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ORIGINAL ARTICLE

Effect of Solvent Extraction System on the Antioxidant Properties of Seven Wild Edible Mushrooms and Identification of Phenolic Compounds by High-Performance Liquid Chromatography

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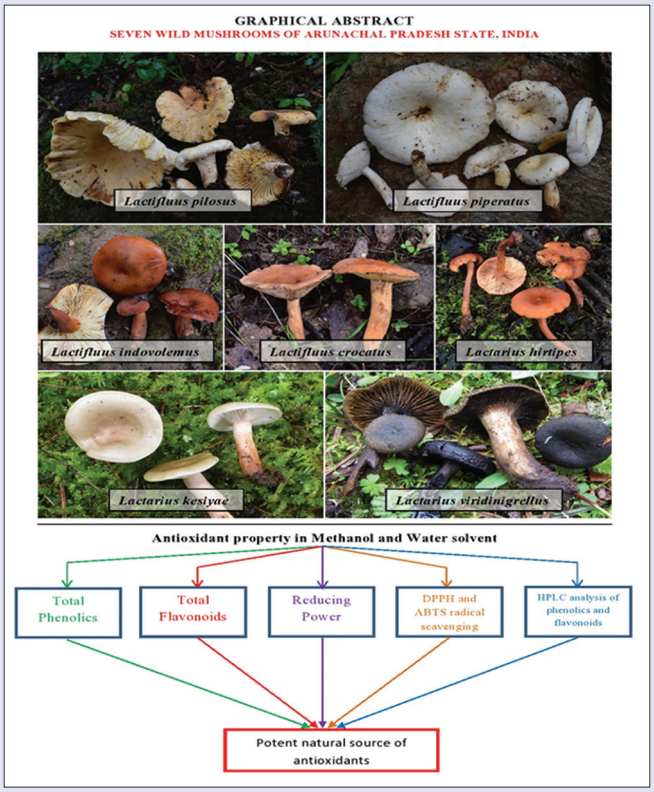
ABSTRACT

Objectives: The main objective of this research was to analyze some selected indigenous wild mushrooms in Arunachal Pradesh, namely, *Lactifluus pilosus, Lactifluus piperatus, Lactifluus indovolemus, Lactifluus crocatus, Lactarius hirtipes, Lactarius kesiyae* and *Lactarius viridinigrellus* for their novel antioxidant components in two different solvent extracts. **Materials and Methods:** The antioxidant properties of water and methanol extracts of these mushrooms were determined by the total phenolics and flavonoid content, reducing power capacity, radical scavenging activity by 1,1-diphenyl-2-picrylhydrazyl and (2,2′-azino-bis [3–ethylbenzothiazoline–6–sulfonic acid]) radicals. The quantification of phenolics and flavonoids was carried out by high-performance liquid chromatography (HPLC) using Acclaim C_{18} column, Dionex Ultimate 3000 liquid chromatograph, and detection was carried out in photodiode array detector. **Results:** The result showed that the total phenolics and flavonoids of the different extracts of the investigated mushrooms ranged from 3.77 ± 0.57 to 65.88 ± 1.81 mg gallic acid equivalents/100 g dry extract (DE) and 1.98 ± 0.002–12.64 ± 0.08 mg rutin equivalent/100 g DE, respectively. Furthermore, the mushroom extracts exhibited good free radical scavenging capacity. The HPLC experiment of both extracts showed the presence of different phenolic acids and flavonoids such as protocatechuic acid, syringic acid, quercetin, and Kaempferol in various amounts. **Conclusion:** The wild mushrooms have been studied to analyze their antioxidant property. These mushrooms may be used as functional additives or can be incorporated into our food regime, representing an alternative source of food to prevent damage caused by oxidation in the human body.

Key words: Antioxidants, Arunachal Pradesh, high-performance liquid chromatography, russulaceae, wild edible mushrooms

SUMMARY

- The current study focused to explore the antioxidant activities of seven underutilized wild mushrooms from Arunachal Pradesh. The antioxidant properties were assessed by using 1,1-diphenyl-2-picrylhydrazyl and 2,2′-azino-bis (3–ethylbenzothiazoline–6–sulfonic acid) radical scavenging activity, reducing power and estimation of total phenolic and flavonoid contents from two solvent extraction system of water and methanol. The quantification of phenolic acids and flavonoids were completed by high-performance liquid chromatography (HPLC). The after effect of this investigation demonstrated that the methanol extracts showed higher antioxidant activity than the water extracts. The HPLC analysis likewise showed the occurrence of phenolic acids and flavonoids in several amounts in these mushrooms which can conclude them as potent natural antioxidant.



Abbreviations used: DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2′-azino-bis (3–ethylbenzothiazoline–6–sulfonic acid); HPLC: High-performance liquid chromatography; PDA: Photo diode array; GAE: Gallic acid equivalents; AAE: Ascorbic acid equivalent; DE: Dry extract; IC: Inhibitory concentration; GPS: Global positioning system.

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INTRODUCTION

End products of metabolic reactions or environmental stress, free radicals are formed in the body which directly damages the living cells. Antioxidants are substances that prevent or delay these damages. When the balance between free radicals and antioxidants is hampered, then physiological condition called oxidative stress occurs. During the stress, the free radicals can directly damage the lipids, proteins, nucleic acids of the cells which will result in various primary and secondary diseases such as malignant growths, Alzheimer's disease, Parkinson's disease, diabetes, cardiovascular conditions such as high blood pressure, atherosclerosis, and stroke.[1] Some synthetic antioxidants, such as propyl gallate, ascorbyl palmitate, butylated hydroxytoluene, butylated hydroxyanisole, and tert–butylhydroquinone, are broadly utilized in the food industry to forestall the induction of oxidative damage in the body due to free radicals. However, the well-being of these synthetic antioxidants was as of late addressed and confined seriously by enactment because of their harmfulness and conceivable carcinogenicity.[2] Along these lines, there is a need to discover alternative economical, sustainable, effective and perhaps more secure wellsprings of compounds with antioxidant activities, such as natural antioxidants. Natural antioxidants can be utilized for therapeutic purposes as well as for food protection, as dietary enhancements or functional foods and in cosmetics.[3]

Polyphenols are one of the most significant gatherings of compounds among the dietary antioxidants. Phenolic compounds are potent antioxidants that are broadly present in fruits, vegetables, and herbs. Subsequently, high utilization of vegetables and fruits can reduce the danger of several illness and shield humans from oxidative damage. Like other bio-sources numerous mushrooms have likewise been accounted for to have antioxidant properties by which they neutralize the free radicals.[4-8]

Mushrooms have been quite popular in culinary activities since antiquity due to its unique taste and flavors. Being low in calories, sugars, fat, sodium, and furthermore being sans cholesterol, mushrooms has been picking up its significance as a significant aspect of the eating regimen. Moreover, they give significant supplements, nutrients, proteins, dietary fibers and are being utilized in the conventional medication and treatment of certain ailments.[9,10] Since they are easily available in the forests and generally fruits in large amount during the monsoon period, it becomes quite accessible for the local people and mushroom hunters. In the local communities of West Kameng in Arunachal Pradesh, India, it has been a tradition to go hunting in the forests and collect the wild fruits and mushrooms. It has been observed that few species of *Lactarius* and *Lactifluus* serve as a major part in the consumable mushrooms. The natives consume the mushrooms without knowing that they are great sources of antioxidants. In recent times, many species of *Lactarius* and *Lactifluus* have been accounted for as an extraordinary wellspring of numerous bioactive constituents and are restoratively significant.[11-17] While undertaking macrofungal foray by one of us (IB) during the rainy season to the mixed forests dominated by *Castanopsis* and *Pinus* sp. in West Kameng district, quite a number of different species of *Lactarius* and *Lactifluus* were collected in the years 2018 and 2019. Of which seven species (*Lactifluus pilosus*, *Lactifluus piperatus*, *Lactifluus indovolemus*, *Lactifluus crocatus*, *Lactarius hirtipes*, *Lactarius kesiyae,* and *Lactarius viridinigrellus*) have been analyzed for their antioxidant property which can help us to evaluate their role and importance in regular diet and consumption.

MATERIALS AND METHODS

Mushroom materials

Fresh basidiomata of seven different species of the family *Russulaceae* (*L. pilosus* [Verbeken, H. T. Le and Lumyong] Verbeken, *L. piperatus* [L.] Roussel, *L. indovolemus* I. Bera and K. Das, *L. crocatus* [Van de Putte and Verbeken] Van de Putte, *L. hirtipes* J. Z. Ying, *L. kesiyae* Verbeken and K. D. Hyde, *L. viridinigrellus* K. Das, I. Bera and Uniyal) were collected from forests of West Kameng district, Arunachal Pradesh state, India, identifications were authenticated based on macro–and micromorphology and phylogenetic inferences in Botanical Survey of India, Kolkata, India. The voucher specimens (*L. pilosus*: BSI-IB-18001, GPS data: 1590 m, N 27°22.510' E 092°16.257'; *L. piperatus*: BSI-IB-18-002, GPS data: 1590 m, N 27°22.510' E 092°16.257'; *L. indovolemus*: BSI-IB-18-003, GPS data: 1590 m, N 27°22.510' E 092°16.257'; *L. crocatus*: BSI-IB-18-015, GPS data: 1611 m, N 27°22.400' E 092°16.240'; *L. hirtipes*: BSI-IB-19-008, GPS data : 2408 m, N 27°07.623' E 092°13.628'; *L. kesiyae*: BSI-IB-19-019, GPS data: 1968 m, N 27°21.155' E 092°18.942'; *L. viridinigrellus*: BSI-IB-19-058, GPS data: 3547 m, N 27°37.964', E 091°50.267') were preserved in the CAL Herbaria. The dried materials were then crushed into their powder form with a mixer grinder and put away in a hermetically sealed container for further extraction.

Chemicals and equipment

To investigate the antioxidant activities, the phenolic and polyphenolic standards such as gallic acid, protocatechuic acid, catechin, rutin, gentisic acid, vanillic acid, caffeic acid, syringic acid, p–coumaric acid, ferulic acid, sinapic acid, salicylic acid, chlorogenic acid, p–hydroxy benzoic acid, ellagic acid, myricetin, quercetin, naringin, apigenin and kaempferol along with (2,2'-azino-bis (3–ethylbenzothiazoline–6–sulfonic acid) [ABTS]) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were procured from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals such as Folin–Ciocalteus's phenol reagent, potassium ferricyanide, potassium per sulfate, aluminum chloride, ferric chloride, sodium carbonate, sodium dihydrogen phosphate, and trifluoroacetic acid and the high-performance liquid chromatography (HPLC) grade solvents such as acetonitrile, methanol, and water, were obtained from Merck (Germany). Reversed-phase HPLC analysis was achieved using Dionex Ultimate 3000 liquid chromatography attached with a diode array detector (DAD) with 5 cm flow cell and gradient elution of the mobile phase system, using Chromeleon system manager as the data processor. The separation was achieved by a reversed-phase Acclaim C_{18} column (5-micron particle size, 250 mm × 4.6 mm).

Antioxidant activities determination in different solvent extracts

Extraction

About 10 g of fresh basidiomata of the seven different specimens were dried with the aluminum field dryer. The final weights of the samples were noted after completely drying. The dried mushrooms were then pummeled in a processor machine and put away in a sealed shut holder. Five hundred milligrams (500 mg) powdered sample of each specimen was absorbed twice in water and methanol, respectively, with consistent stirring for 24 h at room temperature and filtered to get the water and methanol extricate. Concentrates acquired from the first and the ensuing extractions were combined and concentrated using a rotary evaporator under reduced pressure to obtain viscous extracts which were additionally dried using a freeze drier. The dry concentrates were stored at –20°C until use. The dry extract (DE) acquired with every dissolvable was weighed. The percentage yield was communicated as the weight of the extract/weight of plant material multiplied by one hundred. One hundred far as air-dried weight of the mushroom material.

Antioxidant activities determination

Estimation of total phenolic content

The total phenolic content in the investigated samples was determined by using Folin–Ciocalteu procedure.[18] In a test tube, 200 µl of the tested extract and 2.5 ml of Folin–Ciocalteu reagent was added. After 5 min, 2.5 ml of sodium carbonate (7.5%) was added. The tube was allowed to

stand for 30 min. The absorbance of the resulted blue-colored solution was measured at 765 nm in a UV–visible spectrophotometer (Shimadzu UV 1800). Gallic acid was used as the reference standard and the total phenolic content was expressed as gallic acid equivalents (GAEs) in milligram per gram (mg/g) of extract using the regression equation y = 0.0013 x + 0.0498, R^2 = 0.999 (y denotes the absorbance, x signifies, the GAE), obtained from the gallic acid standard curve.

Estimation of total flavonoids

Total flavonoid content in the samples was assayed following the protocol used by A. A. Ordonez and the team in 2006.[19] About 2 ml of sample extract was mixed with an equal volume of 2% aluminum chloride in ethanol solution in a test tube. After incubation at room temperature for 1 h, the absorbance of the reaction mixture was measured at 420 nm in an ultraviolet (UV)–visible spectrophotometer (Shimadzu UV 1800). The appearance of a yellow color confirmed the presence of flavonoids. Total flavonoid contents were measured as rutin equivalent (RE) (mg/g) utilizing the rutin standard calibration equation: y = 0.0182 x – 0.0222, R^2 = 0.9962, where y means the absorbance and x denotes the RE (mg/g).

Measurement of reducing power

The reducing ability of the concentrates was resolved by the strategy for M. Oyaizu in 1986.[20] Two hundred microliters of mushroom extricates were added in 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. 2.5 ml aliquots was taken from the mixture and added to the equal volume of 10% trichloroacetic acid, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the resulting (2.5 ml) was added to the equal amount of distilled water and 0.5 ml freshly prepared ferric chloride solution (0.1%) and kept in dark for 5 min. The absorbance of the resulting solution was estimated at 700 nm in a UV–visible spectrophotometer (Shimadzu UV 1800). The diminishing intensity of the sample extricates were given in ascorbic acid equivalent (AAE) in milligram per gram (mg/g) of dry material utilizing the calibration equation based ascorbic acid standard curve: y = 0.0023 x – 0.0063, R^2 = 0.9955 where y represents the absorbance and x shows the AAE (mg/g).

Determination of 1,1-diphenyl-2-picrylhydrazyl free radical scavenging activity

The free radical scavenging activity of the mushroom extracts on the stable radical DPPH was assayed using the standard protocol.[21] The inhibition rate of DPPH was examined in the presence of the sample. 200 µl of the tested extracts were mixed in with 3.8 ml of freshly prepared DPPH solution (25 mg/L) in methanol. The mixture was kept for 30 min in darkness and the absorbance was measured at 517 nm in a UV–visible spectrophotometer (Shimadzu UV1800). The capability to scavenge the DPPH radical was calculated, using the following equation:

DPPH scavenged (%) = $(A_{control} - A_{test})/A\text{control} \times 100$

Where $A_{control}$ and A_{test} are the absorbance of the control (DPPH) and the reaction mixture of control in the presence of the extracts, respectively. The antioxidant activity of the extract was communicated as half-maximal inhibitory concentration (IC_{50}). The IC_{50} value was defined as mg of DE that prevents the formation of DPPH radicals by 50%. Values are expressed as mean ± standard error mean of three replicates.

Scavenging activity of 2,2'-azino-bis (3–ethylbenzothiazoline–6–sulfonic acid) radical cation

The scavenging activity of the tested extracts on ABTS radical cation was measured according to the method described by Re Roberta in 1999.[22] Both ABTS and potassium persulfate was dissolved separately in distilled water at a concentration of 7 mM and 2.45 mM, respectively. These two solutions were mixed in equal amount and the mixture was allowed to kept in the dark at room temperature for16 h to produce ABTS radical cation. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.70 ± 0.02. To determine the scavenging activity, 2.5 ml of diluted ABTS solution was added to 200 µl of mushroom extract (or water for the control) and the absorbance at 734 nm was measured 15 min after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

ABTS scavenged (%) = $(A_{control} - A_{test})/A_{control} \times 100$

Where $A_{control}$ and A_{test} are the absorbencies of the control and of the test sample in the association of control, respectively. From a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC_{50} value of the tested extracts.

Estimation of phenolic acids and flavonoids by high-performance liquid chromatography

Preparation of the standard solutions

The phenolics acids, for example, gallic acid, gentisic acid, protocatechuic acid, chlorogenic acid, vanillic acid, caffeic acid, p–hydroxy benzoic acid, syringic acid, p–coumaric acid, ferulic acid, sinapic acid, salicylic acid, ellagic acid, and flavonoids such as rutin, myricetin, quercetin, naringin, naringenin, apigenin, and kaempferol were taken as the reference standard for the quantification of phenolics and polyphenolics in the mushroom extracts. 10 mg of each standard was separately dissolved in 10 ml HPLC grade methanol to make 1 mg/ml stock solution. Each standard solution was diluted with the mobile phase solvent and thus the working solutions were prepared. Before to inject in the HPLC instrument, both the standard and working solutions were filtered through 0.45 mm PVDF syringe filter.

Chromatographic analysis of phenolic acids and flavonoids

The chromatographic investigation was completed following the strategy as portrayed by Seal *et al.* in 2017.[23] Dionex Ultimate 3000 liquid chromatography including a DAD with 5 cm flow cell and with Chromeleon system manager as data processor was used for HPLC analysis. Separation was accomplished by a reversed-phase Acclaim C_{18} column (5-micron particle size, 250 mm × 4.6 mm). The strategy was adopted as per the USP and ICH guidelines. A total of 20 mg dry methanol and water extract of seven plant samples *L. pilosus*, *L. piperatus*, *L. indovolemus*, *L. crocatus*, *L. hirtipes*, *L. kesiyae*, *L. viridinigrellus* were separately dissolved in 20 ml mobile phase solvent (methanol: 0.5% acetic acid in water: 1:9) and the sample solution was filtered through 0.45 µm membrane filter before injection into HPLC system. The HPLC analysis was carried out using the mobile solvent phase-containing methanol (Solvent A) and 0.5% aq. acetic acid solutions (Solvent B) and for each sample, 105 min time was taken for the analysis. A photodiode array UV detector was attached with HPLC system and chromatograms of standards and test samples were detected at three different wavelengths, for example, 272, 280, and 310 nm. Each phenolic in the test sample was identified by its retention time, absorption spectra and by mixing with reference standards under the same experimental conditions. The quantification of phenolic acids and flavonoids in the extracts was measured using the calibration curve by plotting peak area against the concentration of the respective standard sample. The data were reported as means ± standard error means of three independent analyses and the method was validated according to the USP and ICH guidelines. Various parameters were studied to validate the reproducibility of the method, namely, the effectiveness, the linearity, the limit of detection, the limit of quantitation, the precision, and the accuracy.

Statistical analysis

All the experiments were carried out using triplicate samples and results are presented as mean ± standard error by using the Statistical Package for the Social Sciences (SPSS variant 7.5) (SPSS Inc, International Business Machines (IBM), USA). Statistical analysis was performed by Tukey's test at 95% confidence level and statistical significance was established at the $P < 0.05$ level.

RESULTS

The total phenolics and flavonoid content, reducing ability, radical scavenging activities along with the extractive value of *L. pilosus*, *L. piperatus*, *L. indovolemus*, *L. crocatus*, *L. hirtipes*, *L. kesiyae* and *L. viridinigrellus* in two distinctive dissolvable fractions are shown in Table 1.

The extractive value

Two different solvents like methanol and water were used to extract the active constituents from seven wild mushroom specimens. From the extractive values shown in Table 1, it can be observed that extraction by the water is higher than the methanol with respect to all samples except *L. pilosus*, where the extractive value with both solvent is almost identical. The highest extractive value is of *L. crocatus* in the water (295.86 ± 0.6). Water extracts of *L. kesiyae* (265.37 ± 0.56) also showed considerably high extractive value. The methanol extract of *L. viridinigrellus* showed the minimum extractive value (59.52 ± 0.51) in this present investigation.

The antioxidant property

The quantities of total phenolic content obtained from the investigated samples are communicated as mg GAE/100 g DE and is depicted in Table 1. The methanol extracts displayed higher phenolic contents than the water extracts. *L. kesiyae* showed the maximum amount of phenolics in its methanol extract (65.88 ± 1.81 mg GAE/100 g DE). Methanol extracts of *L. hirtipes* (61.23 ± 3.67 mg GAE/100 g DE) and *L. indovolemus* (46.53 ± 1.17 mg GAE/100 g DE) also showed higher phenolic contents as compared to others.

The amount of total flavonoids in the investigated samples is expressed as equivalent mg RE/100 g DE and exhibited in Table 1. The results showed that flavonoids were better extracted in methanol than in water. The methanol extract of *L. viridinigrellus* showed the maximum flavonoid content (12.64 ± 0.08 mg Rutin/100 g DE). The rest of the species did not show much variation in their flavonoid contents.

The reducing ability of the extracts is conveyed as AAE in mg/100 g of DE and represented in Table 1. Except for *L. pilosus*, the reducing power has been best detected in the methanol extracts. *L. viridinigrellus* showed the maximum reducing power in its methanol extract (38.56 ± 1.12 mg AAE)/100 g DE). Similarly the methanol extracts of *L. indovolemus* (32.96 ± 0.26 mg AAE)/100 g DE) and *L. hirtipes* (27.36 ± 1.45 mg AAE)/100 g DE) also revealed high reducing power. The decreasing order of reducing power of samples detected in the way *L. viridinigrellus* > *L. indovolemus* > *L. hirtipes* > *L. piperatus* > *L. pilosus* > *L. kesiyae* > *L. crocatus.*

In the current investigation, the most noteworthy DPPH radical scavenging activity was shown by the methanol extract of *L. indovolemus* (IC_{50} = 0.93 ± 0.04 mg DE), whereas the water concentrate of *L. crocatus* showed the least movement (IC_{50} = 5.17 ± 0.14 mg DE).

The methanol extract of *L. viridinigrellus* showed the highest ABTS radical scavenging activity (IC_{50} = 0.11 ± 0.0008 mg DE) in this study, whereas the lowest scavenging activity (IC_{50} = 0.74 ± 0.008 mg DE) was exhibited by the water extract of *L. crocatus*.

Quantification and identification of phenolic acids and flavonoids by high-performance liquid chromatography

The HPLC analysis was carried out with the methanol and water concentrate of the investigated mushrooms to quantify the phenolic and flavonoid components. The quantities of reference compound phenolic acids like gallic acid, protocatechuic acid, gentisic acid, chlorogenic acid, p– hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, p–coumaric acid, ferulic acid, sinapic acid, salicylic acid, ellagic acid and flavonoids such as catechin, rutin, myricetin, quercetin, naringin, naringenin, apigenin, and kaempferol were expressed as µg/100 mg DE and are represented in Table 2. All the analyzed compounds responded at 260 nm, where they were successfully separated. The active constituents under investigation were also identified by the recorded absorption

Table 1: Antioxidant properties of the wild edible mushrooms

Sample	Extract	Extractive value (%)	Total Phenolic Content (mg GAE/100g dry extract)	Total Flavonoid Content (mg RE/100g dry extract)	Reducing Power (mg AE/100g dry extract)	DPPH radical scavenging activity (IC_{50} mg dry extract)	ABTS radical scavenging activity (IC_{50} mg dry extract)
L. pilosus	Water	198.80±0.67^{d}	17.68±1.58^{l}	4.03±0.003^{d}	22.36±0.20^{d}	2.26±0.16^{e}	0.24±0.006^{g}
	Methanol	200±0.65^{c}	22.08±0.66^{j}	5.39±0.02^{b}	19.40±0.30^{e}	2.10±0.07^{f}	0.35±0.011^{e}
L. piperatus	Water	266.02±0.01^{b}	26.41±0.42^{i}	2.54±0.001^{h}	14.51±1.33$^{h, i}$	1.51±0.027^{h}	0.52±0.01^{c}
	Methanol	129.16±0.02^{h}	28.41±0.42^{g}	4.78±0.02^{c}	22.51±0.43^{d}	1.40±0.021^{i}	0.60±0.05^{b}
L. indovolemus	Water	196.9 ± 0.022^{e}	20.96±0.56^{k}	4.05±0.01^{d}	17.66±0.25$^{f, g}$	1.61±0.06^{h}	0.27±0.004^{f}
	Methanol	98.81±0.015^{k}	46.53±1.17^{c}	5.53±0.01^{b}	32.96±0.26^{b}	0.93±0.04^{l}	0.20±0.001^{g}
L. crocatus	Water	295.86±0.6^{a}	5.72±0.32^{m}	1.98±0.002^{i}	9.54±0.11^{j}	5.17±0.14^{a}	0.74±0.008^{a}
	Methanol	198.41±0.8^{d}	27.13±0.82^{h}	3.10±0.013^{g}	15.70±0.25$^{g, h}$	1.75±0.03^{g}	0.44±0.0005^{d}
L. hirtipes	Water	195±2.1^{f}	39.88±0.48^{d}	3.46±0.01$^{e, f}$	13.92±1.02$^{h, i}$	1.60±0.08^{h}	0.35±0.002^{e}
	Methanol	102.09±2.13^{j}	61.23±3.67^{b}	4.62±0.022^{c}	27.36±1.45^{c}	1.19±0.1^{j}	0.21±0.004^{g}
L. kesiyae	Water	265.37±0.56^{b}	32.52±2.11^{e}	2.50±0.015^{h}	12.22±1.30^{i}	2.86±0.095^{c}	0.34±0.006^{e}
	Methanol	112.27±0.61^{i}	65.88±1.81^{a}	3.48±0.04^{e}	17.82±0.48^{f}	2.54±0.34^{d}	0.34±0.008^{e}
L. viridinigrellus	Water	164.34±0.48^{g}	3.77±0.57^{n}	3.27±0.015$^{f, g}$	12.51±1.06^{i}	4.55±0.5^{b}	0.35±0.009^{e}
	Methanol	59.52±0.51^{l}	32.18±2.61^{f}	12.64±0.08^{a}	38.56±1.12^{a}	1.09±0.07^{k}	0.11±0.0008^{h}

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean±SEM. Statistical analysis were carried out by Tukey's test at 95% confidence level and statistical significance were accepted at the $P<0.05$ level. The superscript letter a, b, c d, e, f and so on denotes the significance of various parameters. Letter a is significant to b, c, d, e, f, etc . . . .

Table 2: Quantitative estimation of phenolic acids and flavonoids in the 70% aq. ethanol extract of wild mushrooms by HPLC (µg/100 mg dry extract)

Phenolic acids and flavonoids	*L. pilosus*		*L. piperatus*		*L. indovolemus*	
	Water extract µg/100mg	MeOH extract µg/100 mg	Water extract µg/100 mg	MeOH extract µg/100 mg	Water extract µg/100 mg	MeOH extract µg/100 mg
Gallic acid	5.7±0.001^{h}	3.68±0.001^{i}	0.11±0.002^{j}	0.6±0.001^{j}	28.13±0.001^{e}	68.28±0.006^{c}
Protocatechuic acid	8.37±0.004^{c}	3.15±0.005^{e}	0.76±0.005^{g}	ND	0.18±0.006^{i}	ND
Gentisic acid	ND	ND	0.01±0.001^{b}	ND	0.27±0.001^{a}	ND
p-Hydroxy benzoic acid	0.56±0.015^{c}	0.65±0.015^{b}	0.48±0.019^{d}	ND	ND	ND
Catechin	10.44±0.008^{b}	11.08±0.007^{a}	1.21±0.004^{i}	4.07±0.008^{d}	1.19±0.01^{i}	6.80±0.006^{c}
Chlorogenic acid	2.18±0.002^{h}	2.21±0.007^{h}	1.05±0.002^{k}	8.20±0.001^{c}	3.96±0.003^{g}	ND
Vanillic acid	ND	1.82±0.01^{a}	0.63±0.008^{e}	0.47±0.008^{g}	0.62±0.005^{e}	ND
Caffeic acid	2.47±0.001^{a}	1.72±0.001^{b}	0.96±0.002$^{d, e}$	0.73±0.001^{f}	0.91±0.003^{e}	2.43±0.007^{a}
Syringic acid	19.96±0.096^{b}	16.24±0.01^{c}	1.48±0.007^{j}	1.05±0.008^{l}	1.91±0.087^{i}	5.85±0.09^{e}
p-Coumaric acid	1.12±0.005^{b}	0.52±0.001^{e}	0.13±0.008^{h}	ND	0.099±0.001$^{h, i}$	2.19±0.003^{a}
Ferulic acid	2.14±0.004^{b}	ND	0.19±0.002^{j}	0.09±0.004^{k}	0.18±0.001$^{j, k}$	2.39±0.001^{a}
Sinapic acid	0.19±0.006^{d}	0.50±0.002^{c}	0.03±0.002^{e}	0.15±0.004^{d}	ND	ND
Salicyclic acid	ND	ND	0.44±0.003^{d}	1.17±0.003^{c}	ND	ND
Naringin	0.86±0.001^{a}	0.52±0.002^{b}	ND	ND	ND	ND
Rutin	ND	0.72±0.001^{c}	0.16±0.007^{d}	ND	ND	2.16±0.001^{b}
Ellagic acid	3.83±0.005^{a}	1.45±0.003^{d}	0.08±0.001^{h}	0.93±0.001^{e}	ND	ND
Myricetin	2.50±0.006^{b}	2.25±0.001^{c}	0.37±0.001^{h}	1.05±0.004^{e}	0.23±0.002^{i}	ND
Quercetin	33.54±0.002^{a}	27.07±0.005^{b}	3.69±0.001^{g}	14.39±0.001^{c}	3.50±0.001^{g}	13.77±0.007^{d}
Naringenin	1.42±0.001^{h}	1.31±0.001^{h}	0.37±0.002^{i}	1.13±0.001^{h}	2.40±0.005^{f}	2.08±0.009^{g}
Apigenin	4.80±0.012^{h}	8.3±0.012^{g}	10.47±0.011^{e}	31.04±0.014^{b}	0.48±0.008^{l}	11.07±0.007^{d}
Kaempferol	145.81±0.001^{a}	122.66±0.002^{b}	3.009±0.001^{i}	13.64±0.001^{e}	2.81±0.001$^{l, j}$	8.94±0.005^{f}

Phenolic acids and flavonoids	*L. crocatus*		*L. hirtipes*		*L. kesiyae*		*L. viridinigrellus*	
	Water extract µg/100 mg	MeOH extract µg/100 mg	Water extract µg/100 mg	MeOH extract µg/100 mg	Water extract µg/100 mg	MeOH extract µg/100 mg	Water extract µg/100 mg	MeOH extract µg/100 mg
Gallic acid	8.23±0.002^{g}	20.80±0.002^{f}	77.09±0.001^{b}	173.04±0.012^{a}	8.57±0.004^{g}	47.38±0.002^{d}	3.91±0.013^{i}	5.57±0.001^{h}
Protocatechuic acid	2.19±0.007^{f}	ND	10.34±0.004^{b}	29.01±0.003^{a}	0.21±0.006^{h}	7.51±0.008^{d}	ND	ND
Gentisic acid	ND	ND	ND	ND	ND	ND	ND	ND
p-Hydroxy benzoic acid	0.19±0.012^{e}	ND	ND	ND	0.09±0.013^{f}	1.26±0.014^{a}	0.62±0.015^{b}	ND
Catechin	0.26±0.006^{j}	1.92±0.007^{h}	2.12±0.01^{g}	2.84±0.008^{e}	ND	2.31±0.008^{f}	0.25±0.007^{j}	ND
Chlorogenic acid	ND	1.20±0.001^{j}	1.78±0.007^{i}	43.10±0.006^{b}	6.38±0.002^{f}	6.62±0.005^{e}	6.97±0.007^{d}	56.58±0.007^{a}
Vanillic acid	1.26±0.007^{c}	1.57±0.008^{b}	0.22±0.009^{h}	0.98±0.006^{d}	ND	ND	ND	0.57±0.007^{f}
Caffeic acid	0.20±0.006^{g}	1.10±0.001^{c}	1.08±0.003$^{c, d}$	1.76±0.002^{b}	ND	0.81±0.001^{f}	0.025±0.001^{h}	0.26±0.004^{g}
Syringic acid	1.18±0.01^{k}	2.63±0.012^{g}	2.16±0.012^{h}	3.20±0.011^{f}	1.05±0.007^{l}	10.43±0.085^{d}	0.56±0.004^{m}	65.01±0.004^{a}
p-Coumaric acid	0.08±0.004^{i}	ND	0.34±0.001^{f}	0.85±0.001^{c}	0.07±0.001^{i}	0.57±0.002^{d}	0.18±0.001^{g}	0.86±0.005^{c}
Ferulic acid	0.51±0.005^{h}	1.20±0.001^{f}	0.67±0.001^{g}	1.33±0.002^{e}	0.15±0.004$^{j, k}$	1.87±0.001^{c}	0.27±0.007^{i}	1.61±0.001^{d}
Sinapic acid	ND	ND	0.06±0.001^{e}	0.17±0.001^{d}	ND	0.08±0.006^{e}	2.88±0.001^{b}	13.80±0.001^{a}
Salicyclic acid	ND	ND	1.94±0.008^{b}	6.32±0.006^{a}	ND	ND	ND	ND
Naringin	ND	ND	ND	0.30±0.001^{c}	ND	ND	ND	ND
Rutin	ND	ND	0.75±0.002^{c}	5.80±0.002^{a}	ND	ND	ND	ND
Ellagic acid	0.23±0.001^{g}	ND	1.44±0.002^{d}	1.98±0.001^{c}	ND	0.84±0.001^{f}	ND	3.36±0.007^{b}
Myricetin	0.10±0.008^{j}	ND	1.49±0.001^{d}	5.23±0.001^{a}	ND	0.43±0.005^{g}	ND	0.64±0.001^{f}
Quercetin	4.63±0.001^{f}	9.74±0.001^{e}	0.85±0.003^{k}	1.88±0.001^{j}	ND	1.07±0.002^{i}	ND	2.98±0.001^{h}
Naringenin	0.40±0.001^{i}	1.13±0.001^{h}	8.61±0.098^{c}	22.90±0.011^{a}	4.57±0.001^{e}	22.26±0.001^{b}	4.22±0.003^{e}	7.95±0.001^{d}
Apigenin	4.89±0.009^{h}	8.86±0.01^{f}	1.86±0.078^{j}	34.37±0.08^{a}	1.38±0.01^{k}	21.84±0.011^{c}	0.36±0.009^{l}	2.96±0.009^{i}
Kaempferol	0.82±0.001^{l}	2.91±0.001$^{i, j}$	2.91±0.008$^{i, j}$	15.31±0.001^{d}	3.83±0.002^{h}	6.70±0.007^{g}	1.28±0.001^{k}	67.56±0.003^{c}

ND : Not detected. Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean±SEM. Statistical analysis were carried out by Tukey's test at 95% confidence level and statistical significance were accepted at the $P<0.05$ level. The superscript letter a, b, c d, e, f and so on denotes the significance of various parameters. Letter a is significant to b, c, d, e, f, etc...

spectra, which were comparable both for mushroom extracts and standard substance.

Gallic acid, syringic acid, naringenin, apigenin and kaempferol are present in all the seven studied specimens in their both the extracts. Methanol extract of *L. hirtipes* shows the highest gallic acid (173.04 µg/100 mg DE), protocatechuic acid (29.01 µg/100 mg DE), salicyclic acid (6.32 µg/100 mg DE), rutin (5.80 µg/100 mg DE), naringenin (22.90 µg/100 mg DE) and apigenin (34.37 µg/100 mg DE). Likewise, *L. viridinigrellus* reports the maximum chlorogenic acid (56.58 µg/100 mg DE) and syringic acid (65.01 µg/100 mg DE). The methanol extract of *L. pilosus* exhibits the maximum catechin (11.08 µg/100 mg DE) but shows the maximum quercetin (33.54 µg/100 mg DE) and kaempferol (145.81 µg/100 mg DE) in the water extracts. Gentisic acid is present only in the water extracts of *L. piperatus* and *L. indovolemus*.

DISCUSSION

Mushrooms have been reported to be a very important source of bioactive compounds and secondary metabolites. Antioxidant compounds such as phenolics, flavonoids, ascorbic acid can be extracted from their fruit bodies, mycelium, or even culture broth. Previously studied antioxidant

property of different edible mushrooms reports that the use of methanol/ethanol as a predominant or better extraction solvent which is in accordance to our result.[7,8,14,24]

The extractive value

The outcomes portrayed in Table 1 show that extraction by the water is higher than the methanol. It very well may be presumed that the yields of extraction by the two solvents decreased the accompanying way: Water > methanol giving that water has the higher measure of % yield substance. These confer that water improves extraction yield maybe because of the higher dissolvability of proteins and starches in water than methanol. In any case, extraction yield is not the same as the extraction capacity and subsequently methanol separates show higher antioxidant property than that of water extricates.[25]

The total phenolic and flavonoid content

Phenols are the aromatic organic compounds and probably the largest group of secondary metabolites found in plants and mushrooms. Natural phenolic compounds accumulate as end products from the shikimate and acetate pathways and ranges from simple molecules to highly polymerized compounds.[26] Polar solvents like methanol/ethanol are the strong extracting solvents for the phenolics since methanol has the ability to dissolve the associated biomolecules and also to inhibit the phenoloxidase.[27-31] From the present study, it can be comprehended that both the extracting solvents (water and methanol) essentially ($P < 0.05$) influenced the polyphenol content but the polar antioxidant agents were more extracted by the methanol than the water. Thus methanol demonstrated to be a better extracting solvent in this case also. The methanol extracts of the studied specimens exhibited higher total phenolic and flavonoid content. The decreasing order of phenolic content will be *L. kesiyae* > *L. hirtipes* > *L. indovolemus* > *L. viridinigrellus* > *L. piperatus* > *L. crocatus* > *L. pilosus*.

Flavonoids are the class of polyphenolic compounds found in mushrooms. They are also responsible for chelating the harmful free radicals and protect our health from the toxins. From the results in Table 1, the flavonoid content is quite less in compared to the phenol content. The decreasing order of flavonoid content will be *L. viridinigrellus* > *L. indovolemus*> *L. pilosus*> *L. piperatus*> *L. hirtipes*> *L. kesiyae*> *L. crocatus.*

This might be inferable that more non-phenolic compounds such as carbohydrate and terpene are dissolved in water extracts than in methanol extracts. It might likewise be brought about by the conceivable complex development of some phenolic mixes in the concentrate that are dissolvable in methanol however not in water. These phenolic compounds in methanol may possess more phenol groups or have higher molecular weights than the phenolics in the water extract. In light of the after-effects that methanol was comparative better solvent for displaying higher antioxidant activities than water.

It has been recognized that the medicinal properties of phenolics are mostly attributed due to their antioxidant capacity. Phenolic compounds showed potent antioxidant activities by adsorbing and neutralizing the free radicals.[32] Flavonoids and flavonols are considered as one of the very well-known groups of natural constituents found in the plants and mushrooms. It has been perceived that both flavonoids and flavonols show antioxidant activity through scavenging or chelating process.[33] The results unequivocally recommend that phenolics are significant components of these mushroom specimens. The other phenolic compounds for example flavonoids, flavonols, which contain hydroxyls, are liable for the radical scavenging effect in the specimens. As indicated by our examination, methanol was the most appropriate solvent to segregate the phenolic compounds and the flavonoids from the mushrooms.

The reducing power

The reducing ability is the most helpful approach to test the antioxidant potential. It decides the capacity of an antioxidant to donate a hydrogen atom to the free radical and accordingly stabilizing them and breaking the free radical chain.[34] The reducing power of all the studied mushrooms were better noted in their methanol extracts. The decreasing order will be *L. viridinigrellus* > *L. indovolemus* > *L. hirtipes* > *L. piperatus* > *L. pilosus* > *L. kesiyae* > *L. crocatus.*

The free radical scavenging activity by 1,1-diphenyl-2-picrylhydrazyl assay

DPPH radical scavenging assay was undertaken to evaluate the anti-radical properties of wild mushrooms under investigation. The 50% inhibition of DPPH radical (IC_{50}) by the different extracts was resolved, a lower value would reflect more noteworthy antioxidant activity of the sample. DPPH stable free radical technique is simple, rapid, and sensitive way to assay the antioxidant activity of a specific compound or mushroom extracts.[35] The antioxidant molecules can reduce DPPH free radicals by providing hydrogen atom or by electron donation and a colorless stable molecule 2,2– diphenyl–1–hydrazine is formed and because of which the absorbance (at 517 nm) of the solution is decreased. The antioxidant effect is related to the disappearance of the purple color of DPPH in test samples.

Henceforth, the more potent antioxidant, more decrease in absorbance is observed and thus the IC_{50} value will be minimum. In the current investigation, the highest DPPH radical scavenging activity was appeared by the methanol extract of *L. indovolemus* (IC_{50} = 0.93 ± 0.04 mg DE), while the water extract of *L. crocatus* showed the lowest activity (IC_{50} = 5.17 ± 0.14 mg DE). A strong inhibition was detected for methanol extract of other mushrooms when contrasted with the water extracts. The high radical scavenging property of these mushrooms might be because of the presence of hydroxyl groups that make available the essential component as a radical scavenger.

The free radical scavenging activity by 2,2′-azino-bis (3–ethylbenzothiazoline–6–sulfonic acid) assay

By means of the widely used ABTS assay, the anti-radical activities in various extracts of seven mushrooms were measured and depicted in Table 1. The disappearance of the color of ABTS determines the anti-radical properties of the studied extracts. At which concentration the extracts could inhibit the 50% ABTS free radical (IC_{50}), was used to determine the antioxidant capacity of the sample. It is established that samples that had IC_{50} <50 ppm, considered as very strong antioxidant, while IC_{50} <50-100 ppm IC_{50} <101-150 ppm and IC_{50} >150 ppm might be recognized as strong antioxidant, medium antioxidant, and weak antioxidant, respectively.[21]

The methanol extract of *L. viridinigrellus* (IC_{50} = 0.11 ± 0.0008 mg DE) showed the highest ABTS radical scavenging activity in our study was shown, whereas the least activity (IC_{50} = 0.74 ± 0.008 mg DE) was displayed by the water extract of *L. crocatus.* All extracts obtained by using methanol solvent gave stronger radical scavenging capacity than that of the water extract except *L. pilosus* and *L. piperatus.* The methanol and water extract of both *L. pilosus* and *L. piperatus* were found to display similar nature of radical scavenging activities.

The high-performance liquid chromatography analysis of the phenolics and flavonoids

HPLC analysis of the phenolic compounds of some of the previously studied well–known edible mushrooms such as *Boletus edulis*, *Cantharellus cibarius*, *Lactarius deliciosus*, *Pleurotus ostreatus*, *Agaricus bisporus*, *Lentinus edodes* showed the presence of various phenolic acids like gallic acid, protocatechuic, p–hydroxybenzoic, p–coumaric, ferulic, sinapic, vanillic acid and cinnamic acids as their major phenolic compounds.[4,5,7,36] From our study, it is revealed that *L. pilosus*, *L. piperatus* and *L. hirtipes* showed the maximum diversity of the reference phenolic and flavonoid compounds [Table 2.] and can be considered as a good food supplement. Gallic acid, syringic acid, naringenin, apigenin, and kaempferol are the dominant phenolic compounds in our specimens. Gallic acid is present either in the free–state or as ester and is a very noteworthy anticancer agent.[37] Methanolic extract of *L. hirtipes* showed a very noteworthy amount of gallic acid (173.04 µg/100 mg DE). Protocatechuic acid is known to treat cardiovascular diseases.[38] The methanol extract of *L. hirtipes* expressed the maximum amount (29.01 µg/100 mg DE). Gentisic acid is only reported from water extracts of *L. piperatus* and *L. indovolemus*. p–hydroxybenzoic acid is considerably low in the studied mushrooms and also absent in some of them. It has been reported that other edible mushrooms like *Agaricus bisporus* and *Lentinus edodes* also showed low p–hydroxybenzoic acid. *L. pilosus* reported maximum catechin in both of its extracts (water: 10.44 µg/100 mg DE; methanol: 11.08 µg/100 mg DE) making it a potent source of natural antioxidant. Chlorogenic acid is antidiabetic[39] and found maximum in methanol extract of *L. viridinigrellus* (56.58 µg/100 mg DE). The hepatoprotective vanillic acid[39] is also quite low and found majorly in *L. pilosus* and *L. crocatus*. Water extract of *L. pilosus* (2.47 µg/100 mg DE) and methanol extract of *L. indovolemus* (2.43 µg/100 mg DE) shows almost equal quantity of caffeic acid which is a good source of energy–booster and anti-cancer. Syringic acid is well known for fighting malignancy and have hepato–defensive activities.[40] *L. viridinigrellus* reports the maximum syringic acid content (65.01 µg/100 mg DE). p–coumaric acid is present in both the extracts of all the specimens except methanol extracts of *L. piperatus* and *L. crocatus* and it exhibits strong antioxidant activity.[41] The anti–aging and cholesterol preventive ferulic acid[42] is moderately found in all the studied plants but maximum in *L. indovolemus* (2.39 µg/100 mg DE). Sinapic acid is quite low and completely absent in *L. indovolemus* and *L. crocatus*. The anti-inflammatory and one of the best known antioxidants salicyclic acid[43] is only present in extracts of *L. piperatus* and *L. hirtipes*. Likewise, naringin is only present in *L. pilosus* and *L. hirtipes*. The anti–diabetic[44] and anti–cancer[45] rutin is almost absent in the studied specimens but maximum present in *L. indovolemus* (2.16 µg/100 mg DE). Ellagic acid and myricetin is only moderately present. Maximum quercetin is present in water extract of *L. pilosus* (33.54 µg/100 mg DE). Quercetin is a popular anti-cancer,[46] anti–inflammatory, anti–viral flavonoid compound.[47] *L. hirtipes* again exhibited the maximum flavonoids naringenin (22.90 µg/100 mg DE) and apigenin (34.37 µg/100 mg DE). Naringenin is anti–diabetic[48] whereas apigenin prevents cardiovascular infirmities, neurological disorders, and mutagenesis.[49] Kaemferol helps in fighting cardiovascular maladies, malignancy, arteriosclerosis[50] and *L. pilosus* shows a considerable high and maximum (145.81 µg/100 mg DE) amount of it.

CONCLUSION

In light of the outcomes, it very well may be presumed that the extracts of the studied seven mushrooms showed the noteworthy antioxidant property and can be a decent wellspring of natural antioxidants. Hence, consumption of them will give us a protective shield against the everyday toxins, cell-damaging reactive ions, malignancy, fatigue, and heart diseases. Moreover, since mushrooms are easily available in the forests as well as in the markets, they are quite cost-effective too. When compared to some of the vegetables like broccoli, spinach, carrots, cabbage which are quite good sources of antioxidants, these mushrooms can be equally noteworthy. More extensive study is required furthermore to establish this ground.

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Conflicts of interest

There are no conflicts of interest.

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