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Original Article

Functional Ingredients and Medicinal Prospects of Ethanol Extract from *Macrocybe lobayensis*

Somanjana Khatua, Krishnendu Acharya*

ABSTRACT

Introduction: Mushrooms are a good source of bioactive components and have been traditionally consumed across the globe for maintaining health. However, information concerning medicinal activity of ethnically popular macrofungi still remains limited. **Aim:** Therefore, the present study was aimed for determination of functional constituents and therapeutic efficacy of a less explored mushroom, *Macrocybe lobayensis* that has been habitually used in local diets. **Methods:** In this context, an ethanol extract was prepared from dried basidiocarps and characterized by spectrophotometry and HPLC to identify bioactive constituents. Alongside, the fraction was also subjected for evaluation of medicinal properties namely antioxidant and antibacterial effects. **Results:** The formulation was found to be consisted mainly of phenolic compounds (pyrogallol> cinnamic acid> *p*-coumaric acid> *p*-hydroxybenzoic acid). As a result, the fraction exhibited strong antioxidant activity evident by the ability of quenching DPPH and ABTS radicals, chelating capacity of Fe^{2+} as well as reducing components with EC_{50} of 1000 to 2264 µg/ml. Besides, the formulation also emerged as a potent source of antibiotic as it inhibited growth of investigating microbes in order of *Staphylococcus aureus*> *Escherichia coli*> *Bacillus subtilis*> *Salmonella typhimurium*> *Listeria monocytogenes*. **Conclusion:** Thus, the outcome might encourage use of *M. lobayensis* as natural antioxidant and antibacterial agent in pharmaceutical, cosmetic or food industries.

Key words: Antibacterial activity, Antioxidant potential, Ethanol extract, Wild edible mushroom

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INTRODUCTION

Oxidation is an essential process in living organisms that enables body to transform nutrients into energy. However, a significant portion of oxygen is incompletely reduced during respiration leading to generation of reactive oxygen species (ROS).[1] These free radicals are not harmful at low concentration as they play necessary role in normal physiological conditions such as signal transduction and immune function.[2] Nevertheless, the situation becomes bizarre when external factors like ozone, tobacco smoke and stress also take part in radical generation inside body. Gradually, level of ROS exceeds the defence mechanism and the circumference becomes extremely destructive to organism. These radicals can then damage nucleic acid, oxidize proteins and cause lipid peroxidation affecting many cellular functions.[3] Thus, neutralizing free radicals is important for cell protection and antioxidants play an essential role in this context. Consequently, extensive evidence indicates that ample intake of dietary antioxidants may be of great benefit in improving quality of life.[4]

Besides, worldwide escalation in antibiotic resistance has also become a major public health problem. It has recently been estimated that about 70% bacteria causing hospital acquired infections are now resistant to at least one drug used for treatment.[5] These medicine resistant infection is spreading devastatingly that it resulted about 700,000 deaths in last year and will cause more if left unaddressed.[6] Therefore, search for novel antimicrobial agent with acceptable efficacy and safety has emerged as one of the key strategies for the management of contagious diseases.[7]

Several such therapeutic components have been identified from countless biological sources and among them phenolic constituents have currently gained importance due to their large array of physiological properties. These compounds belong to a diverse group of secondary metabolites characterised by an aromatic ring with one or more hydroxyl substituents and play a major role in protection of oxidation processes.[8] Corresponding structure makes them ideal for trapping free radicals; therefore they can easily act as reducing agents, ROS scavengers, singlet oxygen quenchers or metal ion chelators.[9] Besides, phenolic compounds have also been evidenced to execute antibacterial efficacy

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where the number and site(s) of hydroxyl groups determine their toxic effect to microorganisms.[10]

Recently, numerous studies have conclusively demonstrated that many bioactive phenolics are an important constituent of mushrooms. Consequently, edible macrofungi have attracted more and more attention in recent times as commercial source of antioxidant and antibacterial agent.[11-19] In spite of the increasing research, information concerning therapeutic activity of ethnically popular mushrooms still remains limited. One such less examined fungus is *Macrocybe lobayensis* that has long been accepted as food in Kerala, Uttaranchal and West Bengal of India.[20] In this backdrop, the present work was carried out to evaluate putative therapeutic abilities namely antioxidant and antibacterial effects of an ethanol extract prepared from its basidiocarps. Besides, the fraction was also assessed qualitatively and quantitatively for estimation of bioactive phenolic compounds.

MATERIALS AND METHODS

Collection and authentication

Fresh basidiocarps were assembled from natural habitat at coastal area of West Bengal, India and identified following standard literature.[21] A voucher specimen was deposited in the same department with accession number of CUH AM483 following the method of Pradhan *et al.* (2015).[22]

Preparation of ethanol extract

Lyophilized powder (10 gm) was extracted by stirring with 200 ml of ethanol for overnight and subsequently separated by Whatman filter paper. Residue was then re-extracted with 100 ml of ethanol and the combined ethanolic formulation was evaporated at low temperature to reduce volume. The fraction was stored at -20°C in dark bottle until analysis, for no more than one month.

Determination of major bioactive compounds

To estimate total phenolic compounds, ethanol extract was mixed with Folin-Ciocalteu reagent and 35% sodium carbonate solution. Absorbance was measured at 725 nm where gallic acid was used as standard. Flavonoid was quantified using potassium acetate as well as aluminium nitrate and quercetin was considered as a reference. The amount of ascorbic acid was determined by titration method using 2, 6-dichlorophenol indophenol dye. Contents of carotenoids like β-carotene and lycopene were detected by measuring absorbance at 453, 505 and 663 nm.[20]

HPLC profiling

Dried extract dissolved in HPLC grade methanol was filtered and 20 µl of the filtrate was analysed by HPLC system equipped with an Agilent Eclipse Plus C_{18} column (100 mm × 4.6 mm, 3.5 µm). Elution was carried out by using eluent A (acetonitrile) and eluent B (0.1% v/v aqueous phosphoric acid) through a gradient procedure: 0-5 min, 5% A; 5-10 min, 15% A; 10-15 min, 40% A; 15-20 min, 60% A; 20-22 min, 90% A. The compounds were identified by comparing UV spectra and retention time with authenticate standards namely gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, ferulic acid, myricetin, salicylic acid, quercetin, cinnamic acid and pyrogallol.[23]

Determination of total antioxidant capacity by phosphomolybdenum method

The reaction mixture consisted of 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium sulphate and 4 mM ammonium molybdate) and 0.3 ml sample. The resultant solution was incubated at 95°C for 90 min and absorbance was measured at 695 nm against blank. Different concentrations of vitamin C (1–30 µg) were used to procure a standard curve. Total antioxidant effect was depicted as the number of equivalents of ascorbic acid.[24]

DPPH radical scavenging assay

To determine DPPH radical scavenging activity, ethanol extract at various concentration (100-1000 µg/ml) was mixed with 0.004% DPPH solution in a reaction mixture of 200 µl in 96 well microtiter plate. The plate was incubated for 30 min in dark followed by detection of final colour at 595 nm using microplate reader (Bio-Rad iMarkTM Microplate Reader, USA).[25]

ABTS radical scavenging assay

Extent of radical quenching potentiality of the fraction was again tested by using ABTS. Accordingly, radicals were generated freshly by adding 2.45 mM of potassium persulfate in 7 mM ABTS solution and the mixture was incubated overnight. Further, the reactant was diluted to 0.7 absorbance and radicals were allowed to react with ethanol extract at variable doses (100-1000 µg/ml) in 200 µl reaction mixture in 96 well plate. The plate was shaken for 10 seconds at medium speed and absorbance was noticed at 750 nm.[25]

Chelating ability of ferrous ion

In addition, the assay of chelating ability of ferrous ion was also followed; hence 5 µl ferrous chloride (3 nM) was mixed with ethanol formulation at different level (100-1000 µg/ml). Then, 10 µl ferrozine (0.12 nM) was added and absorbance was estimated at 595 nm following incubation for 10 min using microplate reader.[25]

Estimation of antibacterial action

Bacillus subtilis ATCC® 6633™ (MTCC 736), *Listeria monocytogenes* ATCC® 19111™ (MTCC 657), *Staphylococcus aureus* ATCC® 700699™, *Escherichia coli* ATCC® 25922™ and *Salmonella typhimurium* ATCC® 23564™ (MTCC 98) were utilized for the experiment. After 24 h incubation, antibacterial effect was estimated by determining minimum inhibitory concentration (MIC) values.[26]

Statistical analysis

All values are expressed as mean ± standard deviation (SD) of triplicate values. Statistical analysis was implemented using Student`s *t* test and $p < 0.05$ was denoted as being statistically significant. The treatment was carried out using Microsoft® Office Excel (Microsoft®, USA).

RESULTS AND DISCUSSION

Natural antioxidants in mushroom such as phenols and flavonoids are polar in nature, so efficient extraction could definitely be achieved by using any polar solvent like ethanol, methanol and acetone. Among them, ethanol is known as a better choice for phenol extraction and considered safe for human consumption.[27] Likewise, ethanol was selected as an isolation solvent to prepare fraction from *M. lobayensis* and the formulation was found to be yellowish in colour with moderate extractive yield (3 ± 0.5). The major chemical component was detected to be phenol (12.58 ± 0.57 µg gallic acid equivalent/mg of extract) followed by flavonoid (10.95 ± 0.89 µg quercetin equivalent/mg of extract). In addition, ascorbic acid was also determined to be present in moderate extent i.e. 1.09 ± 0.38 µg/mg of extract. However, insignificant amount of β-carotene and lycopene were estimated such as 0.30 ± 0.07 µg/mg and 0.28 ± 0.08 µg/mg of dry extract respectively. Overall results indicated that the ethanol fraction from *M. lobayensis* consisted of phenolics in higher magnitude than its methanol extract.[20]

Further, a molecular fingerprint was generated with the help of HPLC using eleven standard phenolic compounds. The chromatogram indi-

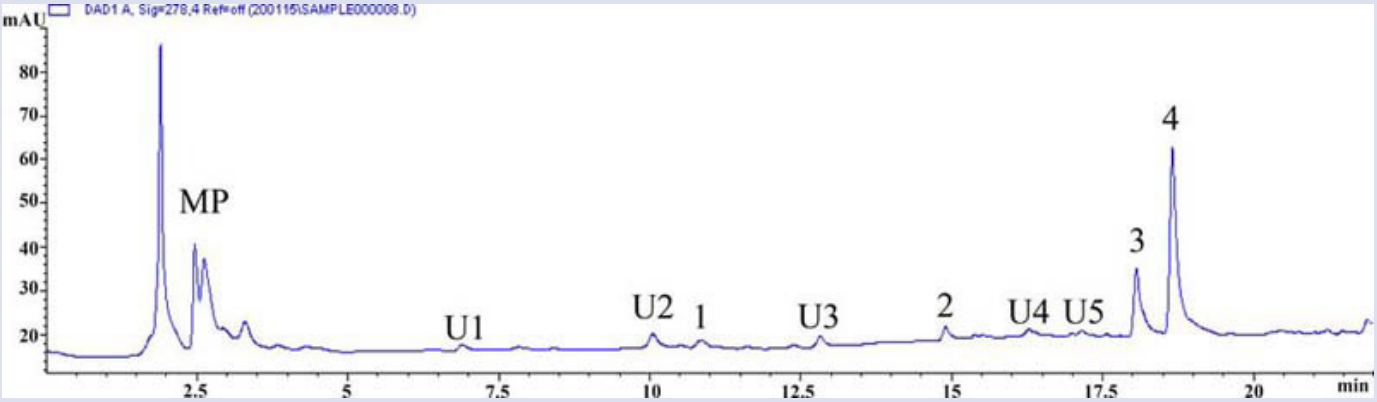

Figure 1: HPLC chromatogram of ethanol extract from *Macrocybe lobayensis*
(MP: mobile phase, peaks: 1: *p*-hydroxybenzoic acid (Rt 10.84 min), 2: *p*-coumaric acid (Rt 14.89 min), 3: cinnamic acid (Rt 18.05 min), 4: pyrogallol (Rt 18.65 min). Peaks designated as U1 to U5 represent unidentified phenols that were eluted at retention time of 6.88, 10.04, 12.81, 16.27 and 17.15 min respectively.

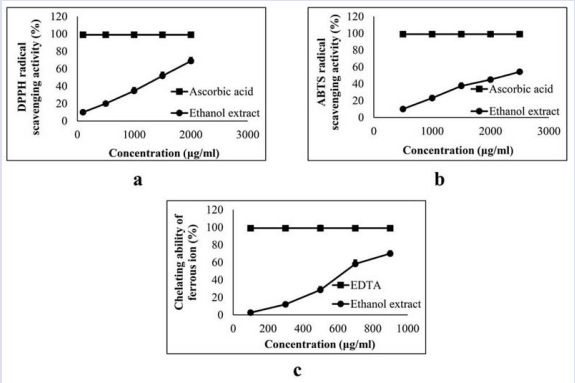

Figure 2: Antioxidant activity of ethanol extract prepared from *Macrocybe lobayensis*
(a) DPPH radical scavenging activity (b) ABTS radical scavenging activity (c) Chelating ability of ferrous ion. Results represent mean ± standard deviation of at least three independent experiments.

cated presence of at least 11 constituents in ethanol fraction of *M. lobayensis* of which four have been tentatively identified (Figure 1). Analysis showed that pyrogallol was the major element (4.72 ± 0.79 µg/mg of extract) present in the extract, followed by cinnamic acid (0.25 ± 0.01 µg/mg of extract). Besides, *p*-hydroxybenzoic acid (0.06 ± 0.01 µg/mg of extract) and *p*-coumaric acid (0.08 ± 0.01 µg/mg of extract) were also detected. Thus, the main ingredients in the fraction were pyrogallol and cinnamic acid which have also been identified in other edible mushrooms.[11,19-20]

Total antioxidant capacity in extracts provide an integrated parameter rather than a simple sum of measurable antioxidants as it considers a cumulative action of all antioxidants present in sample.[28] Phosphomolybdenum method is a good system for evaluation of total antioxidant capacity. The assay is based on formation of green phosphate/Mo (V) complex by reduction of Mo (VI) to Mo (V) by the antioxidant compound and at acidic pH.[28] The formulation showed 8.15 ± 1.44 µg ascorbic acid equivalent/mg of dry extract antioxidant capacity.

DPPH· is organic nitrogen radical and accepts hydrogen or electron to achieve stable diamagnetic form. The advantage of this assay is that DPPH· does not have to be generated, in contrast to other scavenging assays. DPPH· can be deactivated by antioxidative agent owing to ability of donation electron without being free radical itself. Thus the degree of DPPH· scavenging can be used to magnitude hydrogen donating ability of antioxidants. In methanol solution, DPPH· produces violet colour which upon reduction fades to yellow coloured diphenylpicrylhydrazine. Thus, a lower absorbance at 517 nm indicates a higher radical scavenging activity of extract.[4] As presented in Figure 2a, DPPH· scavenging ability of the ethanol extract increased sharply from 34.54 to 51.81% when the concentration was incremented from 1000 to 1500 µg/ml. Ascorbic acid showed the most potent scavenging activity with extremely low EC_{50} value (Table 1). However, EC_{50} value of the ethanol

Table 1: Antioxidant activity of ethanol extract isolated from *Macrocybe lobayensis*.
The effects are presented in terms of EC_{50} values (mean ± standard deviation; n= 3) corresponding to 50% of antioxidant activity except total antioxidant capacity assay. Ascorbic acid was used as standard in DPPH radical quenching, ABTS radical scavenging and total antioxidant capacity protocols, while EDTA was adopted as a positive control in chelating ability of ferrous ion method. In each row, different letters mean significant differences between the sample and standard ($p< 0.05$).

Antioxidant assays		Ethanol extract	Standard
EC_{50} value (µg/ml)	Scavenging ability of DPPH radical	1050.21 ± 49.01[a]	2.15 ± 0.25[b]
	Scavenging ability of ABTS radical	2264.25 ± 10.9[a]	3.18 ± 0.01[b]
	Chelating ability of ferrous ion	1000.38 ± 30.19[a]	2.54 ± 0.5[b]
Total antioxidant activity (¼g ascorbic acid equivalent/mg of dry extract)		8.15 ± 1.44	_

Table 2: Antibacterial activity of ethanol extract isolated from *Macrocybe lobayensis* as determined by minimum inhibitory concentration value (µg/ml) (mean ± standard deviation; n= 3).

In each row, different letters mean significant differences between the sample and standard ($p< 0.05$).

Type of bacteria	Name of bacteria	Ethanol extract	Streptomycin
Gram positive	*Listeria monocytogenes*	380.7 ± 23.86[a]	4.68 ± 0.17[b]
	Bacillus subtilis	209.49 ± 43.61[a]	5.61 ± 0.01[b]
	Staphylococcus aureus	49.85 ± 10.1[a]	6.29 ± 0.16[b]
Gram negative	*Escherichia coli*	171.6 ± 33.29[a]	5.41 ± 0.11[b]
	Salmonella typhimurium	268 ± 19.84[a]	5.09 ± 0.03[b]

extract was found to be lower than that of *Macrocybe crassa*[11] indicating higher activity of *M. lobayensis*.

Further, $ABTS^{\cdot-}$ was also used herein to estimate antioxidant activity of the ethanol extract. In this assay, $ABTS^{\cdot-}$ were produced by persulfate oxidation of $ABTS^{2-}$ and reduced in presence of antioxidant constituent causing decolourization.[29]Analysis specified that the fraction consisted of strong radical scavenging activity that incremented in a dose-dependent mean (Figure 2b). As the concentration ranged from 500, 1000, 1500, 2000 to 2500 µg/ml, inhibition activities of the fraction amplified from 10.69, 23.54, 37.4, 44.96 to 54.27%. Literature review implied that the fraction presented effective antioxidant potential than *Grifola frondosa*, *Lentinula edodes*, *Pleurotus scitrinopileatus*, *Pleurotus eryngii*, *Pleurotus salmoneo-stramineus*, *Trametes versicolor.*[30]

Ferrous ion functions as catalyst for generation of harmful elements like hydroxyl, peroxyl and alkoxyl radicals. Chelating agents possess the ability to stabilize the transition metal and reduce damages caused by free radicals. So moderate Fe^{2+} chelating ability would be beneficial to protect human beings. To determine the effect, ferrozine was used that forms complexes with Fe^{2+} resulting in violet colour solution. When chelating agent is added in the reaction mixture, the complex formation is interrupted, thus colour intensity reduces. Reduction therefore allows estimation of chelating ability of coexisting chelator.[31] As presented in Figure 2c, the fraction chelated 28.56, 58.18 and 70.17% ferrous ions at 500, 700 and 1000 µg/ml respectively. However, the extract demonstrated lower Fe^{2+} ion chelating capacity than that of *M. crassa*.[11]

Antibacterial activity of the ethanol extract from *M. lobayensis* was evaluated against some pathogenic bacteria where streptomycin was used as a standard drug for comparison. The fraction was found to be inhibitory against all the examined Gram positive and Gram negative species (Table 2). The MIC values indicated that the most susceptible bacteria were *S. aureus*, *E. coli* followed by *B. subtilis*. While treatment against *L. monocytogenes* and *S. typhimurium* presented higher MIC values indicating lesser susceptibility. According to literature data, *S. aureus* and *B. subtilis* are the most vulnerable microorganisms to inhibitory action of mushroom extracts.[10] However, the ethanol formulation of *M. lobayensis* exhibited better antibacterial effect against all aforementioned microorganisms than its methanol extract.[20]

CONCLUSION

In conclusion, ethanol extract from *M. lobayensis* emerged as a potential source of biomedicine with respect to antioxidant and antibacterial effects. These putative bioactive potential might be attributed to phenols and flavonoids as they were detected as key ingredients in the fraction. Thus, the studied formulation could be used for dermatological applications, cosmetics and also as supplements in food industry.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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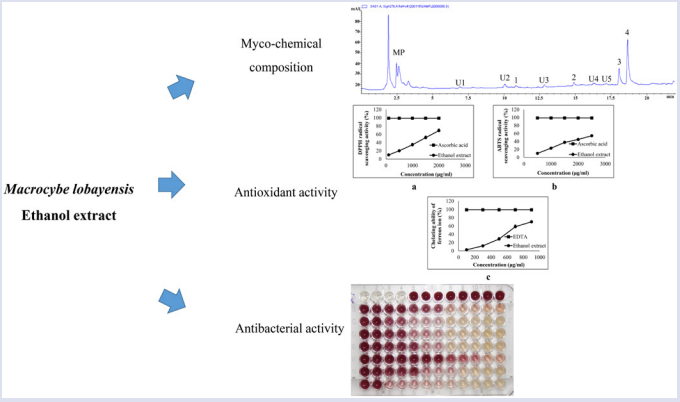
GRAPHICAL ABSTRACT



SUMMARY

- Certain wild macrofungi are popular in indigenous people from time immemorial, although they are not even systematically investigated. Research on them may provide exciting evidences for use of such traditional treasures in favor of human as they have certain biological properties. In the present work, we have highlighted chemical composition and potential therapeutics of ethanol extract from *Macrocybe lobayensis*, a wild edible macrofungus. Our findings revealed that the fraction was effective against various pathogenic microorganisms and free radicals in *in vitro* system. Thus the present study demonstrated *M. lobayensis* as a source of active medicinal ingredients which would greatly inspire scientific world for its further application.

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