Hydromethanolic, infusion and decoction extracts from *Hypholoma fasciculare*: A comparative study on mycochemical composition and antioxidant activity

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Abstract
The Union Territory of Jammu and Kashmir offers a wide variation in the climatic conditions which further invites a number of organisms including mushrooms into different regions. Growing in tufts among the coniferous forests of Kishtwar High Altitude National Park, Hypholoma fasciculare is a very common mushroom that can attract anyone from a distance. The present study hence aimed for a comparative exploration of the chemical profile and antioxidant activity of the specimen using three water-based extraction systems. Comparative analysis revealed that the decoction fraction was enriched with phenols and flavonoids. However, ascorbic acid was present in all three fractions with a better quantity in infusion formulation.

In contrast, β-carotene and lycopene were detected only in hydromethanolic extract. In terms of bioactivity, the hydromethanolic extract presented better antioxidative potential owing to potent quenching ability of DPPH radical, metal ion chelation ability and total antioxidant capacity as compared to infusion and decoction formulations. Thus, the study suggests use of the less explored mushroom for development of pharmaceuticals to treat radical induced disorders.

Keywords: Jammu and Kashmir, Macro-microscopic characterization, Radical scavenging activity, Secondary metabolites, Wild mushroom.

Introduction
Humanity has valued mushrooms for their flavour, taste and nutritional and culinary benefits since antiquity. Later, they also became a common household remedy used for therapeutic and health-improving purposes all across the world, particularly in Asia[10]. Thus, macrofungi drew scientists' attention as they created unique nutraceuticals to shield the human body from a variety of diseases. As a result, mushrooms have become well-known bio-resources for the synthesis of a wide range of chemicals that can be used to displace rival organisms that co-exist in their ecosystem[16]. *Hypholoma fasciculare*, a saprophytic fungus, is one of these matrices that is frequently employed for biocontrol of Armillaria root disease[5].

Typically found on decomposing wood, this yellow-coloured agaric is commonly referred to as 'sulphur tuft' due to its growth habit (tight clusters or tufts) and the vivid sulphur yellow colour of its pileal surface.[3] Among the many mycochemicals that mushrooms create, polyphenols have drawn more attention because they have a number of therapeutic benefits that are partially attributed to their antioxidant activity[18]. The antioxidative property is currently extremely important since it can prevent the generation of free radicals. When synthesised at low levels, these highly reactive chemicals are crucial components of biological systems. However, oxidative stress[15] results when they are produced in excess amounts as a result of some internal and external stimuli. As a result, cellular elements including DNA, RNA, lipid and protein are harmed which have an impact on the emergence of numerous health risks. In this situation, antioxidative chemicals found in nature act as a reliable saviour to maintain homeostasis[13].

However, antioxidant activity depends on the extraction method such as solvent type, temperature, duration and so forth[11]. In this context, bio-based infusion and decoction extracts are gaining increasing attention as consumers prefer these preparations in order to avoid and treat several non-communicable diseases through diet, rather than taking medicines that in turn bring unavoidable side-effects on human's health[14]. The infusion process involves steeping or soaking bio-resources as a whole or some specific part(s) in hot water. The method of decoction comprises of extraction of the essence of a bio-resource by boiling the sample and thus the technique is considered as the most common preparation procedure in herbal medicine systems.

In addition to these methods, a spectrum of secondary metabolites, particularly phenolic compounds, might be separated using an aqueous methanol solvent system[17]. In light of these, the current study sought to compare the antioxidant capacities of *H. fasciculare* decoction, infusion and hydroalcoholic extracts. Additionally, composition of the fractions was also evaluated.

Material and Methods
Study area and collection of fruiting bodies: Mushroom collection was done from the famous National Park viz.

Kishtwar High Altitude National Park (KHANP) from Jammu and Kashmir, situated 250 km away from Jammu city. The park's altitudinal range was from 2300 m to 6000 m above mean sea level.

Since there has been steady water flow across the area for millions of years, it has been thoroughly eroded and transformed into valleys and gorges in every direction. KHANP harbours diverse flora and fauna. Conifers predominate in the park's forests, providing a favourable habitat for the luxuriant growth of related fungi.

Hypholoma fasciculare is one among the many collected agarics from the forests. Macro-morphological details like colour, shape, size, gill attachments, etc. of the collected macrofungus were noted in the field. The collected fruiting bodies were sundried for further microscopic details like basidiospores, basidia, cystidia, hyphae etc. in the laboratory. Identification of the specimen was done on the basis of its macro and microscopic features.

Preparation of hydromethanolic, infusion and decoction extracts: Three hundred milligram of dried mushroom powder was steeped in 20 ml of methanol: water (4:1, v/v) at room temperature and mixed thoroughly. The extract was filtered through Whatmann no. 4 and the filtrate was reduced in volume using a rota-evaporator (Rotavapor R-3, Butchi, Switzerland). The preparation was collected as hydro methanolic fraction of *H. fasciculare.* To prepare the infusion, 300 mg of dried powder was again weighed and suspended in 20 ml of boiling distilled water. The mixture was allowed to stand for 30 min at room temperature and filtered. In order to prepare decoction, 20 ml of distilled water was added to 300 mg of the sample and the mixture was allowed to boil for 20 min. After cooling down at room temperature, the fraction was isolated by filtration and the residue was discarded.

The yield percentage of all these extracts was calculated based on dry weight as:

$$\text{Yield (\%)} = (W_1 \times 100) / W_2$$

where W_1 = weight of extract after solvent evaporation and W_2 = Weight of the minced mushroom.

Estimation of bioactive compounds: The extent of phenolic compounds in each extract was estimated following Folin-Ciocalteu (FC) method. For that, one ml of the preparation was mixed with one ml of FC reagent and one ml of sodium carbonate solution (35%). The volume was raised up to 10 ml, incubated for 90 min and absorbance was recorded at 725 nm. Gallic acid was used as a standard and results were expressed as µg of gallic acid equivalents per mg of extract[12]. For detection of flavonoids, one ml of each extract was mixed with 80% aqueous ethanol, 1 M potassium acetate and 10% aluminium nitrate. After 40 min incubation at room temperature, the absorbance was detected at 415 nm. Quercetin was used to calculate the standard curve and results were expressed as µg of quercetin equivalents per mg of extract[9]. For unveiling the amount of carotenoids, 100 mg extract was mixed with 10 ml acetone-hexane solution (4:6) and filtered. Absorbance was detected at three different wavelengths simultaneously at 453, 505 and 663 nm. The content of carotenoids was calculated as per our earlier studies[9]. Finally, vitamin C content was determined following a titration method[11].

Determination of antioxidant activity: To estimate 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, all the extracts under investigation were mixed with 0.004% DPPH solution at various concentrations. Final volume of the reaction mixture was made up to 200 µl in 96 well plates and incubated for half an hour in dark. The absorbance was recorded at 595 nm using a microplate reader (iMark™ Microplate Absorbance Reader, Bio-Rad, USA)[6]. The magnitude of radical quenching potentiality of all the preparations was further tested by using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical. For that, the radicals were generated freshly by mixing 2.45 mM of potassium persulfate in 7 mM ABTS solution and the mixture was incubated overnight. Further, the solution was diluted to achieve 0.7 absorbance and the radicals were then allowed to react with the extracts at variable concentrations. The reaction volume was made up to 200 µl in a microtiter plate and absorbance was perceived at 750 nm[6].

Further, the chelating ability of the fractions under study was also evaluated. Accordingly, 5 µl ferrous chloride was mixed with the formulations at different levels and then 10 µl ferrozine was added to the solution. The mixture was shaken well, incubated for 10 min and absorbance was estimated at 595 nm using the same microplate reader as mentioned earlier[6].

Finally, total antioxidant activity was determined and for that, a reaction mixture was prepared by mixing 0.6 M sulphuric acid, 28 mM sodium sulphate and 4 mM ammonium molybdate. Total volume of the reaction mixture was made up to 3 ml where 300 µl sample solution was added. The tubes were incubated for 90 min at 95°C and absorbance was enumerated at 695 nm. Total antioxidant activity was expressed as the number of the equivalents of ascorbic acid[1].

Statistical analysis: All data are presented herein as mean ± standard deviation of three independent experiments each in triplicate. Calculations were performed using statistical package for Microsoft® Office Excel (Microsoft®, USA) and differences were evaluated by means of One-way Analysis of Variance (ANOVA).

Results and Discussion

Taxonomic details

Hypholoma fasciculare (Huds.) P. Kumm., Führ. Pilzk. (Zerbst): 72 (1871).

Synonymy: *Agaricus fascicularis* Huds., Fl. Angl., Edn 2 2: 615 (1778).

Hypholoma fasciculare var. *luteolamellatum* Blanco-Dios, Tarrelos 19: 20 (2017) (Fig. 1).

Pileus: 2.5 to 4.6 cm, convex, then becoming flattened or plane, yellowish (4A6) to brownish yellow (5C7), surface smooth, dry, flesh thin, pale cream to yellow, margins incurved.

Gills: Adnate, closely attached, initially yellow then light greenish to olive green (28E4), unequal, thin.

Stipe: 4.5 to 8.5 cm long and upto 1.0 cm wide, solid, yellowish (4A6), often curved, firm, dry.

Basidia: 24.0-24.8 × 4.8-6.4µm, clavate, hyaline.

Sterigmata: 2.4 to 3.2 µm long.

Cystidia: 27.2-43.2 × 5.6-8.0.

Basidiospores: 4.8-7.2 × 3.2-4.8 µm, a_v L=6.0, a_vW=4, Q=1.5, apiculate, hyaline, smooth, greenish brown.

Spore print: Olive green.

Stipe hyphae: 3.2 to 4.8 µm, hyaline, septate, branched.

Edibility: Not edible in the study area.

Collection examined: Jammu and Kashmir, Kishtwar High Altitude National Park, Janakpur, Soil, Dead wood, fasciculate, in large tufts. Accession number HBJU 834, July-September 2018-2019.

Distribution: Reported from Nagaland[4].

Remarks: *Hypholoma capnoides* is very similar to *Hypholoma fasciculare* but can be differentiated in having greyish gilled portion rather than yellowish or greenish.

Estimation of mycochemicals: Bioactive components i.e. phenol, flavonoid, β-carotene, lycopene and ascorbic acid were detected in *H. fasciculare* as presented in table 1. Overall results showed that phenol and flavonoids were presented to the highest extent in decoction fractions. However, it was found that the hydromethanolic extract included β-carotene and lycopene that were absent from the other two fractions. Additionally, all three formulations contained ascorbic acid which was shown to be present in greater quantities in the infusion formulation.

Determination of antioxidant activity: We used four *in vitro* test methods to measure antioxidant capability. Scavenging activity using DPPH was one of them and was one of the most widely used techniques. The method is based on the conversion of $DPPH^{\bullet}$ to diphenyl picrylhydrazine in presence of an antioxidative compound. Consequently, the colour of the reaction mixture turns yellow resulting in decreased absorbance[7]. As presented in fig. 1A, all three fractions of *H. fasciculare* showed effective $DPPH^{\bullet}$ scavenging activity. At the concentration of 10 µg/ml hydromethanolic, infusion and decoction extracts quenched the radical 8.08%, 10.77% and 9.42% respectively.

The activity was raised to 79.65%, 73.32% and 76.64% by hydromethanolic, infusion and decoction extracts at the level of 100 µg/ml. Overall, the hydromethanolic fraction presented higher potential followed by decoction and infusion formulations (Table 2). ABTS radical quenching activity is another popular method that has also been performed in the present study. $ABTS^{\bullet+}$ radicals can easily be generated by a reaction of persulfate oxidation of $ABTS^{2-}$ resulting in a violet coloured solution.

The presence of antioxidant substances in the mixture causes decolourization that can be monitored spectrophotometrically[8]. Results showed that all three fractions presented higher potential in a dose-dependent manner as depicted in fig. 1B. At the level of 10 µg/ml hydromethanol, infusion and decoction extracts inhibited 9.35%, 17.51% and 19.01% radicals respectively. The effect was found to be more prominent at higher concentrations where these fractions quenched 98.46%, 73.32% and 99.59% respectively at the level of 100 µg/ml. As a whole, the investigation depicted that the decoction extract possesses the highest potential than the other two fractions as evident by its low EC_{50} value.

The antioxidative power of a compound is not only dependent on radical scavenging activity but also reliant on the chelating ability of metal ions. Such ions like Fe^{2+} help in the generation of free radicals, thus chelating these ions which further inhibit the propagation of radical-induced damage. The method is based on the binding of ferrozine with Fe^{2+} resulting in violet coloured reaction mixture. The presence of antioxidative compound inhibits such complex formation thereby the colour intensity of the reaction mixture decreases[7]. As represented in fig. 2C, all three extracts demonstrated their ability of binding with Fe^{2+}. However, hydromethanolic fraction exhibited better ability as it chelated 32.6%, 48.36% and 57.67% at the concentrations of 200, 300 and 400 µg/ml respectively.

Infusion and decoction fractions at the dosage of 400 µg/ml presented 51.37% and 53.15% of binding capacity respectively. Thus, it could be suggested that hydromethanolic formulation possessed better metal ion binding capacity.

The phosphomolybdenum method is based on the reduction of Mo (VI) in presence of any antioxidant compound to Mo (V) resulting in green phosphate/Mo (V) complex in acidic pH[2]. Following the assay, the total antioxidant capacity of

the three fractions was investigated and compared against ascorbic acid. Results exhibited that the antioxidant capacity of that hydromethanolic extract exhibited a better effect than infusion and decoction fractions (Table 2)

Table 1
Mycochemical composition of hydromethanol, infusion and decoction extracts from *Hypholoma fasciculare*.
ND: Not detected

Parameters	Hydromethanol	Infusion	Decoction
Phenol (µg GAE/mg of extract)	42.8 ± 0.58	44.08 ± 1.51	51.45 ± 2.11
Flavonoid (µg QE/mg of extract)	47.21 ± 0.75	23. 6 ± 1.11	51.5 ± 1.73
β-carotene (µg/mg of extract)	0.69 ± 0.05	ND	ND
Lycopene (µg/mg of extract)	1.4 ± 0.04	0.5 ± 0.01	0.11 ± 0.02
Ascorbic acid (µg/mg of extract)	1.9 ± 0.01	3.47 ± 0.44	3.45 ± 1.21

Figure 1: *Hypholoma fasciculare* (a) Fruiting bodies growing densely in natural habitat (b) Basidia (c) Basidiospores (d) Cystidia (e) Pileus hyphae (f) Stipe hyphae; Scale bars: (a) = 3 cm (b-f) = 10 µm

Table 2
Antioxidant activity of hydromethanol, infusion and decoction extracts from *Hypholoma fasciculare*. The results are presented in EC_{50} values (mean ± standard deviation; n = 3) corresponding to 50% of antioxidant activity in case of radical scavenging property. Ascorbic acid was considered as standard in ABTS radical inhibition, DPPH radical quenching and total antioxidant capacity techniques. EDTA was adopted as a positive control in chelating ability of ferrous ion method. In each row, different letters mean significant differences between sample and standard (p <0.05).

	Antioxidant Assays	Hydromethanol	Infusion	Decoction	Standard
EC_{50} value (µg/ml)	Scavenging ability of DPPH radicals	62.16 ± 5[a]	64.71 ± 3.1[b]	64.21 ± 2.56[c]	4.5 ± 0.5[d]
	Scavenging ability of ABTS radicals	40 ± 1.5[a]	47.17 ± 0.78[b]	31.87 ± 2.45[c]	2.58 ± 0.09[d]
	Chelating ability of ferrous ion	330 ± 3.64[a]	371.6 ± 2.87[b]	369.63 ± 1.54[c]	2.54 ± 0.5[d]
Total antioxidant activity by phosphomolybdenum method (µg ascorbic acid equivalent/mg of dry extract)		26.05 ± 2.57[a]	20.19 ± 1.19[b]	21.05 ± 1.28[c]	NA

NA: Not applicable

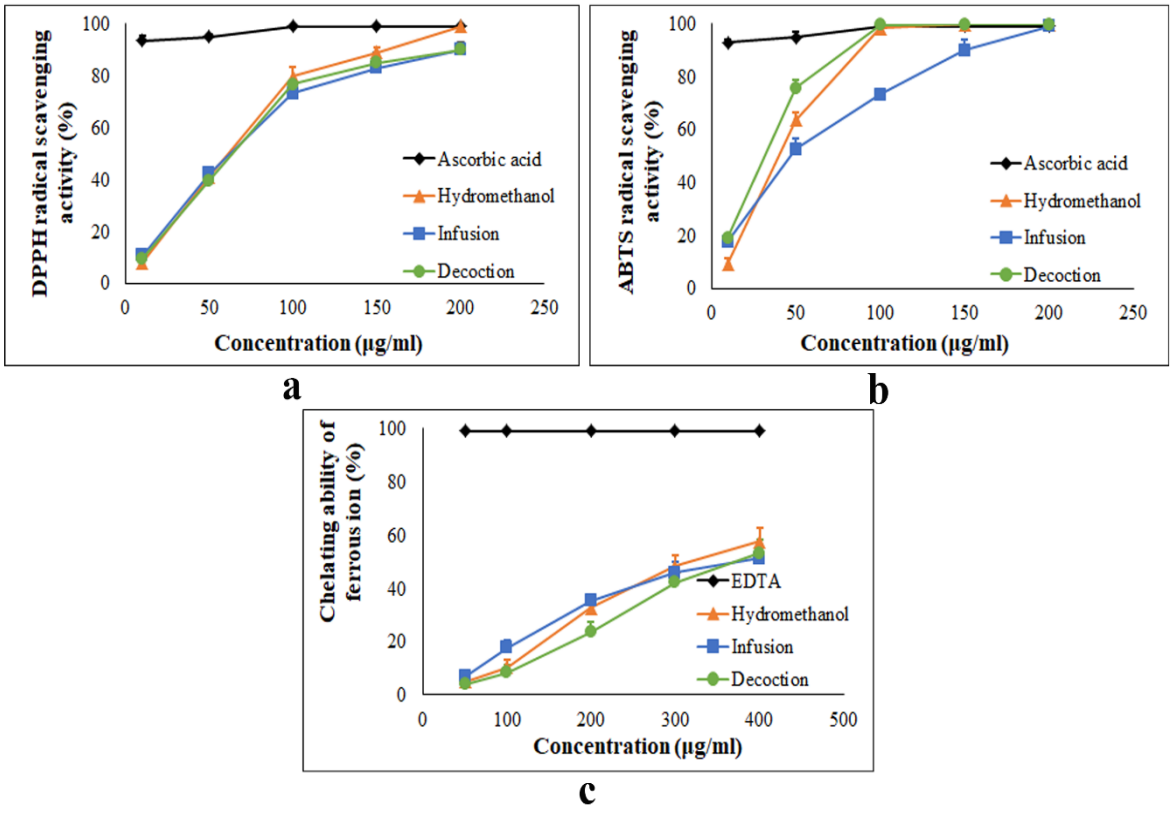

Figure 2: Antioxidant activity of hydromethanol, infusion and decoction extracts from *Hypholoma fasciculare*. (a) DPPH radical scavenging activity (b) ABTS radical scavenging activity (c) Chelating ability of ferrous ion

Conclusion

The hydromethanolic preparation showed greater antioxidant activity as the EC_{50} values ranged from 40-330 µg/ml in the current investigation, which examines the medicinal potential of three distinct extracts from *H. fasciculare*. The decoction fraction on the other hand, likewise demonstrated strong therapeutic potency with EC_{50} values ranging from 31.87 to 369.63 µg/ml. The impact could be attributed to the significant concentrations of a number of secondary metabolites including phenolics, carotenoids and ascorbic acid. Therefore, the current study emphasizes *H. fasciculare's* potential as a natural remedy that needs more research.

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