Mushroom varieties found in the Himalayan regions of India: Antioxidant, antimicrobial, and antiproliferative activities

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Abstract This study evaluates the antioxidant, antimicrobial, and antiproliferative activities of mushroom varieties (*Agaricus bisporus*, *Pleurotus ostreatus*, and *Coprinus atramentarius*) found in the Himalayan regions of Jammu and Kashmir, India. All three mushroom varieties exhibited antioxidant activity. *A. bisporus* showed the highest diphenyl-2-picrylhydrazyl inhibition activity ($87.88 \pm 5.6\%$), *C. atramentarius* showed the highest reducing power (0.49 ± 0.01) and metal chelating ability ($48.57 \pm 0.38\%$), and *P. ostreatus* contained the highest phenolic (5.25 ± 0.14 mg gallic acid equivalent per gram) and flavonoid content (64.39 ± 0.73 μ g rutin equivalent per gram). *C. atramentarius* showed maximum antibacterial activity against all the pathogenic strains and the highest zone of inhibition was against *S. typhi* (7.45 ± 0.5 mm). *P. ostreatus* exhibited the highest cell proliferation inhibition against Colo-205 and MCF-7 cancer cell lines. The structural analysis by Fourier transform infrared spectroscopy indicated the presence of β -configuration in carbohydrates, phenolic compounds, and protein, which impart nutraceutical properties to mushrooms.

Keywords: mushroom, antioxidant, antibacterial, antiproliferative, FTIR

Introduction

Mushrooms are considered as one of the prime functional food for human consumption that has been cultivated and gathered for hundreds of years (1,2). Mushrooms synthesize a number of bioactive compounds including various polysaccharides, glycoproteins, terpenoids, and lectins (3). Oxidation is vital for all living organisms for the production of energy and for carrying out various biological processes. But at the same time, oxygen-centred free radicals and other reactive oxygen species that are continuously being formed *in vivo* result in cell death and tissue damage, which thereby results in various ailments such as atherosclerosis, diabetes, cancer, and cirrhosis (4). Hence, antioxidants (both synthetic/natural) are used to nullify the damage caused by the reactive oxygen and nitrogen species (5). Artificial antioxidants such as butylated hydroxyl anisole, butylated hydroxyl toluene, tert-butyl hydroquinone, and propyl gallate, which are used in various food formulations, have lethal and carcinogenic effects (6). Therefore, there is a need for the expansion and consumption of more valuable natural antioxidants from plant sources such as green tea, cereals, and herbal extracts (7–16) and

mushrooms are classed among them. A number of mushroom extracts of various species have been reported to possess antioxidant activities (17). Mushroom extracts have also been reported to possess antimicrobial activity with strong antibacterial and antifungal properties (18). Seth *et al.* (19) reported that mushrooms containing various active components have numerous therapeutic effects including antitumor and immune-modulating properties. Mushrooms, particularly *A. bisporus*, *P. ostreatus*, and *C. atramentarius*, have various culinary uses besides their usage in cosmetic and pharmaceutical industries in Asian countries, especially India, China, and Japan (20).

Thus, the present study was carried out to explore the *in vitro* antioxidant activities of methanolic extract from *A. bisporus*, *P. ostreatus*, and *C. atramentarius*, including diphenyl-2-picrylhydrazyl (DPPH) radical, reducing power, metal chelating ability of ferrous ions, total phenolics, and flavonoid content. The antimicrobial activity was tested against pathogens, Gram-positive bacteria including *Bacillus cereus* and *Staphylococcus aureus*, and Gram-negative bacteria including *Escherichia coli*, *Salmonella typhi*, and *Proteus vulgaris*. The antiproliferative activity of these extracts was also carried out using colon and breast cancer cell lines.

Materials and Methods

Sample collection Wild and cultivated varieties of mushroom were procured from the Kashmir Valley in the spring season of the year 2015. The cultivated varieties, namely, *A. bisporus* and *P. ostreatus*, were collected from Mushroom Research and Training Centre, Division of Plant Pathology, SKUAST-K, and the wild variety, *C. atramentarius*, sample was randomly collected from the outskirts of Srinagar.

Chemicals and pathogens All the analytical grade chemicals were purchased from HIMEDIA, Sigma-Aldrich (St. Louis, MO, USA) and Merck Co. (Darmstadt, Germany). Water used for chemical preparation was from Milli-Q water purification system (model A10; Millipore, Billerica, MA, USA). The pathogenic bacteria were procured from the Department of Microbiology, Sheri Kashmir Institute of Medical Sciences, Jammu and Kashmir, India.

Sample preparation Fifty grams of dried powder of each mushroom sample was extracted and defatted with petroleum ether in a Soxhlet extractor (extract was collected later, weighed, dried, and kept for further usage). The defatted material was oven dried at 40°C and extraction was carried out in Soxhlet using absolute methanol. The extract collected was later dried, weighed, and kept for further usage in sterilized capped vials at 4°C.

Antioxidant activity assays

Assay for DPPH radical scavenging activity: The radical scavenging activity of mushroom extracts was carried out based on the method of Fu *et al.* (21). The DPPH solution (0.1 mM) was prepared in methanol and 2 mL of this solution was added to 2 mL of aqueous extract of mushrooms having different concentrations (1–4 mg/mL). The absorbance of solution was measured at 517 nm using a UV-vis spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan). The positive control used was butylated hydroxyl toluene. The DPPH radicals scavenging rate of the sample was calculated based on the following equation.

$$\text{Inhibition \%} = (A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100\%$$

where A_{sample} is absorbance with sample and A_{blank} is absorbance without sample.

Reducing power assay: Reducing power was performed according to Oyaizu (22). Mushroom extract of 2.5 mL (1–4 mg/mL) was dissolved in milli-Q water and mixed with 2.5 mL (0.2 M) sodium phosphate buffer (pH 6.6) and 2.5 mL (1%) potassium ferricyanide. The solution was vortex mixed and incubated at 50°C for 20 min. After incubation, 2.5 mL (10%) trichloroacetic acid was added and the solution was centrifuged for 10 min at 2,000× *g*. Five milliliters of the upper layer was mixed with equal volume of milli-Q water and 1 mL (0.1%) ferric chloride. Using ascorbic acid as the positive control, absorbance was read at 700 nm using a spectrophotometer (UV-1650 PC; Shimadzu).

A higher absorbance value indicates a higher reducing power.

Chelating ability on ferrous ions: Metal chelating ability was determined according to Dinis *et al.* (23). One milliliter of mushroom extract (1–4 mg/mL) was mixed with 3.7 mL methanol and 0.1 mL (2 mM) ferrous chloride; 0.2 mL (5 mM) ferrozine was added to initiate the reaction. Using EDTA as a positive control, absorbance was measured at 562 nm after 10 min.

Total phenolics: The total phenolic content was determined according to Singleton and Rossi (24). The results are expressed as milligram gallic acid equivalents per gram (mg GAE/g) of dried mushroom extract.

Total flavonoid content: The total flavonoid content was determined according to Meda *et al.* (25). Five milliliters (2%) of AlCl_3 was mixed with equal volume of the mushroom extract. Absorbance was recorded at 415 nm using a spectrophotometer after 10 min. The total flavonoid content is expressed as rutin equivalent per gram ($\mu\text{g RE/g}$) of mushroom extract.

Assay of antimicrobial activity

Bacterial strains: The antibacterial activity of the mushroom extracts was evaluated using a disc diffusion method against Gram-positive bacteria (*B. cereus* and *S. aureus*) and Gram-negative bacteria (*E. coli*, *Sal. typhi*, and *P. vulgaris*).

Disc diffusion method: The disc diffusion method was carried out for studying the antimicrobial susceptibility following Bauer *et al.* (26). The discs impregnated with a series of mushroom extracts (1 mg/mL) were placed on the Mueller-Hinton agar surface. Each test plate comprised three discs, positive control, negative control (dimethyl sulphoxide), and the treated disc with sample solution. The standard antibiotic discs were erythromycin 5 μg /disc for *S. aureus*, gentamycin 10 μg /disc for *Sal. typhi*, amikacin 30 μg /disc for *E. coli* and *P. vulgaris*, and vancomycin 30 μg /disc for *B. cereus*. The plate was then incubated at 37°C for 18–24 h depending on the species of bacteria used in the test. After incubation, the plates were examined for an inhibition zone. The inhibition zone was then measured using calipers and recorded.

Antiproliferative assay

Cell lines and culture conditions: Human colon cancer cell line (Colo-205) and breast adenocarcinoma cell line (MCF7) were procured from National Centre for Cell Science, Pune, India. Cells were grown on monolayers in Dulbecco's minimum essential medium supplemented with (10%) fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg /mL) and sterilized by filtering through a 0.2- μm filter in a laminar air flow hood in a CO_2 incubator (Galaxy 170R; Eppendorf, Hamburg, Germany) at 37°C in 5% CO_2 with 98% relative humidity. The cells were allowed to adhere and grow for 24 h in a culture medium prior to exposure of extract.

MTT assay: Cell proliferation inhibition was determined using the MTT assay (27). Cells were plated in 96 well plates at 5×10^3 – 7×10^3 cell density/well and grown overnight in 100 μL of 10% fetal bovine

serum. After 24 h, cells were replenished with fresh media and sample solution was added to the cells. Hundred microliters (1–4 mg/mL) of each sample were added to wells in triplicates. Cells were incubated with the extract for 24 h. After 24 h, 20 μ L of MTT dye (5 mg/mL) was added to each well and further incubated for 3 h under dark conditions. Before readout, precipitates formed were dissolved in 150 μ L dimethyl sulphoxide using a shaker for 15 min. The percentage cell inhibition was calculated by measuring absorbance at 590 nm using the following equation:

$$\text{Cell proliferation inhibition (\%)} = 1 - \frac{A_{\text{sample 590}}}{A_{\text{control 590}}} \times 100$$

Fourier transform infrared spectroscopy (FTIR) The attenuated total reflection (ATR)-FTIR spectrum of mushroom extracts was recorded using an ATR-FTIR spectrophotometer (CARY 630; Agilent Technologies, Santa Clara, CA, USA) at room temperature. The spectra were recorded within the range 400–4,000 cm^{-1} .

Statistical analysis Analysis of variance was applied to the data for using a commercial statistical package SPSS ver. 10.1 (SPSS Inc., Chicago, IL, USA). The mean values were compared using Duncan's multiple range test at 5% level of significance.

Results and Discussion

DPPH inhibition activity DPPH is a free radical compound widely used to determine the free radical scavenging ability of various samples by providing proton; its color changes to yellow in the presence of an antioxidant (28). As shown in Fig. 1A, the scavenging activities on DPPH radical of methanolic extract obtained from the three mushroom varieties, *A. bisporus*, *P. ostreatus*, and *C. atramentarius*, increased with an increase in the extract concentrations and the inhibition increased significantly ($p \leq 0.05$) in the ranges 55.75–87.88%, 49.66–85%, and 57.05–85.95%, respectively. The difference in the DPPH scavenging activity of the three mushrooms might be the effect of special bioactive substances present in varying amounts (29). A number of studies have reported the antioxidant activities of various wild and cultivated mushroom varieties. Smolskait *et al.* (30) reported the DPPH scavenging activity of the methanolic extract of *A. bisporus* and *P. ostreatus* to be 0.13 and 0.74 mM TE/g (Trolox equivalent per gram) of the sample, respectively. Liu *et al.* (31) reported the DPPH inhibition activity of the ethanolic extract of button mushroom to be 90.13% at a concentration of 2 mg/mL.

Reducing power assay The reducing power assay measures the electron-donating ability of an antioxidant using the potassium ferricyanide reduction method and serves as an important sign of its antioxidant activity (32). The higher the absorbance of the reaction mixture, the better the reducing power of the compound. The

Fig. 1. (A) DPPH inhibition activity, (B) Reducing power (C) Metal chelating activity of mushroom extracts. Different alphabetical letters above the bars indicate significant difference among samples ($p \leq 0.05$). BHT and EDTA indicates butylated hydroxyl anisole and ethylene diamine tetraacetic acid, respectively.

reducing power of the three mushroom varieties is shown in Fig. 1B. A significant increase in absorbance was observed ($p \leq 0.05$) as the concentration of the extracts increases from 1 to 4 mg/mL, and the highest absorbance was shown by *C. atramentarius* extract (0.49 at 700 nm) at the concentration of 4 mg/mL. To the best of our information, the reducing power of *C. atramentarius* extract has not been investigated so far. The above results indicated that the marked ferric reducing power activity of extract might be due to the occurrence of polyphenols, which work in a parallel fashion as reductones react with free radicals to turn them into more stable products (33). However, Liu *et al.* (31) reported that the reducing power of ethanolic extracts of *A. bisporus* was 0.32 at 0.2 mg/mL.

Metal chelating ability of ferrous ion The metal chelating ability of the three mushroom varieties is shown in Fig. 1C. Among all varieties, *C. atramentarius* showed the highest metal chelating ability ($48.57 \pm 0.38\%$) at 4 mg/mL. As the extract concentration increased from 1 to 4 mg/mL, there is a significant increase ($p \leq 0.05$) in the metal chelating ability. The ability of the mushroom extracts to

Fig. 2. Total phenolic content of mushroom extracts. Different alphabetical letters above the bars indicate significant difference among samples ($p \leq 0.05$).

interfere with the formation of ferrous and ferrozine complexes indicates their metal chelating ability as they capture ferrous ion before ferrozine. Thus, an entire compound formation is interrupted, which results in the reduction of the red color (34). Woldegiorgis *et al.* (35) studied the metal chelating ability of various wild mushrooms of Ethiopia. Among all the studied mushrooms, *A. campestris* shows the highest inhibition of 69.7% at 0.25 mg/mL, exhibiting its superior scavenging and reducing activity.

Total phenolic content Mushrooms are an excellent source of phenolic compounds that can be used in different food formulations to prevent oxidative stress (36). The antioxidant potential of natural compounds is due to the presence of phenolic hydroxyl groups that donate hydrogen to free radicals, and thus in turn, terminates the chain reaction (37). The total phenolic content of the three edible mushrooms is shown in Fig. 2. Results are expressed as mg GAE/g of dried mushroom. *P. ostreatus* had the highest phenolic content (5.25 mg GAE/g) followed by *A. bisporus* (4.15 mg GAE/g) and *C. atramentarius* (3.51 mg GAE/g). Smolskait *et al.* (30) reported the total phenolic contents of *A. bisporus* and *P. ostreatus* to be 4.64 and 5.32 mg GAE/g, respectively.

Total flavonoid content Flavonoids are the most significant phenolics having various biological and chemical activities in addition to radical scavenging properties (38). In alkaline media, flavonoids form red complexes with $AlCl_3$. The flavonoid content of the three mushrooms is expressed as μg RE/g dried mushroom and is shown in Fig. 3. To the best of our knowledge, the estimation of flavonoids has not been reported so far. The flavonoid content varied with the mushroom types that were analyzed. *P. ostreatus* showed the highest flavonoid

Fig. 3. Total flavonoid content of mushroom extracts. Different alphabetical letters above the bars indicate significant difference among samples ($p \leq 0.05$).

concentration of 64.39 μg RE/g as compared to *A. bisporus* (56.76 μg RE/g) and *C. atramentarius* (61.64 μg RE/g).

Antimicrobial activity of mushroom extracts The antibacterial activity of the above analyzed mushroom varieties is shown in Table 1. Among all the three mushroom varieties, *C. atramentarius* showed the highest antibacterial activity against all the pathogenic bacteria tested. The highest zone of inhibition (7.45 ± 0.5 mm) was shown by *C. atramentarius* against *Sal. typhi*. The results are in conformity with the work of Sharma *et al.* (39) who has reported the antibacterial activity of *A. bisporus* using methanol and acetone as solvents against *S. aureus* and *E. coli*. The methanolic extract showed maximum inhibitory effect against the growth of each test bacterium. Llaurodo *et al.* (40) reported the antimicrobial activity of aqueous extract of *Pleurotus sp.* against four bacterial and four yeast strains, as this extract has been reported to combat simple and multiple drug resistant microbial strains (41). Ndyetabura *et al.* (42) reported the antibacterial activity of ethyl acetate extract of *C. cinereus* against *E. coli*, *C. albicans*, and *A. niger*. Hence, mushrooms can be used as potential natural antimicrobial agents.

Effect of mushroom extracts on cell viability Antioxidants are known to restrain cell proliferation and apoptosis, which depends on the nature of antioxidants, types of tumor cells, and treatment program (43-45). The present study showed that all the mushroom varieties possess antiproliferative activity against Colo-205 and MCF7, as shown in Fig. 4A and 4B, respectively. The cell proliferation inhibition activity varied significantly ($p \leq 0.05$) in all mushroom samples. *P. ostreatus* showed the lowest cell survival (22.83%) of malignant cells at the concentration of 4 mg/mL due to an increased

Table 1. Antibacterial activity (zone of inhibition in mm) of mushroom extracts

Samples	<i>E. coli</i>	<i>S. aureus</i>	<i>P. vulgaris</i>	<i>B. subtilis</i>	<i>Sal. typhi</i>
<i>Agaricus bisporus</i>	5.03 $\pm$ 0.37 ^{a1}	4.85 $\pm$ 0.26 ^{a1}	5.42 $\pm$ 0.54 ^{a12}	6.00 $\pm$ 0.55 ^{a2}	6.02 $\pm$ 0.5 ^{a2}
<i>Pleurotus ostreatus</i>	5.14 $\pm$ 0.03 ^{a1}	5.30 $\pm$ 0.17 ^{ab1}	6.61 $\pm$ 0.33 ^{ab1}	7.08 $\pm$ 0.07 ^{b2}	7.16 $\pm$ 0.22 ^{b3}
<i>Coprinus atramentarius</i>	5.93 $\pm$ 0.50 ^{b1}	5.72 $\pm$ 0.30 ^{b1}	7.28 $\pm$ 0.27 ^{b2}	7.04 $\pm$ 0.25 ^{b2}	7.45 $\pm$ 0.5 ^{b2}
Positive control	9.06 $\pm$ 1.40 ^{c1}	8.31 $\pm$ 0.40 ^{c2}	8.04 $\pm$ 0.30 ^{c3}	8.70 $\pm$ 0.70 ^{c3}	8.25 $\pm$ 0.27 ^{c3}

Different alphabetical letters and the numerics indicate significant difference among samples ($p \leq 0.05$) in the same column and row respectively.

Fig. 4. Antiproliferative activity of mushroom extracts against (A) colon and (B) breast cell lines. Different alphabetical letters above the bars indicate significant difference among samples ($p \leq 0.05$).

phenolic content. The antiproliferative activity of mushroom extracts is also due to the presence of intracellular and extracellular polysaccharides (46).

ATR-FTIR The analytical characteristics of the three mushroom extracts obtained from the fruiting bodies of *A. bisporus*, *P. ostreatus*, and *C. atramentarius* are shown in Fig. 5. To the best of our

knowledge, the FTIR spectrum of these mushroom extracts has not been investigated so far. The spectrum showed notable absorptions in the range 3,200-3,400, 2,800-3,000, 1,640-1,670, 1,550-1,640, and 850-890 cm^{-1} , which corresponds to -OH, C-H, C=O, -NH, and β -configuration, respectively. FTIR spectra of mushroom extracts showed the presence of typical carbohydrate, as indicated by the absorption bands ranging from 3,200 to 3,600 cm^{-1} , and the absorption peaks ranging from 3,200 to 3,500 cm^{-1} are also indicative of various phenolic compounds present in various mushroom extracts. Carbonyl groups (C=O) showed an asymmetrical stretching band around 1,640-1,670 cm^{-1} (47). All the extracts had only a small amount of protein, as observed by the absorption of an amide bond ranging from 1,650 to 1,580 cm^{-1} . A characteristic peak of β -configuration ranging from 850 to 890 cm^{-1} imparts mushrooms most of the nutraceutical properties (48). Apart from the presence of β -configuration in carbohydrates, various phenolic compounds and protein provide mushrooms their nutraceutical properties (20).

It can be concluded that edible wild or cultivated varieties of mushrooms have many nutraceutical applications and can be used in food processing units for making functional foods.

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Fig. 5. FTIR spectra of mushroom extracts

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