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Research Article

Chemical and biological activity of mushrooms naturally occurring in Bangladesh

Mohammad Shoeb[a,b]*, Prithu Mondal[a], Henrik Kylin[b] and Nilufar Nahar[a]

[a]*Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh*
[b]*Department of Thematic Studies-Environmental Changes, University of Linköping, SE 58183, Sweden*

* Mohammad Shoeb: shoeb71@yahoo.com

Abstract

Five freeze-dried mushroom species namely *Agaricus bisporus, Coprinus micaceus, Pleurotus. ostreatus, Termitomyces clypeatus* and *T. heimii* were extracted separately with dichloromethane:methanol followed by partitioned using hexane, dichloromethane and methanol. Silica gel column chromatography of hexane extract of *T. heimii* and dichloromethane extract of *P. ostreatus* afforded 2,4-di-hydroxy-9-octadecenoic acid and 2-hydroxy-9-octadecenoic acid, respectively. The n-hexane extracts of mushrooms were analyzed for the presence of n-alkanes and fatty acid compositions. n-Alkanes were found in *T. heimii* only. Different extracts of mushrooms were evaluated for antimicrobial activity against a wide range of Gram-positive and Gram-negative bacteria and fungi, general toxicity and antioxidant activity.

Keywords: *Agaricus bisporus,* n-Alkane, *Coprinus micaceus,* Fatty acid, Mushroom, *Pleurotus. ostreatus, Termitomyces clypeatus, Termitomyces heimii*

Introduction

Mushrooms are macrofungi belonging to the class Agaricomycetes with unique and visible fruiting bodies that usually grow above ground (Miles & Chang, 1997). Mycorrhiza-interactions between the fungal mycelia and plants roots are essentials for the functions in many terrestrial ecosystems (Merckx, 2013). When conditions are favourable, the mycelium, the vegetative part of the fungus, produces fruiting bodies, which may be collected and consumed as food by humans (Chang, 2008**,** Guillamón et al., 2010)]. Mushrooms are rich in protein, minerals, vitamins and essential amino acids (Sadler, 2003), and have been utilized for human consumption since ancient times as a normal part of the diet due to their desirable taste and aroma (Largeteau et al., 2011)**.** Edible mushrooms can be cultivated or collected

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grown naturally. Collection and uses of wild mushrooms as vegetables are common in Europe, South America, Canada, China, Nepal and India. Approximately, 300 species of mushrooms have documented uses as human food in the African continent and Mexico (Rammeloo & Walleyn, 1993, Garibay-Orijel et al., 2006). Food and Agricultural Organization (FAO) reported that about 3.4 million tonnes of cultivated mushrooms were produced worldwide in 2007, a 56% increase from 1997 (Food and Agriculture Organization of the United Nations). The production was about 6 million metric tonnes in 2010 and China claimed the highest production with an estimated amount of 1.5 million tonnes in 2007 (Kalac, 2013, Aida et al., 2009). Mushrooms are also used for medicinal purposes with purported anticancer, antibiotic, antiviral, immunity boosting, and blood lipid lowering activities (Alam et al., 2008). There are medicinal products derived from mushrooms in Brazil, China, Japan, Korea and USA (Russell et al., 2014), (Kerrigan, 2005) and commercialized products in the form of capsules, tablets or extracts as dietary supplements are also available in the market for potential therapeutic effects (Wasser et al., 2000). Different strains of *Agaricus bisporus*, *Lentinula edodes*, *Pleurotus ostreatus*, and *Flammulina velutipes* are the most cultivated mushrooms worldwide (Aida et al., 2009), Chang & Miles, 2004). Annual yields of *A. bisporus* reach more than 1 million tonnes (Zhang et al., 2014). Medically active species are, e.g., *A. subsufescence* (due to a misidentification often sold as *A. blazei* (Kerrigan, 2005), which is used for a wide range of diseases, including cancer, hepatitis, atherosclerosis, hypercholesterolemia, diabetes and dermatitis (Wasser & Weis, 1999, Huang, 1997). *P. ostreatus* is reported to have antitumor activity (Yoshioka et al., 1985) and hypoglycaemic effects in experimentally diabetic induced rats (Chorvathoba et al., 1993). *P. ostreatus* were reported to be active in reducing the total plasma cholesterol and triglyceride level (Alam et al., 2007). During rainy seasons, mushrooms grow naturally in the forest of Bangladesh. The uses of mushrooms as vegetables, however, started only recently, but are increasing rapidly with a large proportion used in Chinese restaurants. Further, both *P. ostreatus* and *A. bisporous* are cultivated commercially, and thus helping to start business in many rural areas of the country. The quality and nutritional value of the mushrooms depends on the geological region, species, maturity, cultivation, and time of harvesting. This study was performed to investigate chemical and biological activity of five mushroom species naturally occurring in Bangladesh.

Materials and Methods

Material: fresh naturally growing fruiting bodies of *A. bisporus, Coprinus micaceus, P. ostreatus, Termitomyces clypeatus* and *T. heimii* were collected from Dhaka University Campus in June 2014 and were processed immediately.

Extraction of mushrooms: each mushroom was washed with de-ionized water, chopped into small pieces, and freeze-dried. The dried samples were extracted separately with 100 mL of dichloromethane (DCM):methanol (1:1) for 3 days (at 60 °C). The whole mixture was filtered through precleaned cotton and the filtrate thus obtained was concentrated below 40 °C on a rotary evaporator. The extract was repeated two more times with the same amount of solvent. Each extract of mushrooms were partitioned using hexane, DCM and aqueous methanol following modified Kupchan's partition method (Sarker et al., 2006) and finally hexane, DCM and methanol extracts were obtained (Table 1).

Instruments: UV and IR spectra were recorded with Shimadzu UV 160A and Shimadzu IR-470 spectrometers (Shimadzu, Asia Pacific Pte Ltd., Singapore), respectively. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer (Bruker, Switzerland) using tetramethylsilane (TMS) as the internal reference. The presence of fatty acids and n-alkanes were analyzed by GC-MS using

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Agilent 6890 GC interfaced with a 5973 mass spectrometer (Agilent Technologies, Avondale, PA, USA) with a DB5-MS column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, J & W Scientific).

Table 1. Amount of different extractives of mushroom

Species	Fresh mass (g)	Dry mass (g)	Hexane fraction (mg)	DCM fraction (mg)	Methanol fraction (mg)
A. bisporus	114	2.7	200	21	275
C. micaceus	108	2.5	200	250	300
P. ostreatus	106	2.1	570	250	35
T. clypeatus	75	0.72	330	90	80
T. heimii	87	0.93	180	27	12

The MS was operated with electron ionization at 70 eV under full-scan mode (m/z 50–500). For fatty acids, the GC temperature program was 35 °C held isothermally for 1 min, 20 °C min^{-1} to 130 °C, 4 °C min^{-1} to 320 °C, held isothermally for 15 min. For alkanes, the temperature program was at 35°C held isothermally for 1 min, 20 °C min^{-1} to 130 °C, 6 °C min^{-1} to 320 °C, held isothermally for 15 min.

Isolation of secondary metabolites: the hexane extract (110 mg) of *T. heimii* was loaded on the top of a silica gel (70-230 mesh size) column (30 cm x 2.5 cm) equilibrated with n-hexane. The column was first eluted with hexane (30 mL) and then with mixtures of hexane with increasing amount of dichloromethane (20% to 100%) and DCM in ethyl acetate (20% to 100%), and finally with methanol (100%). Elution was 30 mL in each case with a drop rate of 2 mL/min. The fraction eluted from DCM:ethyl acetate (1:1) (5.5 mg) gave a single spot in TLC. It was purified by repeated washing with n-hexane and a pure Compound 1 (5.0 mg) was obtained. Similarly, Compound 2 (13.0 mg) was isolated from other fraction DCM:ethyl acetate (3:2) after washing with n-hexane.

Compound 1: light yellow, gummy (5.0 mg), UV (DCM): λ_{max} 229 nm, IR (KBr pellets): 3460, 2980, 1750, 1400, 1250 cm^{-1}. ^{1}H NMR ($CDCl_3$): 5.33 (m), 5.25 (m), 4.28 (m), 4.12 (m), 2.26 (m), 2.00 (m), 1.60 (m), 1.24 (m) and 0.86 (t, *J*=7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$):178.8, 128.4, 128.0, 66.7, 62.1, 34.1, 32.0, 29.7, 29.7, 29.5, 29.4, 29.1, 28.9, 27.2, 24.9, 23.8, 22.7 and 14.1 ppm.

Compound 2: light yellow, gummy (13.0 mg), UV (DCM): λ_{max} 230 nm, IR (KBr pellets): 3400, 2918, 1710, 1450, 1265 cm^{-1}. ^{1}H NMR ($CDCl_3$): 5.51 (multiplet), 5.50 (multiplet), 3.57 (multiplet), 2.35 (triplet), 1.61 (doublet), 1.40 (broad singlet), 1.26 (broad peak), 0.86 (triplet, *J*=7.2 Hz) ppm. ^{13}C NMR ($CDCl_3$): 178.8, 128.4, 128.0, 72.1, 33.9, 31.9, 30.7, 29.7, 29.3, 29.1, 29.0, 25.7, 25.6, 24.7, 23.9, 23.8, 22.9 and 14.1 ppm.

Analysis of fatty acid compositions and n-alkanes: The hexane extract (free fatty acids) of each mushroom was evaporated to dryness under nitrogen. Boron trifluoride-methanol (BF_3-MeOH, 1 mL) complex was added to the dried extract, ultra sonicated (vortexed 30 sec.), heated in a water bath (70 °C) for 2 hours and evaporated into dryness. n-Hexane (~ 1 mL) was added to the dried methyl esters, filtered through Pasteur pipette containing cotton, transferred to a vial and analyzed by GC-MS. n-Hexane extracts of five mushrooms were dissolved in hexane and analyzed by GC-MS for n-alkanes.

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Antimicrobial assay: the *in vitro* antibacterial and antifungal activities of the extracts were determined by the disc diffusion technique [Bauer et al., 1966]. Different concentrations (µg/mL) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents (hexane or chloroform). Five Gram-positive (*Bacillus cereus, B. megaterium, B. subtilis, Staphylococcus aureus, Sarcina lutea*), eight Gram-negative antibacterial (*Escherichia coli, Pseudomonas aureus, Salmonella paratyphi, Sal. typhi, Shigella boydii, Sh. dysenteriae, Vibrio mimicus, V. parahemolyticus*) and three fungal strains (*Candida albicans, Aspergillus niger, Sacharomyces cerevacae)* were used. Nutrient agar media was used for the culture of bacteria and potato dextrose agar media was used for the culture of fungi. Dried and sterilized filter paper discs (7 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Antibiotic, ciprofloxacin (5 µg/disc) and blank discs (impregnated with solvents) were used as positive and negative control, respectively. The plates were incubated at 37 °C for 24 hours to allow maximum growth of the organisms. The antimicrobial potential was measured for each extract in terms of zone of inhibition around the disc expressed in mm.

General toxicity: Brine shrimp lethality assay was carried out to evaluate the general toxicity [Meyer et al., 1982]. Test samples was dissolved in DMSO and varying concentrations of samples: 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781 µg/mL were prepared and each sample solution (100 µL) was added to vial containing 5 mL of sea water and 10 *Artemia salina* shrimp nauplii. The lethal concentration LC_{50} of the test samples after 24 hours was obtained by a plot of percentage of the shrimps killed against the logarithm of the sample concentration.

Antioxidant activity: The antioxidant activity (free radical scavenging activity) of the test samples on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the modified method of Takao (Takao et al., 1994). Test samples (2.0 mg) was dissolved in methanol and varying concentrations of samples such as 500, 250, 62.50, 32, 16, 8, 4 and 2 µg/mL were prepared. Each sample solution (2 mL) was mixed with a DPPH methanol solution (3 mL; 20 µg/ mL) and allowed to stand in the dark for 30 min for the reaction to occur. Then the absorbance was determined at 517 nm against methanol as blank by UV spectrophotometer and from these values, the corresponding percentage of inhibitions was calculated. Standard tert-butyl-1-hydroxy toluene (BHT) was used as positive control.

Results

Compounds **1** (5.0 mg) and **2** (13.0 mg) were isolated from the hexane extract of *T. heimii* (110.0 mg) and DCM extract of *P. ostreatus* (120.0 mg), respectively by silica gel column chromatography (Figure 1). The absorption at 1750 $cm^{-}1$ in the IR spectrum indicated that both compounds may contain carboxylic acid group. The ^{1}H NMR spectrum of the Compound **1** gave signals at 5.33 (m), 5.25 (m), 4.28 (m), 4.12 (m), 2.26 (m), 2.00 (m), 1.60 (m), 1.24 (m) and 0.86 (t, *J*=7.2 Hz) ppm. Signals at 5.33 and 5.25 ppm were due to the presence of olefinic protons while 4.28 and 4.12 ppm were for hydroxyl groups, 2.00 and 1.24 for methylene groups and 0.86 for methyl group. The ^{13}C NMR spectrum of Compound **1** displayed 18 carbons and signals at 178.8 ppm for carbonyl group, 128.0 and 128.4 ppm for olefinic carbons, 66.7 ppm for CH-OH group, 22.7-38.9 ppm for methylene group and 14.1 ppm for methyl carbon. From the UV, IR, ^{1}H and ^{13}C-NMR spectral data the structure of Compound **1** was elucidated as 2,4-di-hydroxy-9-octadecenoic acid (2,4-dihydroxyoleic acid). The ^{1}H NMR spectrum of **2** gave signals at 5.51 (m) & 5.50 (m), 3.57 (m), 2.35 (m) & 1.26 (m) and 0.86 (t, *J*=7.2 Hz) ppm for

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the presence of olefinic protons, hydroxyl group, methylene groups and methyl group, respectively. The ^{13}C NMR spectrum of Compound **2** gave signals at 178.8, 128.4, 72.1 22.9-37.5, 14.1 ppm for carbonyl carbon, olefinic carbon, -CH-OH, methylene and for methyl carbon, respectively. From the spectral data of UV, IR, ^{1}H and ^{13}C-NMR, the structure of Compound **2** was elucidated as 2-hydroxy-9-octadecenoic acid (2-hydroxyoleic acid). The n-hexane extracts of five mushrooms were analyzed for the presence of n-alkanes by GC-MS. The presence of n-alkanes in mushrooms was identified and confirmed by comparing the retention time and mass fragmentation patterns of standard n-alkanes. Out of five mushroom species, n-alkanes were found in *T. heimii* only (Figure 2). The nonacosane was found at the highest percentage (15%) and the trend showed decreasing order octacosane (14%), triacontane (13%), heptacosane (12%), hentriacontane (10%), hexacosane (9.3%), dotricontane (7.0%), pentacosane (6.0%), tritiacontane (4.5%), tetracosane (3.4%), tetratriacontane (2.6%), tricosane (1.7%)

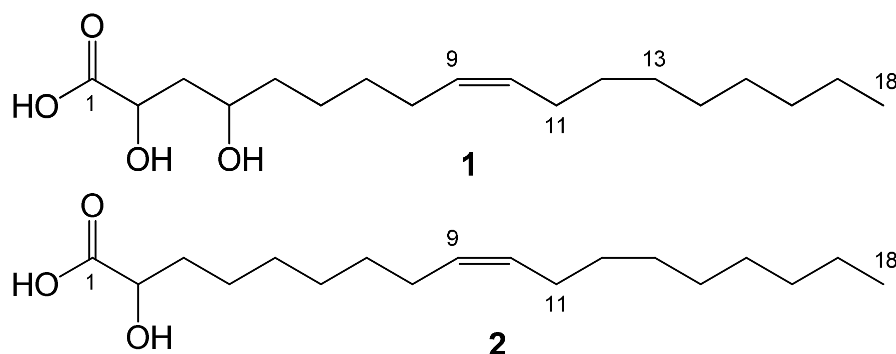

Figure 1: Structures of Compound **1** and **2**

and pentatriacontane (1.5%). The highest percentage of C_{29} alkane in *T. heimii* showed symbiotic relationship with vascular plants and can be used to characterize past terrestrial ecosystems and environments. n-Hexane extracts of five mushrooms were analyzed for the presence of fatty acid. The extracts were *trans*-esterified to make ester of fatty acids using BF_3-MeOH complex, which were finally analyzed by GC-MS by comparing the retention time and fragmentation pattern of standard fatty acid methyl esters. The relative percentage of fatty acids in five mushrooms is shown in Table 2. *A. bisporus* contained 55% 7-octadecenoic acid, 27% hexadecanoic acid. *C. micaceus* contained 70% 9,12-octadecadienoic acid, 25.% hexadecanoic acid. *P. ostreatus* contained 48% hexadecanoic acid, 27% 7-octadecanoic acid and 12% 9-octadecenoic acid. *T. clypeatus* contained 51% 9,12, Octadecadienoic acid, 23% hexadecanoic acid and 17% 9-octadecenoic acid. *T. heimii* mushroom contained 36% hexadecanoic acid and 36% 9,12, Octadecadienoic acid. Other fatty acids are relatively very low in amount. *A. bisporus* contained 66% unsaturated fatty acid and 34% saturated fatty acid (Figure 3).

C. micaceus contained 70% unsaturated fatty acid and 30% saturated fatty acid. *P. ostreatus* contained 45% unsaturated fatty acid and 55% saturated fatty acid. *T. clypeatus* contained 71% unsaturated fatty acid and 29% saturated fatty acid. *T. heimii* contained 55% unsaturated fatty acid and 45% saturated fatty acid. Unsaturated fatty acids are good for human health. Usually, people in Bangladesh get unsaturated fatty acids from fish consumption. But recently it was reported that some species fish contain chemical contaminants which are harmful for health (Zamir et al., 2013). The antibacterial activities of the hexane and DCM extracts of mushroom species were carried out by the disc diffusion method (Table 4).

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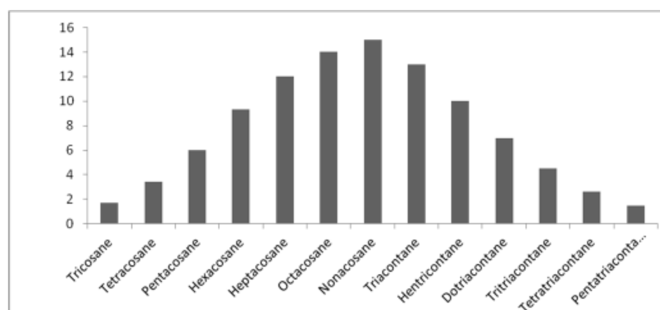

Figure 2: Relative percentage of n-alkane in *T. heimii*

Table 2: Relative percentage fatty acid compositions in five mushroom species

Fatty acids	*A. bisporus*	*C. micaceus*	*P. ostreatus*	*T. clypeatus*	*T. heimii*
Dodecanoic	0.02	0.05	0.05	0.01	0.08
Tetradecanoic	0.20	0.29	0.53	0.25	0.61
9-Hexadecenoic	0.64	0.22	0.93	0.18	1.4
7-Hexadecenoic	1.7	0.20	0.59	0.24	0.35
Hexadecanoic	27	25	48	23	36
9,12-Octadecadienoic	3.3	70	4.3	51	36
9-Octadecenoic	5.7	1.1	12	17	14
7-Octadecenoic	55	0.02	27	1.4	2.9
Octadecanoic	5.4	4.2	6.2	5.0	6.5
Eicosanoic	0.50	0.09	0.15	0.20	0.24
Tetracosanoic	0.71	0.61	0.63	0.80	1.3

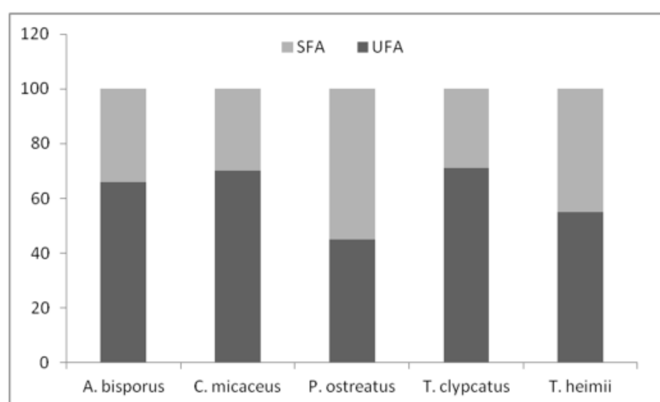

Figure 3. Comparison of percentage of saturated (SFA) and unsaturated (UFA) fatty acid

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Table 3. Antimicrobial activity of hexane and DCM extracts of mushroom species

Microorganisms	*A. bisporus*		*C. micaceus*		*P. ostreatus*		*T. clypeatus*	*T. heimii*	Ciprofloxacin
	Hexane	DCM	Hexane	DCM	Hexane	DCM		DCM	
Bacteria									
B. cereus	-	-	8	9	-	8	8	8	40
B. megaterium	-	-	8	9	-	8	8	8	40
B. subtilis	-	-	8	10	-	8	8	8	40
S. aureus	-	-	-	9	-	-	-	-	40
S. lutea	-	-	-	9	-	-	-	-	40
E. coli	-	-	-	9	-	-	-	8	41
P. aureus	-		-	9	-	-	-	-	40
Sal. paratyphi	-	7	8	9	-	8	8	-	41
Sal. typhi	-	7	-	9	-	-	-	-	40
Sh. boydii	-	-	-	9	-	-	-	-	41
Sh. dysenteriae	-	8	-	9	-	-	-	-	40
V. mimicus	-	-	-	9	-	-	-	-	41
V. parahemolyticus	-	-	-	9	-	-	-	-	40
Fungi								-	
C. albicans	-	8	-	9	-	8	-	-	40
A. niger	-	8	-	9	-	8	-	-	40
Sac. cerevacae	-	8	-	9	-	8	-	-	41

"--" indicates no activity

Table 4. Cytotoxicity and antioxidant assay of extracts

Mushrooms	**LD_{50} (μg/mL)**		**IC_{50} (μg/mL)**	
	Hexane	DCM	Hexane	DCM
A. bisporus	16	34	0.04	0.43
C. micaceus	--	4.8	0.04	2.4
P. ostreatus	37	28	3.0	0.30
T. clypeatus	34	6.9	0.001	0.30
T. heimii	--	19	--	1.9
Vincristine sulphate	1.1		--	
BHT	--		0.002	

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The samples were screened against five gram-positive, eight gram-negative bacteria and three fungi, and zone of inhibition were measured. Ciprofloxacin (5 µg per disk) was used as positive standard. The zone of inhibition of DCM extracts of *A. bisporus*, *P. ostreatus*, *T. clypeatus*, *T. heimii* against the gram-positive bacteria of *B. cereus*, *B. megaterium* and *B. subtilis* were in the range of 8-9 mm and showed mild antibacterial activity. *P. ostreatus* and *T. clypeatus* also showed mild antimicrobial activity against gram-negative bacteria *Sal. paratyphi* whereas *A. bisporus* showed activity against *Sa. paratyphi, Sa. typhi* and *Sh. dysenteriae*. *P. ostreatus* and *A. bisporus* showed mild activity against fungi *C. albicans, A. niger, Sac. cerevacae*. DCM extract of *C. micaceus* showed mild activity against all gram-positive and gram-negative bacteria and fungi. Hexane extract of *C. micaceus* showed antimicrobial activity against *B. cereus, B. megaterium, B. subtilis* and one *Sal. paratyphi*. However, hexane extracts of *P. ostreatus* and *A. bisporus* did not show any activity to any microorganism (Table 3). The general toxicity of n-hexane and DCM extracts of mushrooms were screened by brine shrimp lethality assay (Table 4). LC_{50} values of the DCM extracts of *C. micaceus and T. clypeatus* were 4.8 and 6.9 µg/mL, respectively and were highly cytotoxic among all extracts. LC_{50} values of hexane extract of *A. bisporus* and DCM extract of *T. heimii* were 15.8 and 18.6, respectively and are less cytotoxic than the previous two extracts but more toxic than hexane extract of *T. clypeatus* and DCM extracts of *P. ostreatus* and *A. bisporus*. The higher toxicity of these extracts might be due to the presence of cytotoxic compounds.

The antioxidant activity of extract of mushrooms was tested by DPPH scavenging assay. IC_{50} values of hexane extracts of *A. bisporus*, *C. micaceus, P. ostreatus* and *T. clypeatus* had 0.04, 0.04, 3.00 and 0.001 µg/mL, respectively (Table 4). IC_{50} values of the DCM extracts of *A. bisporus*, *C. micaceus*, *P. ostreatus*, *T. clypeatus* and *T. heimii* had 0.43, 2.4, 0.30 and 0.30 and 1.9 µg/mL, respectively. Extracts containing phenolic natural products like flavonoids and coumaric acid or with hydroxy groups usually show the antioxidant activity, which is predominantly due to their redox properties, i.e. the ability to act as reducing agents, hydrogen donors and singlet oxygen quenchers, and to some extent, could also be due to their metal chelation potential. The highest antioxidant activity of hexane extract of *T. clypeatus* might be due to the presence of hydroxyl acids in the extract.

Conclusion

The cultivation and uses of mushrooms as vegetables started only recently in Bangladesh. The present study of five naturally occurring mushroom species i.e., *A. bisporus, C. micaceus, P. ostreatus, T. clypeatus* and *T. heimii* indicated that they contained high amount of unsaturated fatty acids and extracts are active against a wide range of antimicrobial activity and possessed antioxidants activities. Edible mushrooms can be alternative sources of nutraceuticals and unsaturated fatty acids for the beneficiary of health of mass population of Bangladesh.

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Declaration of conflict of interest

No conflict of interest associated with this work.

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