

Chemical composition and biological activities of methanol extract from *Macrocybe lobayensis*

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ABSTRACT

Over the generations, *Macrocybe lobayensis* has become an integral part of tribal cuisines of India particularly for its aroma and taste. However, no investigation has been carried out so far on its secondary metabolites that may account for therapeutic potential. Therefore, aim of the present study was to identify major constituents of methanol extract isolated from the wild mushroom and examine spectrum of pharmacological activities. Chemical characterization by spectrophotometer and HPLC depicted that the fraction was mainly consisted of phenolic compounds (*p*-hydroxybenzoic acid < *p*-coumaric acid < cinnamic acid < salicylic acid < pyrogallol); while ascorbic acid and carotenoids were detected in lesser extent. In terms of screening medicinal prospects, the extract presented strong radical scavenging activity (DPPH[•] and ABTS^{•+}), chelating ability of ferrous ion, reducing power and total antioxidant capacity. The methanol formulation also exhibited antibacterial properties against both Gram-positive and Gram-negative strains, with MIC values ranging from 245.13-1585.81 µg/ml. Finally, Hep3B human liver carcinoma cells were challenged with the extract to assess cytotoxic effect and result showed concentration dependent decrease in cell viability (IC₅₀ of 234.31 µg/ml). Overall, features described in this work provides the first attempt to establish medicinal prospects of phenolic compounds from *M. lobayensis* empowering its use in pharmaceutical industry.

INTRODUCTION

Free radicals can be defined as any unstable and highly reactive molecular species that contain an unpaired electron in their atomic orbital. They are derived inside human body either through normal metabolic processes or from exogenous sources like air pollutants, X-rays, cigarette smoke, ozone and industrial chemicals (Khatua *et al.*, 2013). Generally, these compounds are beneficiary to us when present in low or moderate level as they play an important role in ion transportation, destruction of invading pathogens and apoptotic cell signalling (Lü *et al.*, 2010). In contrary, when produced in excess quantity, free

radicals attack molecular components of cell responsible for pathogenesis of a large number of diseases (Valko *et al.*, 2007). Consequently, the human body consists of an antioxidant defence system to protect against harmful effects of radicals. Nevertheless, substantial evidence indicated that ample intake of dietary antioxidants in form of foods or nutrients may help to maintain the antioxidant status inside human body (Alam *et al.*, 2013).

Besides, continual growing emergence of antibiotic unaffected bacterial strains is another major threat to human health (Balouiri *et al.*, 2016). It has been estimated that disseminate and indiscriminate use of antibiotics is the main cause of evolvement of drug resistant bacteria, while consequence of acquired mutation assisting bacterial survival also contributes to non-susceptibility of microorganisms. As a result, the prevalence and incidence of opportunistic infections are increasing significantly not only in hospitalised patients, but also in general population (Shen *et al.*, 2017). Thus, treatment with appropriate therapeutic reagent is the sole way to minimise development of drug resistant strains and control infections (Ren *et al.*, 2014).

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Else, continuous exposure of infectious pathogens stimulates the body defence system to generate excess amount of superoxide radical and nitric oxide through a process called inflammation. Repeated inflammatory signalling damages DNA in hepatocytes of regenerating liver and increases chance of gene mutation related to carcinogenesis. Currently the most common type of liver cancer is hepatocellular carcinoma which is one of the fore most prevalent malignancies in Asia, Europe and Africa. Treatment dealing with the disease is very limited due to non-responsiveness to chemotherapy and radiotherapy (Shiraha *et al.*, 2013). In this background, daily intake of anti-carcinogenic food is considered as the most effective mean for preventing cancer in human. Thus, edible anti-mutagenic compounds obtained from nature is of particular importance as it does not produce any xenobiotic effects on living organisms (Burcu *et al.*, 2014).

Consequently, mushrooms have always been appreciated as nutraceutical food across the globe due to their immense role in prevention of various disorders (Valverde *et al.*, 2015). They accumulate a variety of secondary metabolites, such as phenols, polyketides, steroids and terpenes. Among them, phenolics are one of the major group of dietary components that have proved to be associated with antioxidant (Acharya *et al.*, 2015a, Acharya *et al.*, 2015b, Acharya *et al.*, 2016, Acharya *et al.*, 2017a, Acharya *et al.*, 2017b), antimicrobial (Khatua *et al.*, 2015) and anticancer abilities (Yaltirak *et al.*, 2009). Thus, researchers are showing increasing interest to investigate biological activities of macrofungi in order to discover novel therapeutic agent. Findings suggest that 158 species belonging to 88 genera have been recognized so far as new antibiotic sources effective against pathogenic microorganisms (Shen *et al.*, 2017).

Conversely, more than 650 members of higher basidiomycetes have been identified till date that may have potential against various types of cancers (Liu *et al.*, 2013). Besides, a large number of literature also provides extensive data on antioxidant activity of different wild edible mushrooms (Khatua *et al.*, 2013, Mitra *et al.*, 2013, Saha *et al.*, 2013, Nandi *et al.*, 2014, Samanta *et al.*, 2015).

Macrocybe lobayensis (R. Heim) Pegler & Lodge could be one of those matrices by providing a variety of biomolecules including phenolic compounds with diverse pharmacological properties. The species was discovered by French mycologist, Heim, in 1969 and originally described from Central African Republic as *Tricholoma lobayense*. In 1998, Pegler and Lodge created the genus, *Macrocybe*, depending upon several morphological characters to accommodate *Tricholoma lobayensis*. The species could be easily recognized in field owing to its pure white large basidiomes with strong cyanic smell. It is commonly known as Doodh Chhatu (Doodh means milk and Chhatu means mushroom) in West Bengal, India and consumed by several indigenous tribes. The mushroom has also been reported from Uttaranchal and Kerala of India where they have long been accepted as a popular food (Vrinda and Pradeep 2006). However, there is still no systematic investigation on medicinal prospect as well as mycochemical composition from the macrofungus except

the study published by Liu *et al.* (1996) exhibiting immunomodulation and antitumor activity of polysaccharide-protein complex isolated from culture filtrate. In this backdrop, present work was carried out for the first time to provide basic information on bioactive phenolic compounds of methanol extract prepared from fruit bodies of *M. lobayensis*. Further, the fraction was evaluated for putative therapeutic abilities namely antioxidant, antibacterial and cytotoxic effects to predict usefulness of the edible mushroom as a functional food ingredient.

MATERIALS AND METHODS

Standards and reagents

Ferrozine, ferrous chloride, potassium ferricyanide, ferric chloride, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), sodium persulfate, ammonium molybdate, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu reagent, 2,6-dichlorophenol indophenol (DCPIP), ascorbic acid, ethylene diaminetetraacetic acid (EDTA), gallic acid, quercetin, oxalic acid, nutrient broth (NB), *p*-iodonitrotetrazolium chloride (INT) and Dulbecco's Modified Eagles Medium (DMEM) were purchased from Himedia, Mumbai, India. Eleven HPLC standards such as gallic acid, *p*-hydroxybenzoic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, ferulic acid, myricetin, salicylic acid, quercetin, cinnamic acid and pyrogallol were used from Sigma Aldrich (MO, USA). Water-soluble tetrazolium (WST) and fetal bovine serum (FBS) were procured from Takara Bio Inc, Japan and Invitrogen, Carlsbad, CA, USA respectively. PenStrep and amphotericin B were purchased from MP Biomedicals, Santa Ana, CA, USA.

Mushroom collection and authentication

Fresh basidiocarps of *M. lobayensis* (~50 g) were assembled from their natural habitat at coastal area of West Bengal, India during the month of August, 2014 and identified using standard literature (Pegler *et al.*, 1998). A voucher specimen was deposited in the same department with accession number of CUH AM483 following the method of Pradhan *et al.* (2015). Fruit bodies were dried by a field drier at 40 °C to make them crispy. Dried basidiocarps were pulverized using electric blender, sieved through 160 mesh and preserved in air tight container.

Preparation of crude methanol extract

Ten g of dried and powdered fruit bodies were steeped in 200 ml methanol for two days and filtered residue was re-extracted with methanol. The combined fraction was evaporated at 40°C under reduced pressure (Rotavapor R-3, Butchi, Switzerland) to acquire methanol extract of *M. lobayensis*. The yield percentage was calculated based on dry weight:

$$\text{Yield (\%)} = (W_1 \times 100) / W_2$$

Where W_1 = weight of extract after solvent evaporation; W_2 = weight of the minced mushroom

Total polyphenol content determination

To determine total phenolic compounds 1 ml of the methanol extract was mixed with 1 ml of Folin-Ciocalteu reagent. After 3 min incubation, 1 ml sodium carbonate solution (35%) was added and adjusted to 10 ml by water. The reaction was kept in dark for 90 min, and absorbance was measured at 725 nm against blank. Gallic acid (10–40 µg) was considered as standard and results were expressed as µg of gallic acid equivalents per mg of extract (Khatua *et al.*, 2015).

Total flavonoid estimation

1 ml methanol extract was diluted with 4.1 ml of 80% aqueous ethanol, 0.1 ml of 1 M potassium acetate and 0.1 ml of 10% aluminium nitrate to estimate total flavonoid content. After 40 min incubation at room temperature absorbance was detected at 415 nm. Quercetin (5–20 µg) was used to calculate the standard curve and results were expressed as µg of quercetin equivalents per mg of extract (Khatua *et al.*, 2015).

β-carotene and lycopene estimation

100 mg methanol fraction was mixed with 10 ml acetone-hexane solution (4:6) and filtered through Whatman no 4. Absorbance was detected at three different wavelengths simultaneously such as 453, 505, 663 nm (Khatua *et al.*, 2015). Content of carotenoids were calculated according to the following equations:

$$\beta\text{-carotene (mg/100 ml): } 0.216 A_{663} - 0.304 A_{505} + 0.452 A_{453}$$

$$\text{Lycopene (mg/100 ml): } -0.0458 A_{663} + 0.372 A_{505} - 0.0806 A_{453}$$

Ascorbic acid determination

10 ml ascorbic acid (100 µg/ml) was prepared using 0.6% oxalic acid and titrated against 2,6-dichlorophenol indophenol dye. The extent of dye consumed (V_1 ml) represented the quantity of ascorbic acid. Likewise, sample (concentration W µg/ml) was also titrated against dye (V_2 ml) (Khatua *et al.*, 2015) and amount of ascorbic acid present in the fraction was calculated using following formula,

$$\text{Ascorbic acid (}\mu\text{g/mg)} = \left[\left\{ (10 \mu\text{g}/V_1 \text{ml}) \times V_2 \text{ ml} \right\} \times W \mu\text{g} \right] \times 1000$$

Quantification of phenolic compounds by HPLC

10 mg dried extract was dissolved in 1 ml of HPLC grade methanol and filtered through 0.2 µm filter paper. 20 µl filtrate was loaded on the HPLC system (Agilent, USA) and separation was achieved on Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) using a flow rate of 0.8 ml /min at 25°C. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). A gradient program was used for elution: 0-2 min, 5% A; 5-10 min, 15% A; 10-15 min, 40% A; 15-20 min, 60% A; 20-22 min, 90% A. The absorbance of standard and sample solution was measured at 280 nm (Chatterjee *et al.*, 2016). Sample compounds were identified on the basis of retention times and absorption spectra of standard materials.

Components were quantized by comparing their peak areas with those of standard curves.

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 solution. The resultant solution was incubated at 95°C for 90 min and absorbance was measured at 695 nm against blank. Concentrations of ascorbic acid (1–30 µg) were plotted to obtain a standard curve. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid (Prieto *et al.*, 1999).

DPPH radical scavenging assay

To determine DPPH radical scavenging activity, methanol 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 iMark™ Microplate Reader, USA) (Khatua *et al.*, 2017b).

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 methanol 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 (Khatua *et al.*, 2017b).

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 methanol 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 (Khatua *et al.*, 2017b).

Determination of reducing power

Further to that, reducing power of the examined fraction was evaluated by mixing 10 µl of extract solution containing a range of concentrations (500-2500 µg/ml) with 25 µl sodium phosphate buffer and 25 µl potassium ferricyanide (1%) in 96 well plate. After incubation for 20 minutes, 85 µl water and 8.5 µl ferric chloride were added in each well separately. Finally, the optical density was determined at 750 nm using same microplate reader as mentioned earlier (Khatua *et al.*, 2017b).

To compare the effects of extract with a standard, ascorbic acid was used in all assays except chelating ability of ferrous ion method where EDTA was adopted as a positive

control. The sample concentration exhibiting 50% of antioxidant activity or 0.5 of absorbance were determined from graphs of antioxidant activity percentages and regarded as EC₅₀ value.

Estimation of antibacterial potentiality

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), *Klebsiella pneumoniae* ATCC[®] 15380[™] (MTCC 109) were utilized for the experiment. Antibacterial effect was estimated by determining MIC values according to microdilution method (Khatua *et al.*, 2017a).

The six investigating bacteria were cultured freshly and 1×10⁵ CFU/ml concentrated dilutions were prepared separately. Reactions were performed in 96 well plate consisting of 200 µl of NB, 20 µl of inoculum and different dilutions of methanol extract. Following incubation for 24 h at 37°C, 40 µl of INT dye (0.2 mg/ml) was added to each well and incubated for another 30 min. Concentration that inhibited 50% growth of bacteria in comparison with positive control was calculated as MIC value. Streptomycin was used as a standard drug.

Estimation of cytotoxic activity

Hep3B human liver cancer cell line was purchased from NCCS, Pune, India. The cells were maintained in DMEM supplemented with 10% FBS, 0.5% PenStrep (5,000 IU/ml penicillin and 5 mg/ml streptomycin) and 0.25% amphotericin B (250 µg/ml) at 37°C in a humidified atmosphere with 5% CO₂. Inhibition of cell viability was determined using WST assay. Briefly, about 1×10⁴ cells were seeded in 96-well plate before methanolic extract dissolved in sterile DMSO was added. 20 µl WST reagent was added after 48 h treatment and absorbance was measured using ELISA plate reader at a wavelength of 450 nm.

Statistical analysis

All data are presented herein as mean ± standard deviation of three independent experiments each in triplicate. Calculations were performed using statistical package for Microsoft[®] Office Excel (Microsoft[®], USA) and differences were evaluated by means of one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Recovery percentage and mycochemical analysis

In last decades, extensive studies have been conducted on mushrooms with an aim to isolate and identify bioactive compounds. Thus, selection of extraction solvent is a key step to explore the field as it has direct effect on concentration and type of bioactive compounds.

In that view, aqueous condition is widely used for screening pharmacological potential of macrofungi. However, organic compounds specifically phenolic constituents are mainly

recognized as active therapeutics and they could easily be obtained through methanol extraction process (Matijašević *et al.*, 2016). Likewise, methanol was used in the present study for preliminary investigation on bio-organic components of *M. lobayensis* entailing medicinal effects.

As depicted in Table 1, the formulation had high recovery percentage and that could be attributed to different types of constituents extracted in appreciable amount. Briefly, phenols were determined as the major component which was present two times higher than flavonoids. In contrary, carotenoids like β-carotene and lycopene were detected in the lowest extent and existed about ten times less than ascorbic acid. Thus, it could be said that the elements were present quantitatively in the following order of phenol > flavonoid > ascorbic acid > carotenoids. The finding was detected to be in accordance with previous publication (Acharya *et al.*, 2017a).

Table 1: Extractive yield and mycochemical analysis of methanol extract prepared from fruit bodies of *Macrocybe lobayensis*. ND: Not detected

Parameters	Methanol extract
Extractive yield (%)	16 ± 0.29
Phenol (µg GAE/mg of extract)	10.96 ± 0.11
Flavonoid (µg QE/mg of extract)	5.12 ± 1.51
β-carotene (µg/mg of extract)	0.29 ± 0.07
Lycopene (µg/mg of extract)	0.20 ± 0.05
Ascorbic acid (µg/mg of extract)	2.22 ± 0.28
Gallic acid (µg/mg of extract)	ND
<i>p</i> -hydroxybenzoic acid (µg/mg of extract)	0.04 ± 0
Chlorogenic acid (µg/mg of extract)	ND
Vanillic acid (µg/mg of extract)	ND
<i>p</i> -coumaric acid (µg/mg of extract)	0.06 ± 0
Ferulic acid (µg/mg of extract)	ND
Myricetin (µg/mg of extract)	ND
Salicylic acid (µg/mg of extract)	0.26 ± 0.01
Quercetin (µg/mg of extract)	ND
Cinnamic acid (µg/mg of extract)	0.19 ± 0.01
Pyrogallol (µg/mg of extract)	8.04 ± 2.9

Further to that, HPLC profile of the fraction was recorded to provide a phenolic fingerprint and identify major molecular components (Figure 1). The chromatogram depicted that at least 11 components were present in the extract of which five were tentatively recognized and quantified (Table 1). Briefly, *p*-hydroxybenzoic acid, *p*-coumaric acid, salicylic acid, cinnamic acid and pyrogallol were positively ascertained according to their retention time as well as UV spectrum characteristics comparable with commercial standards. Among them, pyrogallol was noted as the major constituent and presented approximately eight times higher than salicylic acid, the second most abundant organic component in the formulation.

In contrary, cinnamic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid were detected in vestigial amounts. Surprisingly, pyrogallol, cinnamic acid and *p*-hydroxybenzoic acid have also been detected in methanol extract of other *Macrocybe* sp. namely *M. crassa* and *M. gigantea* (Khatua and Acharya 2014, Chatterjee *et al.* 2016); hence these hydroxybenzoic acid derivatives might be regarded as the most frequently found phenolic component in *Macrocybe* members.

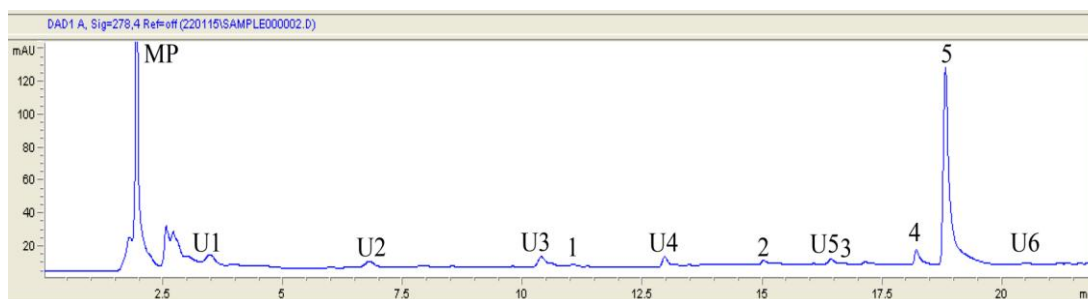


Fig. 1: HPLC chromatogram of methanol extract isolated from *Macrocybe lobayensis* (MP: mobile phase, peaks 1: *p*-hydroxybenzoic acid (Rt 11.05 min), 2: *p*-coumaric acid (Rt 15.02 min), 3: salicylic acid (Rt 17.14 min) 4: cinnamic acid (Rt 18.22 min) 5: pyrogallol (Rt 18.82 min). Peaks designated as U1 to U6 represent unidentified phenols that were eluted at retention time of 3.47 min, 6.8 min, 10.38 min, 12.96 min, 16.43 min and 20.47 min respectively.

Table 2: Antioxidant activity of methanol extract isolated from *Macrocybe lobayensis*. The results are presented in EC₅₀ values (mean ± standard deviation; n = 3) corresponding to 50% of antioxidant activity or 0.5 of absorbance for reducing power assay. Ascorbic acid was used as standard in DPPH and ABTS radical scavenging methods, reducing power and total antioxidant capacity assays; while EDTA was adopted as a positive control in chelating ability of ferrous ion technique. In each row, different letters mean significant differences between sample and standard ($p < 0.05$).

Antioxidant parameters		Methanol extract	Standard
EC ₅₀ value (µg/ml)	Scavenging ability of DPPH radical	610.93 ± 10.66 ^a	7.69 ± 0.02 ^b
	Scavenging ability of ABTS radical	659.94 ± 1.12 ^a	3.18 ± 0.01 ^b
	Reducing power	1785.83 ± 319.16 ^a	18.74 ± 0.01 ^b
	Chelating ability of ferrous ion	990.5 ± 30.3 ^a	11.81 ± 0.58 ^b
Total antioxidant activity by phosphomolybdenum method (µg ascorbic acid equivalent/mg of dry extract)		19.35 ± 0.14	–

Evaluation of antioxidant activity

In the present study, five *in vitro* methods were performed in order to evaluate antioxidant ability of methanol extract from *M. lobayensis* and the effects have been depicted in Table 2. Initially, total antioxidant activity of extract was determined by following phospho-molybdenum method and the activity was compared with a positive control, ascorbic acid. Principle of the technique was based on reducing ability of antioxidant compounds to form Mo (V) from Mo (VI) that eventually generates green colored phosphate/Mo (V) complex. According to the result, reducing capacity of 1 mg of studied methanol fraction was equivalent to 19.35 µg ascorbic acid.

DPPH is a commercially available stable organic nitrogen radical and accepts an electron or hydrogen to become stable diamagnetic radical. The advantage of this assay is that DPPH does not have to be generated before the assay, in contrast to other scavenging methods. The radical can be neutralized by antioxidants as they have ability to donate electron without being free radical itself (Lü *et al.*, 2010). Thus, the degree of DPPH scavenging can be used to magnitude hydrogen donating ability of antioxidants. Hence, DPPH radical scavenging model was implemented herein to evaluate antioxidant potentiality of the natural compound. Method was based on reduction of the radical to DPPH-H in presence of proton-donating element resulting reduction in absorbance from purple to yellow coloured reaction mixture. Thus a lower absorbance at 517 nm indicates higher radical scavenging activity of extract (Kedare and Singh 2011). Eventually, the methanol extract from *M. lobayensis* displayed a

significant dose dependent scavenging pattern at the tested concentration ranges and results were plotted in Figure 2A. At the level of 100, 500 and 1000 µg/ml of extract the inhibition rates were 16.55%, 44.01% and 76.07% respectively demonstrating high scavenging abilities. In this experiment, ascorbic acid was used as a positive control for comparison which exhibited much powerful activity.

Nevertheless, ABTS radical cation was used in the present study for further assessment of antioxidant activity of methanol extract. In this method, ABTS^{•+} were generated by persulfate oxidation of ABTS²⁻ and neutralized in presence of antioxidant component resulting decolourization (Huang *et al.*, 2005). Analysis showed that the fraction possessed strong radical scavenging capacity that incremented in a dose-dependent manner (Figure 2B). As the concentration ranged from 100, 500 to 1000 µg/ml, inhibition activities of the formulation amplified from 23.92%, 36.18% to 79.46%. However, the synthetic antioxidant, ascorbic acid, exhibited about 99% radical quenching activities at those tested concentrations and thus proved to be an excellent scavenger.

Ferrous ions can act as catalysts in formation of hydroxyl, peroxy and alkoxy radicals. Chelating agent stabilizes this transition metal and reduces damage caused by free radicals. So moderate ferrous ion chelating abilities would be beneficial to protect human beings (Lü *et al.*, 2010). In this assay, ferrozine forms complexes with Fe²⁺ resulting in violet colour. In presence of chelating agent, the complex formation is disrupted, thus colour intensity reduces. Reduction therefore allows estimation of chelating ability of coexisting chelator.

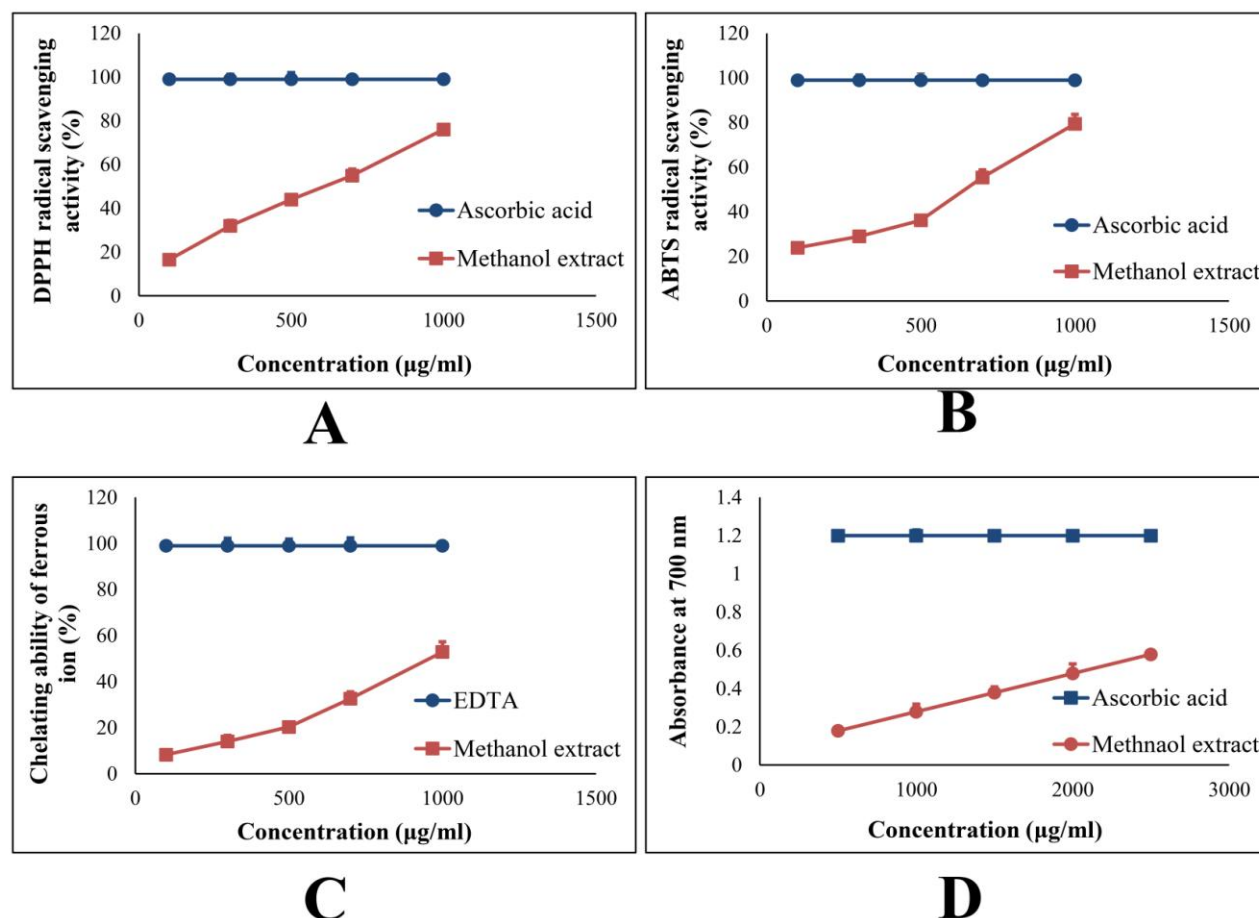


Fig. 2: Antioxidant activity of methanol extract prepared from *Macrocybe lobayensis* (A) DPPH radical scavenging activity (B) ABTS radical scavenging activity (C) Chelating ability of ferrous ion (D) Reducing power. Results represent mean \pm standard deviation of at least three independent experiments.

Figure 2C revealed that at the concentration of 100, 500 and 1000 $\mu\text{g/ml}$ the extract was able to chelate 8.29%, 20.3% and 52.88% ferrous ions respectively whereas EDTA chelated >95% at these same concentration.

Finally, the ferricyanide/prussian blue assay was carried out to determine reducing power of methanol extract. The method was based on action of electron-donating antioxidants to reduce yellow ferric form to blue ferrous form. However, the reducing ability of a sample is not directly correlated to its radical scavenging activity but it is considered as an important parameter of antioxidants (Huang *et al.*, 2005). According to results, the fraction exhibited moderate reducing power which incremented with rise of concentration. At level of 1500 and 2000 $\mu\text{g/ml}$ concentration reducing power of the formulation were 0.34 and 0.48 that gradually elevated to 0.58 at the dose of 2500 $\mu\text{g/ml}$ (Figure 2D). In the present assay, ascorbic acid was selected as a standard for comparison and it exhibited obvious superior antioxidant potentiality.

In the last decade, several reports have been published regarding antioxidant activity of methanol fraction extracted from mushroom. Based on the outcome, it can be said that *M. lobayensis* may possess stronger radical scavenging and total antioxidant potentiality than methanol extract isolated from

M. crassa (Khatua and Acharya 2014), *Pleurotus djamor* (Acharya *et al.*, 2017b) and *Grifola frondosa* (Acharya *et al.*, 2015a). The data also implied that fraction exhibited enhanced reducing power than *Ramaria flava*, *Rhizopogon roseolus* and *Russula delica* (Gursoy *et al.*, 2010). In contrary, the fraction exhibited less effective antioxidant activities than methanolic extract prepared from *Laetiporus sulphureus* (Acharya *et al.*, 2016) and *Ramaria subalpina* (Acharya *et al.*, 2017a). Overall, it can be conferred that the fraction owns relatively strong antioxidant ability as reflected in all aforementioned assays and can therefore be used as a potent antioxidant.

Evaluation of antibacterial activity

Activity of the methanol extract from *M. lobayensis* was tested against some pathogenic bacteria by microdilution method where streptomycin was considered as a standard drug for comparison. The fraction was found to be active against all the examined Gram positive and Gram negative species (Table 3). The MIC values obtained indicated that the most susceptible bacterium was *L. monocytogenes*, followed by *S. aureus* while *K. pneumoniae* showed the least sensitivity. The result was in accordance with previous reports that, generally, Gram positive bacteria are considered more sensitive to different compounds.

Gram negative organisms are difficult to be inhibited because of structure of their cell walls (Khatua *et al.*, 2017a). Further to that, the fraction exhibited low activities in comparison with streptomycin towards all the investigating microorganisms. However, the potentiality of studied fraction was found to be more effective than methanol extract of *Amanita rubescens*, *Cantharellus cibarius*, *Lactarius piperatus*, *Russula cyanoxantha* (Kosanic *et al.*, 2013), *Lycoperdon perlatum*, *Clavaria vermiculris*, *Ramaria formosa*, *Marasmius oreades* and *Pleurotus pulmonarius* (Ramesh and Pattar 2010).

Table 3: Antibacterial activity of methanol extract isolated from *Macrocybe lobayensis* as determined by minimum inhibitory concentration (MIC). Each value is expressed as mean \pm standard deviation (n = 3). In each row, different letters mean significant differences between sample and standard ($p < 0.05$).

Type of bacteria	Name of bacteria	MIC value ($\mu\text{g/ml}$)	
		Methanol extract	Streptomycin
Gram positive	<i>Listeria monocytogenes</i>	245.13 \pm 26.1 ^a	4.68 \pm 0.17 ^b
	<i>Bacillus subtilis</i>	311.29 \pm 43.54 ^a	5.61 \pm 0.01 ^b
	<i>Staphylococcus aureus</i>	309.3 \pm 11.21 ^a	6.29 \pm 0.16 ^b
Gram negative	<i>Escherichia coli</i>	659.5 \pm 29 ^a	5.41 \pm 0.11 ^b
	<i>Klebsiella pneumoniae</i>	1585.81 \pm 201.5 ^a	5.29 \pm 0.14 ^b
	<i>Salmonella typhimurium</i>	1238.66 \pm 115.67 ^a	5.09 \pm 0.03 ^b

So far, many phenolic compounds isolated from mushrooms have been detected to be responsible for diverse biological activities, including antimicrobial effects. For phenolic compounds, it was evidenced that the number and site(s) of hydroxyl groups determines their toxic effect to microorganisms which is possibly executed by enzyme inhibition. Further, the other secondary metabolites, for instance flavonoids, are proposed to act against microbial infections by inhibiting cell membrane, cell wall and nucleic acid synthesis along with energy metabolism (Matijašević *et al.*, 2016). On the other hand, the mode of action of alcohols and terpenes involves disruption of microbial membranes. Thus, it could be assumed that the antibacterial activity of *M. lobayensis* would be related to its mycochemicals particularly phenolic components.

Estimation of cytotoxic activity

To study the growth inhibitory activity *in vitro*, Hep3B cells were incubated for 48 h with varied doses of methanol extract from *M. lobayensis*. Potential toxic effect of the fraction was evaluated using WST assay based on generation of orange coloured formazan dye in direct proportional to number of living cells. Result showed that the percentage of inhibition was 2.77, 24.74, 68.25 and 70.15 when the concentration was 50, 100, 300 and 500 $\mu\text{g/ml}$ respectively (Figure 3). Thus, the formulation displayed effective growth inhibitory activity on the human liver cancer cell line with IC_{50} 234.31 \pm 7.96 $\mu\text{g/ml}$.

Generally, fungal anticancer substances can be divided into two major groups such as high and low molecular weight components. Polysaccharides and protein-bound polysaccharides comprise most of the high molecular weight compounds; whilst phenolics, steroids, sesquiterpenes, triterpenes, sterols and polyketides form the second group. These low molecular weight

ingredients are able to penetrate cell membrane and function on specific signal transduction cascade (Liu *et al.*, 2013). As a result, discovery of new molecules extracted by organic solvent from higher basidiomycetous members is a global trending currently. For instance, anticancer activity of methanol, ethanol and boiled water extract from *Suillus collinitus* was investigated on several human cancer cell lines where the methanolic fraction was found to be the most effective (Vaz *et al.*, 2012). On the other hand, methanol formulation of *Antrodia cinnamomea* exhibited high hepatocellular activity with IC_{50} value of $<150 \mu\text{g/ml}$; though hot water fraction prepared from the same mushroom did not show promising activity even after treatment of 48 h (Cha *et al.*, 2009).

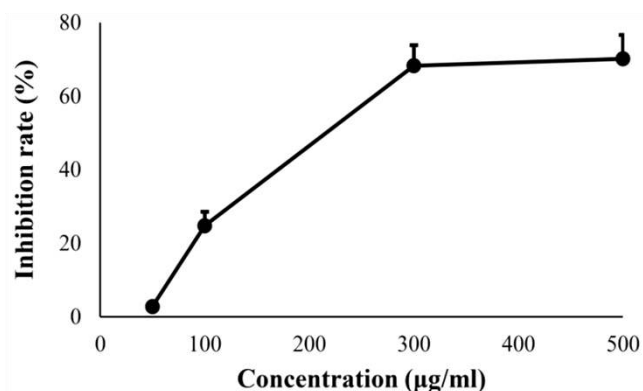


Fig 3: Cytotoxic effect of methanol extract from *Macrocybe lobayensis*: Hep3B human liver cancer cells were treated with various concentrations of the fraction for 48 h and the cell growth inhibition was quantified by WST assay.

CONCLUSION

In conclusion, methanol extract from *M. lobayensis* could be considered as a potential source of biomedicine with respect to antioxidant, antibacterial and cytotoxic effects. These putative therapeutic abilities might be attributed to phenols and flavonoids as they were present predominantly in the fraction. However, isolation and evaluation on key component(s) that has the direct effect on medicinal prospects need to be elucidated further; such studies may lead to potential discovery of novel, natural and bioactive drugs.

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