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Pharmacognostic standardization of a well known edible mushroom, *Volvariella volvacea*

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ABSTRACT

From ancient times, *Volvariella volvacea* has been recognized as a dietary addition in many Asian and European countries. Clinical evaluations have also revealed that the fruit body contains a great diversity of pharmacologically active constituents that posses remarkable medicinal activities. However pharmacognostic studies of the mushroom have not been carried out so far. So the present study was conducted with a view to establish a pharmacognostic standard of the dried powder of *V. volvacea*. Study was executed on the basis of microscopic, physical characters and phytochemical parameters using standard methods. Fluorescence analysis of the dried powder was also conducted using different chemical reagents. On the other hand, a fresh methanol soluble extract was prepared and prelimiminary screening of phytochemicals revealed the presence of phenols, flavonoids, ascorbic acid, β-carotene and lycopene. In addition, HPLC profile was recorded and results showed existence of 12 peaks as detected at 278 nm which might be of phenolic compounds. Furthermore, DPPH radical scavenging activity (EC_{50}-0.12 mg/ml) and total antioxidant capacity (23.75±1.25 µg ascorbic acid equivalent/mg of extract) were conducted to evaluate antioxidant potentiality of the methanol extract. All these above mentioned standards developed from this study will help in maintaining quality and purity as well as ascertain the identity and authenticity of dried powder of *V. volvacea*.

INTRODUCTION

In modern civilizations, natural products are becoming an effective alternative to the toxic chemicals used in preparation of synthetic medicine. Recently, many pharmaceutical companies have started paying their attention towards the exploitation of natural products in medicine formulation. The outputs are quite successful as because the bioactive compounds from natural resources are considered to be safe with no side-effects. But the main hinderance in acceptance of alternative medicine is the lack of standard documentation (Dahanurkar *et al.*, 2000). Thus, there is a need for quality control standardization of these products which not only helps in proper identification but also in authentication of genuine material (Kushwaha *et al.*, 2010). However no single method can provide the absolute result. The best solution is compilation of several different parameters of pharmacognosy including qualitative as well as quantitative measure to get a detailed and multidimensional view of the product. Nowadays researchers have relied more on the therapeutic values of mushrooms.

Different mushroom metabolites and active components have shown strong medicinal prospects (Khatua *et al.*, 2013; Mitra *et al.*, 2016; Chatterjee *et al.*, 2014; Chatterjee *et al.*, 2016; Mallick *et al.*, 2014; Mallick *et al.*, 2015; Nandi *et al.*, 2014; Nandi *et al.*, 2013). *Volvariella volvacea* is one such potential mushroom, commonly known as paddy straw mushroom belongs to the family Pluteaceae (Order: Agaricales).

It is a tropical cosmopolitan edible macrofungi and also cultivated commercially throughout the Southeast Asia. This mushroom is considered a delicacy in Asian cuisines because of its delightful taste and flavor.

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Besides this, the medicinal impact of this mushroom has been scientifically evaluated by a number of scientists around the globe establishing its antioxidative (Rai and Acharya, 2012, Sudha *et al.*, 2008, Ramkumar *et al.*, 2012; Tripathy *et al.*, 2016), antimicrobial (Perera *et al.*, 2001; Ayodele and Idoko, 2011), hepatoprotective (Kalava *et al.*, 2012a; Kalava *et al.*, 2012b; Acharya *et al.*, 2012), anticancer (Wu *et al.*, 2011; Maiti *et al.*, 2008) and immunomodulatory (Jeurink *et al.*, 2008; Hao-Chi *et al.*, 1997) properties. Additionally, a variety of biologically active compounds have been isolated from this mushroom (Hao-chi *et al.*, 1997).

As the demand of this mushroom is increasing day by day for its immense medicinal importance, a strong pharmacognostic profiling is of utmost needed. Therefore, the main objective of the present work is to establish the pharmacognostic parameters of this mushroom using standard methods, which could be helpful in preparation of a monograph for the proper identification of the mushroom.

MATERIALS AND METHODS

Mushroom materials

Basidiocarps of *V. volvacea* were collected during the rainy season from coastal villages of West Bengal. Identification and authentication of the basidiocarp was done using standard literature (Shaffer 1997, Dutta et al. 2011) and the voucher specimen present in CUH herbarium. A representative specimen (CUH AM036) was deposited in the same herbarium for future reference as per the method by Pradhan *et al.*, 2015. For powder analysis basidiocarps were first dried by field drier at 40°C for overnight, then pulverized to make them crispy using electric blender and this coarse powder was sieved into a fine powder by using a160 mesh. Then the finely sieved powder was stored in an airtight container for future use.

Microscopic and organoleptic characterization of powdered basidiocarps

Microscopic features were obtained from dried powder of the basidiocarps in 5% KOH, Melzer's reagent, and Congo red. The slide was then examined under Carl Zeiss AX10 Imager A1 phase contrast microscope and images were captured with a digital camera at desired magnifications. Several other features like colour, odour, taste, and nature of dried powder were also evaluated.

Fluorescence analysis

Fluorescence study of dried sieved powder was performed as per standard procedure (2007). A small quantity of sieved powder was placed on a grease free slide and treated with 16 different chemical agents and mixed properly by gentle tilting the slide. Then the slide was placed inside the UV viewer chamber and exposed to visible, short (254 nm) and long (365 nm) ultraviolet light to study their fluorescence behavior.

Preparation of methanolic extract

5 g of pulverized dried powder was soaked in 100 ml methanol for 72 h before extraction. Thereafter, it was filtered through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure at 80°C using rotary evaporator and preserved in refrigerator at 4°C in a dark bottle for further use. Extractive value was also recorded.

Quantitative estimation of mycochemicals

Methanolic extract was subjected for quantitative estimation of mycochemicals such as phenol, flavonoids, ascorbic acid, β carotene and lycopene. The content of total phenolic compounds in extract was estimated using Folin-Ciocalteu reagent and gallic acid as standard. The results were expressed as µg of gallic acid equivalents per mg of dry extract. Total flavonoid content was determined using aluminium nitrate and potassium acetate. Quercetin (5–20 µg/ml) was used to calculate the standard curve.

The results were expressed as µg of quercetin equivalents per mg of dry extract. β- carotene and lycopene were estimated by measuring absorbance at 453, 505 and 663 nm. Ascorbic acid was determined by titration against 2, 6-dichlorophenol indophenol dye (Khatua *et al.*, 2015).

High performance liquid chromatographic (HPLC) profile of methanolic extract

The extract was filtered through 0.2 µm filter paper and 20 µl filtrate was loaded on the HPLC system (Agilent, USA). Separation was achieved on an Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) using a flow rate of 0.8 ml/min at 25°C using 11 standards. The mobile phase consisted of eluent A (acetonitrile) and eluent B (aqueous phosphoric acid solution, 0.1% v/v). A gradient program was used for elution: 0-2 min, 5% A; 2-5 min, 15% A; 5-10 min, 40% A; 10-15 min, 60% A; 15-18 min, 90% A. The absorbance of sample solution was measured at 280 nm (Khatua *et al.*, 2015).

DPPH radical scavenging activity

A solution of DPPH was freshly prepared by dissolving DPPH dye in required volume of methanol (about 0.1 mM). 2 ml reaction mixture consisted of freshly prepared methanol solution of DPPH (0.1 mM) and various concentrations of extract. After 30 minutes of incubation in dark at room temperature absorbance was measured at 517 nm against blank (Shimada *et al.*, 1992). The percentage inhibition of radicals was calculated using the following formula:

$$\% \text{ inhibition} = (A_{control} - A_{sample}) \times 100 / A_{control}$$

Where $A_{control}$ is the absorbance of DPPH solution without extract and A_{sample} is the absorbance of sample with DPPH solution. The EC_{50} value was reported as the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison. All tests were performed at least in triplicate, and graphs were plotted using the average of three determinations.

Total antioxidant assay for methanolic extract

The reaction mixture consisted of 0.3 ml sample solution and 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium sulphate and 4 mM ammonium molybdate), according to the method by Prieto *et al.*, 1999. Tubes were capped and incubated at 95ºC for 90 min. Samples were cooled at room temperature and absorbance was measured at 695 nm against blank. Concentrations of ascorbic acid (1-30 µg/ml) were used to obtain a standard curve. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid.

Statistical analysis

All assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). Results were compared by means of a Student's t test to determine the significant difference among samples. The analysis was carried out using Microsoft® Office Excel (Microsoft®, USA), where values of $p \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Authentication of natural material is a crucial prerequisite step before using in research field or for medicinal purpose. Therefore, in this study we have performed the different parameters of pharmacognostic investigation to standardize this macrofungus to some extent. Organoleptic characterization of dried powder is considered as an important part of pharmacognostic standardization. The crude sieved powder was light brown in colour, salty taste without any significant odour and powdery in texture. The dried sieved powder of *V. volvacea* under microscopic investigation with 5% KOH and Congo red showed following characters (Figure 1): Several fragmented thin-walled hyphae lacking clamp connection, spores ovoid to ellipsoid (5.13 µm x 2.38 µm) ochraceous salmon, smooth with thickened wall and guttulate contents, basidia (14.08 µm - 4.95 µm x 2.61 µm - 1.24 µm) clavate, tetrasterigmatic. Melzer's reaction showed negative result which indicates that the spores are non-amyloid in nature.

Fluorescence analysis is also an important tool for qualitative assessment of crude drug and it provides an idea about their chemical nature (Sonibare and Olatubosun, 2015, Bhattacharya and Zaman, 2009). If substance themselves do not give fluorescence; they may often be converted into fluorescent derivatives or decomposition products by applying different chemical reagents. This distinguishing character is useful in identification of authentic samples and recognizing adulterates. The fluorescence characteristics of dried powder of *V. volvacea* are summarized in Table 1.

Freshly prepared methanolic extract was subjected for organoleptic characterization and quantitative estimation of phytochemicals. The fraction was brownish in colour, sticky in nature with extractive value of 15 ± 2.3%.

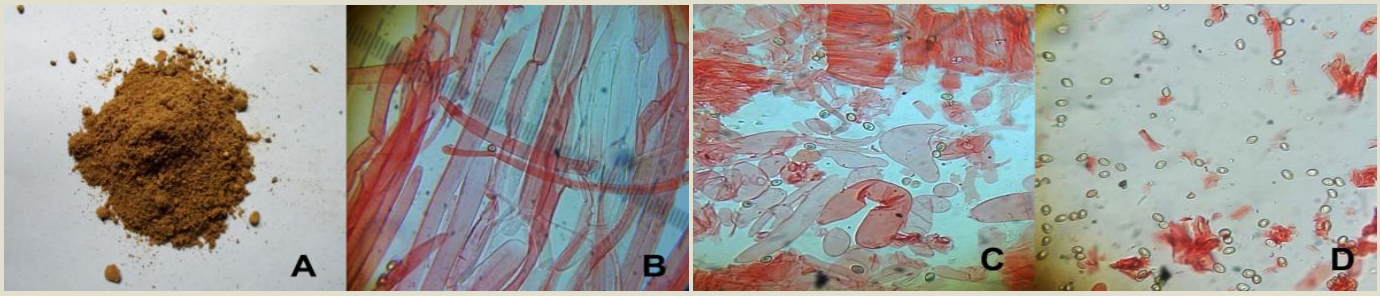

Fig. 1: A) Sieved powder of *Volvariella volvacea*. Microscopic characterization: B) Hyphae C) Fragmented hyphae, basidia and spores D) Spores.

Table 1: Fluorescence profile of dried powder of *Volvariella volvaceae*.

Serial No.	Reagents	Visible	UV	
			Long (365 nm)	Short (254 nm)
1	Powder as such	Brownish-yellow	Blackish brown	Deep brown
2	Hager's	Brown	Brownish black	Dark brown
3	Mayer's	Rust brown	Greyish violet	Rust brown
4	Dragendroff's	Brownish yellow	Greyish magenta	Greenish brown
5	Iodine solution	Brownish orange	Purplish black	Greenish brown
6	1(N) HNO_3	Reddish brown	Purplish black	Brown
7	50% HNO_3	Golden brown	Brownish black	Greenish brown
8	Phloroglucinol	Chocolate brown	Greyish brown	Greenish brown
9	Barfoed	Brownish green	Purplish black	Greyish brown
10	Sodium nitroprusside	Light brown	Reddish brown	Greenish brown
11	$FeCl_3$	Muddy brown	Purplish Brown	Brownish green
12	1(N) NaOH	Rust brown	Purplish black	Brownish green
13	Acetic acid	Brown	Greenish black	Yellowish-brown
14	1(N) HCl	Brown	Blackish brown	Blackish brown
15	Methanol	Rust brown	Purplish black	Blackish brown
16	1(N) NaOH in Methanol	Brown	Blackish brown	Brownish green

Quantification of different signature phytochemicals present in the methanolic extract of *V. volvacea* showed three major antioxidant compounds that are present in the following order: ascorbic acid > phenol > flavonoid. β carotene and lycopene were found in negligible amount (Table 2).

Table 2: Mycochemical estimation of methanolic extract of *Volvariella volvaceae*.

Mycochemical constituents	Amount
Total phenols (µg GAE/mg of extract)	8.75 ± 0.44
Total flavonoids (µg QE/mg of extract)	1.56 ± 0.22
β carotene (µg/mg of extract)	0.286 ±0.022
Lycopene (µg/mg of extract)	0.231 ± 0.021
Ascorbic acid (µg/mg of extract)	9.7125 ± 0.528

In comparison with already published reports of mushrooms such as *Griffola frondosa* (0.37 µg/mg of extract) (Acharya *et al.*, 2015a), *Ganoderma lucidum* (2.77 µg/mg of extract) (Acharya *et al.*, 2015b), *Macrocybe crassa* (1.81 µg/mg of extract) (Acharya *et al.*, 2015c), *Lentinula edodes* (3.96 µg/mg of extract) (Acharya *et al.*, 2015d), *Pleurotus ostreatus* (11.1 µg/mg of extract) (Acharya *et al.*, 2016a), and *Laetiporus sulphureus* (2 µg/mg of extract) (Acharya *et al.*, 2016b), ascorbic acid content of this mushroom was found to be the highest one. According to earlier reports, besides fruits and vegetables, mushrooms are also considered as a huge resource antioxidant compounds. As a part of this category, ascorbic acid is one of the strong water soluble antioxidant.

Chromatographic study also can be categorized as a part of chemical fingerprint. The HPLC fingerprint showed a characteristic fingerprint profile that serves as markers for quality control standardization of the crude drug. Qualitative analysis of the chromatogram revealed existence of 12 peaks with two mobile phase peaks as presented in Figure 2. R_f value of each corresponding peak helps to identify the chemical compounds present in the drug. R_f value along with area of each corresponding peak are given in the Table 3. In order to investigate the antioxidant activity, the methanolic fraction was subjected for two different in vitro assays. DPPH radical scavenging assay constitutes the most simple and widely used method to evaluate antioxidant activities in comparatively short time than others. Stable DPPH radical shows absorption maxima at 517 nm. Antioxidant compounds present in the extract decreases its absorbance due to their hydrogen donating ability. It can be visualized by discoloration of purple colored solution to yellow. As shown in Figure 3, scavenging effect of methanolic extract of *V. volvacea* increased in a concentration dependent manner. EC_{50} value of the methanol extract was 0.12 mg/ml. Our result also coincides with the earlier works which proves that the methanolic extract of different mushrooms have strong antioxidant capacity (Khatua *et al.*, 2013).

Another assay was also carried out to test antioxidant potentiality of the methanolic extract termed as total antioxidant capacity assay using phosphomolybdenum method. The theory is based on the reduction of Mo (IV) to Mo (V) and formation of green phosphate / Mo (V) complex with characteristic absorption at 695 nm. The total antioxidant capacity was determined using linear regression equation expressed as the number of equivalent of ascorbic acid which was found to be 23.75±1.25 ascorbic acid equivalent (AAE) per mg of extract. Therefore high content of ascorbic acid can justify the good antioxidant potentiality of the extract.

Table 3: HPLC profile of methanol extract of *Volvariella volvacea* at 278 nm.

Peak no.	Retention time (min)	Area (AU)
1	4.949	724.45807
2	5.207	2581.14868
3	6.165	155.25165
4	8.688	376.34503
5	10.037	314.59995
6	10.354	128.91489
7	10.504	259.02283
8	10.834	392.47763
9	11.282	582.46271
10	12.355	18.17178
11	12.597	10.77141
12	15.806	216.32178

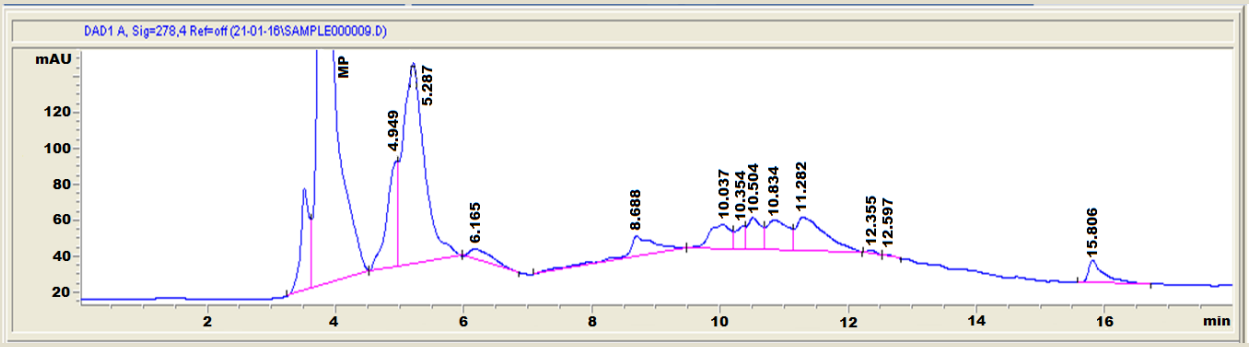

Fig. 2: Enlarged HPLC chromatogram of methanol extract from *Volvariella volvacea* at 278 nm.

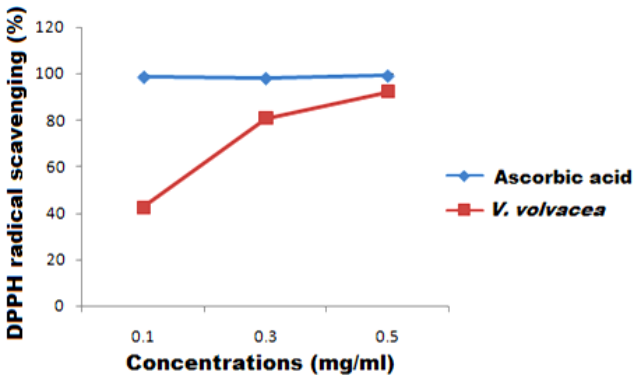

Fig. 3: DPPH radical scavenging activity of methanol extract from *Volvariella volvacea*.

CONCLUSION

The present study provides pharmacognostic standards of *V. volvacea* powder with the help of modern sophisticated techniques and relevant parameters. Different pharmacological parameters including microscopy, organoleptic, mycochemical quantification as well as chromatographic profiling were reported for the first time for this mushroom. All these aggregated data will help to authenticate the crude powder before processing for drug formulations and also to identify the genuine species.

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