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RESEARCH ARTICLES

Mycochemical composition and antioxidant activity of *Flammulina velutipes*: a comparative study on hydromethanol, decoction and infusion extracts

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Abstract
Jammu and Kashmir provide a pleasant environment for the lavish growth of diverse macrofungal groups where many members are ethnically appraised. Local people enjoy edible mushrooms due to their health beneficial effects ensuring food security for inhabitants. In this regard, a recent field survey was carried out by our team in Kishtwar High Altitude National Park, India gifting us several traditionally popular edible mushroom species. One of the collected samples was identified as *Flammulina velutipes*, as described herein, which was further subjected to isolation of various fractions to study mycochemical composition and bioactivity. Our results showed that the decoction preparation was capable of better radical scavenging followed by hydro-methanol extract; while the infusion exhibited a promising effect on the chelating ability of metal ions. The outcome might be justified by the quantity of phenol which was in the utmost in decoction (14.85 µg gallic acid equivalent/mg of extract) followed by infusion preparation (12.02 µg gallic acid equivalent/mg of extract). On the other hand, lycopene (1.4 µg/mg of extract) and ascorbic acid (1.56 µg/mg of extract) were present in higher amounts in the hydromethanol fraction. Thus, the studied mushroom could be considered as an easily accessible source of natural antioxidants that could be used in the pharmaceutical industry.

Keywords Ethnic use · Macro-microscopic characterization · Radical scavenging activity · Secondary metabolites · Wild edible mushroom

Introduction

Ethnomycology is a subject of great worth that uncovers the interactions between mankind and fungi in a given environment, both in the past and present (Molares et al. 2019; Sitotaw et al. 2020). Thus, the extent of traditional knowledge is quite comprehensive and profound in Indian tribal communities, consuming closely 283 species of wild mushrooms out of 2000 overall known globally. Besides food source, mushrooms have long been appreciated for their broad spectrum of therapeutic uses. In fact, a large number of mushrooms are highly regarded in traditional Chinese medicine due to their health promoting and anti-ageing effects (Tang et al. 2016). Considered as one among the delicious foodstuffs packed with medicinal benefits, mushrooms are now frequently produced and consumed internationally (Debnath et al. 2019). Additionally, they have been prized as nutraceutical food across the globe particularly in Asiatic societies (Kumar and Sharma 2009; Lalotra et al. 2016; Khatua et al. 2017a). As a result, researchers have always been keen to explore their medicinal attributes for the development of therapeutic drugs. Recently, it has been estimated that mushrooms possess different types of bioactive compounds exhibiting beneficial health effects, of which antioxidant property is of special significance (Kozarski et al. 2015).

Free radical, a molecule with unpaired electrons is metabolically generated inside the human body and plays a beneficial role. However, the production can exceed the threshold level due to certain exogenous sources like smoking,

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pollution, pesticides, UV radiation, and overexercise, among others resulting in oxidative stress (Valko et al. 2007). Consequently, these free radicals attack cellular components including nucleic acid, lipid, and protein to gain stability. The effect leads to the development of a number of disorders like cardiovascular diseases, atherosclerosis, several kinds of cancer, lung diseases, cirrhosis, neurological disorders, diabetes, etc. (Khatua et al. 2013). Herein, antioxidants can protect the human body from free radical-induced damage and maintain homeostasis. Few synthetic chemicals are available in the market, although they have shown health hazards discouraging their use. Thus, it is important to search for biomedicine with more efficiency and less toxicity where mushrooms could be regarded as an ideal candidate (Lourenço et al. 2019).

Such macrofungal health benefits are generally attributable to their metabolites, especially the phenolic compounds (Khatua et al. 2015). These myco-chemicals can be extracted in adequate amounts by using an appropriate solvent with a suitable level of polarity. However, identification of a single solvent that can efficiently extract most of the bioactive compounds from any bio-resource often becomes challenging. Researchers now thus have adopted the concept of using a different extraction condition to target isolation of different components in appreciable quantity (Ukaegbu et al. 2018).

Flammulina velutipes (Curtis) Singer (velvet shank, enoki, winter, or golden needle mushroom) is one of the most known edible mushrooms that retains an inclusive range of biological activities. Historically, the taxon is being cultivated for regular cuisine and medicinal usage, particularly in China since 800 AD. Presently, it is one of the foremost globally acknowledged mushrooms owing to its supreme delicacy, nutritional value, and pharmacological properties (Tang et al. 2016). However, the medicinal potential of the mushroom still remains under-explored. Against this backdrop, the present work was aimed to study myco-chemical composition and antioxidant activity of different extracts namely infusion, decoction, and hydro-methanolic fractions prepared from the fruit bodies of *F. velutipes*.

Materials and methods

Collection of fruiting bodies and traditional usage

Located in the north-western Himalayas region of Jammu and Kashmir, Kishtwar High Altitude National Park (KHANP) is contiguous in nature and tract is situated on the high altitudes i.e., sub-alpine and alpine zones with an altitude range of 2300–6000 m above the mean sea level (Fig. 1). The main activities in KHANP are agriculture and stockbreeding. Mushroom picking is one of the common activities practised by the local people during rainy seasons in forests and meadows. Owing to a wide range of elevation, slopes and moisture regime, the region harbours a variety of temperate conifer forests that further supports a number of mycorrhizal mushroom species. Six different study sites were selected from the Park for the collection of *F. velutipes.* Morphological details (size, shape and colour of the fruiting body) of the species were studied in the field. For the collection of traditional data, people living in the peripheral areas of the Park were asked questions regarding the collection, usage, vernacular names, and methods of preservation of different macrofungal species. They have also been enquired about the medicinal uses of mushrooms by either showing them fruiting bodies or photographs taken in the field. Once data on conventional knowledge was collected, the samples were brought to the laboratory for studying their microscopic details (basidia, basidiospores, cystidia, hyphae etc.) with the help of a compound microscope. Identification of the specimen was performed based on morphological characters.

Preparation of infusion, decoction and hydro-methanolic extracts

After collection, the specimens were subjected to sun-drying followed by drying in the self-designed electric drier. Dried fruit bodies were first pulverized using an electric blender and sieved through 160 mesh. For hydro-alcoholic extract preparation, 300 mg of the powder was soaked in 20 ml of methanol: water (80:20, v/v) for one hour with constant shaking. The fraction was then isolated using Whatman filter paper. The preparation was further dried by evaporation (Rotavapor R-3, Butchi, Switzerland) at 40 °C. For infusion preparation, the sample (300 mg) was added to 20 ml of boiling distilled water, incubated for around 30 min at room temperature and then filtered. To isolate a decoction preparation, the same amount of sample was added to 20 ml of distilled water and the mixture was boiled for around 20 min. The preparation was cooled down for 5 min and then filtered. The obtained infusion and decoction were then frozen as well as lyophilized (Sharma et al. 2019). The recovery percentage of all the studied extracts was estimated following an established formula:

$$\text{Yield}\ (\%) = (\text{W1} \times 100)/\text{W2}$$

W1 and W2 represent the weight of the fraction after solvent evaporation and the weight of the minced mushroom, respectively.

Determination of major bioactive compounds

For estimation of total phenolic compounds, Folin-Ciocalteu (FC) assay was followed where the preparations were mixed

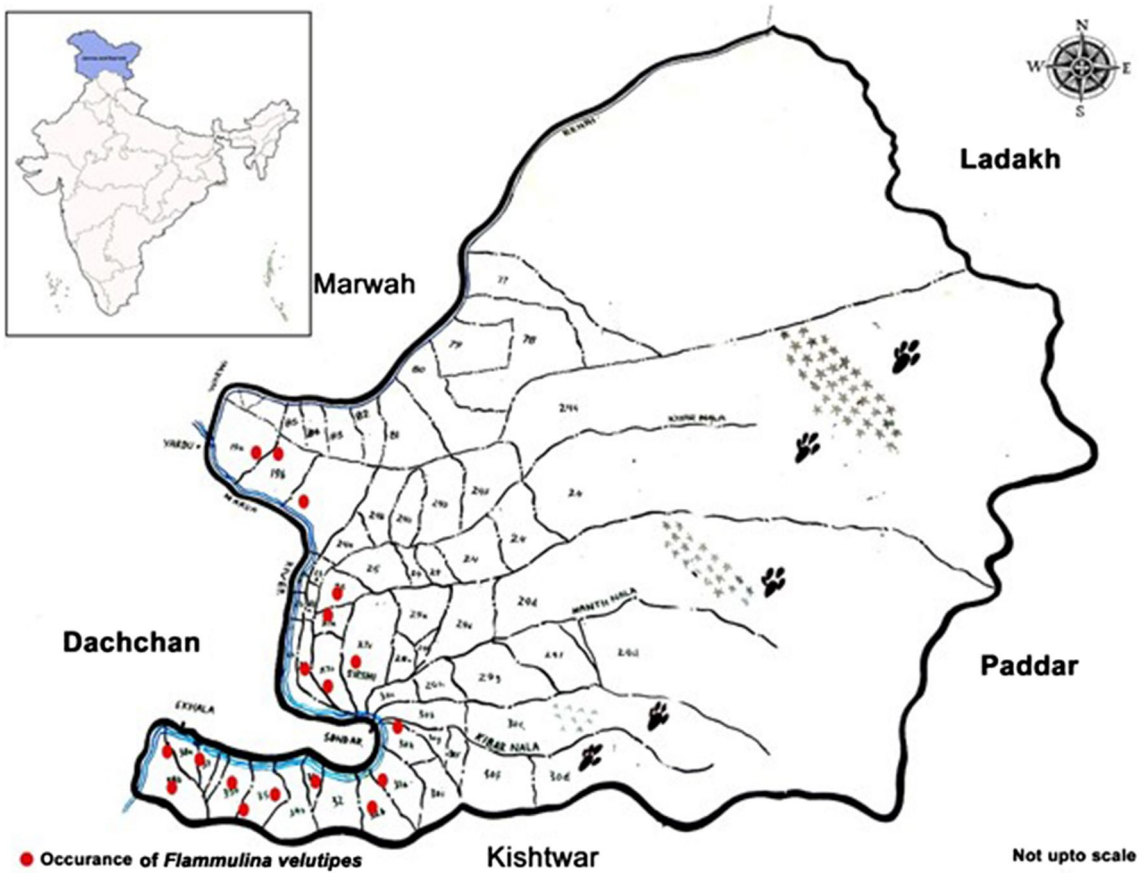

Fig. 1 Map showing occurrence of *Flammulina velutipes* in different regions of Kishtwar High Altitude National Park (KHANP)

with FC reagent, allowed to stand for 3 min at room temperature and then sodium carbonate solution was added. Finally, absorbance was recorded at 725 nm and gallic acid (10–40 µg) was used as a standard. The amount of total flavonoid was quantified by mixing the extract with aluminium nitrate, potassium acetate, and 80% ethanol. Following 40 min incubation in dark, absorbance was measured at 415 nm. Quercetin (5–20 µg) was considered as a reference. Further, the amount of ascorbic acid was quantified following a modified titration method where vitamin C was mixed with oxalic acid and titrated against 2, 6-dichlorophenolindophenol dye which was prepared by mixing sodium bicarbonate and the stain in water. Contents of carotenoids were determined by mixing the extract with an acetone-hexane solution and recording the absorbance at 3 different wavelengths such as 453, 505 and 663 nm (Khatua et al. 2017b).

Evaluation of antioxidant activity

The ability of the fractions to chelate ferrous ions was estimated in the microtiter plate using ferrozine as well as ferrous chloride and absorbance at 595 nm was estimated with the help of a microplate spectrophotometer (iMark™ Microplate Absorbance Reader, Bio-Rad, USA). To estimate radical scavenging activity, 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals were produced freshly by adding potassium persulfate in ABTS solution and after 12–16 h incubation, $ABTS^{\bullet+}$ solution was adjusted to the absorbance of 0.7 ± 0.02 at 750 nm. The radicals were then permitted to react with the extracts at variable doses in a 200 µL reaction mixture in a 96 well plate and absorbance was noted. Further, methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was evaluated against various dosages of the studied extracts in 96 well plate and absorbance at 595 nm was determined (Khatua et al. 2017c). The method of total antioxidant capacity was carried out in the present study following phosphomolybdenum method. For that, the mixture of sample, sulphuric acid, sodium sulphate as well as ammonium molybdate was heated at 95 °C for 90 min and the activity of all the extracts was depicted compared with that of standard, ascorbic acid (Khatua et al. 2018).

Statistical analysis

The results presented herein are expressed as mean ± SD of three independent experiments. The analysis of statistical

data was procured with Student's t-test by $p < 0.05$ as the minimal level of significance using IBM SPSS Statistics, v. 23.0. (IBM Corp., Armonk, New York, USA).

Results and discussion

Traditional information

Indigenous people including, elderly folk, shepherds, traditional healers, local tribes belonging to *Gaddis, Bakarwal*, and *Gujjar* were interviewed regarding the edible and medicinal importance of *F. velutipes*. It is found to be traditionally collected and used in the study area. Locally known as 'Sirer' (mushroom-like) in Kashmiri, Kishtwari, and 'Haildii Chaltii' (Bhaderwahi), this species is found to be widely consumed by villagers situated around the Park. Besides, its wide consumption it is thought to cure the problem of diabetes among the native people. Early morning mushroom hunting was preferred by the local people and trowels or sharp knives were used for uprooting their fruiting bodies. Large containers locally called 'Balti' or 'Tokri' were also carried by them to safely keep the collected fruiting bodies. After collection, the fructifications were washed properly in order to remove the debris, if any. Thereafter, at home, the fruiting bodies were chopped with a sharp knife, and some portions were left for drying in open sunlight for future consumption. For storage, large glass jars were used. Besides, the delicious preparations from these fruit bodies, available in abundance locally, could further help in feeding the whole family. As a simple and energetic diet, generally, the consumption of such mushrooms was preferred during breakfast to enable them to easily carry out their whole day's work.

Morpho-taxonomic details

Flammulina velutipes (Curtis) Singer, Lilloa 22: 307 (1951) [1949] (Figs. 2 and 3a–e).

Synonymy: *Flammulina velutipes* var. *radicans* Wichansky, (1968).

Flammulina velutipes var. *lupinicola* Redhead & R.H. Petersen, Mycotaxon 71: 292 (1999).

Collection Examined: Jammu and Kashmir (J&K), Kishtwar High Altitude National Park (KHANP), found in dense clusters, Roshi Sharma and Y.P. Sharma, HBJU 606, September 2017–2018.

Pileus: creamish brown, 1.0–2.5 cm wide, planoconvex to nearly flat, surface smooth, sticky when moist, flesh thin; **Gills:** creamish, adnate, unequal, sub-crowded; **Stipe:** 2.5–4.5 cm long and 0.2–0.3 cm wide, brownish, velvety, slender, solid, striated; **Basidia:** clavate, 15.2–21.6 × 2.4–5.6 µm, hyaline, guttulated, basal clamps present; **Sterigmata:** 1 to 4 in number, 1.6–2.4 µm long;

Fig. 2 Fructifications of *Flammulina velutipes* in natural habitat

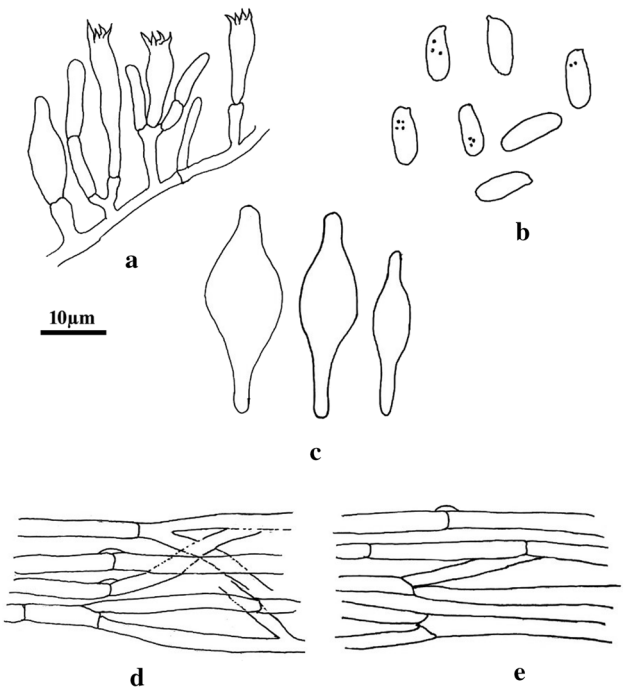

Fig. 3 Characterization of *Flammulina velutipes*. **a** basidia **b** basidiospores **c** cystidia **d** pileus hyphae **e** stipe hyphae

Basidiospores: 5.6–8.8 × 2.4–3.2 µm, a_v L = 7.2, a_v W = 2.8, Q = 2.3–2.7, hyaline, smooth, monoguttulated, thin-walled; **Cystidia:** clavate, 14.4–34.4 × 4.0–12.8 µm wide, hyaline, guttulated; **Pileus hyphae:** 0.8–2.4 µm wide, inflated up to 6.4 µm, branched, septate, clamped; **Stipe cuticle hyphae:** 1.6–24.0 µm wide, clamped, branched, septate; **Stipe context hyphae:** 6.4–10.4 µm wide, branching absent, septate, hyaline.

Edibility: Consumed in the study area.

Distribution: Previously reported from Darjeeling, Sikkim (Berkeley 1856), Calcutta (Banerjee 1947), and J&K (Watling and Gregory 1980; Kumar and Sharma 2008).

Remarks: *Flammulina velutipes* is popularly known by different names like velvet mushroom, golden needle, etc. It is often seen to grow in large tufts or clusters, thus making it convenient to harvest. It is mostly found to occur in the winter months, thus known by the name of winter mushroom. The present species was seen to prop up from the heap of coniferous needles along with the fallen twigs that are known to provide nutrients for the luxurious growth of the fruiting bodies.

Mycochemical analysis

A range of bioactive substances like phenols, flavonoids, carotenoids, and ascorbic acid content was measured in the studied three extracts prepared from *F. velutipes* and the outcome has been depicted in Table 1. In summary, phenol was detected to the highest extent in decoction followed by infusion preparation. Though flavonoid was estimated in better quantity in infusion formulation than that of other extracts. Conversely, the hydro-methanol fraction was found to be enriched in lycopene and ascorbic acid followed by infusion extract. However, β-carotene was not detected in any of the preparation. Thus, it could be inferred that heating favours leaching out of phenolic compounds, although the parameter was not suitable for the extraction of carotenoid as well as vitamin C.

Estimation of antioxidant potential

In order to understand the antioxidative behaviour of the isolated fractions from *F. velutipes*, a range of in vitro assays was performed (Table 2). Initially, the ferrous ion chelating ability method was performed as Fe^{2+} is known as the most important pro-oxidant that causes the generation of highly reactive free radicals. Therefore, the iron-chelating activity of a substance is considered to be related to antioxidant activity. To determine the effect, ferrozine was used in the present study that eagerly forms complexes with Fe^{2+} resulting in a violet colour solution. When another chelator is added to the reaction mixture, complex formation is disrupted and thus the solution loses its distinct colour (Khatua and Acharya 2018). As presented in Fig. 4a, all extracts from *F. velutipes* demonstrated a similar trend of affinity towards iron. At 300 and 500 µg/ml concentrations, the infusion presented 33.84% and 57.36% binding capacity, respectively. The outcome was as per the decoction preparation. While hydro-methanol extract exhibited a lower effect as it depicted 24.73% and 54.11% chelating ability, respectively at the above-mentioned levels.

Further, a free radical cation namely $ABTS^{\bullet+}$ was used in the present study for better understanding the antioxidant activity of three extracts from *F. velutipes*. ABTS salt can produce blue-green coloured $ABTS^{\bullet+}$ by reacting with potassium persulfate. However, the chromophore can rapidly be converted back to its colourless neutral form in presence of antioxidative compounds (Khatua et al. 2017c). Our analysis indicated that the studied fractions possessed strong

Table 1 Extractive yield and mycochemical composition of hydromethanol extract, infusion and decoction from *Flammulina velutipes*

Parameters	Hydromethanol	Infusion	Decoction
Extractive yield (%)	33.33	40	43.33
Phenol content (µg gallic acid equivalent/mg of extract)	7.5 ± 0.56	12.02 ± 1.54	14.85 ± 2.14
Flavonoid content (µg quercetin equivalent/mg of extract)	0.73 ± 0.05	5.71 ± 0.78	3.51 ± 1.11
Ascorbic acid content (µg/mg of extract)	1.56 ± 0.6	0.94 ± 0.07	0.92 ± 0.03
Lycopene content (µg/mg of extract)	1.4 ± 0.01	0.44 ± 0.08	0.11 ± 0.04

Table 2 Antioxidant activity of hydromethanol extract, infusion and decoction from *Flammulina velutipes*

Antioxidant assays	Hydromethanol	Infusion	Decoction	Standard
EC_{50} value (µg/ml)				
Chelating ability of ferrous ion	441.35 ± 19^{a}	389.22 ± 22^{b}	416.88 ± 14^{c}	2.54 ± 0.5^{d}
Scavenging ability of ABTS radicals	291.87 ± 28^{a}	308.52 ± 44^{b}	221.52 ± 31^{c}	2.58 ± 0.09^{d}
Scavenging ability of DPPH radicals	536.52 ± 31^{a}	421.91 ± 20^{b}	421.54 ± 13^{c}	4.5 ± 0.5^{d}
Total antioxidant activity by phosphomolybdenum method (µg ascorbic acid equivalent/mg of dry polysaccharide)	12.04 ± 0.08^{a}	7.11 ± 0.32^{b}	11.71 ± 1.44^{c}	NA

The outcomes are depicted in EC_{50} values ($n = 3$, mean ± standard deviation) portraying 50% of antioxidant activity in case of chelating ability and radical scavenging property. For chelating ability of ferrous ion method, EDTA was used a standard; whereas ascorbic acid was regarded as a positive control in rest of the assays. Dissimilar letters in each row designate significant alterations between the sample and standard ($p < 0.05$)

NA not applicable

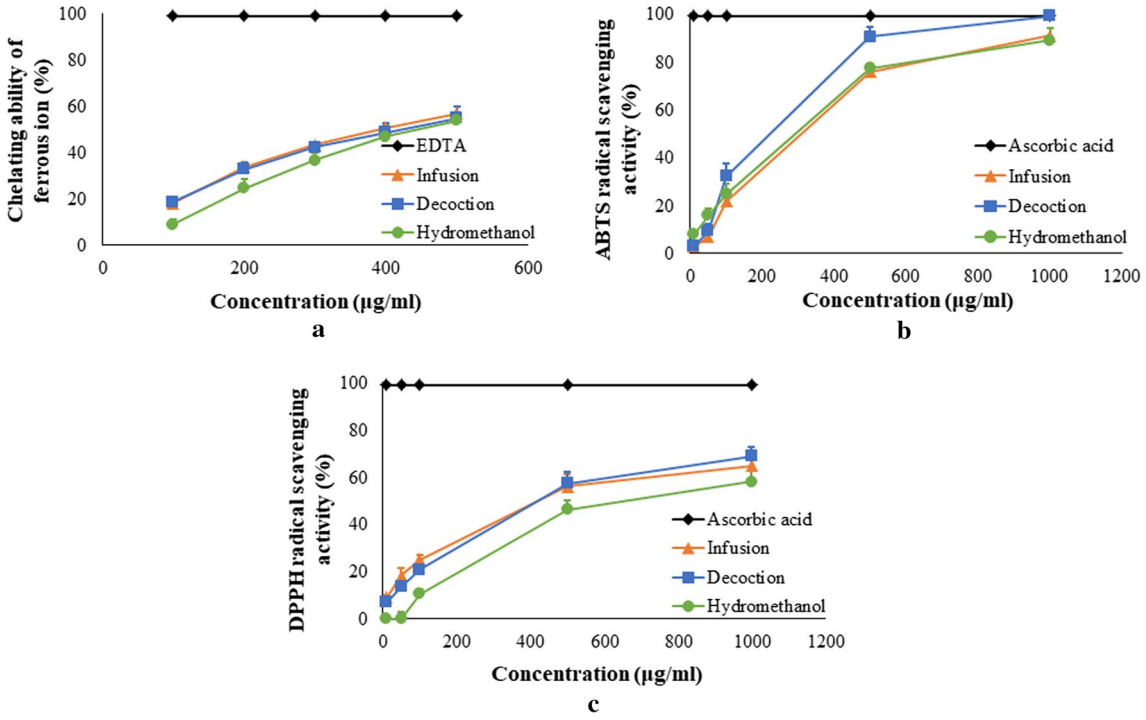

Fig. 4 Antioxidant activity of hydromethanol extract, infusion and decoction from *Flammulina velutipes*. **a** Chelating ability of ferrous ion **b** ABTS radical scavenging effect **c** DPPH radical scavenging activity

radical scavenging activity where the potentiality increased in a steady-state manner (Fig. 4b). At the levels of 100 and 500 µg/ml, hydro-methanol extract quenched 24.9% and 77.21% radicals, respectively. A similar trend of effect was also detected in the case of infusion preparation. Whilst, the decoction fraction exhibited the best potential as it inhibited 32.34% and 90.45% radicals, respectively resulting low EC_{50} value.

Besides, DPPH$^{\bullet}$ was also used to understand the antioxidant property of the fractions under the present study, being an easy-to-perform and economic means to evaluate the bioactive potential of the investigating drug. This commercially available nitrogen-bearing radical is characterized by its intense violet colour. When an antioxidative substance is added to the solution, the radical is quickly transformed to pale yellow coloured diphenyl–picrylhydrazine (Alam et al. 2013). A decrease in absorbance is therefore directly proportional to increased antioxidant potency. As presented in Fig. 4c, all three examined extracts possessed effective DPPH$^{\bullet}$ scavenging ability that incremented with the increase of concentrations. At the level of 100 µg/ml, aqueous-alcohol fraction quenched 10.43% radicals which reached to inhibition of 46.14% radicals in presence of 500 µg/ml concentrated sample. On the other hand, infusion preparation exhibited 25.26% and 56.18% radical scavenging effects at the above-mentioned dosages, respectively. A similar trend of outcome has also been recorded in the case of decoction preparation. Overall, decoction extract exhibited the best activity; although the effect was lower than the positive control.

Finally, phosphomolybdenum assay was implemented to determine the reducing ability of the antioxidative compounds converting Mo (VI) to Mo (V) (Khatua and Acharya 2018). The effect was further compared with a standard, i.e. ascorbic acid. The comparative analysis portrayed that the hydro-methanolic extract might possess better reducing power followed by the decoction fraction. Thus, infusion preparation exhibited lower efficacy in this assay (Table 2).

Conclusions

All the studied fractions isolated from *F. velutipes* exhibited effective antioxidant potential where the decoction depicted a better radical scavenging effect. On the other hand, hydro-methanol extract portrayed superior reducing capacity; whilst a high ability to chelate metal ions was found in infusion. A study on mycochemical analysis showed that all three preparations consisted of bioactive secondary metabolites making the studied mushroom an ideal nutritional supplement. Further study is required aiming isolation of bioactive compounds that could be used to relieve oxidative stress-related disorders in humans.

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Declarations

Conflict of interest Authors state that they have no conflict of interests and proper permission has been sought from the Wildlife Warden of Kishtwar High Altitude National Park before the collection of *F. velutipes*.

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