclusion method.

Splenocyte proliferation assay

Splenocytes were isolated from male inbred BALB/c mice (NIN, Hyderabad, India)[9,10] and were suspended in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine,100 µg streptomycin mL^{-1}, 100 units penicillin mL^{-1}, 50 µM 2-mercaptoethanol, and 1 mM HEPES. Cells were adjusted to 4 x10^5 cells well–1 in 96-well flat bottom tissue culture plates and treated with various concentrations of *P. eous* extracts along with solvent control (DMSO). After being incubated for 48 h at 37^{0}C in a 5% CO_2 incubator, Cell viability was determined using methylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide (MTT) assay. About 20 µl of MTT solution (2 mg ml–1) was added to 180 µl of cell suspension for 4 h. After sucking the supernatant out, the insoluble formazan product was dissolved in 200 µl DMSO[11,12,13,14]. The optical density of the culture wells was then measured using an ELISA reader at 570 nm. Cell viability was determined by trypan blue exclusion. The number of cells and viability did not vary significantly among the experimental conditions.

Assay for NO production

Peritoneal macrophages were isolated and stored in appropriate condition[5]. Macrophages were seeded in to the wells of 96 well tissue culture plates at a density of 1 x 10^5 cells/well and allowed to attach to bottom of the well at 37 ^{0}C 5% CO_2 for 1hr.

Macrophages in different culture plates were treated with various extracts of *P. eous* with LPS (1 µg mL^{-1}) for 24 h. The amount of NO production in the medium was detected with the Griess reaction. Fifty microliter of the cell-free culture media were removed and placed in a new 96-well flat-bottom plate. About 100 µl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% of Naphthylethylenediamine dihydrochloride in distilled water) were added to each well and incubated for 10min at room temperature (light protected). The absorbance was measured at 540 nm using an ELISA reader. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0–100 µM).

Statistical Analysis

Multiple extracts from Mushroom *P. eous* were prepared and analyzed on multiple environments. The results for the splenocyte proliferation assay are presented of three independent experiments performed in triplicate wells. The results for the NO production assay expressed as the mean ± SD. An analysis of variance (ANOVA) was used to determine differences between the control and test wells. When statistically significant differences ($p<0.05$) were found between the groups, unpaired t-test was used to determine the level of significant difference between the control and the test group.

Results and Discussion

Splenocyte proliferation assay

The splenocyte proliferation assay was carried out for the chloroform, ethyl acetate, methanol, water, and boiling water extracts of *P. eous* (mushroom fruiting body). The cell viability was determined by MTT assay and tryphan blue dye exclusions technique and shown in Table 1 Ethyl acetate extract of *P. eous* show lower CTC_{50} value at 160 µg mL^{-1} and the remaining extracts showed CTC_{50} vales in the range from 190-380 µg/ml. The aqueous extract show less toxicity towards the splenocyte as compare to other extracts.

Nitric Oxide Production Assay

The cell viability was determined by MTT assay and tryphan blue dye exclusion method. The CTC_{50} value found to be in the range of 298 – 665 µgmL^{-1} in macrophages also the aqueous extract of *P. eous* show less toxicity when compared to other extracts.

Effect of different concentrations of various extract *P. eous* on NO production in murine macrophages was shown in Figure 1.

Table 1: Cytotoxcity study of *P. eous* extract on mouse splenocyte and macrophage cells.

	CTC_{50} µg/ml	
Pleurotus eous extract	Splenocyte	Macrophage
Chloroform	190±2.30	453±1.93
Ethyl acetate	160±2.00	298±3.23
Methanol	230±1.73	320±1.23
Water	450±2.30	665±2.83
Hot water	380±2.16	580±2.42

Note: Values are expressed as mean ± SEM of three experiments. **P < 0.01, when compared with each groups (One way ANOVA using Tukey t-test

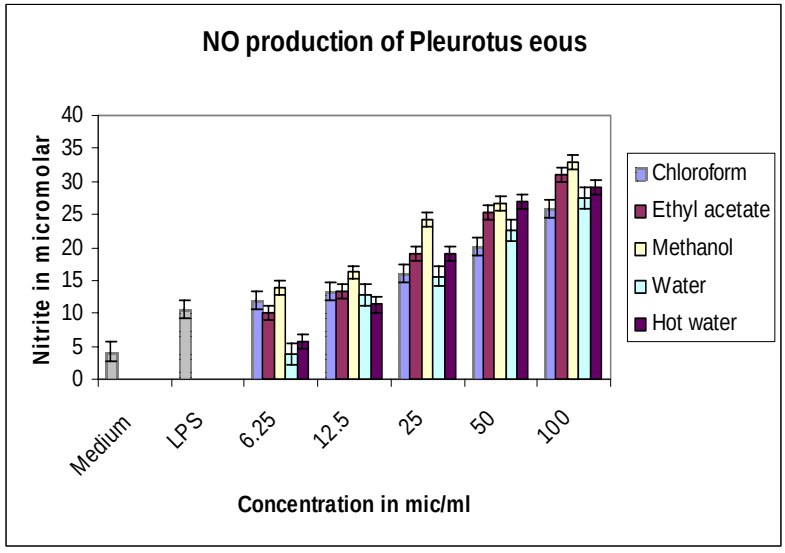


Figure 1: Nitric oxide production of *P.eous* on in mouse macrophage

Based on the cell viability study the various concentrations(less than CTC_{50} value) of extracts were used for NO stimulation in peritoneal macrophage (6.25 μgmL^{-1} to 100 μgmL^{-1}). The amount of NO was measured by Greiss reagent after 24 h of incubation. Data reported as the mean ± S.E. of 3 different observations. P value of H_20 <0.0001 is considered to be significantly different from the STD (*P < 0.0001).

To enhance iNOS expression and NO production was stimulated with LPS and the nitrite level was measured in cell culture supernatants. All the extracts were able to stimulate NO production in macrophage at concentration 6.25 to 100 μgmL^{-1} (Figure 1) as compare to LPS control. The production of NO increased with the increase of concentration. Methanol soluble fraction increases 2-3 fold of NO production at the concentration of 100 μgmL^{-1}.

Mushrooms have long been suggested to possess immunomodulatory properties[16,17]. The ability of lymphocytes to respond to mitogen by undergoing mitotic proliferation is a distinctive characteristic and this reflects the immune potential of the organism[5].The present investigation shows the effects of *P. eous* extract on the lymphoproliferative response of splenocytes shows that the various extracts of *P. eous* modulated the proliferation of splenocytes and inhibit the splenocyte proliferation at higher concentration.

Production of nitric oxide (NO) in activated macrophages, which is an important cytotoxic mechanism of nonspecific immunity, has been very well documented. This process is initiated by 'induction' of the inducible NO synthase (iNOS), involved in the synthesis of both mRNA for the protein and the protein itself. The induction of iNOS and NO synthesis in macrophages may be caused by activators of these cells, including such bacterial products as lipopolysaccharide, muramyl dipeptide, some exotoxins and host cell products, especially cytokines such as interferon, tumour necrosis factor-α (TNFα) and interleukin-1[4]. However, little is known about induction of NO synthesis by factors which do not belong to these groups, in particular mushroom products. The present study shows that *P. eous* methanolic extract can enhance NO production by mice peritoneal macrophages. A recent report shows that alteration of potassium channel inhibitors because alteration in potassium chanels play major role in NO production and induction of Inos[18].

Previous studies by other investigators have shown that the increased levels of IL-1β and IL-12 correspond to the amount of NO produced by

peritoneal macrophages[19], mentioned inducing the NO production lead to inhibit the tumor cells.

The constituent responsible for the splenocyte proliferation and NO enhancement activity of edible PE mushroom has not been identified. A phytochemical analysis of the mushroom revealed that it contained D-glucan, ergosterol, mannitol, phenolic compounds, linoleic acid, peptides, and carbohydrates most of which are antioxidants. The polyphenolic compounds flavonoids, phenolic acids and their derivatives, and coumarin and its derivative have antiproliferative activity[20] and also reported the antiproliferative effect of *P. eous* Mushroom Lectin (PEL) with Mixed Carbohydrate in MCF-7, K562 and Hep2 cell lines at the lowest CTC_{50} concentration at 2 μgmL^{-1}, In our cytotoxicity study shows that all the *P.eous* extracts were non toxic to splenocyte and macrophages.

In the results of this study demonstrate that dietary supplementation with *P. eous* mushrooms enhances NO production in mice macrophage cell. This effect of mushrooms may be mediated through increased production of IFN-γ and TNF-α. These results suggest that consumption of *P. eous* mushrooms may increase innate immunity to tumors via splenocyte antiproliferation and viral infections. Future studies are needed to determine the clinical significance of these findings, particularly in those with impaired immune functions, such as elderly, and in those with cancer.

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