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ORIGINAL ARTICLE

Inducible Nitric Oxide Synthase Downregulation and Apoptotic Activity of *Pleurotus florida* (Oyster Mushroom)

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ABSTRACT

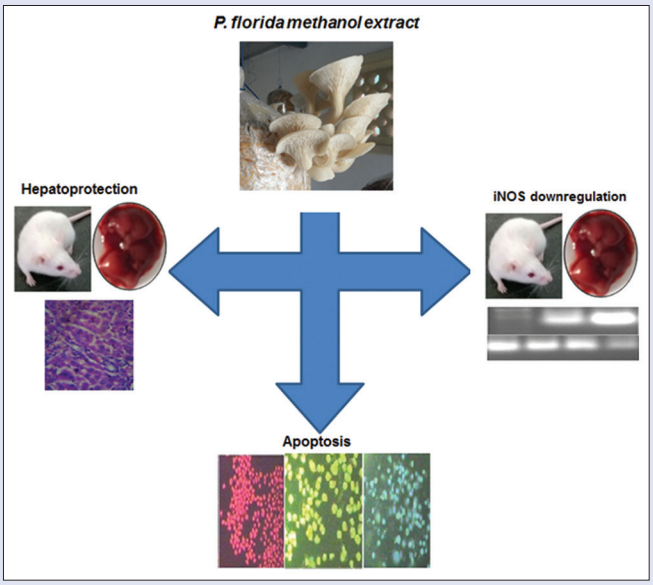
Background: *Pleurotus florida* is an edible delicious mushroom grown on large scale in different parts of the world. **Objective:** The objective of this study is to test the hepatoprotective, cytotoxic, and apoptotic activities of methanol extract of *P. florida*. **Materials and Methods:** 1.5 µg lipopolysaccharide (LPS)/25 g body weight was used to induce hepatic inflammation in Baggs albino mice strain c followed by treatment with varied concentrations of *P. florida* methanol extract. Hepatoprotective activity of mushroom extract was studied examining the liver sections. Total RNA of hepatocytes was isolated and reverse transcriptase-polymerase chain reaction (RT-PCR) of inducible nitric oxide synthase (iNOS) gene was performed. The mushroom extract was evaluated for its cytotoxic and apoptotic activity against human cervical carcinoma cells using 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide and 4′-6-diamidino-2-phenylindole assay, propidium iodide, acridine orange/ethidium bromide staining methods. **Results:** High hepatoprotection with no abnormalities was observed at 125 µg of mushroom extract. RT-PCR showed significantly reduced transcriptional expression of iNOS at 125 µg concentration of mushroom extract. The methanol extract of *P. florida* showed cytotoxic activity with IC_{50} of 47.11 ± 0.01 µg/mL and effectively induced apoptosis in a concentration-dependent manner. **Conclusion:** The results of the present study revealed that *P. florida* is not only a nutritionally valuable food but also an important source for therapeutic compounds.

Key words: Apoptosis, inflammation, mushroom, *Pleurotus*, reverse transcriptase-polymerase chain reaction

SUMMARY

- *Pleurotus florida* methanol extract protected the Baggs albino mice strain c liver from lipopolysaccharide-induced inflammation
- Upregulated nitric oxide synthase gene has been inhibited by *P. florida* extract in a concentration-dependent manner
- 47.11 ± 0.01 µg/mL concentration of *P. florida* extract induced cytotoxicity against human cervical carcinoma cells followed by apoptosis.

Abbreviations used: iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; MTT: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide; PI: Propidium iodide; AO: Acridine orange; EtBr: Ethidium bromide; DAPI: 4′-6-diamidino-2-phenylindole; PBS: Phosphate-buffered saline; COX-2: Cyclooxygenase-2; NO: Nitric oxide; BALB/c: Baggs albino mice strain c; RT-PCR: Reverse transcriptase-polymerase chain reaction; HeLa: Human cervical carcinoma; DMEM: Dulbecco's modified Eagle's medium



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INTRODUCTION

Mushrooms are nutritionally functional food and a rich source of physiologically beneficial and nontoxic medicines. Many mushroom species have been discovered to produce hundreds of novel metabolites such as polyphenolics, terpenoids, ergosterols, and volatile organic compounds.[1] Mushroom extracts have anticancer,[2] antibacterial, antiviral,[3] antihypoglycemic,[4] and immunomodulatory[5] applications. As mushroom metabolites act as adaptogens and immunostimulants, it could be considered as antitumor agents for clinical usage.

Mushroom contains various bioactive compounds namely polysaccharides, vitamins, terpenes, steroids, and amino acids.[6] Polysaccharides, mainly α or β glucans, protein-bound polysaccharides, and glycoproteins exhibit immunomodulatory activity by involving in

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enhancing the production of cytokines, activation of natural killer cells, and expression of inducible nitric oxide synthase (iNOS).[7]

Oyster mushrooms (*Pleurotus*) are a popular food used for many years by humans worldwide.[8] It has been used as medicinal mushrooms as it contains bioactive compounds, namely, lectins, phenolics, and polysaccharides with wide spectrum of pharmacological properties including immunomodulatory, antiproliferative, antioxidant, antitumor, immunoenhancing, and anticancer activities. One such important species, *Pleurotus florida,* is grown on a large scale in different parts of the world including India. *P. florida* exhibits biological activities such as antioxidant, immunostimulator, antitumor, anti-inflammatory, anticataract and anthelmintic.[9-12]

MATERIALS AND METHODS

Source and extraction of mushroom

P. florida was collected from mushroom hut, Kongunadu Arts and Science College, GN Mills, Coimbatore, Tamil Nadu, India. A sample of 500 g of fresh material was shade dried and powdered. A sample of 100 g of mushroom powder was extracted with 500 mL of methanol at room temperature to yield crude extract in Soxhlet apparatus.

Animal handling and experimental design

Animals were cared and handled as per the regulations of Council Directive (CPCSEA no: 659/02/a) about good laboratory practice. All animal experiments were performed in the laboratory according to the ethical guidelines suggested by the Institutional Animal Ethics Committee. Adult Baggs albino mice strain c (BALB/c) weighing 25 g were used. Mice were provided by KMCH Hospital, Coimbatore, micro isolators with autoclaved bedding and cages were used for the maintenance and fed with autoclaved food pellets and deionized water. The mice were kept under standard humidity conditions, temperature (25°C ± 2°C), and light (12 h light/dark). Sterile procedures were used in handling these animals to prevent unintentional introduction of microbes that could activate iNOS production.

Adult BALB/c mice were randomly assigned into four groups: normal group ($n = 3$), mushroom extract treated ($n = 3$), lipopolysaccharide (LPS) treated ($n = 3$), and experimental group (LPS + mushroom extract) ($n = 15$). Mice were maintained on restricted once a day diet and administered with LPS (1.5 µg/25 g body weight) and varied concentrations of mushroom extract (25, 50, 75, 100, and 125 µg/25 g body weight). The schedule for feeding and mushroom extract treatment was illustrated in Figure 1.[13] After 4–5 h LPS treatment, animals were sacrificed and livers were excised. A part of liver was fixed in 10% formalin for histopathological analysis, and another was taken for iNOS gene downregulation studies.

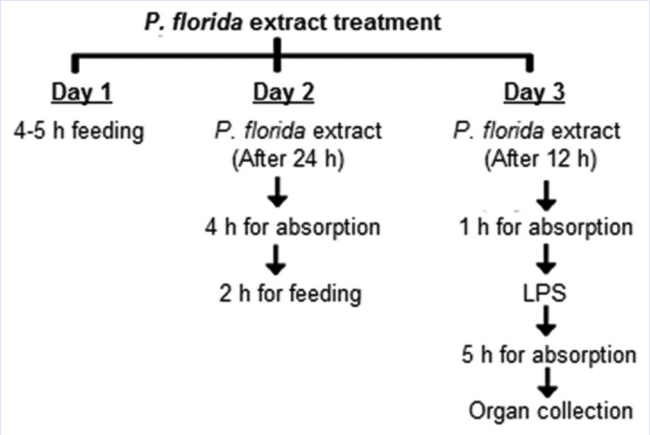

Figure 1: Schematic representation of feeding and *Pleurotus florida* extract treatment

Histopathological analysis

The tissues were impregnated with histology grade paraffin wax (melting point 58°C–60°C) at 60°C for two changes of 1 h each. The tissues impregnated with wax were embedded in paraffin blocks, mounted, and cut with rotary microtome at 3 µM thickness. The sections were stained in Ehrlich's hematoxylin (0.75%) for 8 min. Further, they were counter stained in 1% aqueous eosin (1 g in 100 mL water) for 1 min, and the excess stain was removed by washing in tap water and allowed to dry. When the sections cooled, they were mounted in DPX (Distrene, Plasticiser, and Xylene) mount (the sections wetted in xylene were inverted on to the mount kept on cover slip). The architecture was observed at low power objective lens with ×10. The hepatocyte injury and other tissue morphology were observed under high-power dry objectives.

Inducible nitric oxide synthase gene down regulation

Total RNA extraction and cDNA synthesis

Total RNA was extracted from 300 mg of liver tissue sample using Trizol reagent (GeNei, Bengaluru). Synthesis of cDNA from isolated total RNA was performed using a cDNA kit (HELINI, Chennai). Briefly, 20 µL reaction mixture was prepared through the addition of 6 µL of cDNA reaction mix (1X), 2 µL of oligo dT, 2 µL of random hexamer, 2 µL of enzyme mix, 2 µL of isolated RNA, and 6 µL of nuclease-free water in a 0.2 mL PCR tube. Reverse transcription was carried out for one cycle at 42°C for 30 min and inactivation at 95°C for 2 min. Finally, the cDNA was stored at-20°C for further use.

Reverse transcriptase-polymerase chain reaction analysis

The reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was performed on the aliquots of the cDNA preparations for detecting iNOS gene expression. The sequence of oligonucleotide primers used was as follows for iNOS: [14] iNOS_F (5'-ATGGACCAGTATAAGGCAAGC-3') and iNOS_R (5'-GCTCTGGATGAGCCTATATTG-3'). Amplification reactions with the following cycles were carried out in a gradient thermocycler (Applied Biosystems, California): initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation for 30s at 92°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C, followed by final extension for 10 min at 72°C. PCR reaction mix contained 5 µL of PCR master mix (×2) (Merck Specialties, Mumbai) solution, 1 µL of forward primer, 1 µL of reverse primer, 1 µL of cDNA, and 2 µL of nuclease-free water.

Cell line studies

The human cervical carcinoma (HeLa) cell line, purchased from NCCS, Pune, was cultured in a 25 cm^2 cell culture flask-containing DMEM supplemented with 10% fetal bovine serum, penicillin (100U/mL), and streptomycin (100 µg/mL). The cell lines were incubated in humidified incubator at 37°C with 5% CO_2.

3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay

Each well of 96 well plate was added with 1×10^4 cells/100 µL medium and incubated for 24 h. Then, varied concentrations of mushroom extract were added to wells and further incubated for 48 h. 20 µl of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide

(5 mg/mL) in phosphate-buffered saline (PBS) was added to each well and incubated for 4 h at 37°C. After removing the medium, 100 µL of dimethyl sulfoxide was added to each well and incubated for 10 min at 37°C. The plate was read at 570 nm using a microplate reader.[15] % of cell viability = ([AB − AA]/AB) × 100, where AB, absorption of blank sample, AA, absorption of test sample.

Propidium iodide staining

Apoptosis was assessed using uptake of fluorescent dye propidium iodide (PI).[16] In a 24-well plate, HeLa cells (1×10^4 cells/well) were seeded and grown until confluent (80%). The cells were treated for 24 h with varied concentrations (25–125 µg/mL) of mushroom extract. The cells were washed with ice-cold PBS and fixed for 30 min with 70% ethanol. The plates were washed again and stained with 200 µL of PI (500 µM) for 1 h; further, the plates were washed twice, and the apoptotic cells were observed under Olympus CKX42 fluorescence microscope.

Acridine orange/ethidium bromide dual staining

Morphological analysis of apoptosis by acridine orange/ethidium bromide (AO/EtBr; Hi-media, India) dual staining was performed.[17] Briefly, 2×10^4 cells per well was seeded in a 24-well plate and treated with different concentrations of mushroom extract (25–125 µg/ml) for 24 h. After incubation, 10 µL of 1 mg/ml AO and EtBr mixture was added to each well. Nuclei were visualized and photographed under Olympus CKX42 fluorescent microscope.

4′-6-diamidino-2-phenylindole staining

Cells (1×10^4 cells/well) were seeded in a 24-well plate and treated with varied concentrations of mushroom extract (25-125 µg/ml) for 24 h. After treatment, the cells were collected and fixed with acetic acid/methanol (1:3) solution at room temperature for 10 min and then incubated in 4'-6-diamidino-2-phenylindole (DAPI) (1 µg/ml) for 5 min. Further, the cells were washed thrice with PBS, and the cells were examined using Olympus CKX42 fluorescence microscope.[18]

RESULTS

In vivo hepatoprotective studies

Histopathological study of the liver of BALB/c mice control group showed no obvious abnormality. The hepatocytes were seen intact with central vein presenting normal architecture [Figure 2a]. In the present study, 1.5 µg LPS/25 g body weight induced hepatic tissue damage with severe hemorrhage and cell necrosis. The section revealed the distended hepatic central vein and severe inflammation with lymphocytic cellular infiltration [Figure 2b]. Mushroom extract alone (1 mg/25 g body weight) treated liver section showed normal architecture with the absence of inflammation or any other cell necrosis [Figure 2c]. 25 µg of mushroom extract did not show any positive effect on hepatoprotection in LPS-treated animals. Severe necrosis with inflammation, sinusoidal dilatation, congestion, and hepatic lesions was observed [Figure 2d]. The histology of the hepatocytes was slightly altered with mild inflammation, cellular necrosis, and sparse lymphocytic infiltration at 50 µg of the mushroom extract [Figure 2e]. At 75 µg of mushroom extract, the hepatocytes appeared with normal architecture and no visible inflammatory cell infiltration in tissue section [Figure 2f]. High hepatoprotection with no abnormalities was observed at 100 and 125 µg of mushroom extract [Figure 2g and h].

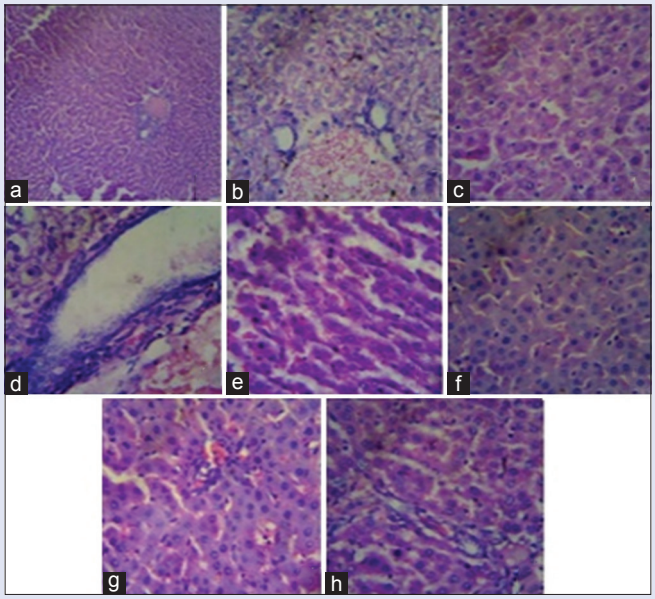

Figure 2: Hepatoprotective effect of *Pleurotus florida* extract: (a) Normal liver, (b) lipopolysaccharide treated, (c) extract treated, (d-h) 25, 50, 75, 100, and 125 µg/mL of *Pleurotus Florida* extract-treated lipopolysaccharide-induced mice

In vivo Inducible nitric oxide synthase downregulation

Total RNA was isolated from hepatocytes, and RT-PCR was performed to study the downregulation of iNOS. The results of RT-PCR revealed that 1.5 µg LPS-treated liver cells had overexpression of iNOS [Figure 3A], which was then downregulated at transcriptional level followed by treatment with mushroom extract. While increasing the concentration of mushroom extract, the iNOS gene was downregulated dose-dependently [Figure 3B].

In vitro anticancer studies

Mushroom extract showed a considerable cytotoxicity against HeLa cells and its 50% inhibitory concentration was estimated as 47.11 ± 0.01 µg/mL. A remarkable elevation in the number of apoptotic cells was observed in all staining techniques including PI, Ao/Etbr, and DAPI staining based on increasing concentrations of mushroom extract with highly induced apoptosis at 125 µg/mL mushroom extract [Figures 4-6].

DISSCUSSION

LPS is widely used to examine the mechanism of inflammation that produces hepatic necrosis followed by hepatic failure.[19] Histopathology data showed that mushroom extract is a potent hepatoprotective agent. The hepatoprotective activity of mushroom extract observed in this study may be in relation with the reduction of LPS-induced nuclear factor-κB-mediated mitogen-activated protein kinase (MAPKs).[20] A similar result reported the suppressed inflammation with decrease in paw thickness by *P. florida* extract in carrageen and formalin-induced inflammation.

Overproduction of the inflammatory mediator NO, synthesized by iNOS, is involved in hepatic inflammation. Therefore, suppressing iNOS expression is believed to be a good approach for the treatment of inflammatory diseases.[21] Hepatoprotection of *P. florida* might be attributed by the presence of bioactive constituents in methanol extract.[22] This is supported by the earlier study reporting that phenolic compounds and flavonoids showed antiinflammatory activity by controlling the levels of various inflammatory cytokines and mediators including iNOS and cyclooxygenase-2.[23]

Apoptosis is an important mechanism for the tumor cell growth inhibition.[24] Deepalakshmi and Mirunalini[25] revealed the cytotoxic effect of *P. ostreatus* against mammary carcinoma cells attributed by the presence of bioactive constituents such as tetradecanoic acid and triacontane.

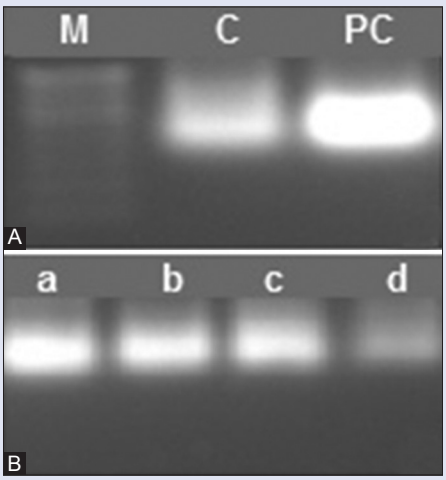

Figure 3: Inducible nitric oxide synthase gene downregulation capacity of *Pleurotus florida* extract: (A) Overexpression of inducible nitric oxide synthase on lipopolysaccharide treatment, (B) *Pleurotus florida* extract treatment showed dose-dependent downregulation of inducible nitric oxide synthase gene, M, 100bp ladder; C, control; PC, positive control; (a-d) 25, 50, 75, and 100 µg/mL of *Pleurotus florida* extract-treated lipopolysaccharide-induced mice

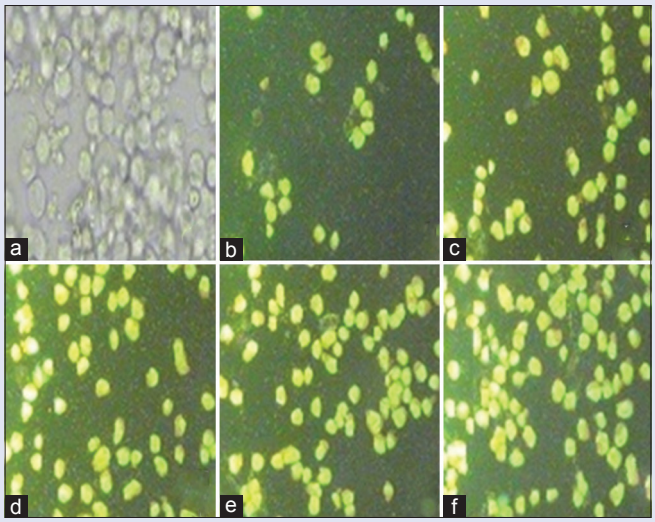

Figure 5: Apoptosis induction by *Pleurotus florida* extract on human cervical carcinoma cells was observed using acridine orange/ethidium bromide staining: (a) Control, (b-f) cells treated with *Pleurotus florida* extract of 25, 50, 75, 100, and 125 µg/mL, respectively

A similar finding of positive Ao/Etbr staining in HepG2 cells have been reported earlier on treatment with the polysaccharide from *P. nebrodensis*.[26]

CONCLUSION

The results of the present study revealed that *P. florida* is not only a nutritionally valuable food but also an important source for therapeutic compounds.

Acknowledgements

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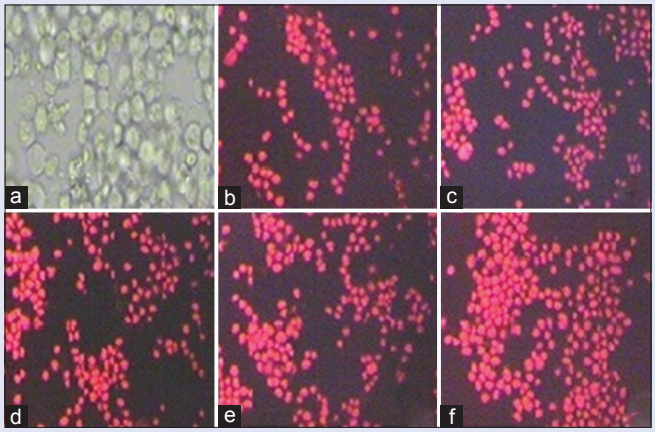

Figure 4: Apoptosis induction by *Pleurotus florida* extract on human cervical carcinoma cells was observed using propidium iodide staining: (a) Control, (b-f) cells treated with *Pleurotus florida* extract of 25, 50, 75, 100, and 125 µg/mL, respectively

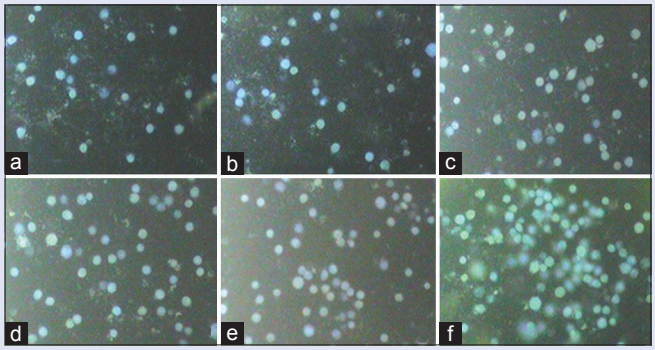

Figure 6: Apoptosis induction by *Pleurotus florida* extract on human cervical carcinoma cells was observed using 4′-6-diamidino-2-phenylindole staining: (a) Control, (b-f) cells treated with *Pleurotus florida* extract of 25, 50, 75, 100, and 125 µg/mL, respectively

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Conflicts of interest

There are no conflicts of interest.

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