Research Paper

Polyporus leptocephalus (pat.) (Polyporaceae): A safe medicinal mushroom stimulating the immunological functions of macrophage and neutrophil

Accepted 23rd December, 2021

ABSTRACT

In the absence of therapies for various infections, the use of traditional medicine has been approved in various countries and has led to increased emphasis of immunostimulators consumption. This study exposed the potential immunomodulatory effects of the water and ethanol extracts from Polyporus leptocephalus, to support the pharmacological and safety of this mushroom. Water and ethanol extracts from fruit body of P. leptocephalus. Extracts were tested on LPS-induced response of peritoneal macrophages and neutrophils isolated from rats to assess the effect on phagocytosis and related activities. Water extract and ethanol extract of P. leptocephalus increased the immunological capacities as demonstrated by improvement of phagocytic index, production of nitric oxide and reactive oxygen species, lysosomal acid phosphatase of LPS-induced response of macrophages and neutrophils myeloperoxidase activity and adherence property of neutrophils. The water extract did not show a cytotoxic effect against peritoneal macrophages and neutrophils, while ethanol extract showed a little cytotoxic effect against macrophages inhibiting the metabolic viability seen at high concentration. The results and findings of the study demonstrate the immune heightening activity of fruit extracts of P. leptocephalus in vitro on LPS-induced macrophages and neutrophils activation.

Key words: Immunomodulation, neutrophils, macrophages, Polyporus leptocephalus.

INTRODUCTION

In December 2019, a novel coronavirus disease occurred in Wuhan, China, and rapidly spread to other areas. In severe form, the disease is characterized by infiltration of a large number of inflammatory cells in the lungs (Xu et al., 2020; Tian et al., 2020). Adverse outcomes of the disease were associated with various comorbidities, including immunodepression. Currently, there is no targeted therapy available, medical management is largely supportive and it varies from country to another. Presumptive anti-Covid-19 drugs, include lopinavir-ritonavir, remdesivir, hydroxychloroquine, and azithromycin (Zhou et al., 2020; Cao et al., 2020; Gautret et al., 2020). So far, the plant-derived compound chloroquine phosphate, previously used against malaria, is being promoted for the treatment of COVID-19. Ongoing research supports plants as bio-factories for the production of eagerly-awaited SARS-CoV-2 vaccines. Plants that the preparations harness host immune responses via phagocytosis stimulation, lymphoid cell stimulation, and cellular immune function enhancement, are therefore to support the patients.

Immunomodulators drugs has long had high importance in control of infectious diseases (Soumya et al., 2018), and natural product resources are excellent raw materials for the discovery and development of novel
immunomodulatory compounds (Wahab et al., 2014). P. leptocehalus (pat.) belongs to the Polyporacea family and distributes in the tropical and subtropical zone. Various species of this family have shown various pharmacological properties (Zhang et al., 2014). For instance, P. rhinocerus have been proved scientifically in treating liver cancer, chronic hepatitis and gastric ulcer, and it is one of the most important medicinal mushroom used to relieve fever, cough, asthma, cancer, food poisoning and as a general tonic (Liu et al., 2016). Though P. leptocehalus has been used in folk medicine in treatment of fever and cancer, there are no scientific data supporting the health benefits of this mushroom.

Diseases that negatively affect the immune system usually alter both the innate and adaptive immunity. In the innate immunity, polymorphonuclear leukocytes, more commonly known as neutrophils, along with monocytes/macrophages are essential professional phagocytes carrying out various roles in the host’s innate defense against pathogen (Butterfield et al., 2006; Silva, 2010). For their cellular phagocytic activities, neutrophils and macrophages co-express similar antigens and readily produce effect or molecules such as granular proteins, oxidants, chemokines and cytokines (Nauseef, 2007). Boosting the phagocytic activities of neutrophils and macrophages might help to minimize the pathogenesis of many infectious diseases. This study therefore aims at evaluating the safety and immunomodulatory potential of the water and ethanol extracts of P. leptocehalus on peritoneal neutrophils and macrophages of rats.

MATERIALS AND METHODS

Chemical reagents

Various reagents obtained from sigma Aldrich (Germany) were used in the present study. They include: 3-(4,5-dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate buffered saline (PBS), acetate buffer, Ethylenediaminetetraacetic acid (EDTA), Dimethyl sulfoxide (DMSO), L-glutamine, lipopolysaccharides (LPS), neutral red, o-phenylenediamine and Nitrobluetetrazolium solution (NBT), Bovine saline albumin (BSA), Triton X-100 et streptomycin/penicillin antibiotic.

Collection of Polyporus leptocehalus

A specimen of P. leptocehalus was collected from Bambili village in Tubah subdivision, Mezam division in the Nord West Region of the country. The specimen was identified by a mycologist in the Department of Biological Sciences, Faculty of Sciences. The collected specimen was washed using distilled water and kept at 4°C until the day of use.

Preparation of extracts of Polyporus leptocehalus

The aqueous extract (AEP) of P. leptocehalus was prepared using distilled water. Fresh entire P. leptocehalus (100g) was added in 500 ml of distilled water, incubated at 100°C for 1 h and filtered using the Whatmann filter paper No. 1. The filtrate was oven dried at 38.5°C to obtain the aqueous extract (2.57 g).

The ethanol extract was prepared using absolute ethanol (Merck). Fresh P. leptocehalus (100g) was macerated in 500 ml of absolute ethanol and allowed to stand for 4 days during which it was daily homogenized for 10 min. The maceration was filtered using the Whatmann paper No. 1. The filtrate was evaporated at 38.5°C to obtain the ethanol extract (2.3 g).

Experiment animals

Male albino Wistar rats procured from the animal house of the Department of Biochemistry were used for isolation of the peritoneal macrophages and neutrophils. The animals were raised under natural conditions (12 h light/ 12 h dark). They were fed with the standard diet and water ad libitum.

Isolation of peritoneal macrophages

Peritoneal macrophages and neutrophils were obtained from rats that had been injected intraperitoneally 3 days and 12 h prior with 2 ml, 5% BSA respectively (Sigma, Germany) (Yuki et al., 2020; Kühtreiber et al., 2005). Three days later, rats were sacrificed by dislocating cervical vertebrae and immersed in 70% ethanol for 2 min. The peritoneal membrane was collected and washed in 5 ml of RPMI-1640. The medium containing the cells was centrifuged (1800 rpm, 10 min). Cells in the pellets were suspended in RPMI-1640. The cell numbers were determined by a hemocytometer and cell viability was tested by Trypan blue dye exclusion technique.

Cytotoxicity assays

Cells (1.5 × 10^6 cells/ml) were seeded on 96 well tissue culture plates in RPMI-1640 containing 10% FBS, 100 mg/l L-glutamine and 5% streptomycin/penicillin. Extracts were added to the cultures at the concentrations indicated in the figure. The cultures were incubated at 37°C, for 48 h. Cell viability was examined using the MTT assay and neutral red assay (Abdullah et al., 2017; Repetto et al., 2008).
In MTT assay, at end time of incubation, 20 μl of 0.05% (w/v) solution of MTT in PBS buffer was added to the plates, and incubated for 3 hours. Later, 100 μl of dimethyl-sulfoxide containing 1% (v/v) 1 N hydrochloric acid was added to the plates and homogenized. The absorbance of the plate was read at 550 nm using a Microplate reader.

In the neutral red (NR) assay, after discarding the supernatant followed by washing with PBS, 100 μl of neutral red in PBS (0.075%) were added to each well. The plates were incubated at 37°C for 30 min. 100 μl of a solution of methanol (50%) and acetic acid (1%) in phosphate buffer saline was added to each well and the plates incubated for a further 15 minutes. The OD value of each well was measured at 540 nm. Cell viability was calculated as follows:

\[ \text{Cell survival rate} \% = \frac{\text{OD sample}}{\text{OD control}} \times 100, \]

Where: OD sample is the optical density value of test samples with cells and OD control, the optical density value of untreated cells (medium control).

**Immunological assays**

Cells (1.5 \times 10^4 viable cells/ml) were seeded on 96-well tissue culture plates in RPMI-1640 containing 5% FBS, 100 mg/L L-glutamine and 5% streptomycin/penicillin. Samples and LPS were added at different concentration indicated in each figure, and the cellular functions of macrophages and neutrophils such as: index phagocytic, nitric oxide and reactive oxygen species production, lysosomal phosphatase activity, myeloperoxidase and neutrophil adherence were evaluated (Mahamat et al., 2020; Aoyama et al., 2001; Chan-Zapata et al., 2018).

For the phagocytic index and nitric oxide production, after 48 hours of incubation of cells at 37°C, the supernatant was taken apart to measure the nitric oxide levels. Cells were gently rinsed twice with PBS and the phagocytic index was determined using the neutral red engulfment assay. For this, a hundred microliter (100 μl) of 0.075% neutral red solution was added to each well and the plate was incubated for 4 hours. The unphagocytized neutral red was washed out with PBS, and 100 μl of cell lysis buffer (10% acetic acid/ 40% ethanol = 1:1, ml/ml) were added to each well following by 30 min incubation, and the Optical density of each well was measured at 570 nm.

The estimation of nitric oxide concentrations was carried out by mixing 100 μl of supernatant with the same volume of Greiss reagent (1% sulfuranilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride (1:1 ratio [vol/vol]]), and incubation for 30 min. The Optical density value of each well was then measured at 545 nm.

In nitrobluetetrazolium (NBT) dye reduction assay determining the superoxide anion production, after incubation for 24 h, the supernatant was discarded and 50 μl of NBT solution in PBS (0.15%) were added. After incubation for 1 hour, the adherent macrophages and neutrophils were rinsed vigorously with PBS and washed 4 times with 200 μl of methanol, and air-dried. The formazan deposits were solubilized in 120 μl of 2M KOH and 140 μl of DMSO. After homogenization of the content of the wells, the optical density was read at 630 nm using a microplate reader.

To assess the lysosomal enzyme activity, after 48 hours of incubation of cells, intracellular acid phosphatase activity and that in the culture medium were evaluated. To this effect, 100 μl of medium were harvested from each well and cells were washed twice with PBS. To the cells, 25 μl of cold lysis buffer (0.2% Triton X-100 in 0.05M acetate buffer) were added and the plates were placed on ice for 30 minutes. Cell lysate and the harvested medium were incubated with 20 μl of glacial acetic acid and 50 μl of 6 mg/ml solution of p-nitrophenyl-phosphate in 0.1M acetate buffer for 65 minutes at 37°C, and the reaction was stopped with the addition of 100 μl of 1N NaOH. The optical density was read at 405 nm.

In evaluating my eloperoxidase activity, neutrophils (1×10^6 cells/well) were stimulated with LPS (4 μg/ml) and incubated for 24 hours. Thereafter, 100 μl of o-phenylenediamine (1g/200 ml) and 100 μl of 0.002% H2O2 inophosphate-citrate buffer (pH5.0) were added to each well. The reaction was stopped after 10 min using 0.1 NH2SO4 and the Optical densities were measured at 490 nm.

The neutrophil adherence assay was assessed by culturing neutrophils (1×10^6 cells/well) with various concentrations of the mushroom extracts into 96-well and incubation at 37°C for 6 hours and the culture medium (containing non adherent neutrophils) was discarded. The number of adhered neutrophils was determined using MTT assay.

The activity of the extract on phagocytosis, myeloperoxidase, acid phosphatase, NO, ROS production and neutrophil adherence was expressed as percentage of stimulation and calculated follows:

\[ \%\text{Stimulation} = \frac{\text{OD sample} - \text{OD control}}{\text{OD control}} \times 100, \]

Where: OD sample is the optical density value of test samples with cells and OD control, the optical density value of untreated cells (medium control).

**Statistical analysis**

Experimental values were expressed as the mean ±
Standard deviations (SD) of three experiments in triplicate. Data were analysed using one-way analysis of variance, followed by post hoc Turkey’s tests. Levels of p < 0.05 were considered as indicative of significance.

RESULTS

Cytotoxic effect of extracts of Polyporus leptocephalus

Using the MTT assay, the results demonstrated that ethanol extract showed significant inhibitory effect at 8.33 mg/ml (p<0.05), while aqueous extract does not affect cell viability of peritoneal cells. In contrast, no cytotoxic was observed using NR exclusion test assessing the membrane integrity as shown in Figure 1.

Effect of extracts of Polyporus leptocephalus on LPS-induced response of peritoneal macrophages

The results of this study show that the EEP and AEP of P. leptocephalus increased the different activities of LPS-activated macrophages. Treatment with the EEP as well as the AEP of P. leptocephalus on LPS-activated macrophages increased the phagocytic index (Figure 2A), NO production (Figure 2B), MPO activity (Figure 2C) and lysosomal enzyme activity (Figure 2D) of these cells in a concentration-dependent.

Effect of extracts of Polyporus leptocephalus on LPS-induced response and adherence on plastic surface of peritoneal neutrophils

Neutrophils were treated with various concentrations of EEP and AEP of P. leptocephalus, and results show that the EEP and AEP significantly increased the phagocytic index of these cells with high increase at low concentrations as shown in Figure 3A. In contrast, the ROS production (Figure 3B), NO production (Figure 3C), lysosomal enzyme activity (Figure 3D), MPO activity (Figure 3E) as well as the % adherence on plastic surface (Figure 3F) of these LPS-activated neutrophils incubated with the EEP and AEP of P. leptocephalus increased in concentration dependent.

DISCUSSION

Macrophages and neutrophils are recruited to the site of infection where they engulf and kill the invading microorganisms, clear the microorganisms from a body and prevent any diseases (Cutolo, 1999; Gordon, 2002; Srinivasan, 2013). Studies to investigate the effect of products on the immune system have been long done through the assessment of immune cells functions as well as macrophages and neutrophils (Mahamat et al., 2018). Results demonstrate enhanced efficacy of immune response by targeting macrophages and neutrophils with immunomodulatory products.

Mushroom species are well known to be immunomodulator products but which species is immunomodulators requires future experiments. Various mushrooms species of the genera Polyporus are known for their medicinal properties such as in cancer treatment or in treatment of infectious diseases (Zhang et al., 2014). It is believed that the immunomodulator activities of Polyporus mushrooms are of large importance in their therapeutic use (Liu et al., 2016; Pan et al., 2015). In this study, a hot-water extract and ethanol extract of P. leptocephalus was investigated. Our results demonstrated that the LPS-induced response of macrophage and neutrophil was stimulated by extracts of P. leptocephalus favoring the intracellular killing in phagocytosis. Both water and ethanol extracts of P. leptocephalus increased the phagocytic index in macrophage and neutrophil. Phagocytosis is the fundamental process used by macrophages and neutrophils to eliminate invading pathogens while maintaining
Figure 2: Effect of extracts of *P. leptocephalus* LPS-activated macrophages phagocytic index (A), ROS production (B), NO production (C) and lysosomal enzyme activity (D). EEP: ethanol extract. AEP: aqueous extract. Data are means ± S.D. (n=3); the letters indicates the concentration response difference. Significant differences are indicated by different stars * P < 0.05. ** P < 0.01. *** P <0.001 vs LPS-treated control. (Tukey’s test for multiple comparisons).

Figure 3: Effect of extracts of *P. leptocephalus* LPS-activated neutrophils phagocytic index (A), ROS production (B), NO production (C) and lysosomal enzyme activity (D), MPO activity (E) and Neutrophils’ adherence (F). EEP: ethanol extract. AEP: aqueous extract. Data are means ± S.D. (n=3); the letters indicates the concentration response difference. Significant differences are indicated by different stars * P < 0.05. ** P < 0.01. *** P <0.001 vs LPS-treated control. (Tukey’s test for multiple comparisons).
homeostasis (Eileen and Carlos, 2017). Thus, the result above showed that *P. leptocephalus* stimulate immune defense, and it is consistent with the effect of another species, *Polyporus rhinoceros*, whose isolated polysaccharides is a potential immunomodulatory agent for cancer immunotherapy (Liu et al., 2016).

Phagocytosis process is clearly fundamental to macrophage and neutrophil function because they destroy pathogens in part through ROS as H₂O₂ or reactive nitrogen species (RNS) as NO (Chan-Zapata et al., 2018; Flannagan et al., 2015). In this study, results showed that these extracts of *P. leptocephaalus* upgraded the production of ROS and NO, in macrophage and neutrophils. This has been reported for many mushroom species such as *Agaricus blazei* Murrill, which promotes production of ROS and NO production in granulocytes of human whole blood (Bernardshaw et al., 2007).

During an infection, innate activation of macrophages or phagocytosis results in secretion or synthesis of lysosomal enzymes, such as lysosomal acid phosphatase and myeloperoxidase. MPO is an essential enzyme in the production of one of the most potent compounds, hypochlorous acid, which is particularly important in the process of removing pathogens phagocytized by neutrophils and monocytes (Liu et al., 2017; Dale et al., 2008; Klebanoff et al., 2013). Lysosomal acid phosphatase is a degradative enzyme, present in the lumen of the phagolysosome which degrade most phagocytized microorganisms (Uribe-Querol and Rosales, 2017). In this study, results showed that the extracts of *P. leptocephaalus* elevated the production of lysosomal acid phosphatase in macrophage and neutrophil, and production of myeloperoxidase in neutrophil. This demonstrates the potent stimulatory activity of at the extracts of *P. leptocephaalus* in immune response.

Adherence process is evidently essential to phagocytosis or destruction of pathogens because the engulfment phase is initiated later after adherence of the pathogen to the surface of phagocytes through recognition receptor (Zarbock, and Ley, 2009; Boudoukha et al., 2016; Kuntz and Kunz, 2014). In the present study, the effect of *P. leptocephaalus* extracts on neutrophil adherence was investigated. Exposure of peritoneal neutrophils to the AEP and EEP exerted an important stimulatory effect on neutrophil adherence. These findings indicate that AEP and EEP appear endowed with the ability to stimulate the adherence of neutrophils depending on the concentration of the extracts. Given that adherence is important for the phagocytic/microbicidal activity of neutrophils, the upgrade in this quality supports the idea of the extracts-induced increase in the phagocytic indexes.

*Polyporus* species are used in folk herbal medicine for its high content of bioactive compound that gives significant medicinal properties (Liu et al., 2016). In this study, ethanol extract of *P. leptocephaalus* were found to be a little toxic to peritoneal cells as assessed by the MTT test, but this extract exhibited several immune-stimulatory properties in low doses. Based on the results of this study, *P. leptocephaalus* extracts was more potent in stimulation of immune response than its cytotoxicity.

**CONCLUSION**

Results of the present investigation clearly indicate that water and ethanol can increase the activity of macrophages and neutrophils. *P. leptocephaalus* may be considered as an interesting source of immunomodulatory agents with potential pharmacological applications in pathologies in which an unhealthy activation of macrophages and neutrophils are involved.

**ACKNOWLEDGEMENTS**

The authors are thankful to the Institute of Research and Agricultural Development (IRAD), Bambui in Bamenda, Cameroon, for offering the facilities used to carry out these researches. The authors are also indebted to Dr. Tonjock Rosemary Kinge for helping with the identification of mushroom species.

**REFERENCES**


