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Antimicrobial and antioxidant properties of *Morchella* spp. collected from Udhampur district of J&K

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ABSTRACT

***Morchella* have long been known for their medicinal properties. In J&K,*Morchella* locally known as "guchhi" are found in the forests and are being utilized in the healthcare industry as medicinal mushroom because it has a wide range of pharmacological activities and anti- inflammatory effects. In the present study, the fruiting bodies of*Morchella* spp. were collected from five regions of district Udhampur of J&K and tissue culture of the five isolates were designated i.e., Isolate-101, Isolate-102, Isolate-103, Isolate-104 and Isolate-105. Results of preliminary investigation against human pathogens by disc diffusion method revealed that Isolate-102 showed best inhibition against human pathogens (*Pseudomonas aeruginosa*, *Salmonella typhi* and *Klebsiella pneumonia*). Secondary metabolite of fruiting bodies and mycelium of potential Isolate-102 was extracted using ethanol and ethyl acetate. The crude extracts of mycelium and fruiting bodies were further studied for antimicrobial and antioxidant activities. The results of antimicrobial study showed that the mycelium inhibited *Pseudomonas aeruginosa* and *Salmonella typhi* (4.41mm and 11.16mm) whereas fruiting bodies didn't show any inhibition. The antioxidant activity (DPPH method and Carotene/Linoleic acid test) showed more inhibition (IC_{50}) in mycelium whereas the phenolic and flavonoid content was higher in fruiting bodies. The investigation showed that the *Morchella* mushroom mycelium is an excellent source of antioxidants and capable of imparting protection at different levels.**

Keywords: *Morchella*, antimicrobial, antioxidant, mycelium, fruiting bodies

The state of Jammu and Kashmir is rich in diversity range of flora, especially mushrooms, which have yet to be investigated and catalogued thoroughly (Watling and Abrahim, 1992). Some mushrooms are eaten for their nutritional value, while others have long been considered in traditional medicine (Lindequist *et al*., 2005). Different varieties of mushroom are used as natural sources of nutrients and medicines by humans (Sevindik, 2021). Gucchi, morel, common morel, real morel, morel mushroom, yellow morel, sponge morel, sheep belly, lamb belly mushroom, and other edible mushroom species belong to the Genus *Morchella* (Prasad *et al*., 2002). *Morchella* is the one of the most commercially important wild mushroom species. It is regarded as a valuable edible mushroom around the world. It has excellent healing properties for treating different illnesses, like tissue regeneration at 29.03%, fever at 19.35%, ulcers and colitis at 12.90%, anti-inflammatory at 22.58%, anti-carcinogenic at 9.67%, and immunosuppressive and

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antibiotic treatment at 3.23% (Magrati *et al*. 2011). Recently, it has been proven that morel possess anti-inflammatory, antitumor, antioxidant and antimicrobial activities and various health benefits (Thakur *et al*. 2021).

Morels can be found in timber habitat at altitude of 2500-3500 m and grows naturally in mountainous altitudes with a cold environment. *Morchella* is typically found in hardwood and coniferous forests as a mycorrhizal or saprobic association (Negi, 2006). The two worldwide production, India and Pakistan, each produce approximately 50 tonnes of dry morels, which are all exported. *Morchella* is high in protein, carbohydrates, vitamins, especially vitamin B, and has trace amounts of vitamins A, C, and D, making it a nutrient-rich, tasty, and healthy food. Iron, zinc, potassium, magnesium, sodium, calcium, phosphorus, selenium, copper, and manganese are among the minerals found in morels. Morels have a minimal fat and calorie content (Ajmal *et al*., 2015). *Morchella* spp. (True morels) are delicious mushrooms with exquisite aromas and remarkable appearance. The taxonomic position of morels is *Ascomycota, Pezizomycetes, Pezizales* and *Morchellaceae* (Hibbett *et al*. 2007).

Cultivation of *Morchella* has been impossible in the past and continues to be impossible today due to the inability to cultivate in *in vitro* circumstances. However, efforts are being made to cultivate this species of mushroom, and many studies are being attempted. Mycelium of *Morchella* mushrooms obtained from pure cultures is an ideal choice for developing safe health products. Extracts of this mushroom can be helpful in the treatment of various diseases. Several bioactive compounds can be derived from extracts of these morels and chemically defined molecules obtained from these may be used to develop functional foods and pharmaceutical drugs (Kosanic *et al.,*2013). Keeping in view the importance of morels, present study was designed to investigate the antimicrobial and antioxidant activities of fruiting body and mycelium of different *Morchella* spp. collected from the Udhampur district.

MATERIAL AND METHODS

Collection of mushroom sample

Fruiting bodies of morels was collected from Basantgarh, Ramnagar, Dudu, Kalwanta and Majouri of Udhampur district of J&K. The collected mushroom samples were brought to the Division of Microbiology, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Chatha Campus. The mushroom samples were dried in an oven at 40°C and ground into a fine powder for the extraction of fruiting bodies, while the remaining samples were stored in a refrigerator to obtain pure cultures.

Culture preparation of *Morchella* spp.

Pure cultures of *Morchella* spp. were obtained through tissue culture. The sample was sliced with a sharp knife and then immersed in a 0.1% $HgCl_2$ solution for 30–60 seconds to surface sterilize. With a sterile inoculating needle, small pieces of tissue were placed aseptically on to potato dextrose agar plates. The inoculation tubes were incubated for 10-15 days at 26±2°C, until the medium surface is completely covered with mycelial growth (Gupta *et al*., 2018).

Morpho-cultural identification of *Morchella* spp.

Isolates of *Morchella* spp. was grown on petri plates containing potato dextrose agar media (PDA) and incubated at 26±2°C for 5-6 days and colony as well as spore characteristics was recorded.

Submission of cultures to National repository

Pure cultures of *Morchella* spp. obtained were deposited to gene bank of Directorate of Mushroom Research, Solan India.

Screening of isolates of against human pathogens

Antimicrobial activity of all the isolates were examined using disc diffusion methods against human pathogens such as *Salmonella typhi* (MTCC98), *Pseudomonas aeruginosa* (MTCC741) obtained from IMTECH-Chandigarh, India, and *Klebsiella pneumoniae* (KS-19) obtained from NDRI Karnal. McFarland standards were used to standardize the microorganisms that were tested. The McFarland standard was used as a guide to regulate the turbidity of microbiological suspensions to keep their numbers within a certain range. With an absorbance of 0.132 at wavelength of 600 nm, 0.5 McFarland gives an approximate cell density of 1.5 x 10 CFU/ml. The bacterial suspensions were generated in their respective sterile nutrient broths and visually compared to the standard using Wicker ham cards and absorbance readings (Andrews, 2001). A volume of 100 µL of human pathogenic culture was dispersed over the surface of the agar media using a sterile cotton swab in petri plates containing nutrient agar media for bacterial growth. Using a sterile corkborer, nine-millimeter diameter actively growing fungal culture discs from PDA plates were cut and inoculated on the surface of the nutrient agar media seeded with test microorganisms. The plates were sealed with parafilm and maintained in the refrigerator for 12 hours at 4°C to ensure for complete antimicrobial agent diffusion, after which they were incubated at 26±2°C for another 12 hours. The diameter of the inhibitory zone was measured after incubation as per Zhang *et al.* (2009).

Secondary metabolite extraction from mycelium and fruiting bodies

Extraction from culture

Pure isolate of *Morchella* spp. was inoculated on a PDB in 250 mL Erlenmeyer flask with 100 mL medium. The flasks were incubated at 28 °C with occasional shaking at 150 rpm for two weeks. The fermentation broth was homogenized after the incubation period by adding 10% ethyl acetate to it and filtered. An equal volume of solvent was added to the filtrate, followed by vigorous mixing for 10 minutes and storage for 5 minutes until two clear immiscible layers formed. A separating funnel was used to separate the upper layer of solvent containing the extracted bio-actives. The crude metabolite was obtained by evaporating the solvent and drying the final product (Bhardwaj *et al*., 2015). 1 mg/mL of extract yield is dissolved in Dimethyl sulphoxide and stored at 4°C for further use (Sharma *et al*., 2016).

Extraction from fruiting bodies

The dried powder sample (20 gm) was extracted using 200 ml of ethanol at 30°C at 150 rpm for 24 h and filtered through Whatman No. 4 filter paper. A further 200 ml of ethanol was used to remove the residue. The combined ethanolic extract was then dried at 40°C, dissolved in Dimethyl sulphoxide to a concentration of 1 mg /ml, and kept at 4°C for further use (Turkoglu *et al*., 2006).

Secondary screening of crude extract of mycelium and fruiting bodies of Morchella spp.

The concentrated crude extract of mycelium and fruiting bodies of *Morchella* spp. was evaluated for antibacterial properties using agar well diffusion method. Wells were created using 6mm sterile corkborer. Human pathogens (*Klebsiella pneumonia, Pseudomonas aeruginosa*, *Salmonella typhi*) were swabbed on nutrient agar plates. The wells were filled with 80 µl of crude extracts of *Morchella* spp. and in the control plate, wells were filled with DMSO. The plates were incubated at 37±2°C for 24 hours. Zone of inhibition were recorded and compared with control (Perez *et al.* 1990).

Evaluation of antioxidant properties of *Morchella* spp.

DPPH method

Free radical scavenging activities were determined using DPPH method. 1, 1- diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical with a maximum optical absorbance at 517 nm. The presence of an antioxidant molecule in this radical medium is characterized by the change of the violet purple colour into a light yellow colour. A volume of 2 ml of ethanol solution of DPPH radical, with different concentration of *Morchella* (i.e., 40, 60, 80 and 100 µg) extract were mixed in tubes. The mixture was shaken vigorously and left to stay at room temperature for 30 min. The absorbance was read at 517 nm and radical scavenging activity was calculated as a percentage of DPPH using the following equation:

$$RSA\% = [(A_0-A_1 / A_0)100]$$

Where A_0 was the absorbance of control and A_1 was absorbance in presence of sample. Extract concentration providing 50% inhibition (IC_{50}) were calculated from the plot of inhibition (%) against extract concentration (Kosanic *et al.*, 2013).

Carotene/Linoleic acid test system

A solution of carotene was prepared by dissolving 2 mg of α-carotene in 10 ml of chloroform. A total of 2ml of this solution were pipetted into a 100 ml round bottom flask. After the chloroform was removed at 40°C, 40 mg of linoleic acid, 200 mg of tween 40 emulsifier and 100 ml of distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing different concentrations (i.e., 40, 60, 80 and 100 µg) of mycelial and fruiting body extracts. The tubes were shaken and incubated at 50°C in a water bath. As soon as the emulsion was added to each tube, the zero-time absorbance was measured at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 120 min after incubation at room temperature. Same procedure with Butylated Hydroxytoluene (BHT), with same concentration and a blank of 350 µL of ethanol were repeated. Antioxidant activity was calculated using the following equation:

$$\% AA = 100 [1-(A_o-A_t) / (A_{00}-A_{ot})]$$

Where A_o and A_{oo} is the absorbance measured at the beginning of the incubation for samples and control respectively. A_t and A_{ot} are absorbance measured for samples and control after incubation for 120 min respectively. The extract concentration providing 50% antioxidant activity (IC_{50}) was calculated from the graph of antioxidant activity percentage against extract concentration. BHT was used as standard (Boudoukha *et al.*, 2018).

Determination of total phenolic and flavonoid content of fruiting bodies and mycelium

Total Phenolic content

Folin Ciocalteu's phenol reagent technique was used to evaluate total phenolic content. The sample with different concentration was dissolved in distilled water and mixed with 10 mL Folin-Ciocalteau's reagent diluted 1/10 with ethanol. After few minutes sodium carbonate (8mL) was added to this solution. This solution was stored in dark place for 20 minutes and the absorbance was measured at 760 nm. Using gallic acid calibration as standard, the values were obtained for the concentration of total phenols and expressed as mg of GAE's/gdw of extract (Alispahic *et al.*, 2015).

Total Flavonoid Content

TFC was determined using aluminum chloride method. In 100 µl of extract, 1.25 ml distilled water

with 75 µl of 5% sodium nitrite was added and incubated for 5 minutes. 150 µl of aluminum chloride was added and incubated for 6 minutes, 1M 500 µl sodium hydroxide and 275 µl distilled water was added and mixed well. The absorbance was measured at 510 nm. Using quercetin calibration curve as standard, the values were obtained for the concentration of total flavonoid and expressed as mg of QE's/gdw of extract (Siangu *et al*., 2019).

Statistical analysis

All the results were carried out in triplicates and were expressed as Mean ± SD using excel and software SPSS.

RESULTS AND DISCUSSION

Fruiting bodies of *Morchella* spp. were collected from 5 different regions of Udhampur district of J&K. Pure cultures from the collected samples were obtained by tissue culture method and the isolates obtained were named as Isolate-101, Isolate-102, Isolate-103, Isolate-104 and Isolate-105 (Fig 1). Result of the failure of commercial cultivation of this species, is the reason for its mycelium culturing which is broadly utilized till date (Strapac *et al*. 2019). The cultured mycelium is obtained by many workers (Wang *et al*. 2020, Ozdal *et al*. 2019, Bala *et al*. 2017). All the isolates were grown on potato dextrose agar media (PDA) and incubated at 26±2^{0}C. The full growth of isolates took place in 5-6 days. Cultural characteristics of different isolates is given in Table 1. Microscopically all the isolates have septate hyphae and spores are hyaline and thin walled (Fig 2). Morpho-cultural identification and microscopic characters were seen of all 5 isolates.

All the isolates were screened against human pathogens by disc diffusion method, The results presented in Table 2 showed that Isolate-101 and 102 significantly inhibited all the human pathogens viz., *Pseudomonas aeruginosa* (4.50±1.03 and

Fig. 1. *Morchella* samples collected from Jammu and Kashmir

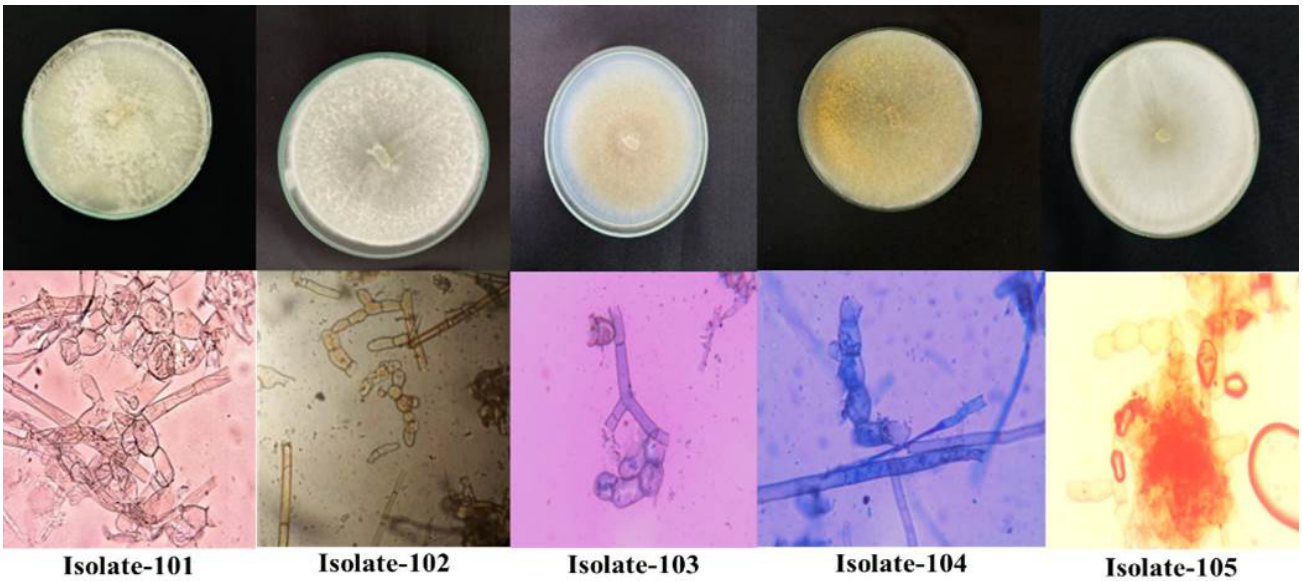

Fig. 2. Macroscopic and microscopic characteristics of collected specimens

Table 1. Macroscopic and microscopic characters of *Morchella* spp.

Characters	Isolate-101	Isolate-102	Isolate-103	Isolate-104	Isolate-105
Culture colour	Creamy white and fluffy like cotton	Dirty whitish	Brownish	Yellowish	Whitish
Spores	Heavy	Heavy	Heavy	Heavy	Poor
Hyphae	Septate	Septate	Septate	Septate	Septate
Texture	Smooth	Smooth	Smooth	Rough	Smooth
Microscopic walled	Hyaline thin walled	Hyaline thin walled	Hyaline thin walled	Hyaline thin walled	Hyaline thin
Margin	Regular	Regular	Regular	Regular	Regular

6.45±1.42mm), *Salmonella typhi* (7.80±1.21 and 7.60±1.15 mm) and *Klebsiella pneumonia* (5.45± 1.42 and 8.80± 0.95mm) respectively, whereas Isolate-103 showed zone of inhibition against *Pseudomonas aeruginosa* (5.30± 1.40mm) and *Salmonella typhi* (4.50± 1.03mm), Isolate-104

Table 2. Preliminary Screening of isolates against human pathogens

Isolates	Zone of Inhibition (mm)		
	Pseudomonas aeruginosa	***Salmonella typhi***	***Klebsiella pneumonia***
Isolate-101	4.50±1.03	7.80±1.21	5.45±1.42
Isolate-102	6.45±1.42	7.601.15	8.80± 0.95
Isolate-103	5.30±1.40	4.50±1.03	0.00±0.00
Isolate-104	0.00±0.00	7.30±1.30	6.30±1.40
Isolate- 105	0.00±0.00	0.00±0.00	7.80±1.21

showed zone of inhibition against *Salmonella typhi* (7.30± 1.30mm) and *Klebsiella pneumonia* (6.30± 1.40 mm). Isolate-105 inhibited the growth of *Klebsiella pneumonia* (7.80± 1.21 mm) only. Among all the treatments, Isolate -102 showed best result and therefore, it was selected for further experiments (Fig. 3).

Pseudomonas aeruginosa causes urinary tract infections and respiratory system infections etc. *Salmonella typhi* infection can lead to liver damage and inflammation of the heart etc. *Klebsiella pneumonia* are usually harmless, they often live in our intestines without giving you any problem. Among all the isolates Isolate-101 and Isolate-102 showed inhibition against all human pathogens in which the Isolate-101 showed the highest antimicrobial activity in the present study. Previous studies showed antimicrobial activities by *Morchella* sp. (Goutam *et al*. 2016; Heleno *et al*. 2009) using disc diffusion method against different human pathogens.

The two best pure cultures of *Morchella* spp. obtained after screening was deposited to gene bank of Directorate of Mushroom Research, Solan India. The accession number of deposited pure culture are mentioned in table-3 which proves that the isolated pure cultures are from *Morchella* species (Table 3).

Isolate -102 was further screened for secondary metabolite extraction. The fruiting bodies were extracted by using ethanol as solvent which produced

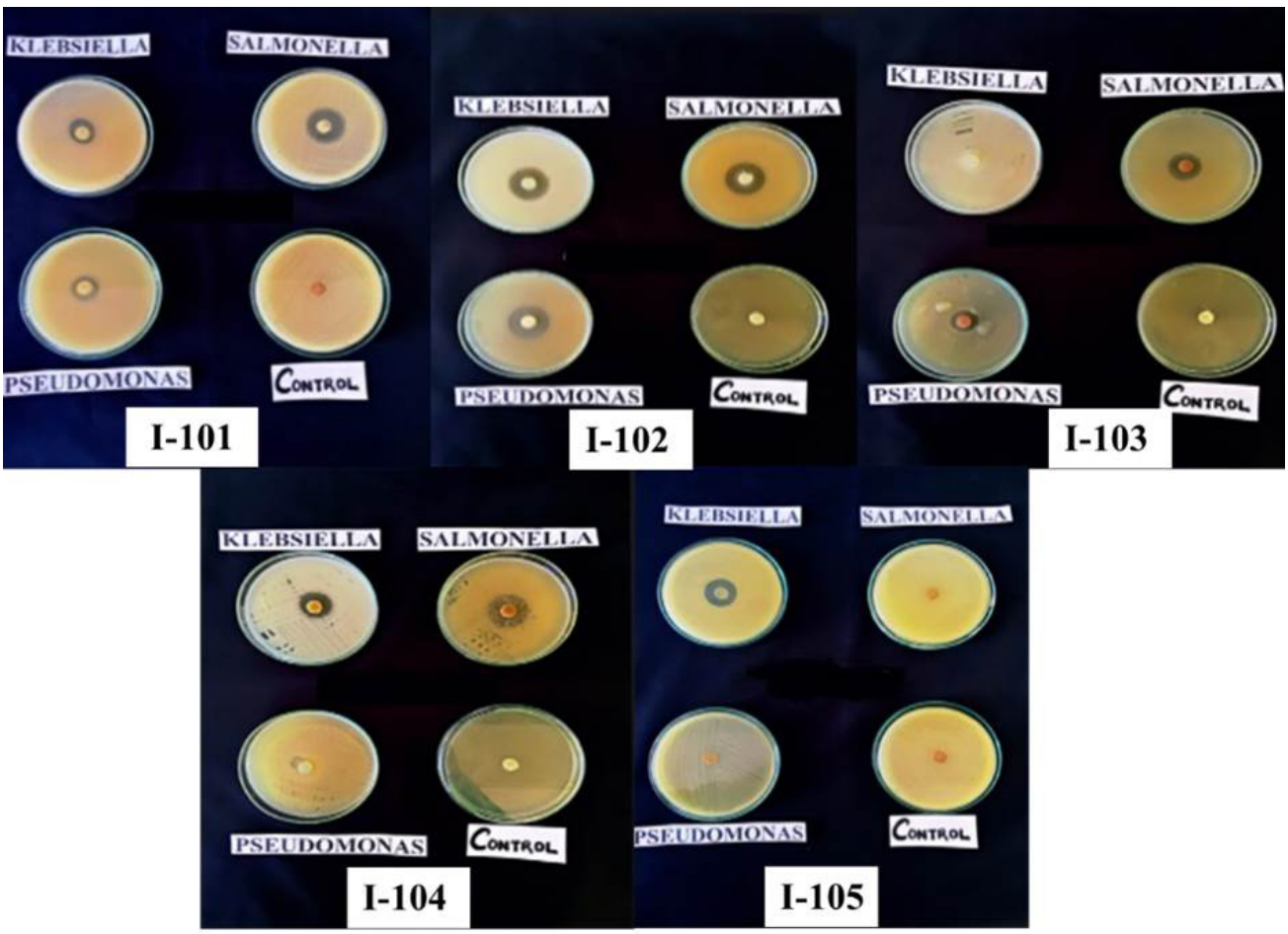

Fig. 3. Preliminary Screening of isolates against human pathogens

Table 3. Accession numbers obtained from directorate of mushroom research

Culture	Accession Number	Deposited Code
Morchella Spp.	DMRX-1937	J-19-20-101
Morchella Spp.	DMRX-1938	J-19-20-102

760 mg yield whereas the fermented cultured mycelium was extracted using ethyl acetate as solvent which produced 80 mg yield. The crude extract showed promising results by exhibiting antibacterial and antioxidant activities (Fig. 4). Secondary metabolites were extracted from cultured mycelium

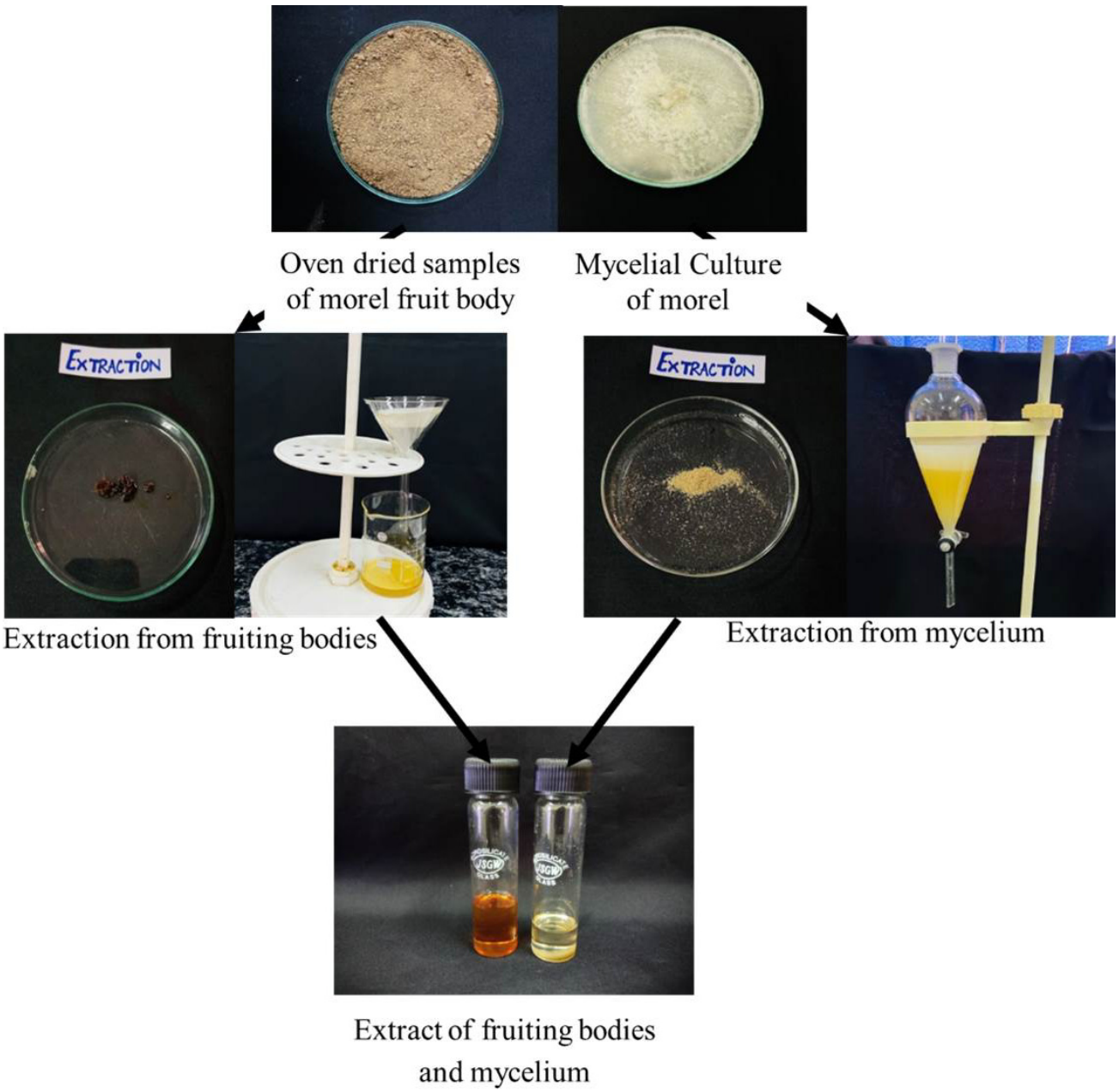

Fig. 4. Extraction of Secondary metabolites from fruiting bodies and mycelium of *Morchella* spp.

by fermentation process using ethyl acetate as solvent and the dried crude extract was dissolved in DMSO. Secondary metabolites were extracted from fruiting bodies by ethanol as solvent and the dried crude extract was dissolved in DMSO. Vamanu (2013) also reported extraction of secondary metabolites from *Morchella* mycelial cultures.

The crude extract of mycelium and fruiting body of Isolate-102 was screened against human pathogens by agar well diffusion method. The extract of mycelium showed zone of inhibition against *Pseudomonas aeruginosa* and *Salmonella typhi* (4.41±2.13 mm and 11.16±1.43 mm respectively). The extract of fruiting bodies didn't show any zone of inhibition against any human pathogens (Table 4). Khan *et al.* (2019) also tested antibacterial properties of *Morchella esculenta* using different solvents and ethyl acetate extract showed inhibition against *Pseudomonas aeruginosa* and *Salmonella typhi* but didn't show any inhibition against *Klebsiella pneumonia*. The ethanolic extract of fruiting bodies didn't show any zone of inhibition against any human pathogens. Previous studies revealed no antibacterial activity of *Morchella conica* against *P. aeruginosa, E. coli, M. morganii* and *K. pneumoniae* ethanol extract. The reason of the differences in results may be due to the difference in the species and the extraction solvents (Canli *et al.* 2019). These results demonstrate that the solubility of mushroom bioactive components varies on the extraction solvents used (Kumari *et al.* 2017).

During studies performed, radical scavenging activity (RSA%) in DPPH was observed in concentration of 40, 60, 80 and 100 µg is 46.50, 50.20, 57.40 and 63.80 per cent, respectively in the extract of fruiting body and 54.90, 62.33, 76.43 and 86.20 per cent, respectively in the extract of mycelium. The IC_{50} of fruiting bodies was 54.94 mg/ml and mycelium were 33.93 mg/ml, which means that mycelium showed maximum inhibition (Fig. 5). Antioxidant activity (AA%) in Carotene/Linoleic acid test resulted in 9.06, 15.26, 27.06 and 36.93 per cent in the extract of fruiting body in concentration of 40, 60, 80 and 100 µg, respectively while 35.33, 48.20, 57.33 and 66.53 per cent, respectively in the extract of mycelium. The IC_{50} of fruiting bodies is 128.5 mg/ml and mycelium 66.40 mg/ml, which showed higher inhibition in mycelium (Fig. 6). The blank of BHT value is 99.2 percent of antioxidant activity. The inhibition in both (DPPH & Carotene/Linoleic acid test system) is more in mycelium than in fruiting bodies showing that the ethyl acetate extract of mycelium of *Morchella* has more free radicals and many therapeutic values (Badshah *et al*. 2021).

During the present study, the total phenolic contents in the examined *Morchella* spp. extracts using the Folin-Ciocalteu's reagent is expressed in terms of Gallic acid equivalent (standard curve equation: $y = 0.007x+0.008$ $R^2 = 0.994$). The values obtained for the concentration of total phenols are expressed as mg of GAE's/gdw of extract. Here, the total phenolic content was shown higher in the fruiting bodies (Table 5). The total flavonoid contents is expressed in terms of quercetin equivalent (standard curve equation: $y=0.002x+0.015$ $R^2 = 0.994$). The values obtained for the concentration of total flavonoid are expressed as mg of QE's/gdw of extract. Here,

Table 4. Antibacterial activity of crude extract of mycelium and fruiting bodies

Isolates	Zone of Inhibition (mm)		
	Pseudomonas aeruginosa	***Salmonella typhi***	***Klebsiella pneumonia***
Mycelium	4.41±2.13	11.16±1.43	0.00±0.00
Fruiting bodies	0.00±0.00	0.00±0.00	0.00±.00

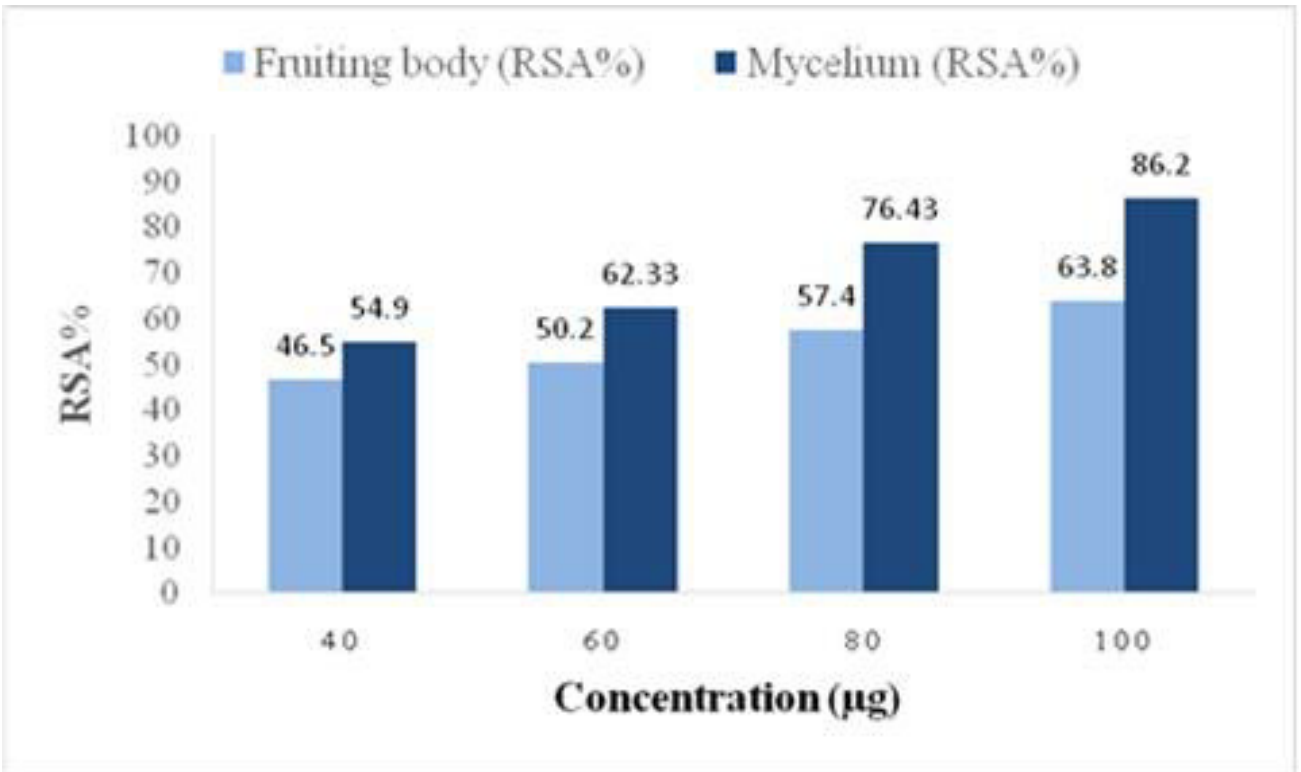

Fig. 5. DPPH Analysis of *Morchella* fruiting bodies and mycelium

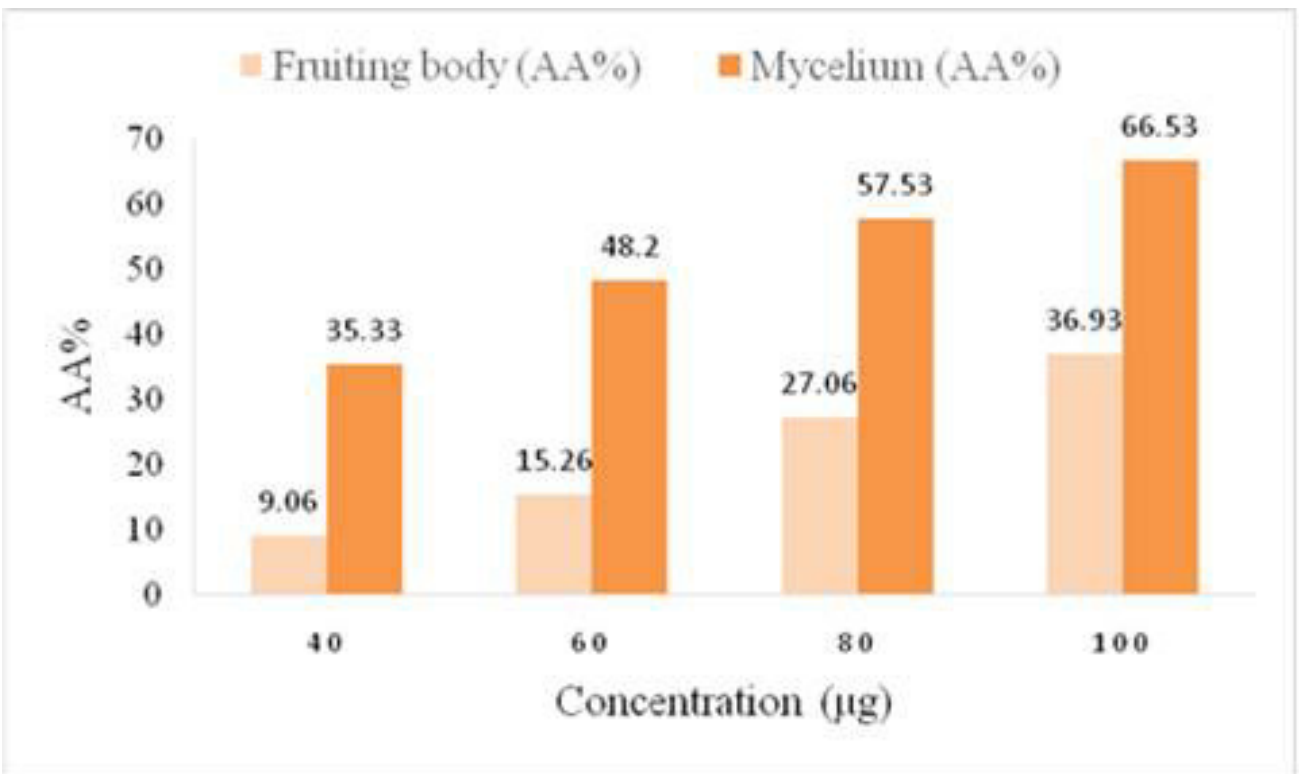

Fig. 6. Carotene/Linoleic Acid Test System of *Morchella* fruiting bodies and mycelium

Table 5. Total phenol and total flavonoid content of fruiting bodies and mycelium

Extract	TPC (mg GAE's /gdw)	TFC (mg QE's /gdw)
Fruiting bodies	29.37±0.67	17.99±0.8
Mycelium	22.05±0.44	8.65±0.64

total flavonoid content was also observed higher in the fruiting bodies (Table 5).

The correlation of total phenol and total flavonoid content with DPPH free radical scavenging activities and β-Carotene /Linoleic acid test of fruiting bodies revealed that both are positively correlated whereas total phenol and flavonoid content are also positively correlated with DPPH free radical scavenging activities and β-Carotene /Linoleic acid test of mycelium. Total phenolic content was shown maximum in the extract of fruiting bodies. Phenols and flavonoids have inhibitory effects on mutagenesis and carcinogenesis in humans, and are also known to reduce the risk of major chronic diseases (Raut *et al*. 2020). In our study, it was observed that antimicrobial and antioxidant activity of *Morchella* depend on the type of extract, i.e. the polarity of solvent used in extraction. High solubility of metabolites in polar solvents provides high concentration of these compounds in the extracts (Nitha *et al*. 2010). Regular eating of this mushroom may aid in the strengthening of learning and memory as well as can be an alternate source of food to help prevent oxidative damage in the human body (Bulam *et al*. 2018).

CONCLUSION

The results of the present study concluded that the extract of fruiting body and mycelium of *Morchella* sp. have significant antioxidant and antimicrobial activity under *in-vitro* conditions whereas the mycelium extract showed significant antibacterial activity against the test microorganisms. These findings suggest that mushrooms are a good and secure natural source of antioxidants. Further investigation should be done on fractionations and chemical characteristics of the antioxidant and antimicrobial components of these extracts.

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