

Biochemical, Antimicrobial, and Antioxidant activities of some wild Mushrooms from Nepal

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Abstract

Wild mushrooms represent a crucial dietary staple for many tribal groups throughout the world since they consist of an excellent amount of biologically active constituents including phenolic compounds, and tocopherol, and act as anti-cancer, anti-allergic, anti-obesity, anti-inflammatory compounds, etc. Wild mushrooms including *Scleroderma citrinum*, *Heterobasidion annosum*, *Coriolus hirsutus*, *Cavimalum indicum*, *Russula sanguinea*, and *Suillus punctatipes* were studied to evaluate their phytochemicals, antimicrobial activity, antioxidant activity, toxicity and relevance as a food source along with safety concerns. Initially, the total phenolic content (TPC), total tannin content (TTC), and total flavonoid content (TFC) along with antioxidant and antimicrobial activity were assessed using ethanolic extracts of mushrooms. Furthermore, a Brine shrimp bioassay was performed, the correlation of which with antioxidant activity, TPC, TFC, TTC, and lethal concentration (LC_{50}) was shown by principal component analysis (PCA). Secondary metabolites such as glucosides, flavonoids, polyphenols, alkaloids, terpenoids, saponins, and quinones were identified using phytochemical investigations. The TPC ranged from 45.98 to 102.3 mg GAE/g for the extracts, TFC from 100 to 225 mg QE/g, and TTC from 80 to 180 mg GAE/g. The findings of the antioxidant studies demonstrated that *S. punctatipes* exhibited the highest antioxidant activity ($IC_{50} = 16.95 \mu\text{g/mL}$), followed by *C. indicum* ($IC_{50} = 22.5 \mu\text{g/mL}$), and *C. hirsutus* ($IC_{50} = 35.34 \mu\text{g/mL}$). Likewise, *S. punctatipes* exhibited strong antimicrobial activity as compared to other extracts. The larvicidal efficacy against Brine shrimp bioassay revealed that three mushrooms; *C. hirsutus*, *C. indicum*, and *S. punctatipes*—contain highly toxic chemicals while the other three are non-toxic and can be consumed to some extent.

Keywords

Wild mushroom, Phytochemicals, Antioxidants, Antimicrobial.

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1 Introduction

Mushrooms are fungi that develop fleshy, spore-bearing fruiting bodies and are members of the higher groups; Ascomycota and Basidiomycota [1]. An ample diversity of mushrooms has long been identified as edible fungi that possess a wealth of nutritional value, low-calorie content, and an abundance of structurally varied and beneficial secondary metabolites [2]. As a beloved delicacy food source, mushrooms have long been used, and recent research into their antioxidant and health advantages further adds to their allure [3]. Bioactive substances derived from fungi have long been recognized, and fungi's secondary metabolites have been the subject of investigation [4]. Medicinal chemists have considered the potential benefits of mushrooms in humans for a variety of diseases to be beneficial in drug discovery and development by offering fundamental template structures and pharmacophores of therapeutically and economically effective products [5].

The therapeutic advantages of mushrooms, which include anti-hyperlipidemic, anti-inflammatory, anti-cancer, antioxidant, immunoregulatory, and cardioprotective properties, are all owing to the presence of secondary metabolites in mushrooms [6-9]. Numerous bioactive constituents including tocopherols, ergosterols, lectins, ergothioneine, glutathione, vitamin D, selenium, Repandiol, Polysaccharide-K, and many more are reported as a source of antioxidant activity in various mushroom species [5,7,10]. Likewise, striatal A, B, C, and D, effective against cancer, were isolated from *Cyathus striatus* and showed antimicrobial properties [11]. Several triterpenoids; ganoderic acid E, lucidumol A, and ganoder-manontriol, found in *Ganoderma lucidum* demonstrated remarkable cytotoxicity against human cancer cells. Poisonous mushrooms that smell bad or cause severe gastrointestinal complications are classified as non-edible species. Wild edible mushrooms are a natural forest resource well recognized for their nutritional, medicinal, economic, and cultural worth [12]. Meanwhile, poisonous mushrooms are frequently mistaken for edible species when ingested as wild mushrooms because of their similarity in form and color. Concern is also raised about the dangerous byproducts that are produced by toxic mushrooms [13]. However, the value of wild mushrooms is increasing as a result of their phenolic compounds, which act as potent antioxidants in addition to their pharmacological, nutritional, and sensory qualities [2]. Thus, it is equally crucial to explore secondary metabolites found in mushrooms.

Mushrooms had been used as a therapeutic agent in civilizations all over the world. It has long been known as a medicinal plant in China and other

Asian countries, including Japan and Korea, and has extended to countries such as the United States as well as Eastern Europe, including Russia [14]. Even though nature has endowed wild mushrooms with diverse economic and therapeutic significance, research on the bioactivity and potential medical benefits of available mushrooms has not been thoroughly conducted extensively. The situation is even direr in developing nations like Nepal where there has only been a scant study on wild mushrooms regardless of the availability of 108 families, 357 genera, and 1291 species of mushrooms with about 159 being edible, and 74 species reported to have medicinal properties [15].

Scleroderma citrinum (genus *Scleroderma*), also called *Scleroderma aurantium* or *Scleroderma vulgare horn*, grows from late summer to early winter frequently seen in Europe and Nepal, has remarkable medicinal properties, and thus, used in the pharmaceutical industries, cosmetics, and nutritious food as well [16]. It is reported to contain bioactive compounds such as sclerocitrin, norbadione A, xerocomic acid, badione A, etc [17].

Heterobasidion annosum (Agaricomycetes species), also known as *Polyporus annosum*, is a species of the Bondarzewiaceae family that can grow a diameter of up to 40 cm and a thickness of 3.5 cm. It is frequently found in North America and Nepal, is inedible, and has been discovered to help prevent colon cancer [18]. Likewise, *Coriolus hirsutus* commonly known as Rau Bhako Chyau is a common hairy beechwood-grown bracket fungus that not only serves as a food source but is also reported to cure wounds in traditional Nepalese communities [19]. *Coriolus hirsutus* is a common hairy bracket fungus that is grown specifically in beechwood. It persists throughout the year, has a slightly zoned crown, is occasionally white-gray with short hairs, and is tomentose and yellowish near the border. Another wild mushroom species, *Cavimalum indicum* mostly grows in bamboo trees and very little study has been reported to date. The Basidiomycota family includes *Russula sanguinea* which grows beside coniferous trees and is mycorrhizal with softwood trees. Moreover, studies on *R. sanguinea*, a vibrantly brightly colored mushroom, exhibit strong enzyme inhibition; anti-amylase and anti-glucosidase activities along with effective antioxidant activities [20]. Bioactive constituents like sangusulactones A-C, blennin A, and 15-hydroxyblennin A responsible for several pharmacological activities are found in this species [21]. *Suillus punctatipes*, often known as puffballs, are characterized by their white-yellow cap meat Mass, a layer of grayish-red-purple skin, and pores that are greenish-yellow in color. It has been demonstrated for the production of cytokinins like zeatin and ribosylzeatin [22]. The human adenosine A2A

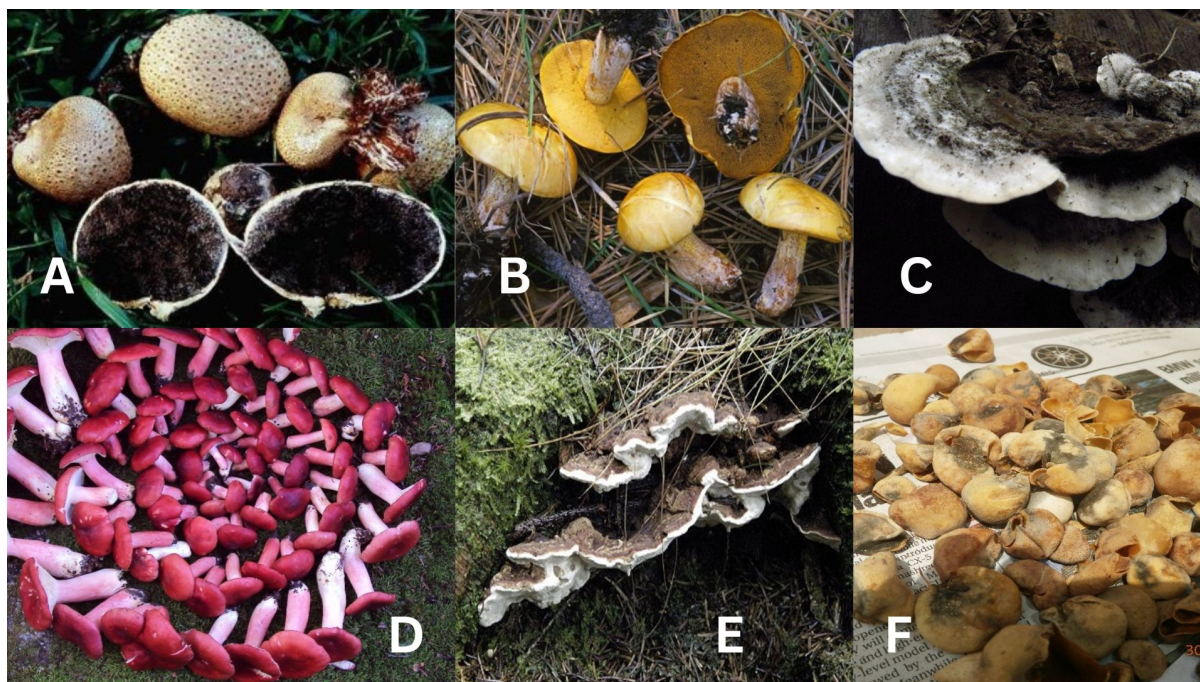


Figure 1: List of some collected mushrooms. A- *Scleroderma citrinum*, B- *Suillus punctatipes*, C- *Coriolus hirsutus*, D- *Russula sanguinea*, E- *Heterobasidion annosum*, F- *Cavimalum indicum*.

receptor, which is widely represented by many different types of cells and has an important function in controlling the activity of cells engaged in both adaptive and innate immunity, has recently been shown to be activated by the naturally occurring cytokinin zeatin riboside [23].

Numerous species of mushrooms have been the subject of various research throughout the world, but only a small number of studies have focused on specific wild mushrooms. The key purpose of this research is to investigate the phytochemical content, evaluation of toxicity of mushroom extracts to some extent, antioxidant activity, antimicrobial activity, TPC, TFC, and TTC of six selected wild mushrooms from Nepal along with their correlation to each other have been investigated by PCA.

2 Materials and Methods

2.1 Collection and Extraction of Mushroom

The six different mushroom species were primarily collected during the rainy season (July-August) from different locations and were identified taxonomically at the National Academy of Science and Technology (NAST), Khumaltar, Lalitpur. Figure 1 represents the lists of collected mushroom species and their botanical description along with phar-

macological importance are mentioned in Table 1. Each mushroom species was thoroughly cleaned, freed of contaminants, and dried in the shade before the ethanol-based soxhlet extraction method was used to extract the mushroom powder. The extracts were concentrated using a rotary evaporator, and each extract was thoroughly extracted for 12 hours. By using this formula, the percentage yield of dried mushroom extracts was calculated:

$$\text{Percentage Yield} = \frac{\text{Dry weight of extract}}{\text{Dry weight of sample}} \times 100\% \quad (1)$$

The metabolites found in these mushrooms were surveyed thoroughly, thereby the chemical structures of some metabolites reported previously are illustrated in Figure 2.

2.2 Screening

Identification of the phytochemicals found in the mushroom extracts was done by chemical method. As per the previously specified procedures [24], various tests including flavonoids, glycosides, alkaloids, steroids, phenolic compounds, terpenoids, saponins, tannins, carbohydrates, fats, and fixed oils were carried out.

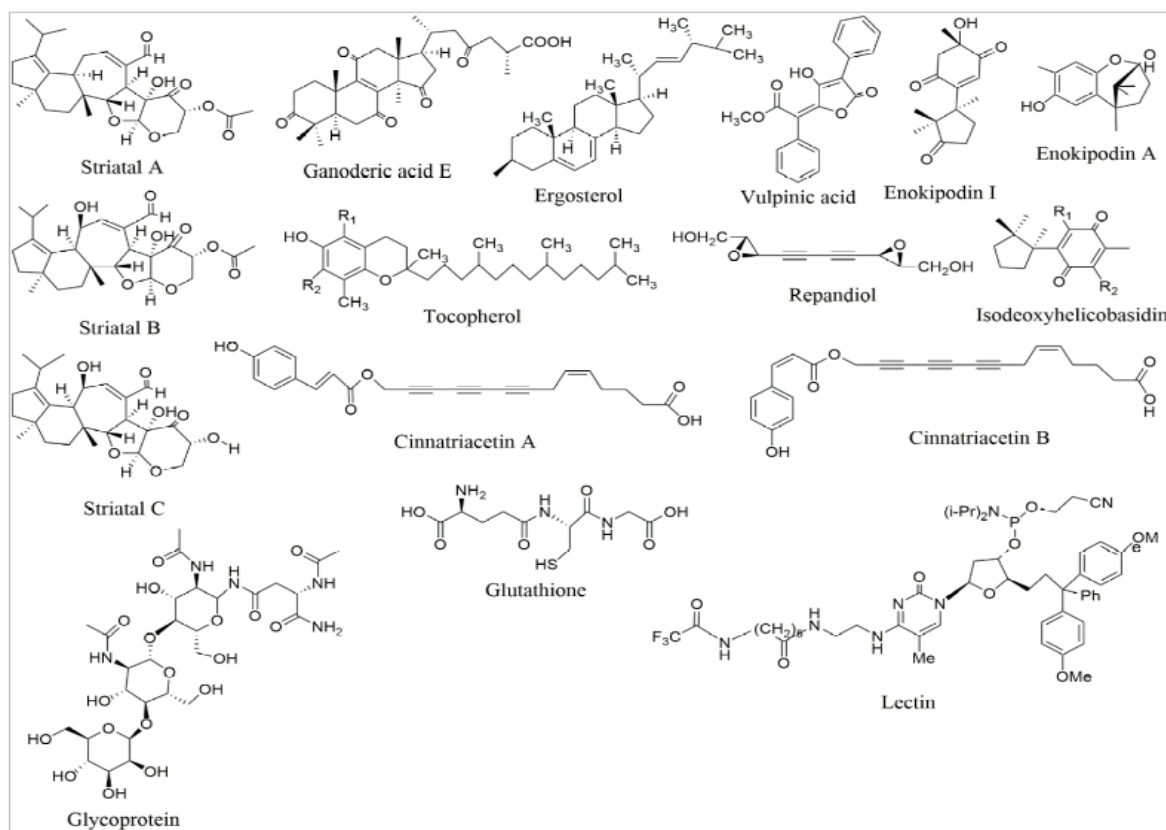


Figure 2: Some potential phytochemicals found in wild mushrooms.

Table 1: Name of Mushroom, Collection site, Common Name, Family, and Pharmacological Application

Name of Mushroom	Collection site	Common Name	Family	Pharmacological Application	References
<i>S. citrinum</i>	Nagarkot, Kathmandu	Common earth Ball	Sclerodermataceae	Anti-inflammatory, antiseptic properties	Lopusiewicz, 2018)
<i>H. annosum</i>	Dhulikhel, Kavre	Root rot fungus	Bondarzewiaceae	Used as potential candidates for bioremediation and act as antioxidants, and anti-inflammatories	(Sadowska et al., 2020)
<i>C. hirsutus</i>	Sundarijal, Kathmandu	Turkey tail mushroom	Polyporaceae	Potential immunomodulatory properties, anti-cancer properties	(Adhikari et al., 2005)
<i>C. indicum</i>	Sundarijal, Kathmandu	Indian mallow	Clavicipitaceae	-	-
<i>R. sanguinea</i>	Godawari, Lalitpur	Bloody brittle gill mushroom	Russulaceae	Anti-inflammatory, antimicrobial, antioxidant, and analgesic properties	(Alkan et al., 2020)
<i>S. punctatipes</i>	Matatirtha, Kathmandu	Puffball mushroom	Suillaceae	Anti-inflammatory, antioxidant, and immune-modulating properties	(Crafts & Miller, 1974)

2.3 Phenolic Content

The total phenolic content (TPC) present in the mushroom extracts was assessed by the Folin-Ciocalteu colorimetric method [25]. Initially, the gallic acid stock solution was made by dissolving 10 mg of the acid in 10 mL of ethanol (1 mg/mL). Gallic acid was produced in different quantities, including 125, 250, 500, and 1000 g/mL. The stock solution was made by dissolving 10 mg of each extract in 1 mL ethanol. The concentrations of 125, 250, 500, and 1000 g/mL were obtained by sequential dilutions. Then, 1 mL sample solution was taken in a test tube, to which 4 mL of 7% Na₂CO₃ and 5 mL of 10% FCR were added, shaken well, and kept at ambient temperature in the dark for half an hour. The absorbance of this blue mixture was then taken at 760 nm against control, with each experiment being carried out in a triplicate manner. To plot calibration curves at various concentrations, Gallic acid was employed as the reference and written as Gallic acid equivalent (mg GAE/g).

2.4 Flavonoid Content

An aluminum chloride colorimetric test was used to assess the total flavonoid content (TFC) of mushroom extracts [26]. To make a quercetin stock solution, quercetin stock solution was made by dissolving its 10 mg in 10 mL ethanol (1 mg/mL). Different quercetin concentrations, including 125, 250, 500, and 1000 g/mL, were produced. Ten milligrams of each extract were dissolved in one mL of ethanol to create the stock solutions. To attain the concentration of 500 g/mL, several dilutions were performed. The sample solution of concentration 10 mg/mL in ethanol was diluted to 1 mL, and successively 0.3 mL of 5% NaNO₃, 0.3 mL of 10% AlCl₃, and 2 mL of 1 M NaOH were added. Five minutes later, 0.3 mL of 10% AlCl₃ was introduced after the addition of double-distilled water to the mixture. The absorbance of the pink color was then compared to the control (quercetin) at 510 nm. The experiment for each concentration was carried out thrice.

2.5 Tannin Content

The total tannin content (TTC) was assessed by the Folin and Ciocalteu technique based on the work carried out by this procedure [27]. A gallic acid stock solution was prepared by dissolving 10 mg of gallic acid in 10 mL ethanol (1 mg/mL). Gallic acid was produced in a variety of quantities, including 125, 250, 500, and 1000 g/mL. Following the initial mixing of 0.1 mL sample solution with 7.5 mL distilled water, 0.5 mL of 10% FCR and 1 mL of 35% Na₂CO₃ were added and diluted to 10 mL by distilled water. After a thorough shaking, the blue-colored mixture was maintained at room

temperature for 30 minutes in the dark. Then, the absorbance at 725 nm was compared to the control. Each experiment was performed three times.

2.6 Activity on DPPH for Antioxidant Assay

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical test was performed to determine the antioxidant activities of six different mushrooms employing the earlier-mentioned approach [28]. The molecular weight of 2, 2-diphenyl-1 picrylhydrazyl (DPPH) is 394.32 Da. As a result, 100 mL of 0.1 mM solution of DPPH was produced by precisely weighing 0.4 mg of DPPH, and dissolving it in ethanol, and was then maintained the volume at 100 mL. At first, a 100 mL solution of 0.1 mM DPPH was made in ethanol along with ethanolic solutions of Ascorbic acid and mushroom extract were prepared in a range of concentrations (15-500 g/mL). After thorough vortexing, 1 mL of DPPH solution and 1 mL of sample were combined and left at ambient temperature for roughly 30 minutes in the dark. The absorbance was compared to the control at 517 nm. The experiment for each concentration was carried out thrice. Finally, the determination of antioxidant activity was done by the equation below:

$$\text{DPPH free radical scavenging (\%)} = \left[\frac{A_{517} \text{ of control} - A_{517} \text{ of sample}}{A_{517} \text{ of control}} \right] \times 100 \quad (2)$$

2.7 Antimicrobial Activity

The antibacterial activity of the mushroom extracts was assessed by the Agar well diffusion method following the standard protocol [29]. *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, the four tested bacterial strains, were cultured in MHB media with turbidity matched with 0.5 McFarland and swabbed in the MHA plates with 1.5 x 10⁸ CFU/mL bacterial suspension for 24 hours before the antibacterial test at 37 °C incubator. Using a 6 mm cork borer, wells form in these plates, and 100 mg/mL of each extract in DMSO is prepared. About 40 µL of extracts are subsequently kept in the well, along with DMSO as a negative control and Neomycin as a positive control. After incubating overnight at 37 °C, the plates were observed as well as the zone of inhibition was determined.

2.8 Brine Shrimp Lethality Assay

The brine shrimp bioassay, to assess the cytotoxicity, was performed on each ethanolic extract following the procedure [30]. Brine shrimps (*Artemia*

Salina) were incubated by spreading brine shrimp eggs (50mg) on top of the artificial seawater-filled beaker and illuminated with a table lamp (100 watts) at a temperature of 30 °C. In Brine shrimp bioassay, freshly hatched brine shrimp nauplii—the egg of *Artemia salina*—are exposed to various solutions of certain mushroom extracts. To carry out the brine shrimp bioassay, the shrimp's egg must hatch, which required precise equipment sterilization, the making of artificial seawater, and sample processing. The artificial seawater was prepared by following protocol [31]. Thus, newly hatched brine shrimp nauplii were subjected to a categorized solution of mushroom extracts, and based on their cytotoxicity against the nauplii, the bioactivity was assessed. Probit Analysis was used to evaluate the LC₅₀ value (g/mL) at 95% confidence intervals. Meyer and others regarded the substance with LC₅₀ of 100 ppm to be effective (potent). The percentage mortality (%M) was also computed to confirm that the bioactive substances in the mushroom extracts were responsible for nauplii's demise.

$$\text{Percentage mortality (\%M)} = \frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}} \times 100 \quad (3)$$

2.9 Statistical Analysis

The statistical data were analyzed by implementing **R** (version 4.2.2) and **RStudio** (version 2022.10.31). In general, we used antioxidant activity (IC₅₀ value), TFC, TPC, TTC, and LC₅₀ value (for cytotoxicity) for the analysis using this software, along with the correlation of these data together with principal components analysis.

To determine an appropriate correlation method, it was necessary to conduct a normality test. The parameters DPPH, TFC, TPC, TTC, and IC₅₀ were subjected to the Shapiro-Wilk normality test to validate their skewness, normality, and kurtosis. In case the data were not found to be normally distributed, Kendall rank correlation was analyzed. The coefficient of determination (R^2) ranged between 0.9701 and 0.9753 after the data were linearly fitted.

2.9.1 Principal Components Analysis

High-dimensional data can be simplified using principal component analysis (PCA) while maintaining the integrity of underlying trends and patterns. This is accomplished through the compression of obtained data into limited dimensions that serve as feature summaries [32]. Principal components are used to deal with correlated predictors and show data in a two-dimensional space.

3 Results and Discussion

3.1 Phytochemical Screening

The respective percentage yield of ethanolic extract of mushrooms is displayed in Table 2, with *R. sanguinea* having the highest percentage yield (31.90%) and *C. indicum* demonstrating the lowest (5.10%). The differences in the amounts of mushroom extracts may be due to the solvent's ability, which relies on the chemical composition of the mushroom, the extraction technique, and the solvents' polarity utilized for extraction.

Phytochemical investigation of mushroom extracts showed the existence of polyphenols, quinones, terpenoids, saponins, and flavonoids. All mushroom extracts except for *S. citrinum* consist of reducing chemicals, whereas alkaloids predominate in the extracts of *S. citrinum*, *R. sanguinea*, *S. punctatipes*, and *C. indicum*. On the contrary, only two mushroom species, namely *H. annosum* and *R. sanguinea*, demonstrated the presence of glycosides. Preliminary screening of *R. sanguinea* indicates the presence of every phytochemical examined. The list of the phytochemicals contained in the ethanolic extract of the studied mushrooms is represented in Table 3.

Studies on the ethanol extracts of the edible mushrooms *Pleurotus ostearus* and *Coprinus comatus*, by high-performance liquid chromatography (HPLC), reported the existence of bioactive substances such as flavonoids, alkaloids, terpenes, glycosides, and saponins, which is consistent with the current result [33,34].

Table 2: Percentage yield of ethanolic extract of different mushrooms

S.N.	Name of the mushroom species	Dry weight of the sample (g)	Dry weight of extract (g)	Percentage yield (%)
1	<i>C. indicum</i>	40	2.041	5.10
2	<i>C. hirsutus</i>	40	3.979	9.94
3	<i>H. annosum</i>	40	4.897	12.24
4	<i>S. citrinum</i>	40	5.232	13.08
5	<i>S. punctatipes</i>	40	6.235	15.58
6	<i>R. sanguinea</i>	40	12.76	31.90

Table 3: Phytochemical investigation of six wild mushroom species (Note: (+) present and (-) absent)

Group of compounds	<i>S. citrinum</i>	<i>H. annosum</i>	<i>C. hirsutus</i>	<i>C. indicum</i>	<i>R. sanguinea</i>	<i>S. punctatipes</i>
Polyphenols	+	+	+	+	+	+
Reducing compounds	-	+	+	+	+	+
Glycosides	-	+	-	-	+	-
Quinones	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Alkaloids	+	-	-	+	+	+
Terpenoids	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+

Several investigations have indicated that extracts from a wide variety of other mushroom species contain significant amounts of phenols, flavonoids, alkaloids, terpenoids, tannins, steroids, and cardiac glycosides [35–37]. These bioactive substances have been attributed to various biological activities, including anti-diabetic and anti-inflammatory properties by saponins, anti-cancer and antimalarial properties by terpenoids [38], and scavenging, anti-allergenic, antiviral, anti-inflammatory, and vasodilating effects by flavonoids [39]. Moreover, phenolic compounds have also been acclaimed with considerable restorative benefits [34]. Hence, the sufficient utilization of potent compounds could provide an important base for the study of the bioactivity of the mushrooms.

3.2 Total Phenolic Content

Antioxidant activity in plants is mostly attributed to phenolic compounds, which are crucial elements with redox characteristics since hydroxyl groups help to neutralize free radicals [40]. The total phenolic contents of different mushroom extracts, expressed as mg GAEs/g of the extract, are listed in Table 4. Among the examined mushrooms, *S. punctatipes* exhibited the highest phenolic content (102.3 mg GAE/g), and *S. citrinum* was the lowest (45.98 mg GAE/g). This is in contrast to a study that showed *S. citrinum* melanin having a greater polyphenol content responsible for increased antioxidant activity [16]. Such variations in total phenolic content present in the mushrooms could be resolved by various parameters, such as harvest time, growth conditions, and environment. Numerous investigations have demonstrated that phenolic compounds vary in quantity and composition at the subcellular level throughout tissues [41]. Moreover, the content of simple phenolic acids such as Caffeic acid, Ferulic acid, etc., is typically higher in younger tissues since many phenolic acids condense later into complex phenolic compounds like flavonoids, tannins,

and lignins [42].

3.3 Total Flavonoid Content

Flavonoids, possibly the most significant natural phenols, are among the most varied and common groups of natural compounds being beneficial in the context of human health [43]. The total flavonoid content was determined using the aluminum chloride colorimetric technique with Quercetin as a reference and indicated as mg QEs/g of extract. With *S. punctatipes* having the maximum flavonoid content and *S. citrinum* the minimum, Table 4 below shows that the total flavonoid content of six mushroom extracts varied from 100 to 225 mg QE/g. It was determined that *S. punctatipes* with the maximum total phenolic content also contained the maximum flavonoid content (225 mg QE/g). This illustrates the positive correlation between TPC and TFC implying that there is some relationship connecting TFC and antioxidant potential [44]. Our results can therefore be connected with the previous work showing the positive impact of flavonoid and phenolic content in the free radical scavenging activity [45].

3.4 Total Tannin Content

Tannins are a unique group of water-soluble polyphenols, most commonly present in various mushrooms, and are regarded as good supplies of biologically active substances in the human diet [46]. The total tannin content present in the ethanolic mushroom extracts was quantified by the Folin-Ciocalteu technique, where Gallic acid was used as a reference based on the previously described procedure [47]. Table 4 shows that *S. punctatipes* had the maximum total tannin concentration (180 mg GAE/g), and it gradually decreased from *C. indicum*, *C. hirsutus*, and *H. annosum*, to the lowest *S. citrinum* (80 mg GAE/g), respectively.

Table 4: Comparison of IC₅₀, TFC, TPC, and TTC content in various mushroom extracts

Mushroom extracts	Phenolic contents(mg GAE/g TPC)	Flavonoids contents (mg QE/g TFC)	Tannin contents (mg GAE/g TTC)	Antioxidant activity IC ₅₀ Values ($\mu\text{g}/\text{mL}$)
<i>S. punctatipes</i>	102.30	225	180	16.95
<i>C. indicum</i>	82.76	200	160	22.50
<i>C. hirsutus</i>	74.71	175	140	35.34
<i>H. annosum</i>	63.23	175	140	39.89
<i>R. sanguinea</i>	51.73	125	100	53.40
<i>S. citrinum</i>	45.98	100	80	138.00

3.5 Antioxidant Activity

The DPPH free radical scavenging technique was employed to assess the antioxidant activity of several ethanolic extracts of mushrooms in which the decolorization of an ethanol solution of 2,2-diphenyl-1-picrylhydrazyl was observed to evaluate the mushroom extract's ability to donate hydrogen atoms (DPPH). When DPPH is dissolved in ethanol, it gives a violet or purple tint that, when present with antioxidants, fade to varying degrees of yellow (DPPH-H). Antioxidant activity was determined in terms of IC₅₀ value in which antioxidant activity increases as the IC₅₀ value decreases [6]. The antioxidant property of certain prevalent edible wild mushrooms resembles their total phenolic content [48]. Since, phenolic compounds in mushrooms, which include free hydrogen, contribute to the antioxidant capacity of mushroom extracts, many species of wild mushrooms demonstrated antioxidant activity [49]. The IC₅₀ value of Ascorbic acid was low i.e, 15.62 $\mu\text{g}/\text{mL}$, indicating that it exhibits the highest antioxidant activity (Table 4) which implies that, in comparison to other mushrooms, *S.punctatipes* had the greatest antioxidant activity since its IC₅₀ value is close to that of Ascorbic acid. The *Suillus* species are reported to have strong antioxidant activity, and thereby prevent oxidative stress in humans [50]. The mushrooms—*H. annosum*, *C.hirsutus*, and *C.indicum* having less than 50 $\mu\text{g}/\text{mL}$ IC₅₀ possess significant antioxidant properties, in contrast to *R. sanguinea*'s that exhibited greater than 50 $\mu\text{g}/\text{mL}$ IC₅₀ value. *S. citrinum* demonstrated the least level of antioxidant activity. As per the study, the mushroom extracts' varying antioxidant activity was likely caused by the interaction of several phenolic chemicals found in the plant itself [51]. Boletus, a wild culinary mushroom, was shown to correlate with phenolic compounds, the main group of phytochemicals that contribute to the antioxidant properties of mushroom species [52]. A recent study found that consuming 18 g of mushrooms daily might boost cellular antioxidant functions and reduce the likelihood of cancer [53]. Several antioxidant components are frequently used in different foods to defend against

oxidative damage caused by free-radical molecules. Since the beginning of time, people have consumed wild mushrooms as a part of their diets and as a source of nutritional supplements as they exhibit good antioxidant effects [54]. Moreover, the antioxidant property of mushrooms has been reported to prevent oxidative damage of lipids, proteins, and nucleic acids by neutralizing free radicals. The level of oxidative stress could be lowered by supplementing the diet with antioxidant-rich edible mushrooms [3]. Due to these attributes, the use of mushrooms in several nutraceutical products is rising in popularity [55,56]. Likewise, antioxidant activity, nutritional value, and healthy properties of food sources are interrelated to each other [57]. According to this, *S. punctatipes* which exhibited good antioxidant activity could be a potential nutrient dietary source.

3.6 Antimicrobial Activity

The antibacterial activity of six different species of mushroom was analyzed by the Agar-well diffusion technique with Neomycin as standard. Table 5 shows the antimicrobial activity of six mushrooms towards all four test pathogens comprising two gram-positive (*S. aureus* and *B. cereus*) and two gram-negative (*E. coli* and *S. typhimurium*) bacteria. In our experiments, the *S. punctatipes* demonstrated the greatest antimicrobial activity with a zone of inhibition of 12 mm and 11 mm against gram-positive bacteria while *B. cereus* and *S. aureus*, along with a zone of inhibition of 9 mm and 10 mm against gram-negative bacteria *S. typhimurium* and *E. coli*. Likewise, *S.citrinum*, *H.annosum*, *C.indicum*, and *R. sanguinea* showed good antimicrobial efficacy against gram-positive bacteria, while *C.hirsutus*, and *C.indicum*, displayed antimicrobial activity against gram-negative bacteria. The variation in the outcomes of antimicrobial activity could be attributed to several variables, such as the environmental and climatic conditions in which the mushroom was grown, the choice of mushroom extracts, the choice of extraction techniques, the antimicrobial test method, and the test microorganisms. As per a previous study,

the pigment β -carotene, most commonly found in *Suillus* sp., exhibited significant antimicrobial properties towards *S. aureus* and *E. coli* [50]. Numerous secondary metabolites including alkaloids, flavonoids, tannins, and other phytochemicals have been documented to affect antimicrobial activity [58]. As per the prior study, the melanin of *Scleroderma citrinum* showed no antimicrobial effec-

tiveness towards *B. cereus*, *E. coli*, and *S. aureus*, which in contrast to our present study, exhibited good antimicrobial effectiveness towards both gram-positive strains [16]. There's a probability that some of the tested extracts from this investigation will succeed as antimicrobial medicines in the future.

Table 5: In vitro antimicrobial activity of mushroom extracts against selected bacterial strains

Mushroom extract (100 mg/mL)	Zone of Inhibition(mm)			
	Gram-positive bacteria		Gram-negative bacteria	
	<i>S. aureus</i>	<i>B. cereus</i>	<i>E. coli</i>	<i>S. typhimorium</i>
<i>S. citrinum</i>	9	9	6	7
<i>H. annosum</i>	10	9	6	7
<i>C. hirsutus</i>	7	7	10	9
<i>C. indicum</i>	10	10	9	9
<i>R. sanguinea</i>	11	10	7	7
<i>S. punctatipes</i>	12	11	9	10
Neomycin	34	35	24	23
DMSO solution	0	0	0	0

Table 6: LC₅₀ values of different mushroom extracts

Mushroom extracts	LC ₅₀ (g/mL)
<i>S. citrinum</i>	4265.8
<i>H. annosum</i>	14791
<i>C. hirsutus</i>	338.8
<i>C. indicum</i>	338.8
<i>R. sanguinea</i>	16596
<i>S. punctatipes</i>	257.0

Table 7: Kendall rank correlation coefficient

	DPPH	TPC	TFC	TTC	LC ₅₀
DPPH	1	-1	-0.96609	-0.96609	0.69007
TPC	-1	1	0.96609	0.96609	-0.69007
TFC	-0.96609	0.96609	1	1	-0.64286
TTC	-0.96609	0.96609	1	1	-0.64286
LC ₅₀	0.69007	-0.69007	-0.64286	-0.64286	1

3.7 Brine Shrimp Assay

The brine shrimp assay is a preliminary screening method used to assess the bioactivity of crude extracts by measuring their toxicity. It has been employed to identify fungal toxins, plant extract toxicity, and cytotoxicity [59–61]. The LC₅₀ values, which represent the concentration of the extract that causes 50% mortality in the brine shrimp, are calculated using this method [62]. A lower LC₅₀ value indicates higher toxicity. According to Meyer et al., LC₅₀ values of less than 100 ppm are consid-

ered potent, values less than 1000 ppm are toxic, and values greater than 1000 ppm are non-toxic [63].

This study demonstrates the larvicidal activity of ethanolic extracts from different mushrooms against brine shrimp. The calculated LC₅₀ values of the mushroom extracts analyzed in this study are listed in Table 6.

The concentration of these mushroom extracts and their efficiency were directly correlated. At a concentration of 1000 ppm, all mushroom extracts demonstrated high toxicity with the high-

est mortality rates. This suggests that three of the six mushrooms examined, *Coriolus hirsutus*, *Cavimatum indicum*, and *Suillus punctatipes*, might contain highly potent toxic substances, while the other three, *S. citrinum*, *H. annosum*, and *R. sanguinea*, were found to be non-toxic.

According to our study, the three mushrooms *S. citrinum*, *H. annosum*, and *R. sanguinea* were found to be non-toxic, with an LC_{50} value greater than 1000, which is considered non-toxic according to Meyer et al. (1982). This finding of *H. annosum* being moderately or mildly toxic corresponds to previous findings [18]. The non-toxicity or low cytotoxicity of these mushroom species suggests that they are potentially suitable for consumption.

However, when it comes to safety concerns, the edibility of the six mushrooms depends on proper examination. All mushrooms can be consumed, but one mushroom, namely *S. punctatipes*, should only be consumed if the cap is removed. Therefore, it is important to be aware of this and conduct sufficient surveys and examinations when consuming such mushrooms.

Additionally, the study, classification, and production of medicinal mushrooms could benefit from the application of cutting-edge technologies such as metabolomics, proteomics, transcriptomics, and genome sequencing [64]. Further studies on the toxicity and nutritional value of these mushrooms will likely reveal whether they could be a valuable ad-

dition to our diets in the near future.

3.8 Statistical Analyses

3.8.1 Correlation

According to the Shapiro-Wilk test, the W and p -value were found to be 0.98676 and 0.9672, respectively. Since the p -value was greater than 0.05, the data are not normally distributed. Therefore, the Kendall rank correlation coefficient was performed, and the results are shown in Table 7.

3.8.2 Principal Components Analysis of Different Variables

The scree plot of the Principal Component Analysis (PCA) (Figure 3) displayed two principal components that accounted for 97.7% of the overall variation. The variables TPC, TFC, and TTC showed a strong correlation with the formation of axis 1 (79.4%), while DPPH and LC_{50} strongly contributed to the development of axis 2 (18.3%) (Figure 4). This plot indicates that variables such as TFC, TPC, and TTC are negatively correlated with LC_{50} , while variables are positively correlated with LC_{50} values. This means that an increase in the amount of phenolic, flavonoid, and tannin content leads to a decrease in toxicity (lethal concentration).

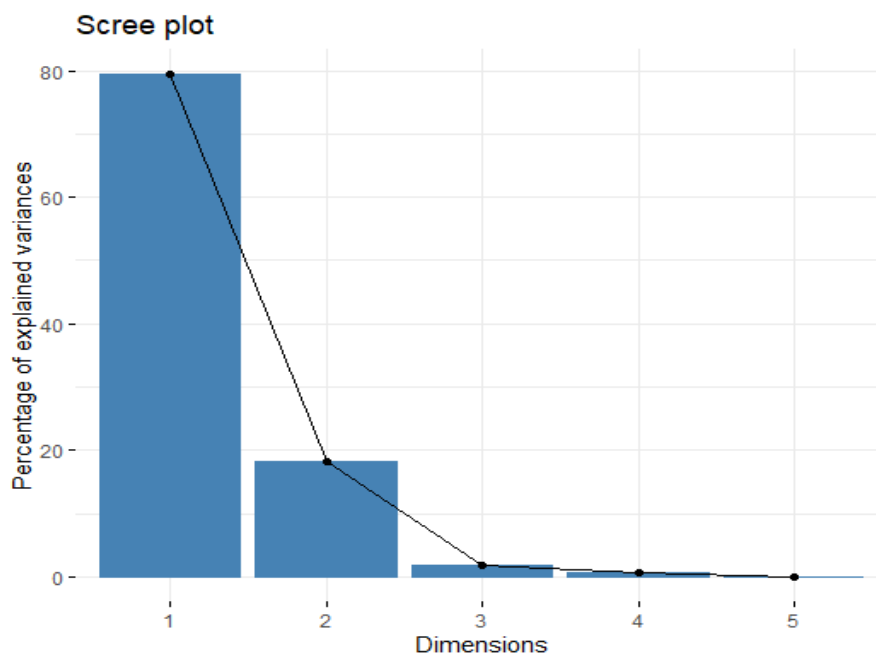


Figure 3: Scree plot of Principal component analysis.

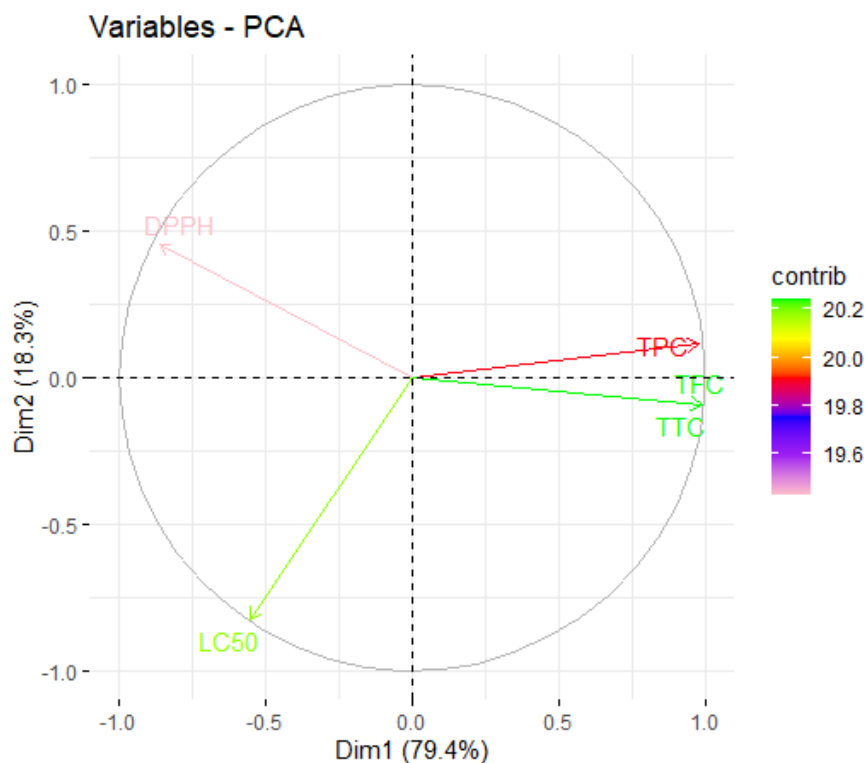


Figure 4: Principal component plot of variables.

4 Conclusion

Mushrooms contain a variety of phytochemicals, protein, dietary fiber, vitamins, and minerals, making them suitable as food supplements for all age groups. This research focused on analyzing the phytochemicals and evaluating the biological properties of the selected wild mushrooms. The preliminary assessment effectively demonstrated their cytotoxic, antibacterial, and antioxidant characteristics. The presence of various phytochemicals can be attributed to the potential antibacterial and antioxidant properties of the examined samples. All mushroom extracts exhibited at least one phytochemical, while only *R. sanguinea* exhibited all tested phytochemicals. The brine shrimp bioassay demonstrated the potential toxicity of *S. punctatipes*, *C. indicum*, and *C. hirsutus* against brine shrimp nauplii, while *S. citrinum*, *H. annosum*, and *R. sanguinea* were found to be non-toxic. Further studies should be conducted to analyze their bioactivity and potential toxicity. *S. punctatipes* exhibited the most notable antibacterial and antioxidant activity among all the mushrooms studied. Additionally, *S. punctatipes* and *C. indicum* contained the highest levels of flavonoids, phenolics, and tannins. Further in-depth research may lead to the discovery of substances that could serve as potential drugs or templates for new drug development. Mushrooms can be a great source of antioxidants

due to their high phenolic content and can be consumed when handled properly.

Abbreviations

DMSO: Dimethyl sulfoxide, DPPH: 2,2-picrylhydrazyl-hydrate, FCR: Folin-Ciocalteu Reagent, GAE/g: Gallic acid equivalent per gram, IC₅₀: Half-maximal inhibitory concentration, LC₅₀: Lethal concentration 50%, TFC: Total Flavonoids Content, TPC: Total Phenolic Content, TTC: Total Tannin Content, QE/g: Quercetin equivalent per gram, ZOI: Zone of Inhibition

Author's contributions

N. P. supervised the research; N. Panth performed lab work; P. C. and B. K. R. conceptualize and analyze data; P. C., B. K. R., N. P., N. S., and S. S. wrote the manuscript; B.B.T. and A.D.M. reviewed and edited the manuscript.

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