

Molecular Identification and Isolation of Antimicrobial, Phytochemical, Antioxidant Properties of Different Solvent Extracts from Wild Mushrooms

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Abstract

The main objective of the present research work was to identify the unknown wild mushrooms based on morphological characteristics and DNA based sequencing by using universal primers ITS 1 and ITS 4 which were collected from Andhra Pradesh and Telangana. To test the potential of various extracts of mushrooms by using three different solvents (Methanol, Hot Water and Hexane). To elucidate antimicrobial properties, qualitative and quantitative estimation of phytochemical properties and antioxidant activity was determined by DPPH free radical scavenging activity. Then, IC₅₀ values were determined. The four isolates were identified on the basis of morphological and molecular characterization was identified as *Trametes versicolor*, *Daedaleopsis confragosa*, *Schizophyllum commune* and *Tyromyces chioneus*. Three different solvents had been shown good antimicrobial activity against all pathogenic microorganisms. The hot water extract of *T. versicolor* exhibited more amount of phenol content (11.13±0.081) and ascorbic acid content (9.62±0.086). Methanol extract of *S. commune* showed high amount of flavonoids content (12.58±0.138). Methanol extract of *T. chioneus* exhibited more amount of β-carotene (3.54±0.010) and hot water extract of *D. confragosa* exhibited more amount of lycopene content (2.29±0.012). The DPPH free radical scavenging activity and IC₅₀ values was much more exhibited by methanol extracts then followed by hot water extracts and hexane extracts of wild mushrooms they all have exhibited stronger antioxidant activity between (40-50 µg/ml). This comprehensive analysis demonstrates that these mushrooms having a viable source of natural antioxidants that can utilized for functional foods, nutraceutical's applications with beneficial biological activities and implementation of new drug discovery.

Keywords: Antimicrobial, Antioxidant, DNA isolation, Mushrooms and Phytochemical.

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ABBREVIATIONS

DNA – Deoxyribonucleic acid

DMSO – Di methoxy sulfoxide

DPPH - 1, 1-diphenyl -2-picrylhydrazyl

MEA – Malt Extract Agar

MHA – Muller Hinton Agar

MHB – Muller Hinton Broth

PCR – Polymerase Chain Reaction

Highlights of the Paper

- The four isolates of Wild mushrooms are *Trametes versicolor*, *Daedaleopsis confragosa*, *Schizophyllum commune* and *Tyromyces chioneus* has shown good antimicrobial activity against pathogenic microorganisms.

- Also, they have exhibited good potential of antioxidant properties.
- Molecular identification is done for these species, therefore DNA is extracted by liquid nitrogen method and sequences are submitted to Gene Bank further they provided with accession numbers and Phylogenetic Tree was constructed.
- Hence, comprehensive analysis demonstrates that these mushrooms having a viable source of natural antioxidants that can utilized as functional foods, nutraceutical's applications with beneficial biological activities and implementation of new drug discovery, if further investigation is processed.

INTRODUCTION

The world of fungi is large and diverse in ecosystem. Wild mushrooms are important sources of natural bioactive compounds and are antioxidant potential. Therefore, mushroom is a type of a fleshy fruiting body of some fungi which are arising from a group of mycelia buried in substratum [1]. Most of them are basidiomycetes and are gilled. Based on their spores, they are called as a basidiospores [2]. Wild mushrooms are either epigeous or hypogeous heterotrophic organisms that belong to the Basidiomycota and Ascomycota divisions [3]. Many of the wild mushroom species are becoming important parts of the livelihoods of humans now days in different parts of the world [4], and being collected as valuable product of Non -Timber Forest Products (NTFPs) [5]. Now a day's microorganisms are becoming serious threat to mankind by becoming more drug resistance. Due to long term indiscriminate use of drugs against antimicrobial agents they are becoming more resistance which results in acquisition of mutation in the genome and genes of disease-causing microorganisms that assist their survival [6]. Therefore, it is not possible to prevent the microbial evolution, but it is important to choose the most appropriate reliable antibiotics and use them properly to reduce the development of drug resistant strains [7]. As we all know wild mushrooms are a good source of nutrition and having wide range of medicinal values [8].

MATERIALS AND METHODS

Collection, isolation and morphological characteristics of wild mushrooms:

Wild mushroom samples are collected from Andhra Pradesh and Telangana forest area and was labelled as sample no. 1/18, 2/18, 3/18, 4/18. The samples were kept in sterile plastic bag. Brought it to laboratory and washed thoroughly with sterile distilled water and 0.001% mercuric chloride and with the help of sterile blade it was brake into length wise of the tissue. The bits aseptically transferred onto a MEA. The four samples replicates were made and the plates incubated at 25°C temperature for 7 days. Sub-culture was done from mother inoculum plates to pure MEA plates to obtain the pure cultures. Therefore, general characters and microscopic features were recorded.

Maintenance of culture of test organisms

Five bacterial strains were taken namely *Klebsiella pneumoniae* (MTCC109), *Salmonella typhi* (MTCC10248), *Staphylococcus aureus* (MTCC737), *Escherichia coli* (MTCC739) and *Pseudomonas aeruginosa* (MTCC741) obtained from Microbial Type Culture Collection (MTCC) IMTECH, Chandigarh (INDIA) and stored at 4°C in refrigerator and sub cultured at regular intervals of 48h until use. One fungal strain was taken namely *Candida albicans* (80CC90028) strain procured from yeast laboratory of Shoolini University.

Preparation of different extracts

Methanol and Hexane extracts

Freeze-dried mycelial biomass (5g) was accurately weighed into a 100 ml sterilin and shaken overnight (18 – 24 hours) with 50 ml of methanol at room temperature. The extract was then filtered through Whatman paper No.1. The residue was then resuspended in another 50 ml of methanol. The process was repeated twice. The combined methanol filtrate was transferred to a pre-weighted sterilin and the solvent was evaporated by applying a constant flow of air. Residual methanol was removed by vacuum pressure at 50°C overnight in an oven and the dry weight recorded. Therefore, hexane extract is also prepared as same as methanol extract. In place of methanol add hexane solvent for preparation of hexane extract [9].

Hot water extracts

Dry mycelial biomass was accurately weighed and 10 ml deionized water was added to it. The mixture was heated at 100°C for 20 minutes and allowed to cool for 20 minutes. After this, the filtrate was separated from the mycelial biomass by using No. 42 Whatman filter paper. The process was repeated twice. The combined filtrate was then freeze-dried and the dry weight of sample was recorded [10].

Antimicrobial activity

MHA plates with 4% NaCl supplementation were prepared. Sterilized swabs were dipped in standardized bacterial suspension with inoculums size of 1.5 X 10⁸cfu/ml prepared above and excess culture was removed by turning the swab against the side of the tube. Inoculum was spread evenly over the entire surface of MHA plates. These plates were allowed to dry for at least 15 min and then wells (7mm diameter) were made on petridish using sterile cork borer. About 25µl extract was introduced into bore agar wells using sterile dropping pipette. These plates were kept inside the refrigerator at 4°C for 6 hours to allow proper diffusion of extracts into medium. The plates were then examined for antibacterial activities of extracts after 24 hours of incubation at 37°C. Antibacterial activity was determined by measuring the diameter zone of inhibitions in mm [11].

Minimal Restrictory concentration for estimating Antimicrobial activity

Resazurin solution was prepared by dissolving 270 mg of resazurin powder in 40 ml sterile distilled water. Each well of 96-well microtiter plate was aliquoted with 50 µl of MHB; 12th well (sterility control) was added with 100 µl of Ciprofloxacin antibiotic as positive control. 11th well (growth control) was added with MHB with 10% DMSO. 50 µl of mycelial extract initially dissolved in 10% DMSO to the concentration of 100 mg/ml was added into the first well and a serial 2-fold dilution was performed by

transferring 50 µl of the suspension to the subsequent wells up till the 10th well; the final 50 µl of the suspension was discarded. Then, of 5 µl bacterial suspension was added to each well and incubated at 37°C for 24 hours. After 24 hours of incubation 5 µl of resazurin was added to each well. Plates were incubated at 37°C for additional 1 hour. After one hour of incubation the plates were read for colour change from blue to purple/ pink. A blue coloured solution indicated the growth inhibition in the test wells, while pink to colourless solution indicated microbial growth or absence of inhibition [12].

Qualitative and Quantitative analysis of bioactive compounds

Qualitative analysis of phytochemical compounds was done for three different extracts of four wild mushrooms. For identification of alkaloids Mayer's and Wagner's test was done [13]. Molisch's and Benedict's test were performed for the identification of carbohydrates [13]. To check the presence of flavonoids alkaline reagent test was done [14]. Salkowski test was done for identification of terpenoid's (Bains and Tripathi, 2016). For identification of phenolic compounds and tannin Ferric chloride and Lead acetate test was done. Detection of glycosides was done by adding 1ml of glacial acetic acid and 5% of ferric chloride was added to 2 ml of extract, presence of greenish blue colour indicates glycosides [10]. Therefore, for detection of saponins 5-10 ml of distilled water was added to 1 ml of extract and shaken gradually cylinder for 15 minutes, formation of foam indicates positive [15]. There by quantitative analysis of bioactive compounds was done for phenolic, flavonoid, ascorbic, β-carotene and lycopene determination.

Phenolic Compound determination

100µl of all extract of mushroom samples mixed with 1.5ml of Folin-Ciocalteu reagent and incubated at room temperature for one minute followed by the addition of 1.5ml sodium carbonate (60g/l). The tubes would be then vortex-mixed for 15s and allowed to stand for 90 minutes in dark at room temperature. Absorbance will be then measured at 725 nm. Gallic acid used as standard curve with concentration ranges from 1 to 100µg/ml ($R^2=0.996$) and the results expressed as mg gallic acid equivalent (GAE)/g. All experiments would be performed in triplicates [16].

Flavonoid determination

250 µl extract of all extract of mushroom samples mixed with 1.25 ml of distilled water and 75 µl of a 5% NaNO₂ solution. 150 µl of 10% AlCl₃.H₂O added after 5 min. Then 275 µl of distilled water and 500 µl of 1 M NaOH added to the mixture after 6 min. The solution then mixed well and the intensity of pink colour measured at 510 nm. The flavonoid compounds estimation would be carried out in triplicate. Quercetin used for calibration curve with concentration range of

20-100 µg/ml ($R^2=0.9938$). The results would be mean values ± standard deviations and expressed as milligrams of (+) quercetin equivalents (QEs) per gram of extract [17].

Ascorbic acid determination

100 mg of all dried extracts of mushroom samples extracted with 10 ml of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No.4 filter paper. The filtrate (1 ml) will be mixed with 9 ml of 2, 6-dichloroindophenol and then absorbance will be measured within 30 min at 515 nm against a blank. Content of ascorbic acid will be calculated on the basis of the calibration curve of authentic L-ascorbic with concentration range of 2- 125 µg /ml ($R^2=0.9929$). All the experiments would be performed in triplicates [18].

β-carotene and lycopene determination

The dried all three extracts of each mushroom samples (100 mg) are vigorously shake with 10 ml of acetone-hexane mixture (4:6) for 1 min and filtered through Whatman No.4 filter paper. The absorbance of the filtrate measured at 453, 505, 645 and 663 nm [19]. Content of β-carotene and lycopene were calculated by using following equations:

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

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

Scavenging activity on DPPH for antioxidant assay

The antioxidant activity of the extracts on the stable radical DPPH was determined by the method developed by [20]. The 0.1 ml of mushroom ethanol extract, at various concentrations was added to 3 ml of a 0.004% methanol solution of DPPH and was allowed to stand for 30 min for the reaction to occur. The absorbance of the resulting solution was measured at 517 nm from these values the corresponding percentage of inhibitions was calculated by using the following equation:

$$I \% = [(A_{\text{Blank}} - A_{\text{sample}}) / A_{\text{Blank}}] \times 100,$$

Where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} is the absorbance of sample/standard. To get the effective concentration IC₅₀, (defined as the concentration of antioxidant required to reducing the concentration of initial DPPH 50%) for each extract, concentration providing 50% inhibition (IC₅₀) was calculated from the graph plotted I% versus concentration curve. The free radical scavenging activity was measured for different concentrations of sample and compared with standard (Ascorbic acid).

Molecular identification of wild mushroom and sequencing

Genomic DNA of wild mushrooms was isolated by manually using chemicals like liquid nitrogen, phenol, chloroform, isopropyl alcohol, ethanol, lysis buffer, SDS (Sodium dodecyl sulphate) and TE buffer. After isolation the DNA was subjected for PCR amplification. The DNA was amplified by using universal primers ITS1-(5'-TCC GTA GGT GAA CCT GCG G-3') Forward primer and ITS4-(5' TCC GCT TAT TGA TAT GC-3') Reverse primer for the region containing ITS1, ITS4 and rDNA. The reaction mixture procedure was followed by the kit (HIMEDIA X-PERT). Amplifications was performed in 25µl reactions of PCR buffer in replicates and PCR reactions consists of an initial denaturation at 94°C for 3 minutes, for annealing 25 cycles at 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 1 minute and final extension at 72°C for 10 minutes. Amplification products was

electrophoresing in 0.8% agarose gel, stained with ethidium bromide and amplicons will be observed under UV light. The final PCR product was subjected to DNA sequencing. The DNA sequence was used in NCBI (National Centre for Biotechnology Information) and search tool BLAST (Basic Local Alignment Search Tool) for bioinformatics analysis, to look homologous nucleotide sequences. Based on the information, genus and species status was assigned.

RESULTS

Collection and Morphological Characters

Four samples of wild mushrooms were collected and maintenance of pure cultures was done aseptically. The microscopic features were studied under microscope by using Congo red stain. Clamp connections and chlamydospores was observed (Fig 1).

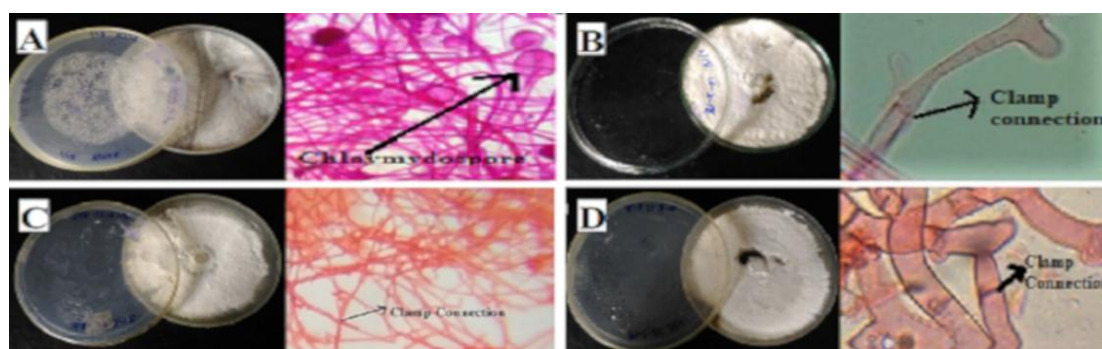


Fig-1: Mycelial and microscopic view of pure cultures of wild mushrooms.

A) *Trametes versicolor*, B) *Daedaleopsis confragosa*, C) *Schizophyllum commune* and D) *Tyromyces chioneus*

Antimicrobial activity for screening

All the methanol extract of cultures was found to be active against all the five pathogenic microorganisms. Among all the cultures, *T. versicolor* shows maximum activity (29.75±0.25) against *K.*

pneumonia, against *E. coli*, *T. chioneus* shows maximum activity (27.80±0.20), against *Staphylococcus aureus*, *S. commune* shows maximum activity (28.00±1.00), against *P. aeruginosa*, *S. commune* shows maximum activity (26.75±0.25), and against *C. albicans*, *T. chioneus* shows maximum activity (27.75±0.25). The results of methanol, hot water and hexane extract of different species were shown below in (Table 1 and Fig 2).

Table-1: Antimicrobial activity of Methanol (M), Hot Water (HW) and Hexane (H) extracts of different wild mushrooms culture against different pathogenic microorganisms

Sr. No	Isolates	Test micro-organisms and concentration of Methanol, Hot water and Hexane Extract used (100mg/ml) Zone inhibition diameter (mm)														
		<i>Klebsiella pneumoniae</i>			<i>Escherichia coli</i>			<i>Staphylococcus aureus</i>			<i>Pseudomonas aeruginosa</i>			<i>Candida sp.</i>		
		M	HW	H	M	HW	H	M	HW	H	M	HW	H	M	HW	H
1	<i>Trametes sp.</i>	29.75±0.25 ^a	24.75±0.25 ^b	19.75±0.25 ^c	25.50±0.50 ^b	22.50±0.50 ^b	24.55±0.45 ^b	26.25±0.25 ^a	23.00±1.00 ^b	18.50±0.50 ^c	24.50±1.50 ^b	25.75±1.25 ^a	21.92±1.58 ^b	23.00±1.00 ^b	22.30±0.30 ^b	22.50±1.50 ^b

2	<i>Daedaleopsis</i> sp.	28.50±0.50 ^a	26.85±0.15 ^a	20.50±1.50 ^c	27.00±2.00 ^a	27.00±1.00 ^a	24.50±0.50 ^b	26.50±0.50 ^a	25.50±2.50 ^a	18.50±0.50 ^c	23.50±0.50 ^b	25.00±1.00 ^a	22.00±1.00 ^b	24.30±0.30 ^b	24.55±0.45 ^b	25.00±2.00 ^b
3	<i>Schizophyllum</i> sp.	27.00±1.00 ^a	23.60±0.39 ^c	23.90±0.10 ^b	25.75±1.25 ^b	22.50±1.50 ^b	23.75±0.25 ^b	28.00±1.00 ^a	19.40±0.60 ^c	25.00±1.00 ^b	26.75±0.25 ^a	23.82±0.17 ^b	24.00±1.00 ^b	24.50±0.70 ^b	26.90±0.10 ^a	23.50±1.50 ^b
4	<i>Tyromyces</i> sp.	28.55±0.45 ^a	25.85±0.15 ^a	22.00±1.00 ^b	27.80±0.20 ^a	24.75±0.25 ^a	21.90±0.90 ^c	24.85±0.15 ^b	23.55±0.45 ^b	22.88±0.12 ^b	25.50±0.50 ^a	25.83±0.16 ^a	26.15±0.85 ^b	27.75±0.25 ^a	22.56±0.44 ^b	22.50±1.50 ^b
5	Ciprofloxacin/ Flucanazole (Control)	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	28.25±0.23 ^a	27.45±0.10 ^a	27.45±0.10 ^a	27.45±0.10 ^a

Different letters of each values in row means significant difference at P<0.05. Values are Mean ± SD (n=2).

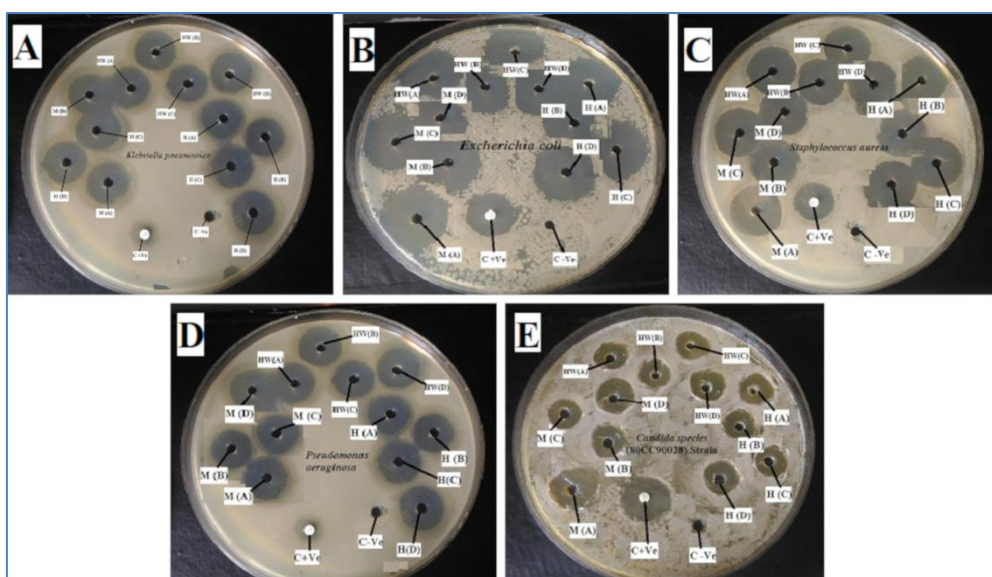


Fig-2: Zone of inhibition of Methanol, Hot Water and Hexane extracts of four wild mushroom cultures against Pathogenic microorganisms.

A) *Klebsiella pneumoniae*, B) *Escherichia coli*, C) *Staphylococcus aureus*, D) *Pseudomonas aeruginosa* and *Candida* sp. M (A) Activity of Methanol extract of *Trametes* sp., M (B) *Daedaleopsis* sp., M (C) *Schizophyllum* sp. and M (D) *Tyromyces* sp., HW (A) Activity of Hot water extract of *Trametes* sp., HW (B) *Daedaleopsis* sp., HW (C) *Schizophyllum* sp., and HW (D) *Tyromyces* sp., H (A) Activity of Hexane extract of *Trametes* sp., H (B) *Daedaleopsis* sp., H (C) *Schizophyllum* sp., and H (D) *Tyromyces* sp. C +ve is

positive control Ciprofloxacin and C -ve is negative control DMSO.

Minimal inhibitory concentration of methanol, hot water and hexane extract

According to results all four-methanol extract of mushroom cultures exhibit a good antimicrobial spectrum activity compared to hot water extract and hexane extract. *S. communae* of methanol extract is best to potentiate the antibacterial activity of *S. aureus* and

P. aeruginosa with MIC 0.39 mg/ml. against fungal strain of *C. albicans* extract of *S. communae* showed good activity with MIC 0.39 mg/ml. Extract of *S. communae* showed high antibacterial potential against

K. pneumoniae and *E. coli* with MIC 1.56 mg/ml. The results of minimal inhibitory concentration of methanol extract shown in below (Table 2).

Table-2: Minimal inhibitory concentration of Methanol (M), Hot Water (HW) and Hexane (H) extracts of different wild mushrooms culture against different pathogenic microorganisms

Sr. No	Isolates	Minimal inhibitory concentration of methanol extracts (mg/ml)														
		<i>Klebsiella pneumoniae</i>			<i>Escherichia coli</i>		<i>Staphylococcus aureus</i>			<i>Pseudomonas aeruginosa</i>			<i>Candida sp.</i>			
		M	HW	H	M	HW	H	M	HW	H	M	HW	H	M	HW	H
1	<i>Trametes sp.</i>	6.25	1.56	3.12	12.5	1.56	3.12	3.12	0.39	12.5	3.12	0.39	6.25	1.56	0.39	6.25
2	<i>Daedaleopsis sp.</i>	12.5	12.5	6.25	25	25	1.56	12.5	12.5	1.56	6.25	6.25	1.56	3.12	3.12	0.78
3	<i>Schizophyllum sp.</i>	1.56	1.56	3.12	1.56	1.56	25	0.39	0.39	25	0.39	0.39	25	0.39	0.39	12.5
4	<i>Tyromyces sp.</i>	6.25	1.56	12.5	25	0.39	25	1.56	0.78	50	1.56	3.12	12.5	0.78	0.39	25

Qualitative analysis of bioactive compounds

The preliminary phytoconstituents analysis of all isolates revealed that the methanol, hot water and

hexane extracts contain Alkaloids, Tannins, Terpeonoids, Carbohydrates, Phenols, Flavonoids, Glycosides and Saponins as shown in (Table 3).

Table-3: Qualitative analysis of methanol, hot water and hexane extracts of wild mushroom cultures

Sr. No.	Phytochemicals	Qualitative analysis of Methanol, Hot Water and Hexane extracts											
		<i>Trametes sp.</i>			<i>Daedaleopsis sp.</i>		<i>Schizophyllum sp.</i>			<i>Tyromyces sp.</i>			
		M	HW	H	M	HW	H	M	HW	H	M	HW	H
1	Alkaloids	+	-	-	+	-	-	+	+	-	+	+	+
2	Carbohydrates	+	-	+	+	+	-	+	+	+	+	+	-
3	Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
4	Terpeonoids	+	+	+	+	+	+	+	+	+	+	+	+
5	Phenols	+	+	+	+	+	+	+	+	-	-	+	+
6	Tannins	+	-	-	+	+	+	+	+	-	+	+	+
7	Glycosides	+	+	+	+	-	-	+	+	+	+	+	-
8	Saponins	+	+	-	+	+	-	+	+	-	-	-	-

Quantitative analysis of phytochemical compounds

Phenolic compounds are known to be powerful chain-breaking antioxidants and they possess scavenging ability due to their hydroxyl groups. There must be phenolic compounds which contribute directly to the anti-oxidative action.

In present study, that is for phenols hot water extract is the best compare to methanol and hexane extracts. The phenol content in hot water extract of *T. versicolor* was (11.13±0.081 µg/ml), *D. confragosa* was (8.27±0.046 µg/ml), *S. communae* was (9.52±0.210 µg/ml) and *T. chioneus* was (5.53±0.105 µg/ml) observed.

For flavonoids methanol extract is having more efficiency compare to hot water and hexane extracts. The flavonoids content in methanol extract of *T. versicolor* was (10.04±0.057 µg/ml), *D. confragosa* was (10.26±0.073 µg/ml), *S. communae* was (12.58±0.138 µg/ml) and *T. chioneus* was (10.76±0.100 µg/ml).

For ascorbic acid content in these mushrooms hot water extract shows best results compare to methanol and hexane extract. The ascorbic acid content in hot water extract of *T. versicolor* was (9.62±0.086 µg/ml), *D. confragosa* was (7.22±0.104 µg/ml), *S.*

communae was (7.48±0.165 µg/ml) and *T. chioneus* was (6.66±0.047 µg/ml).

For β-carotene methanol extract shows efficiency compare to hot water extract and hexane extract. The β-carotene content in methanol extract of *T. versicolor* was (2.41±0.305 µg/ml), *D. confragosa* was (3.10±0.056 µg/ml), *S. communae* was (2.27±0.026 µg/ml) and *T. chioneus* was (3.54±0.010 µg/ml).

For lycopene methanol extract is best compare to hot water extract and hexane extract. The lycopene content in methanol extract of *T. versicolor* was (2.02±0.015 µg/ml), *D. confragosa* was (1.94±0.017 µg/ml), *S. communae* was (2.13±0.018 µg/ml) and *T. chioneus* was (0.18±0.003 µg/ml).

Totals phenols, flavonoids, ascorbic acid, β-carotene and lycopene were the major naturally occurring antioxidant components estimated in this study. The higher amounts of these components in these three extracts (methanol, hot water and hexane) might explain its effectiveness in antioxidant properties.

DPPH Radical Scavenging activity

The DPPH assay is based on the capability of an antioxidant to donate hydrogen radical or an electron to DPPH radical, which is stable free radical with deep

violet colour. As shown in (Table 4), the scavenging ability of the methanol extract was highest for *S. communae* ranges from 11.27±0.14 to 76.59±0.08 followed by *T. chioneus* ranges from 38.66±0.19 to 75.43±0.11, *D. confragosa* ranges from 25.43±0.05 to

73.81±0.08, *T. versicolor* 18.30±0.03 to 65.97±0.32, respectively. Ascorbic acid was taken as standard which showed excellent scavenging activity of 83.57±0.01% as shown in (Table 4).

Table-4: Scavenging ability of Methanol extract from different isolates on 2,2-diphenyl-1-picrylhydrazyl radicals

Isolates	Concentration of Methanol Extract used ($\mu\text{g/ml}$) (Scavenging activity of free DPPH radicals) (%)				
	5	10	15	20	25
<i>Trametes sp.</i>	18.30±0.03 ^a	29.64±0.02 ^b	37.93±0.03 ^b	57.09±0.48 ^a	65.97±0.32 ^b
<i>Daedaleopsis sp.</i>	25.43±0.05 ^b	30.16±0.14 ^b	43.25±0.04 ^a	58.42±0.02 ^a	73.81±0.08 ^b
<i>Schizophyllum sp.</i>	11.27±0.14 ^c	32.62±0.09 ^b	53.43±0.16 ^a	63.47±0.03 ^a	76.59±0.08 ^a
<i>Tyromyces sp.</i>	38.66±0.19 ^a	45.20±0.04 ^a	51.14±0.01 ^a	59.67±0.02 ^a	75.43±0.11 ^a
Ascorbic acid	10.56±0.12 ^c	32.71±0.06 ^b	48.46±0.02 ^a	66.33±0.01 ^a	83.57±0.01 ^a

Different letters of each value mean significant difference at $P < 0.05$. Values are Mean \pm SD (n=3)

According to recent research, polar solvents are the best for the extraction of antioxidants. The IC_{50} value was calculated to determine the concentration of the sample required to inhibit 50% of radical. Therefore, the extracts which possess IC_{50} values ranging from 50 to 100 mg / mL is considered to exhibit intermediate antioxidant activity. Meanwhile, extracts with IC_{50} value ranging between 10 to 50 mg / mL is considered to possess strong antioxidant activity [21]. The IC_{50} value of methanol extract of *Trametes species* is 4.9 mg/ml, *D. confragosa* is 4.8 mg/ml, *S. communae* is 4.9 mg/ml and *T. chioneus* is 4.4 mg/ml.

Molecular identification of wild mushrooms

DNA is successfully was extracted from the cultures of mycelium from wild mushrooms by liquid nitrogen. After centrifugation the genomic DNA is extracted and PCR reactions were carried out successfully by using universal primers (ITS1 forward primer and ITS4 reverse primer) were used for the amplification of the ITS regions of the wild mushrooms. The PCR amplification products showed that *T. versicolor* showed about 723 bp amplified band, *D. confragosa* showed about 577 bp amplified band, while *S. communae* gave about 562 bp amplified band and *T. chioneus* showed about 815 bp amplified bands respectively (Fig 3).

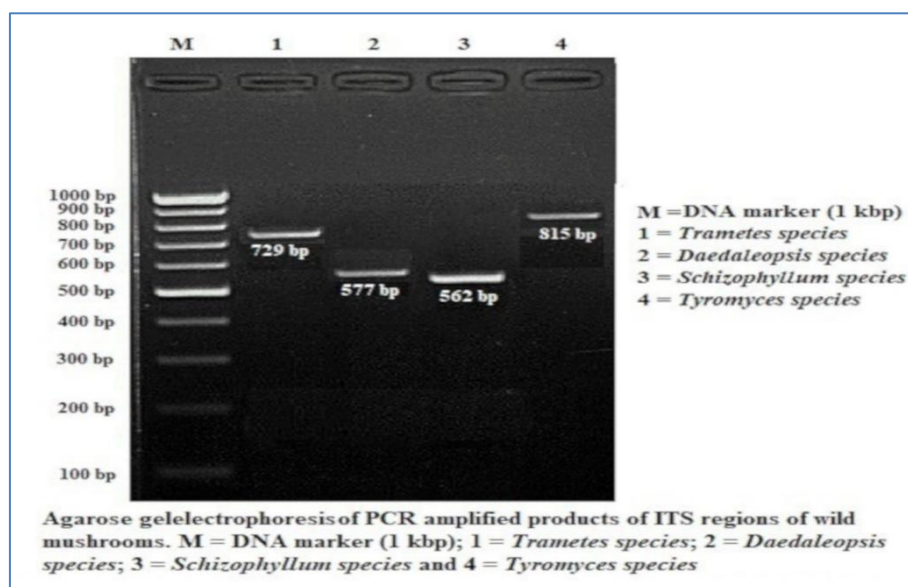


Fig-3: Agarose Gel Electrophoresis of PCR amplified products of ITS regions

The DNA marker which is used is 1 kbp. The PCR products were gel purified, run in 1% agarose gel, and processed for nucleotide sequencing. The nucleotide sequences of four samples were obtained and then analysed for Basic Local Alignment Search Tool (BLAST).

According to BLAST summary of the samples, they were closely resembled to these species. Identification was based on % highest coverage and followed by the Accession no. were presented. The mushrooms may be identified as follows: 1/18-*Trametes versicolor* (100% AY138396.1), 2/18-

Daedaleopsis confragosa (99% KR108001.1), 3/18-*Schizophyllum commune* (99% AF062633.1), and 4/18-*Tyromyces chioneus* (99% KX066001.1). The aligned nucleotide sequence of samples was submitted to NCBI and nucleotide sequences of four samples is provided with gene bank accession number MK249878 (*Trametes versicolor*), MK239266 (*Daedaleopsis confragosa*), MK212358 (*Schizophyllum commune*) and MK239265 (*Tyromyces chioneus*).

Phylogenetic analysis of wild mushrooms

Construction of Phylogenetic Tree of mushroom samples by using NCBI (National Centre for Biotechnology Information and MEGA (Molecular Evolutionary Genetics Analysis) Software shown in (Fig 4)

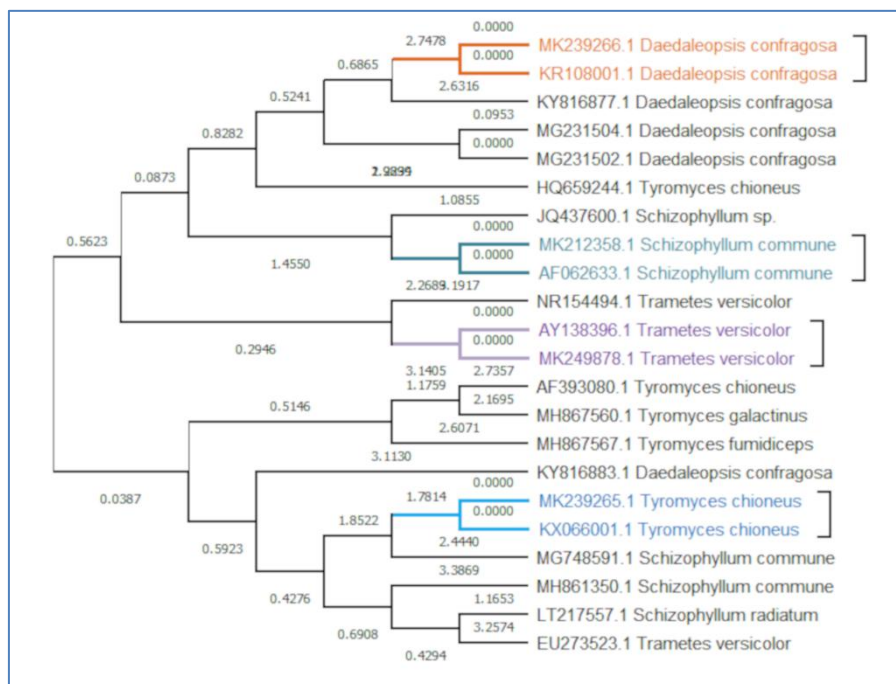


Fig-4: Phylogenetic analysis of four samples (*Daedaleopsis confragosa*, *Schizophyllum commune*, *Trametes versicolor* and *Tyromyces chioneus*)

The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 52.19427294 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 22 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1463 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

DISCUSSIONS

Methanol extract of *T. elegans* produced the highest anticandidal activity with an inhibition zone of 30.00 mm against isolate *C. albicans* [22]. This finding was in the agreement of [23] that the antibacterial activity of polypores and gilled mushrooms was found to be more pronounced than antifungal activity [23]. As in most cases, it appears that the fungal strains are more resistant to antimicrobial compounds than bacterial strains [24, 25]. The filamentous fungus, *S. commune* produced metabolites that possessed antifungal activity

[26]. When an odd electron becomes paired in the presence of free radical scavenger of antioxidant agent, DPPH radicals get reduced to corresponding hydrazine, DPPH-H form [27]. Due to quenching of its free radicals by antioxidants present in the medium the deep purple colour disappears, i.e., by electron donation or by providing hydrogen atom *via* free radical attack on DPPH molecule and convert them to colourless bleached product known as 2,2 diphenyl-1-hydrazine. This results in decrease in the absorption at 517 nm, hence, more rapidly the decrease in absorbance more potent the antioxidant activity of the extract [9]. The lower the IC₅₀ value, the higher the antioxidant activity of samples [28].

CONCLUSION

From the above, study it is concluded that all four mushrooms *T. versicolor*, *D. confragosa*, *S. commune* and *T. chioneus* have potent antimicrobial and antioxidant properties which can be exploited for its medicinal purpose at industrial and pharmaceutical level. The nucleotide sequence of samples was submitted to NCBI and nucleotide sequences of four samples is provided with gene bank accession number MK249878 (*Trametes versicolor*), MK239266 (*Daedaleopsis confragosa*), MK212358 (*Schizophyllum*

commune) and MK239265 (*Tyromyces chioneus*). All species of mushroom has shown good antioxidant activity. The antioxidant potential of the methanolic extracts of the mushroom samples in this study recorded higher values. According to the results, this indicates that the mushrooms have high antioxidant properties and rationalizes further investigation in the potential discovery of new natural bioactive principles from these mushrooms. It also reveals a novel potential to fight against diseases. Mushrooms are rich source of natural antibiotics, in these, the cell wall glucans are well known for their immunomodulatory properties and many of the externalized secondary metabolites. According to research, flavonoids and phenolic compounds contribute largely to antioxidant affect.

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