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Bioactive and Phosphatase Potential of Two Wild Mushrooms of Subtropical Forest consumed by Ethnic Tribes of Meghalaya, India

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ABSTRACT: *Scleroderma polyrhizum* and *Morganella pyriformis* mushrooms are unexplored and inaccessible, rarely found in the subtropical forest of lower Himalayan region of India. These mushrooms are the common source of medicine used by ethnic tribes of eastern India. The current study deals with the evaluation for their efficacy in free radical scavenging, antioxidant, and antimicrobial properties. The total phenolic content was analyzed along with reducing power and 1-Diphenyl-2-picryl-hydrazyle-hydrate (DPPH) assay. The phenolic content showed potent antioxidant components with a concentration range of 0.140 to 0.870 mg/g. The antioxidant activity of the extract of *S. polyrhizum* was higher than that of *M. pyriformis*. The mushroom extracts showed growth inhibition of pathogens, with the average inhibitory zone of 8-11mm for pathogenic bacteria and 13 mm for fungal pathogen, indicating this nutritive and therapeutic supplement of ethnic tribes as potent antioxidant and antimicrobial source.

Keywords: Wild mushrooms, free radical scavenging, antimicrobial, antioxidant activity.

INTRODUCTION

Wild mushrooms are considered to have ethnic relevance, contributing as pricey delicacies, potent medicines, or additional sources of money for ethnic communities. Wild mushrooms are becoming into global commodities and local resources that can open vistas for global exploitation. For the ethnic communities inhabiting in the mountainous ranges, wild mushroom hunting and gathering may be one of their off-farm revenue sources (Hussain and Sher 2023). The fruiting bodies of mushrooms are known to have a high level of micronutrients, including vitamin B complex, and a high level of mineral elements that are crucial for human health. They are also full of unsaturated fatty acids, a good source of fibre, a higher amount of carbohydrates, and high levels of essential amino acids, including both essential and non-essential amino acids (Petraglia *et al*., 2023). With potential effect over the use of such resources as well as livelihood of the indigenous people who rely on these resources in part, there is a global concern over the exploitation and the sustainability of such resources for future use. Strong connections between people and wild mushrooms are observed across all racial, ethnic, and geographic communities, despite the fact that each community exhibits a variety of preferences.

The change in lifestyle and pollution are major causes of oxidative distress in humans caused by DNA, protein, and membrane damages. The presence of antioxidants (flavonoids, ascorbic acid, etc) in the diet might help reduce oxidative damage in the human body (Barros *et al*., 2007; Jerang *et al.*, 2022; Singh *et al*., 2023). Various antioxidant components are used in food and beverage industries to reduce antioxidant damage to the endogenous system by free radicals. Additionally, infectious diseases continue to be a big problem for humanity. The primary concern globally is the emergence of antibiotic resistance in clinical infections. A significant factor driving the quest for novel biological specimens to be used as antimicrobial agents is the emergence of multidrug resistant clinical isolates. (Thomson and Moland 2000). Although fungi generate a broad spectrum of antibiotics, including penicillin, cephalosporin, streptomycin, erythromycin, griseofulvin, and rifamycin, which are commercially used, the occurrence of antibiotics from imperfect fungi is less well documented. Animal and human medications are still mostly derived from natural compounds that affect metabolic pathways (Debnath *et al*., 2023). Since the time of the Romans and Greeks,

people have used mushrooms, or imperfect fungi, as a source of food and medicine (Sagakami *et al*., 1991; Wasser and Weiss 1999). In addition to the cell wall glucans and secondary metabolites, it is also known that mushrooms contain phenolic compounds, flavonoids, triterpenoids, lectins, dietary fibres, terpenes, and alkaloids, which have significant antimicrobial, anti-inflammatory, antiviral, antioxidant, and immunomodulatory activities (Kim and Fung 2004; Barros *et al*., 2007; Akyuz *et al*., 2010). The abundant mushrooms in the virgin forest have a variety of antioxidant and antibacterial properties that make them a powerful source of dietary supplement (de Menezes Filho *et al*., 2022; Teniou *et al*., 2022). The mushroom fruiting bodies, spores, and mycelia have been shown to contain over 400 bioactive compounds, including polysaccharides, steroids, triterpenoids, sterols, fatty acids, alkaloids, amino acids, proteins, nucleosides, and more (Erbiai *et al*., 2023). The development of biotech products produced from mushrooms may also benefit from metabolomics and genomic research into the underutilized biotechnological potential of mushrooms (Badalyan *et al*., 2023). Mushroom phosphatase, the soil microbial enzyme, is important in microbial degradation and decomposition. The ability of mushrooms to produce organic acids or/and proton extrusion, which, through their hydroxyl and carboxyl groups, chelate the cation bound to phosphate, results in the latter being transformed into soluble forms, determines the amount of phosphate that may be dissolved (Yarzábal, 2010; Jena and Rath 2014).

The basidiomycete fungus *Scleroderma polyrhizum*, also known as "star earthball" is a medicinal mushroom (Xianling *et al.,* 2005). It is also known as dead man's hand and is dominant in the climatic zones of Asia, America and Europe. *Morganella pyriformis* also known as the pear ball mushroom, is considered edible.

The use of edible and therapeutic mushrooms has been documented and passed down from generation to generation in the eastern nations of China and Japan, unlike India. Despite this flaw, the majority of Indian ethnic groups have demonstrated vast and profound traditional mycological knowledge, eating roughly 283 species of wild mushrooms without proper documentation (Panda and Tayung 2015). The present study aimed to evaluate the free radical scavenging, antioxidant potential, total phenolics content, antimicrobial potential, and enzymatic efficiencies of scientifically unexplored *S. polyrhizum* and *M. pyriformis*, which are collected by the ethnic tribals from the subtropical forests prevalent in North-Eastern India for consumption and traditional ethnic medicinal sources.

MATERIALS AND METHODS

Sample Collection. *Scleroderma polyrhizum* and *Morganella pyriformis* were gathered from Meghalaya's subtropical woodland range (25°61′N and 91°89′E) in the late monsoon of 2021. Morphological and sporological identification of mushrooms was carried out with the data available on mushroomexpert.com (Fig. 1 and 2). The fruiting body was cleaned to remove the dirt prior to its identification. Fresh and dirt free mushrooms were air-dried, macerated to powder form in a morter and pestle, and stored at 4°C for further use. The black-coloured gleba inside the *Scleroderma polyrhizum* was removed before drying the outer cap of the mushroom.

Sample Preparation. The mushroom extract was prepared in water for various phytochemical, antioxidant, and antimicrobial assays following a standard protocol (Gbolagade and Fasidi 2005; Ugochukwu *et al*., 2013). Finally, the dried biomaterial was dissolved in DMSO and methanol at a stock concentration of 1g mL^{-1}, and its bioactivity was assessed.

Phytochemical Screening. Tests for various phytochemicals, including alkaloids, flavonoids, terpenoids, saponins, glycosides, phenolics, and carbohydrate, were carried out following a standard method (Ugochukwu *et al*., 2013).

Antioxidant Assay. Antioxidant assays, including the total reducing power assay and 1-Diphenyl-2-picryl-hydrazyle-hydrate (DPPH) assay from the mushroom extracts, were assessed:

Reducing power assay of the mushroom extract. The reducing ability of the crude extract was analysed (Chang *et al*., 2002). 0.1 mL of 1% potassium ferricyanide was added to a 0.5 ml aliquot of the crude extract. For 30 minutes, the mixture was incubated at 40°C. Following the incubation period, the mixture was combined with 0.1 mL of trichloroacetic acid and 0.1 mL of 1% ferric chloride. It was then left undisturbed for the following 20 to 30 minutes, during which time the absorbance at 700 nm was measured. Ascorbic acid was used as a standard.

DPPH free radical assay scavenging assay. A gradient concentration of mushroom extract was used, ranging from 1 to 10 mg mL^{-1}, and 0.5 mL of 0.1 mM DPPH in methanol was added to a test tube containing 1 mL of the crude extract. The mixture was vigorously shaken before being left undisturbed in the dark for 30 minutes. After 30 minutes of incubation, absorbance at a wavelength of 517 nm was observed (Nath *et al*., 2012). The inhibition concentration of the extract was calculated as the percentage inhibition of DPPH radicals by the extract and expressed in terms of inhibition concentration (IC_{50}). A standard graph was calculated considering the formula:

DPPH scavenging effect (%) = $[(A_0 - A_1/A_0) \times 100]$

where, A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the mushroom extract.

Total phenolic content assay. The total phenolic content of extracts was analysed by Folin Ciocalteau calorimetric methods, considering the protocol as described by Singleton *et al*., 1999. To a volume of 0.5 mL of test extract, 0.2 mL of Folin Ciocalteau reagent was mixed and left undisturbed for 15 minutes after which 0.6 mL of 20% sodium carbonate was added. The reaction was kept at 40°C for 30 minutes in the dark, and the absorbance was recorded at 765nm after the incubation period.

Antimicrobial Assay. The disc diffusion method was used to analyse *in vitro* antimicrobial assays against two

gram positive, two gram negative, and a fungal clinical pathogen (Borthakur and Joshi 2016). The bacterial strains, *Streptococcus pyogenes* MTCC1925, *Staphylococcus aureus* MTCC96, *Klebsiella pneumoniae* MTCC109, and *Escherichia coli* MTCC730, were grown in Brain Heart Infusion broth at 37°C for 18 to 24 h until they reached 0.5 McFarland standards (10^6 Colony Forming Units mL^{-1}), and the fungal strain, *Candida albicans* MTCC183, was grown in Potato Dextrose broth to reach a turbidity of 10^4 CFU mL^{-1}. Then one millilitre of the bacterial and fungal cultures was swapped for 20µl of Mueller Hilton Agar and Potato Dextrose Agar plates, respectively. The antimicrobial assay using the crude extract was performed using a sterile susceptibility disc (Himedia, Mumbai, India) of diameter 6mm into which 40µl of the crude extract and pure extraction solvent (DMSO) as a negative control were loaded individually. The agar plates were pre-incubated at 4°C for 15min, followed by an incubation period at 37°C for 24 h for bacterial toxicity determination and 28°C for 48 h for fungal toxicity determination. Genistin (10µg) was used to ascertain the strains' sensitivity as a positive control. The zone of inhibition around the paper disc was measured to assess the crude extracts' antimicrobial activity.

Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) of the extracts using micro dilution technique. A 96-well microtiter plate was used to measure the MIC/MFC of mushroom extracts *in vitro* (Nath *et al*., 2014). 50µl each of the Mueller-Hilton broth for bacteria and the Potato dextrose broth for fungi were placed from the second to the twelfth wells in the plate (A2 to A12) for the susceptibility analysis. In the first row of the plate, crude extract of concentration (25mg mL^{-1}) was applied at a volume of 100µl. A micropipette was used to apply serial dilution to the remaining wells (A2-A10). Each of the wells received a single addition of antimicrobial crude extract in a gradual dilution. 50µl of both bacterial and fungal suspension at 10^6 and 10^4 CFU mL^{-1} respectively, were added to each well. In order to prevent the microbial cells on the plates from drying out, they were covered with covers and placed in an incubator with growth conditions of 37°C for bacteria for 24 hours and 28°C for fungus for 48 hours, respectively. After incubation, each well received 10µl of 0.2% 2, 3, 5-triphenyltetrazoliumchloride (TTC). The TTC-containing microtiter plates were then incubated for a further 1 hour at the same temperature. The change in colour from purple to pink in an hour indicated the growth of the microbes. The MIC or MFC of the mushroom extract was determined as the lowest concentration that prevented microbial growth.

Enzymatic Assay

Enzyme extract preparation. Crude enzyme extract was prepared by disrupting the mushroom cell membrane using liquid nitrogen. The samples were treated with 0.01 M phosphate buffer (pH 7.0) along with 2mM EDTA and 1mM $MgCl_2$ for 15mins at a temperature of 4°C. The homogenate was centrifuged at 10,000 rpm for 15mins and the supernatants were filtered and stored at -20°C for further enzymatic analysis.

Acid phosphatase assay. Phosphatase activity was determined in crude enzyme extract by using Paranitrophenol Phosphate (PNPP) as the substrate over time and temperature.

Reaction over time. To 1mL of the 4mM PNPP substrate solution, 1mL of acetate-acetic acid buffer (pH 4.8) was added. The tubes with the reaction mixture were placed in a 37°C water bath for 3 min and marked as tubes 1-6. Crude mushroom enzyme extracts of 1mL were added to tubes 2-6, whereas tube 1 was considered to be blank with water. The exact time of the addition for each tube was recorded. The tubes were allowed to react with the enzyme extract at 37°C, which was later recovered serially after 5 min intervals. The reaction was terminated with 3 mL of the 1.0M NaOH solution.

Reaction over temperature. To 1mL of the buffer solution (pH 4.8), 1mL of 4mM PNPP was added. Tubes in duplicate were placed in water baths of varying temperature with one blank and one sample (0°C, 25°C, 37°C, 50°C, and 70°C) for 3mins. After the incubation time, 1mL of enzyme solution was added to one tube of each temperature, and water as a blank was added to the other tube and allowed to stand undisturbed for the next 15mins for reaction to take place which was further terminated by adding 3mL of 1 M NaOH solution.

Statistical analysis. The data were analysed using one-way analysis of variance and expressed as the mean standard error of the three sets of observations, which is significant ($P< 0.05$) for all assays that were performed in triplicate.

RESULTS AND DISCUSSION

Phytochemical Screening. The seven phytochemicals screened, five were present in both mushroom extracts. Terpenoids and saponins were not present in *M. pyriformis*, whereas carbohydrate content was found to be high. Phenolic content was higher in *S. polyrhizum* than that of *M. pyriformis* (Table 1). The presence of the phytoconstituents in the metabolic extract of the present study corroborates the finding by de Menezes Filho *et al*. (2022).

Antioxidant Assay

Reducing power assay. The reducing power assay of the methanolic crude extract, which is capable of donating free hydrogen, showed that the extracts of *S. polyrhizum* and *M. pyriformis* were found to be constantly increasing with an increase in concentration (Fig. 3). The reducing power of a 5mg mL^{-1} concentration of *S. polyrhizum* showed a higher ability to reduce than that of *M. pyriformis* extract. The reducing power attribute of the extract, which donates the hydrogen atom by breaking the radical chain, is typically caused by the presence of reductones (Ferreira *et al*., 2007; Huang *et al*., 2011). The amount of reducing power in mushrooms rose with higher concentrations (Pereira *et al*., 2012).

DPPH assay. Both extracts possessed good free radical scavenging activity, which increased with increase in

concentration. *S. polyrhizum* extracts have greater free radical scavenging capacity than *M. pyriformis* extracts (Fig. 4). At a concentration of 10mg mL^{-1}, the percentage inhibition was 71.64%, while the inhibition by *M. pyriformis* was only 48.98%. The IC_{50} values of the methanolic extract were recorded as 3.55mg mL^{-1} and 13.92 mg mL^{-1} for *S. polyrhizum* and *M. pyriformis*, respectively. The crude metabolites of *S. polyrhizum* extract showed higher free radical scavenging activity when compared to the extracts of *M. pyriformis*. The study also showed significant correlation to the report of Ferreira *et al.,* 2007, who showed the scavenging effect of BHA at a concentration of 3.6mg mL^{-1} being 96% and that of α-tocopherol (8.6mg mL^{-1}) being 95% (Ferreira *et al*., 2007).

Total phenolic assay. Due to their interaction with active oxygen radicals such as lipid peroxy radicals, superoxide anion radicals, and hydroxyl radicals, phenolic components are known to have direct antioxidant capabilities (Ferreira *et al*., 2007). Total phenolic content in the methanolic extracts, measured as mg gallic acid/gram dry weight (gallic acid equivalent, or GAE), and revealed substantial phenolic content in both extracts with scavenging activity. A significant difference was observed in the phenolic content of *S. polyrhizum* (0.2 ± 0.02mg/g) compared to that of *M. pyriformis* (0.141 ± 0.04 mg/g) at $p < 0.05$ (Table 1). The result of the present study attributes shares similarities with the study by Badshah *et al*., 2015 which states total phenolic content at a range of 0.17 mg/g to 0.4 mg/g.

Antimicrobial Assay. Both mushroom extracts showed higher antibacterial efficacy when compared to antifungal activity. The extracts of *M. pyriformis* and *S. polyrhizum* both had the greatest antibacterial activity against the pathogenic microorganisms. For the extract of *S. polyrhizum*, the zones of inhibition were 12mm, 13mm, 9mm and 11mm against *Streptococcus pyogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumonia*, respectively, and 8mm against *Candida albicans* (Fig. 5a). The zone of inhibition by *M. pyriformis* metabolites was 11mm for *S. pyogenes*, 8mm for both *S. aureus* and *E. coli*, 9mm for *K. pneumoniae*, and 13mm for *C. albicans* (Fig. 5b). There was no indication of an inhibitory zone forming on the DMSO-controlled disc (Table 2). The fruiting cap and the stalk are known to contain a wide range of bioactive compounds that are used to scavenge free radical and as antimicrobial agents (Akyuz *et al*., 2007). The effectiveness of the presence of bioactive compounds in treating bacterial and fungal infections has been significantly boosted by the presence of secondary metabolites such as flavonoids, limonoids, alkaloids, phenolics, and coumarins (Bhowal *et al*., 2022).

MIC/MFC of the extracts. The crude extracts were further evaluated for their minimal inhibitory concentration by 96 well micro-dilution techniques. The MIC value by the extract of *S. polyrhizum* showing a potent antagonistic effect against gram positive *Staphylococcus aureus* was 12.5mg mL^{-1}, followed by a MIC value of 25mg mL^{-1} against *Streptococcus pyogenes* by *M. pyriformis* and a MFC of 12.5mg mL^{-1} and 6.5mg mL^{-1} against *Candida albicans* by *S. polyrhizum* and *M. pyriformis*, respectively (Fig. 6). The MIC value corroborates the finding of the primary screening using the disc diffusion technique. It is also evident from the results that the mushroom extracts showed a better effect against gram positive than gram negative bacteria and pathogenic fungal strains, which may be attributed to the difference in the cell wall composition of gram negative bacteria and the fungus. A study by Bristy *et al*. (2022) states that the antimicrobial aspects of the fungal extracts are attributed by the presence of polyphenols along with the alkaloid, tannins, and saponins content in the metabolic extracts.

Enzymatic assay

Acid phosphatase over time and temperature. Phosphate is considered an important factor for the growth and various biological activities in organisms (Adetunji *et al*., 2017). Acid phosphatase production is regulated directly or indirectly by biotic as well as abiotic factors (Sharma *et al*., 2010). The amount of phosphatase production grew steadily with increased incubation time, according to the rate of reaction over time, and the effect of temperature on phosphatase production revealed that rising temperature boosted the production of the phosphatase enzyme. The extract of *S. polyrhizum* produced 15.3µM to 17.1µM of PNP as a final product by releasing phosphate from PNPP, whereas the enzyme extract of *M. pyriformis* produced 9µM to 16.9µM of PNP over time (Fig. 7a). The PNP formation was from 9 µM to over 100 µM by *S. polyrhizum* extract and from 6 µM to 100 µM by *M. pyriformis* extract over the temperature ranging from 4°C to 70°C (Fig. 7b). Over time and temperature, the enzyme extract had the capability for proton extrusion, which, through its hydroxyl or carbonyl group, helped in chelating the cation bound to phosphate and converting it into a more soluble form (Table 3).

Table 1: Phytochemicals and total phenolic content of the crude mushroom extracts.

Isolates	Alkaloids	Flavonoids	Terpenoids	Saponins	Glycosides	Phenolic	Carbohydrate	TPC
Scleroderma polyrhizum	+	+	+	+	+	++	+	0.2 ± 0.02
Morganella pyriformis	+	+	-	-	++	+	+++	0.141 ± 0.04

+++ indicates intense, ++ indicates moderate, + indicates low, - indicates absence of any components; Total Phenolic Content (TPC): gallic acid equivalent/g dry wt

Table 2: Antimicrobial activity of the extracts against pathogenic microbes.

Isolates	*Streptococcus pyogenes*	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumoniae*	*Candida albicans*
Scleroderma polyrhizum	++	++	+	++	+
Morganella pyriformis	++	+	+	++	++

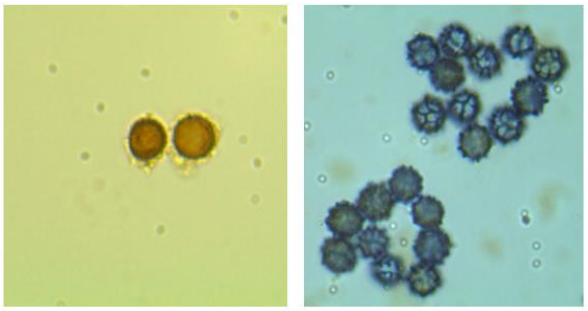
+ indicates inhibition zone between 8-10 mm; ++ indicates inhibition zone between 11-13mm

Table 3: Acid phosphatase activity assessed as reaction over time and temperature.

Isolates	PNP concentration over time (µM)							PNP concentration (µM) over temperature and reaction rate (µM/min)					
Scleroderma polyrhizum	Time (mins)							**Temp (°C)**	4	25	37	50	70
	Time	0	5	10	15	20	25	**PNP**	9	13	25	50	100
	PNP	0	15.3	16.1	16.4	16.5	17.1	**Rate**	0.6	0.8	1.7	3.3	6.0
Morganella pyriformis	Time (mins)							**Temp (°C)**	4	25	37	50	70
	Time	0	5	10	15	20	25	**PNP**	6	13	25	50	100
	PNP	0	9	14.5	14.9	16.4	16.9	**Rate**	0.4	0.9	1.7	3.3	6.7

Scleroderma polyrhizum *Morganella pyriformis*

Fig. 1. Macrographic images of the collected Mushrooms.



S. polyrhizum *M. pyriformis*

Fig. 2. Sporological images of the collected Mushrooms.

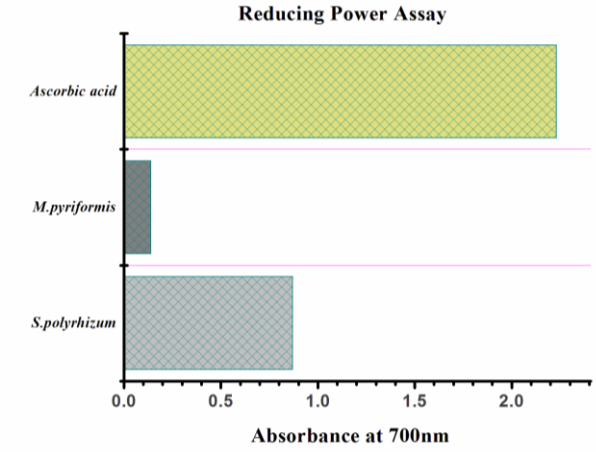


Fig. 3. Reducing power assay of crude extracts.

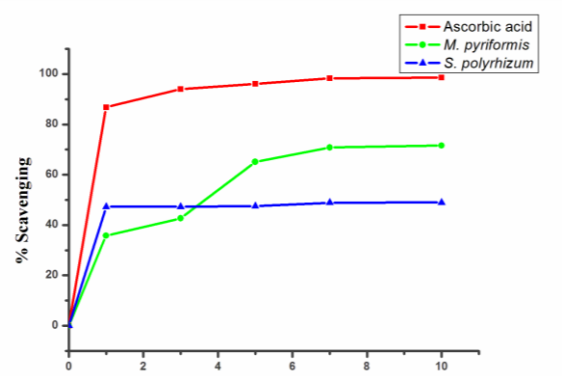


Fig. 4. Percentage scavenging by the crude extracts (DPPH).

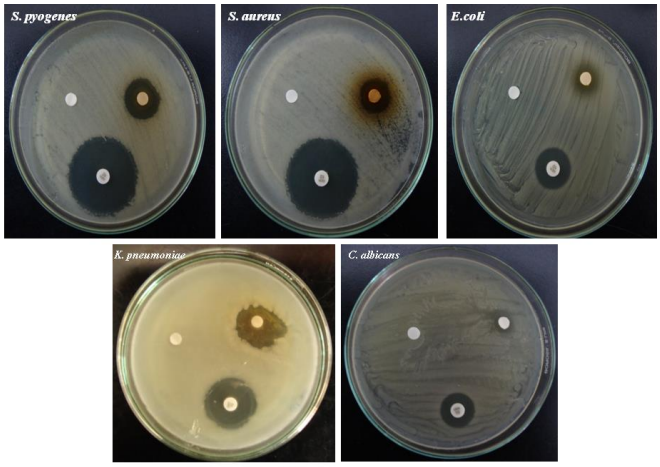


Fig. 5a. Zone of Inhibition of the crude extracts of *S. polyrhizum* against pathogenic strain.

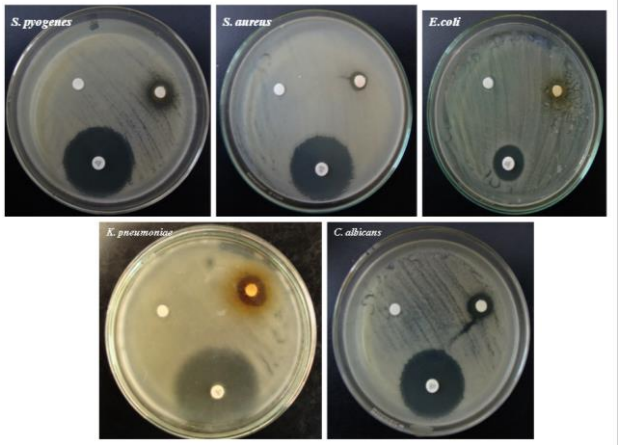


Fig. 5b. Zone of Inhibition of the crude extracts of *M. pyriformis* against pathogenic strain.

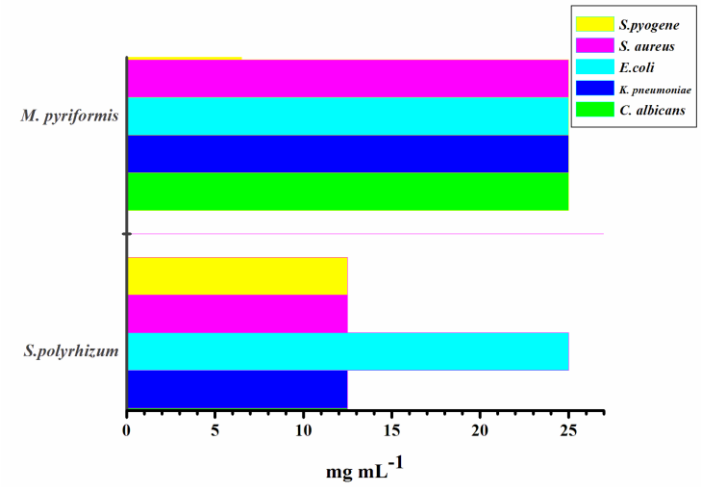


Fig. 6. MIC and MFC of crude extracts against pathogenic strain.

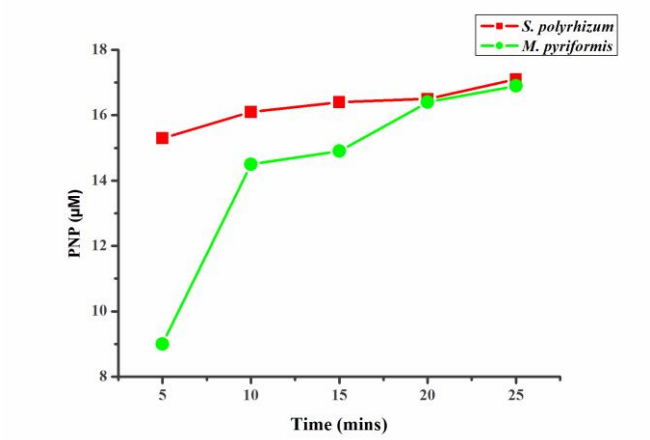


Fig. 7a. Concentration of PNP formation over time.

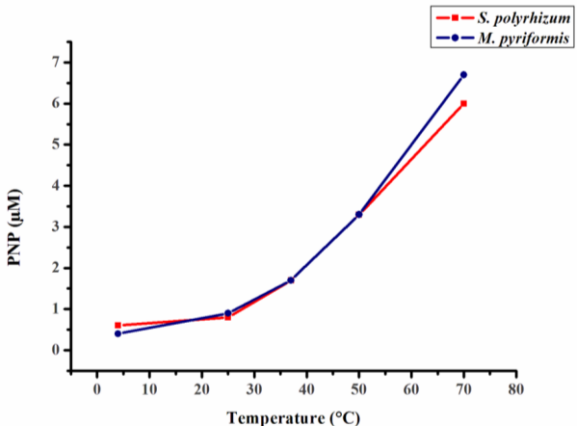


Fig. 7b. Concentration of PNP formation over temperature.

CONCLUSIONS

The metabolites of *S.polyrhizum* and *M. pyriformis* exhibited an effective free radical scavenging effect and reducing power capacity. The methanolic extract of *S.polyrhizum* exhibited better antioxidant properties, with IC_{50} values being 3.55mg $mL^{-1}$and 13.92 mg mL^{-1} for *S. polyrhizum* and *M. pyriformis*, respectively. The extract of *S. polyrhizum* also showed high concentrations of phenolic compounds as compared to *M. pyriformis*. The studied extracts exhibited antimicrobial activity, inhibiting the growth of tested clinical pathogens. The findings reveal that the metabolites of mushrooms can be adapted as a natural converter of bound phosphate into available soluble phosphate that is involved in various metabolic pathways. The two wild edible mushrooms indicate that they are potent sources of bioactivities that can be bio-prospected for human benefits.

This is the first report on the studied mushrooms with bioactive and the antimicrobial properties, along with the cellular deformities, from forest grove's in the northeastern part of India.

FUTURE SCOPE

A large section of the mushroom population is largely unexplored as the information is fragmented about their bioavailability and characterization. The present study will aid in further development of the studied mushrooms for their biological and therapeutic applications and open vistas for their bioprospection and use for human benefits.

Conflict of Interest. None.

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