Original Article

Pleurotus ostreatus extract Enhances the Phagocytic Actions of Neutrophils against *Streptococcus pneumoniae*

Angus Nnamdi Oli[1]*, Akunna Perpetual Emeruwa[1], Obinna Ikechukwu Ekwunife[2], Ebele Linda Okoye[3], Chinekwu S. Anyaoku[4], Gordon C. Ibeanu[5], Ejeatuluchukwu Obi[6], Chibueze Peter Ihekwereme[7] and Monday Obaji[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Agulu, Nnamdi Azikiwe University, Awka, Nigeria; [2]Department of Clinical Pharmacy and Pharmacy Management, Faculty of Pharmaceutical Sciences, Agulu, Nnamdi Azikiwe University, Awka, Nigeria; [3]Department of Applied Microbiology and Brewing, Faculty of Biosciences, Nnamdi Azikiwe University, Awka, Nigeria; [4]Family Medicine/General Outpatient Department, Nnamdi Azikiwe University Teaching Hospital, Nnewi, Nigeria; [5]Department of Pharmaceutical Science, North Carolina Central University, 1801 Fayetteville St., Durham, North Carolina 27707, USA; [6]Department of Pharmacology and Therapeutics, Faculty of Medicine, College of Health Sciences, Nnamdi Azikiwe University, Nnewi Campus, Anambra State, Nigeria; [7]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Agulu, Nnamdi Azikiwe University, Awka, Nigeria

Corresponding author:
Angus Nnamdi Oli,
Department of Pharmaceutical Microbiology and Biotechnology,
Faculty of Pharmaceutical Sciences,
Nnamdi Azikiwe University,
Anambra, Nigeria, Tel: +234-8056306927;
E-mail: a.n.oli@live.com

Abstract

Introduction: Edible mushrooms have played prominent roles in improving the health of people for centuries. *Pleurotus ostreatus* is an edible mushroom with history of medicinal uses. This study was designed to examine, *in vitro* and *in vivo*, the possibility of methanolic and hot aqueous extracts of the mushroom modulating *Streptococcus pneumoniae* infection. **Methods:** Bioactive constituents of the methanolic and hot aqueous extracts were determined quantitatively and qualitatively using standard methods. Isolation, identification and confirmation of *Streptococcus pneumoniae* in infected blood were done using standard protocols. The effect of the extract on cellular immune responses was assessed by phagocytosis evaluation and quantitative Nitroblue Tetrazolium Test. *In vivo* immunological response against Streptococcal infection was evaluated using the neutrophil adhesion test and Hemagglutinating antibody (HA) titre post infection in mice. **Results:** Methanol and aqueous extracts revealed the presence of flavonoids (6.41%; 3.01%), alkaloids (10.01%; 11.01%), saponins (2.02%; 2.02%), phenols (1.55%; 0.01%), tannins (0.02%; 0.03%), carbohydrate (14.05%; 11.09%), and proteins (45.09%; 41.00%). Percentage stimulation of the polymorphonuclear neutrophils (PMNs) was 48% and 53% for the highest concentration (500 mg/mL) of the extracts used, while the quantitative Nitroblue Tetrazolium test score ranged from 58.00 ± 9.64 and 57.33 ± 2.91 for 400 mg/ml to 27.33 ± 4.84 and 25.67 ± 1.40 for 50 mg/ml. Oral administration of the extract in mice significantly increased neutrophil adhesion to nylon fiber when compared to control group and significant increased circulating antibody titer and phagocytic index in a concentration dependent manner. **Conclusions:** *Pleurotus ostreatus* has high carbohydrate and protein content and may help the immune system in defense against infections caused by *Streptococcus pneumoniae*.

Keywords: *Pleurotus ostreatus*; Edible mushroom; Phagocytosis; *Streptococcus pneumonia*; Immune system

Introduction

Mushrooms are fleshy, spore bearing macro-fungi with distinctive visible fruiting body (which can be hypogeous or epigeous) that does not undergo photosynthesis. The genus *Pleurotus* widely known as Oyster mushroom is preferred by many people for its delicate taste, mild yet chewy texture and unique aroma. Nutritionally, the mushroom (Oyster) has been found to contain great arrays of vitamins and other nutrients. [1,2] Medically, the species *Pleurotus ostreatus* have been reported to decrease cholesterol levels. [3] Another research observed that the carpophore of the mushroom is also a potential source of lignin and phenol degrading enzymes and may be applied in mycoremediation, being able to remediate several kinds of pollutants. [4-6] Some secondary metabolites have been found in the oyster mushroom such as the phenolic compounds, [7] flavonoids, terpenoids, sterols, ascorbic acid, ergothioneine and carotenoids. [6] Like other edible mushrooms, *Pleurotus* exhibits high antioxidant properties which ward off cancers, HIV-1 AIDS and other viral ailments. Oxidative stress caused by the presence of free radicals in the body cause cell damage; generation of cancer cells and brain cell aging. [7] However, antioxidants which are abundant in some macro-fungi, including *Pleurotus*, have

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the ability to scavenge these free radicals and so play important roles in maintaining human health. In addition to the antioxidant properties of oyster, reports from many scientists showed that it exhibits significant antibacterial effects against Gram-negative and Gram-positive bacteria. [8-10]

The specific effects of the mushroom on the immune system include: better-functioning γδ T-cells and reductions in inflammatory proteins, [11,12] modification of cytokines within specific cancer cells for better cancer resolution, [13] improvement in cognitive abilities of the aged, [14] stimulation of the innate and adaptive immunological responses, activation of the complement system and synthesis of pro- and anti-inflammatory cytokines and also helping in the passage of blood cells through the intact walls of the capillaries during inflammation. [15,16]

Neutrophils, the most abundant type of granulocytes as well as the most abundant type of white blood cells in most mammals, constitute an essential part of the innate immune system and provide the first line of defense against infections by phagocytosing, killing, and digesting infecting pathogens. [17,18] The susceptibility of the newborns to bacterial infections is attributable to immaturity of innate immunity mainly due to the impaired phagocytic function of neutrophils and monocytes. [19] It has been reported that phagocytosis by neutrophils and monocytes is the main defense mechanism against bacterial challenges. [20]

Streptococcus pneumoniae is a Gram-positive, facultative, catalase-negative anaerobic bacteria belonging to the genus *Streptococcus*. Under aerobic conditions, the bacteria partially break down the red blood cells (is alpha-hemolytic) but under anaerobic conditions, the bacteria completely lyse the blood cells (is beta-hemolytic). The organism is also called pneumococcus and is a common bacterial pathogen of the human respiratory system causing pneumonia and meningitis. Children, immunocompromised individuals and the elderly are most vulnerable to infections caused by the bacteria because they have weaker immune systems. [21] *Streptococcus pneumoniae* infections are known causes of morbidity and mortality in these populations. There is therefore every need to protect these population groups, especially the children.

It is not yet known the effect of *Pleurotus ostreatus* consumption in *Streptococcus pneumoniae* infection prevention. This study is aimed at demonstrating the ability of *Pleurotus ostreatus* to stimulate the phagocytic actions of the neutrophils against *Streptococcus pneumoniae* infected cells using a mice model.

Materials and Methods

Mushroom material source

The oyster mushroom, *Pleurotus ostreatus* was purchased from Dilomat farm, Phalga town, Port Harcourt, River State of Nigeria and authenticated by Mrs. Anthonia Emezie of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences Agulu, Nnamdi Azikiwe University, Awka, Nigeria. A voucher specimen (voucher number PLG/474/A/038) was deposited in the herbarium of the department. The mushroom was air-dried for 2 weeks, pulverized and stored till ready for use.

Methanolic extraction of Stock A

In methanolic isolation for the *in vitro* and *in vivo* investigations of *Pleurotus ostreatus*, 500 g of dried powder of the mushroom samples were macerated in 2.5 litres of methanol and were intermittently shaken for 24 hours. The mixtures were sieved using muslin cloth. They were further filtered with whatman filter paper No. 4 in a separator funnel. The filtrates were concentrated using rotary evaporator and then water bath to 50%. The crude extracts were stored in refrigerator for further analysis.

Hot aqueous extraction of Stock B

Previously boiled hot water was poured into a beaker containing 500 g of air-dried *Pleurotus ostreatus* powder such that the powder was fully covered by the water and stirred. The beaker was then placed on a hot plate (maintained at 70 ± 4°C) and allowed to stand for 4 hours with intermittent stirring. After 4 hours, the boiled sample was allowed to cool to 45°C after which it was filtered using sieve cloth. The extract was stored inside a refrigerator at the temperature 4°C for future use.

Preliminary phytochemical screening

The hot aqueous extract of *Pleurotus ostreatus* was subjected to qualitative preliminary phytochemical screening using the methods described by Sofowora; [22] Trease and Evans [23] and Harborne. [24] Also, quantitative determinations of the phytochemical constituents were carried out.

Test for flavonoids

Qualitative determination: A 0.1 g quantity of the extract was mixed with 2 ml of distilled water and 15 drops of dilute of sodium hydroxide were added in a drop wise manner. An intense yellow colour was produced in the mushroom extract, which became colorless on addition of a few drops of dilute acid; indicating the presence of flavonoids. [25]

Quantitative determination: This was done using modified methods reported previously. [26,27] Briefly; in a 250 ml beaker, 50 ml of 80% aqueous methanol was mixed with 2.50 g of the extract, covered and stored at 25-27°C for 24 h. The sediment was recovered by decanting and re-extraction carried out thrice using 50 ml ethanol. Instead of decanting, Whatman filter paper number 42 (125 mm) was used to filter whole solution of each ethanol extract. The final filtrate was evaporated to dryness in a crucible and over a water bath until constant weight was obtained. The flavonoids content was calculated as

$$\% \text{ Flavonoid} = \frac{(\text{Initial Wt of Crucible - Final Wt of Crucible})\ 100}{\text{Wt of Crude extract used}}$$

Test for alkaloids

Qualitative determination: Qualitative determination was carried out by adding 0.5 g extracts in 5 ml 1% HCL, boiled,

filtered and Mayer's reagent was added. The absence of an orange or red precipitate immediately indicated the absence of alkaloids.

Quantitative determination: Quantitative determination of alkaloid was done as described by previous researchers [28,29] with some modifications. Briefly; in a 250 ml beaker, 200 ml of 10% acetic acid in ethanol was mixed with 2.5 g of extract, covered and stored at 27 ± 2°C for 4 h. After heating on a water bath to one-quarter of the original volume, conc. NH_4OH (15 drops) was added in a drop wise manner until precipitation was completed. The precipitate was recovered by decanting after allowing it to settle for 3 h, washed with 20 ml of 0.1 M NH_4OH and then filtered using Gem filter paper (12.5 cm). The residue was oven-dried until constant weight was obtained. The alkaloid content was computed as

$$\% \text{ Alkaloid} = \frac{(\text{Initial Wt of Gem filter paper - Final Wt})\ 100}{\text{Wt of Crude extract used}}$$

Test for saponins

Qualitative determination: The extract was subjected to frothing test for the identification of saponins. The 0.5 g of extract was diluted with 20 ml of distilled water and agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam showed the presence of saponins.

Quantitative determination: The methods as described in earlier studies [29,30] were used with some modifications. A 5 g quantity of the extract was mixed with 100 ml of 20% aqueous ethanol in a 250 ml conical flask and heated at 55°C over a hot water bath for 4 h with continuous stirring. After allowing it to stand for another 4h, the mixture was filtered and the whole processes repeated. The final extract was heated to 40 ml over water bath at 90°C. Using a 250 ml separator funnel, the concentrate was partitioned between 20 ml diethyl ether layer and the aqueous layer after vigorous shaking. The aqueous layer was recovered and the process repeated. The purification process of the aqueous layer was continued by further partitioning between 60 ml of n-butanol. The butanol extract was washed twice with 10 ml 5% NaCl. After decanting off the sodium chloride layer, the remaining solution was heated in a water bath for 30 minutes and transferred to a crucible for drying in an oven to a constant weight. The Saponin content was calculated as

$$\% \text{ Saponin} = \frac{(\text{Initial Wt of Crucible - Final Wt of Crucible})\ 100}{\text{Wt of Crude extract used}}$$

Phenols/tannins

Qualitative determination: The test for tannins was carried out by adding 3 g of mushroom extract to 6 ml of distilled water, filtered and ferric chloride reagents added. Blue black precipitate indicates the presence of tannins and phenols.

Quantitative determination: The determination of total phenolic contents of the mushroom extract was done using a method previously reported. [30,31]

Briefly, 0.2 g of crude extract was dissolved in 3 mL of distilled water and carefully mixed with 500 µL of Folin–Ciocalteu reagent (a mixture of phosphotungstic and phosphomolybdic acids) for 3-5 min, and then added 2 mL of 20% (w/v) sodium carbonate. The mixture was stored in the dark at 25-27°C for 1-2 h for colour development. The absorbance was measured, using APEL: PD-303 spectrophotometer, made in Japan, at 650 nm against a blank having all the reagents excluding the sample. The total phenolic content was calculated from the calibration curve of gallic acid, and the results expressed as mg of gallic acid equivalent per g dry extract. Folin-Ciocalteu reagent is reduced to blue coloured tungsten oxide and molybdenum oxide during phenol oxidation in the presence of sodium carbonate. The intensity of blue color is proportional to the phenolic content of the extract and can be measured using spectrophotometer.

The determination of tannins: This was done using modified methods in earlier reports. [32-26] Folin-Denis reagent was prepared by dissolving 50 g of sodium tungstate (Na_2WO_4) in 37 ml of distilled water and then adding 10 g of phosphomolybdic acid ($H_3PMo_{12}O_{40}$) and 25 ml of orthophosphoric acid (H_3PO_4). The mixture was refluxed for 2 h, cooled and diluted to 500 ml with distilled water. Then 1 g of the mushroom was mixed with 100 ml of distilled water in a 250 ml conical flask and gently boiled for an hour on an electric hot plate. Using Whatman filter paper No 42 (125 mm) the cooled mixture was filtered into a 100 ml volumetric flask. A 10 ml volume of the filtrate was mixed with 50 ml of distilled water followed by the addition of 5 ml Folin-Denis reagent and 10 ml of saturated Na_2CO_3 solution. After a careful and thorough mixing, the solution was stored in the dark on a water bath at 25°C for 1 hr. The absorbance was measured at 500 nm using APEL: PD-303 spectrophotometer, made in Japan and compared on a standard tannic acid curve. The amount of total condensed tannins was expressed in mg/100 g dry weight of extract used.

The tannin content was calculated as

$$\% \text{ Tannic acid} = \frac{\text{C(Volume of extract)}\ 100}{\text{(Aliquot Vol used) (Wt of Sample used)}}$$

C = concentration of tannic acid read from the standard tannic acid curve.

Carbohydrates (Reducing sugar): Molisch's test

Qualitative determination: A 0.2 g of the extract was mixed with 3 ml of distilled water and then added 2 ml of Molisch's reagent. The resulting mixture was shaken properly and then 2 ml of conc H_2SO_4 was poured carefully down the side of the test tube. A violet ring at the interface indicates the presence of carbohydrate.

Quantitative determination: The method described by DuBois et al. [32] and modified by Fasidi [33] was used. Briefly, ethanol-soluble sugar was extracted by boiling in 80% ethanol for 5 h and quantified using APEL: PD-303 spectrophotometer at 500 nm wavelength.

Test for soluble protein

Quantitative determination: The soluble protein contents were

determined according to the colorimetric methods described by Jose et al. [34] and the authors. Briefly, 10 g of the dried extract was placed in a conical flask (125 mL) containing 25 mL sodium citrate buffer (0.05 mol/L and pH 4.8) and kept in a shaker for 30 min at 150 rpm. Thereafter, the extract was filtered using millipore membranes and the filtrate heated in a water bath for 30 minutes and transferred to a crucible for drying in an oven to a constant weight. The protein content was calculated as

$$\% \text{ dried protein} = \frac{(\text{Initial Wt of Crucible - Final Wt of Crucible})\ 100}{\text{Wt of dry crude extract used}}$$

Test for cardiac glycosides

Qualitative determination: Killer-Kiliani test was adopted. A 2 ml aliquot of the extract was dissolved in 2 ml of glacial acetic acid containing one drop of $FeCl_3$ solution. The mixture was then poured into a test tube containing 1 ml of conc. H_2SO_4. A brown ring at the inter-phase indicates the presence of glycosides.

Quantitative determination: The method reported by Muhammad and Abubakar [35] was used with some modifications. Briefly, 2 g of crude extract was dissolved in 8 ml of distilled water and transferred to a 100 ml volumetric flask. Volumes of 60 ml of H_2O and 8 ml of 12.5% lead acetate were added, mixed and filtered. A volume of 50 ml of the filtrate was transferred into another 100 ml flask and 8 ml of 47% Na_2HPO_4 were added to precipitate out excess Pb^{2+} ion. After filtration, the process of precipitating out Pb^{2+} ion was repeated twice and then, 10 ml of purified filtrate was transferred into clean conical flask and treated with 10 ml Baljet reagent. A blank titration was carried out using 10 ml distilled water and 10 ml Baljet reagent. The two reaction mixtures were allowed to stand in the dark for 1 h for complete colour development. The intensity (absorbance) of the color developed is proportional to the Cardiac glycosides content of the extract and can be measured using spectrophotometer at 495 nm and compared on a standard curve. The amount of cardiac glycoside was expressed in mg/100 g dry weight of extract used.

$$\% \text{ Total glycoside} = \frac{\text{C(Volume of extract) } 100}{\text{(Aliquot Vol used) (Wt of Sample used)}}$$

C = concentration of glycoside corresponding to the absorbance measured.

Test organism

The isolate (*Streptococcus pneumoniae*) used in this study was isolates stored at the post-graduate lab of Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, Agulu, Nnamdi Azikiwe University, Awka. Identification and confirmation were done using standard protocols.

Preparation of stock and graded concentrations of plant extract

800 mg of the crude extract was dissolved in 2 mL of Dimethyl sulfoxide (DMSO) to get a stock concentration of 400 mg/mL. Then, two-fold serial-dilution was made to get 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0.78 mg/mL concentrations.

Animal model

A total of twenty naive laboratory mice, gender matched, aged between 6-8 weeks, and within the weight ranges of 15.1-18.8 g were used. They were sourced from the Department of Zoology, University of Nigeria Nsukka. The mice were fed for 1 week under a pathogen free condition and environment and infected with a sample of *Streptococcus pneumoniae* after being. Used for acute toxicity test Isolation, identification and confirmation of *Streptococcus pneumoniae* (post-infection) in infected blood were done using standard protocols. Seventeen out of the twenty animals were used for acute toxicity test prior to infection.

Phagocytosis evaluation

This was carried out as previously reported by Surana et al. [36] with some modifications. A volume of 0.2 ml of the infected serum from the lab animals' blood was dropped on a sterile microscopic slide and mixed gently with 0.1 ml predetermined concentration of the test sample (400 mg/ml, 200 mg/ml, 100 mg/ml and 50 mg/ml). The slide was then incubated at 37°C for 30 minutes. After draining and fixing with methanol, the slide was stained with Giemsa stain. A standard was tested by preparing the slide in the same way with uninfected mice serum. Positive and negative controls were tested by preparing other slides in the same way using endotoxin activated and phosphate buffer activated mice serum respectively. The mean number of *Streptococcus pneumoniae* cells phagocytosed by the polymorphonuclear neutrophils (PMNS) on the slide was determined microscopically for 100 granulocytes using morphological criteria. This number was taken as the phagocytosis index (PI) and was compared with the basal PI of the standard not containing test extract. This procedure was reported for the different concentrations of the test sample used.

The immune-stimulation in percentage (%) was calculated as below, [37]

$$\text{Stimulation } (\%) = \frac{(\text{PI(test) - PI(Standard)}) \times 100}{\text{PI(Standard)}}$$

Quantitative determination of the Nitroblue Tetrazolium (NBT) Test Score

This was determined using a method previously reported by Surana et al. [36] with some modifications. A 0.2 ml uninfected mice serum suspension in 0.5 ml of phosphate buffer solution (PBS) was prepared in six different test tubes (A-F) as shown in Table 1. Without the addition of the extracts to the test tubes C-F, all the test tubes were first incubated in a water bath at 37°C for 10 minutes. The extracts were then added and all the tubes further incubated in a water bath at 37°C for 20 minutes, centrifuged at 4000 rpm for 3-4 minutes and the supernatant discarded. The PMNs-rich deposits were re-suspended in a little quantity of PBS. From each test tube, a thin film/smear was made with a drop on 4 slides, dried and fixed by heating. A counter stain was made (for each slide) with dilute carbol fuchsin for 15 s followed by washing the slide with tap water, drying and then observed under 100xoil immersion objective lens. A total of 100 neutrophils were examined per smear/slide.

Table 1: Nitroblue Tetrazolium (NBT) Test Score table of constituents.

Test tube	Contents
A	Suspension (0.2 ml) of uninfected mice serum in 0.5 ml PBS + 0.2 ml of 0.15% NBT + 0.1 ml PBS
B	Suspension (0.2 ml) of uninfected mice serum in 0.5 ml PBS + 0.2 ml of 0.15% NBT + 0.1 ml *S. pneumoniae* infected mice blood sample
C	Suspension (0.2 ml) of uninfected mice serum in 0.5 ml PBS + 0.2 ml of 0.15% NBT + 0.1 ml *S. pneumoniae* infected mice blood sample + 0.1 ml of 100 mg/ml Extract
D	Suspension (0.2 ml) of uninfected mice serum in 0.5 ml PBS + 0.2 ml of 0.15% NBT + 0.1 ml *S. pneumoniae* infected mice blood sample + 0.1 ml of 200 mg/ml Extract
E	Suspension (0.2 ml) of uninfected mice serum in 0.5 ml PBS + 0.2 ml of 0.15% NBT + 0.1 ml *S. pneumoniae* infected mice blood sample + 0.1 ml of 400 mg/ml Extract

The neutrophils that phagocytosed the bacterial cell (blue-black granules/lumps) were counted (classified as "NBT positive") and the percentage of positives or the "score" is recorded as the NBT Test score. This is a measure of the number of bacterial cells engulfed by the neutrophils.

Immunological Evaluation

Experimental Design for Acute Toxicity Study

This was estimated using Lorke's method [37] with some modification. [38] The method involved two phases consisting of seventeen animals. The first phase consisted of nine animals divided into three groups of three animals each. Each group of animals was administered different doses (10, 100 and 1000 mg/kg) of test substance. The animals were then placed under observation for 24 hours to monitor their behavior as well as mortality. The second phase involved the use of eight animals, which were distributed into four groups of two animals each. The animals were also administered higher doses (2000, 3000, 4000 and 5000 mg/kg) of test substance and then observed for 24 hours for behavioral changes as well as mortality.

Then the LD_{50} was calculated using the formula: $LD_{50} = \sqrt{(D_0 \times D_{100})}$

D_0 = Highest dose that gave no mortality, D_{100} = Lowest dose that produced mortality.

Neutrophil Adhesion Test

Neutrophil adhesion test used the method of Mallurwar et al. [39] The mice were divided into eight groups of five animals in each group. The negative control group I received 10 ml of 5% DMSO, while group II was administered 250 mg/kg of Noni which served as the positive control. The mice in groups 3-5 received aqueous *Pleurotus ostreatus* extract at doses of 125, 250 and 500 mg/kg/day, while groups 6-8 received the methanolic extract of the mushroom in the concentration as aqueous per body weight daily for 14 days. On the 14th day of treatment, blood samples from all the groups were collected by puncturing the retro-orbital plexus under mild ether anesthesia. Blood was collected in pre-treated disodium EDTA vials and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC). To carry out the total leukocyte count, blood sample drawn was diluted with Turk's fluid in WBC pipette, in which red cells were lysed without affecting the leukocyte population. Leukocytes were counted by using an improved Neubauer's counting chamber.

In differential leukocyte count (DLC), blood smear was prepared on a clean glass slide and stained with Leishman's and Field 1 stains. The different population of leukocytes was differentiated and identified based on the cell size, presence of granules, colour and shape of nucleus under the microscope using immersion oil. After initial counts, blood samples were inoculated with 80 mg/ml of nylon fibres for 15 min at 37°C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and% neutrophil gives neutrophil index (NI) of blood sample. Percent of neutrophil adhesion was calculated as follows:

$$\text{Neutrophil Adhesion (\%)} = \frac{(NLu - NLt)100}{NLu}$$

Where NLu is the neutrophil index of untreated blood samples and NLt is the neutrophil index of treated blood samples.

Hemagglutinating antibody (HA) titre

Hemagglutinating antibody titre was according to Puri et al. [40] The animals were challenged by injecting 0.1 ml of *Streptococcus pneumoniae* suspension containing 0.5×10^9 cells intraperitoneally on day zero. The test samples of extracts were separately administered to all the animals continuously for 7 days. Blood samples were collected in micro-centrifuge tubes from individual animal by retro-orbital puncture on 7th day. The blood samples were centrifuged at 2500 rpm for 10 minutes and serum obtained. [41] Antibody levels were determined by the hemagglutination technique. Equal volumes of individual serum samples of each group were pooled and two fold serial dilutions of samples made in 25 µl volume of normal saline in micro-titration plates were added to 25 µl of 1% suspension of *Streptococcus pneumoniae* in saline. After mixing, the plates were then incubated at 37ºC for 1hour and examined for hemagglutination. The reciprocal of the highest dilution of the test serum agglutination was taken as the antibody titre.

Statistical analysis

Statistical analysis was performed using Graph pad Prism Software Version 3.10. All the results were expressed as Mean ± Standard Error Mean (SEM). Data were analyzed using one-way Analysis of Variance (ANOVA) followed by Dunnett Multiple comparison test. P-values < 0.05 were considered statistically significant.

Results and Discussion

The phytochemical analysis of the hot aqueous extract of *Pleurotus ostreatus* was carried out and the result obtained is shown in Table 2. The study showed that 100 g dry weight of the hot aqueous extract of *Pleurotus ostreatus* studied is almost one-half carbohydrate, one quarter soluble proteins and moderate quantities of saponins, phenols and tannins [Tables 3-5].

Table 2: Preliminary phytochemical screening.

S/N	Bioactive compounds	Qualitative Estimation/100g weight of dry extract	Quantitative Estimation/100g weight of dry extract
1	Flavonoids	++	6.41
2	Alkaloids	++	10.01
3	Saponins	+	2.02
4	Phenols	+	1.55
5	Tannins	-	0.02
6	Carbohydrate	++	14.08
7	Proteins/Amino Acids	+++	45.09

*Gallic acid equivalent per 100 g dry weight of sample. Key: - = absent or undetectable, + = trace, ++ = moderate, +++ = excess

Table 3: Neutrophil adhesion result of the methanolic extract of *Pleurotus ostreatus*.

Treatment	Doses (mg/kg)	TLC (10^3/mm^3) UB	TLC (10^3/mm^3) FTB	N (%) UB	N (%) FTB	N.I UB	N.I FTB	N.A (%)
Negative Control	10 (H_2O)	60.33 ± 2.18	57.33 ± 2.96	51.33 ± 0.88	44.00 ± 5.03	3100.33 ± 158.62	2495.33 ± 191.50	19.51
Positive Control	250 (noni)	63.67 ± 5.84	45.33 ± 6.18	54.00 ± 1.53	43.00 ± 6.56	3455.66 ± 403.45	1922 ± 365.33	44.38
MET A	250	56.66 ± 1.76	54.67 ± 1.67	50.00 ± 0.58	49.33 ± 0.88	2835.33 ± 120.81	2699.66 ± 130.07	4.79**
MET B	500	60.33 ± 6.12	58.33 ± 4.09	51.66 ± 1.76	38.66 ± 7.69	3137.66 ± 418.47	2316 ± 628.64	26.19

The values are expressed as Mean ± SEM. The significance on comparison with the positive control group is indicated by ** mark. ** p<0.01. TLC-Total Leukocyte Count, N- Neutrophils, N.I- Neutrophil Index, N.A- Neutrophil Adhesion, UB- Untreated Blood, FTB- Fibre Treated Blood

Table 4: Neutrophil adhesion result of the aqueous extract of *Pleurotus ostreatus*.

Treatment	Doses (mg/kg)	TLC (10^3/mm^3) UB	TLC (10^3/mm^3) FTB	N (%) UB	N (%) FTB	N.I UB	N.I FTB	N.A (%)
Negative Control	10 (H_2O)	60.33 ± 2.18	57.33 ± 2.96	51.33 ± 0.88	44.00 ± 5.03	3100.33 ± 158.62	2495.33 ± 191.50	19.51
Positive Control	250 (noni)	63.67 ± 5.84	45.33 ± 6.18	54.00 ± 1.53	43.00 ± 6.56	3455.66 ± 403.45	1922 ± 365.33	44.38
Aqueous A	250	64.00 ± 3.05	56.33 ± 5.89	53.00 ± 1.53	51.33 ± 2.33	3396.66 ± 229.14	2919 ± 446.52	14.06*
Aqueous B	500	63.00 ± 5.51	63.00 ± 2.08	52.00 ± 2.52	52.00 ± 0.59	3299.33 ± 437.00	3278.33 ± 144.32	0.62**

The values are expressed as Mean ± SEM. The significance on comparison with the positive control group is indicated by **mark. **p<0.01. TLC-Total Leukocyte Count, N- Neutrophils, N.I- Neutrophil Index, N.A- Neutrophil Adhesion, UB- Untreated Blood, FTB-Fibre Treated Blood

Table 5: Hemagglutination (HA) Titre of the methanol and aqueous extract of *Pleurotus ostreatus*.

Treatment	Doses	HA Titre
Negative Control	10 mg/kg (H_2O)	26.043 ± 13.540
Positive Control	250 mg/kg (Noni)	29.500 ± 1.500
Met A	125 mg/kg	18.753 ± 15.623
Met B	250 mg/kg	21.817 ± 14.319
Met C	500 mg/kg	50.000 ± 0.000*
Aqueous A	125 mg/kg	5.270 ± 1.040**
Aq B	250 mg/kg	26.043 ± 13.540
Aq C	500 mg/kg	27.100 ± 12.659

The values are expressed as Mean ± SEM. The significance on comparison with the positive control group is indicated by * mark. * p<0.05

Phagocytosis, a type of endocytosis, refers to the engulfing of solid particles such as bacterial cells. The neutrophils (a constituent of the white blood cell) normally engulf a bacterium and subsequently destroy it. In investigating cell-mediated immune response, the phagocytic ability of polymorphonuclear neutrophils (PMNs) may be evaluated *in vitro*. PMNs are part of the reticuloendothelial system which is formed by phagocytes (macrophages and neutrophils). These cells are large granular leucocytes that are cytotoxic. Their function is to engulf foreign particles and microorganisms, as well as act as scavengers that rid the body of worn out tissues and cell debris. They do this through the process of phagocytosis and production of an array of chemical substances. [42] Phagocytes may opsonise foreign particulate matter, including pathogens, with antibodies and complement C3b thereby rapidly clearing them from the blood. [43]

Plants have, since antiquity, been useful to man in many ways ranging from food products to countless medicines for the treatment and prevention of diseases. Man has always relied on them for the provision of substances that make him healthier and fitter. This study shows that *Pleurotus Ostreatus* is rich in carbohydrate and proteins signifying a high nutritional value. Similar reports had been published. [44,45] Edible mushrooms generally contain diverse health promoting constituents. Deepak and Deepika [46] confirmed the high protein (32%) and carbohydrate (55%) contents of mushroom and also reported the nutraceutical values of same plant.

These constituents can possible promote human health in a number of ways including enhancement of phagocytic activity.

Plants have been reported as sources of antimicrobial agents. [47-49] Every plant, in fact, has antimicrobial activity depending on the concentration involved. However, useful and acceptable activity is taken at concentrations well below the toxicity range of the plant material. The anti-pneumococcal activity may be useful in promoting the phagocytic stimulatory property of the mushroom against the organism and may be attributed to the tannins/phenolic compounds and/or glycoprotein constituents. [50,51]

Cells in the immune system act as phagocytes to identify and destroy invaders that would otherwise make us sick. Immune cells also act as clean-up crews. They engulf dead cells and cellular debris. [42] The white blood cells (specifically, the phagocytes) attach their membranes to the membranes of the

infecting pathogen using surface receptors specially designed to detect and attach to the pathogens membrane molecules. Up on attachment, the cell membrane of the white blood cell swells outward around the pathogen to engulf it, and then pinches off to form a little pouch, called a phagosome. The white blood cell secretes digestive enzymes into the phagosome to destroy the pathogen. The resulting harmless particles can either be used by the cell or released out of the cell.

Several different cells in the immune system act as phagocytes and play different roles. The neutrophils, for instance, normally aggregate quickly to a site of injury to gobble up any infecting bacteria. The incorporation of the extracts [Figure 1] showed graded levels of stimulation of the polymorphonuclear neutrophils (PMNs) in phagocytosing the bacteria. First, the white blood cell (PMNs) has to recognize the invader and realize that it needs to be destroyed. The results are in allegiance to the mechanism related to phagocytosis by macrophages and by clinical antibody samples. [51,53] These reports from previous works supports the result obtained in this study where the extracts of *Pleurotus ostreatus* showed significant increase in the phagocytic activity of neutrophils during *Streptococcus pneumoniae* infection, *in vitro*. There was increase in the percentage stimulation as the concentration of the test sample increases. Studies carried out on two different polysaccharides (PSP and PSK) extracted from *Trametes versicolor* (an edible mushroom) produced significant increase in the stimulation of neutrophils. [53,54] An insight into receptor-binding in immune cells by β-glucans from fungi was provided by other researchers. [55,56] These authors showed that β-glucans from yeast bind to ic3b- receptors (CR3, CD11b/CD18) of phagocytic cells and natural killer cells, stimulating phagocytosis and/or cytotoxic degranulation. All these reports are in line with the result obtained in our study [Figure 2].

Stimulation or enhancement of phagocytic action can be demonstrated in a number of ways. Phagocytic index is a measure of phagocytic activity. It is measured by counting the

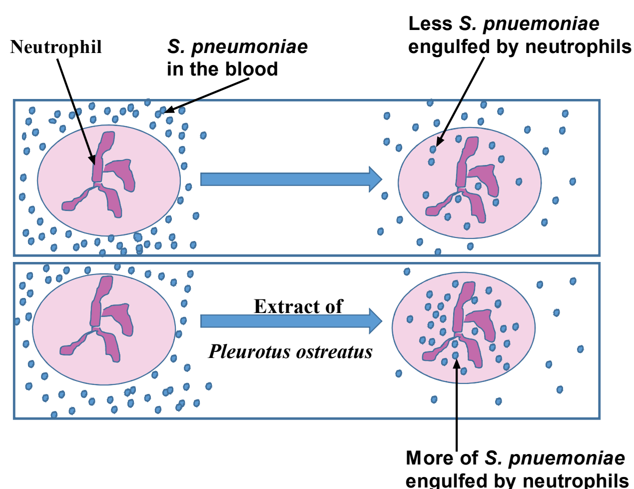

Figure 1: Potentiation action of the extract on Polymorphonuclear neutrophils phagocytic action. The neutrophils (PMNs) are seen mobilized for phagocytosis. The phagocytic action of neutrophils enhanced by the extracts of *Pleurotus ostreatus* against *Streptococcus pneumoniae*. The enhancement was concentration dependent.

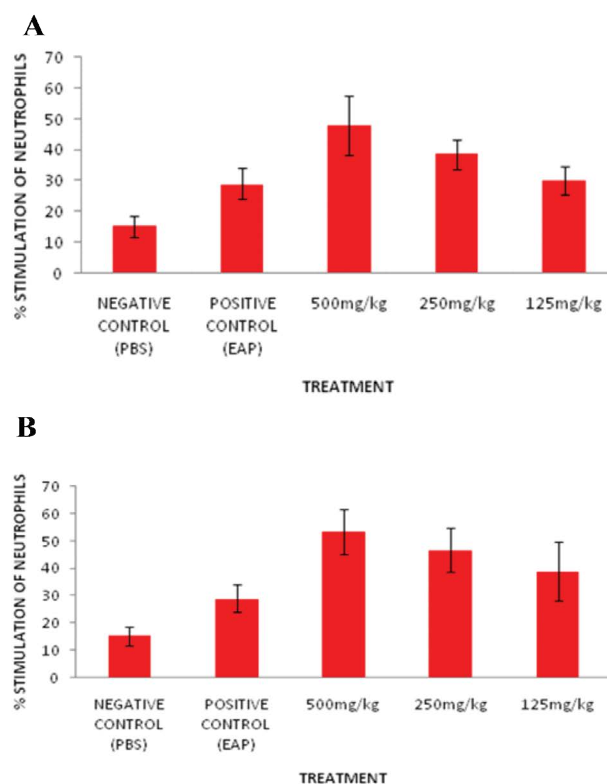

Figure 2: Stimulation (in percentage) of phagocytic action of neutrophils by the methanol (A) and aqueous (B) extract of *Pleurotus ostreatus* (phagocytosis evaluation).

number of bacteria ingested per phagocyte during a determined incubation period of a suspension of bacteria and phagocytes in a serum/blood sample. [52,57] Nitroblue Tetrazolium Test score can be used to determine phagocytic index. Also, nitroblue-tetrazolium (NBT) test is the laboratory diagnostic test for chronic granulomatous disorder or chronic granulomatous disease (CGD). [58] The test is negative in CGD because the defective phagocyte NADPH (nicotinamide adenine dinucleotide phosphate) oxidase does not reduce the NBT to the insoluble blue formazan compound. The major mechanism by which phagocytes (i.e., neutrophils and macrophages) kill ingested (phagocytosed) bacteria is via the production of reactive oxygen species (ROS) in the presence of the enzyme NADPH oxidase, the other mechanisms, being via nitric oxide and proteases. [59] NADPH oxidase is, therefore, critical for phagocyte killing of bacteria through reactive oxygen species. [42] In-born error in any one of the five genes that encode for the subunits of phagocyte NADPH oxidase may precipitate CGD of varying severity. [60] A genetic analysis is needed to determine the exact mutation underlying the cause of the CGD. [61] Our study showed that, increasing concentrations of the aqueous extract *Pleurotus ostreatus* led to proportionate increase in the NBT score indicating that the ability of the neutrophils in killing off the phagocytosed cells of *Streptococcus pneumoniae* was enhanced. A study carried out on the methanolic extract of *Ocimum gratissimum* produced significant increase in the NBT score against *Candida albicans*. [36] This report is in line with the result obtained in our study. Potentiating the host defense system may result in the activation of many kinds of immune cells that are vital for the maintenance of homeostatisis. Polysaccharides

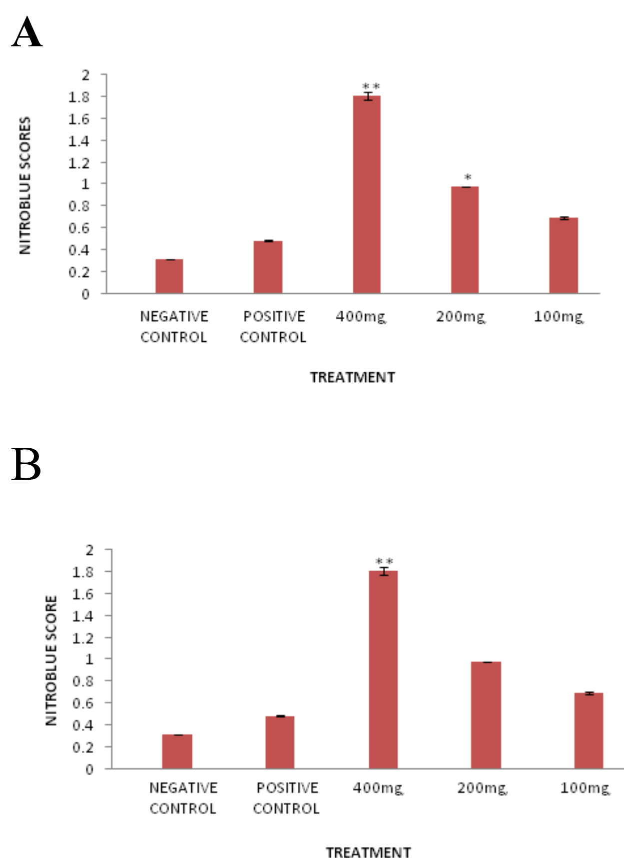

Figure 3: Quantitative Nitroblue Tetrazolium Test score of methanol **(A)** and aqueous (B) extract of *Pleurotus ostreatus*.

and glycoproteins are multi-cytokine inducers. They can induce gene expression of various immunomodulatory cytokines and receptor cytokines largely due to their structural diversity and variability.[62] *S. pneumoniae* can invade and utilize erythrocytes to circumvent killing by neutrophils.[63] Our study proved that even in the presence of the red blood cells, the extracts caused increased opsonophagocytosis of the bacteria by the neutrophils [Figure 3].

Streptococcus pneumoniae is an important pathogen implicated in disease conditions such as pneumonia, otitis media, sinusitis and septicemia. The World Health Organization estimates that over 1 million children especially in developing countries die every year of invasive pneumococcal disease.[64] Our study shows that methanol and aqueous extract of *Pleurotus ostreatus* when compared to the standard (noni) demonstrated excellent activity in stimulating immune response against *S. pneumoniae*. This is similar to a previous work.[65] which demonstrated that *Agaricus blazei Murill* elicited protection in mice against systemic *S. pneumonia* infection and provides control to other diseases.

Conclusion

Pleurotus ostreatus has high carbohydrate and protein content and may help the immune system in defense against infections caused by *Streptococcus pneumoniae*.

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Authors' Contributions

All authors participated equally in manuscript development but OLI Angus conceptualized the study and EMERUWA Akunna also assisted in the laboratory work

Conflict of Interest

The authors disclose that they have no conflicts of interest.

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