



Research Article

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Pharmacognostic standardization of *Calocybe indica*: A medicinally sound mushroom

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ABSTRACT

Calocybe indica is a well known, important medicinal mushroom. But its quality standards are not yet reported. Hence, the present study was conducted which included microscopic, physical and florescent characterisation of dry powder derived from the fruit bodies of the mushroom. A methanolic extract of this fungus was also prepared for analysis of phenol, flavonoid, ascorbic acid, β -carotene and lycopene content. High performance liquid chromatography was used to determine the phenolic fingerprinting. The chromatogram indicated presence of atleast 6 phenolic compounds in the extract. In-vitro antioxidant assays revealed that the extract also posses DPPH radical scavenging activity (EC_{50} was 0.957 mg/ml) and total antioxidant capacity of 19.0 μ g AAE/ mg of extract. In the end data depicted in this investigation is quite significant towards future identification as well as authentication of this mushroom material establishing pharmacognostic standards. Its good antioxidant activity indicates its potentiality to be used as a therapeutic drug.

Key words: *Calocybe indica*, HPLC, mushroom, pharmacognosy

INTRODUCTION

Pharmacognosy is a simple and reliable tool for characterization of crude drug or potential drug of natural origin. It deals with the study of physical, chemical, biochemical and biological properties of drugs. The main purpose of pharmacognosy is to authenticate and quality control of crude drugs by investigating various aspects such as macroscopic, microscopic, physico-chemical and fluorescence analysis along with phytochemical screening. Proper identification and quality assurance of the starting material are essential prerequisites to ascertain reproducible quality of herbal drugs.

Since antiquity edible mushrooms are a perennial component of human diet. Its use in ancient oriental therapies seems to date back to the Neolithic age. After decades of serious obsession with modern medicinal system due to adverse effects of synthetic drugs, people have started looking for alternatives. Contemporary research has just accelerated this traditional knowledge for the well being of human. Mushrooms are thought to possess more than 100 different medicinal function as they have already proved themselves to posses good antioxidant [1-2], anti-diabetic [3], antimicrobial [4], hepato-protective [5], antiulcer [6] and anticancer [7-8] properties because of the unique physiological and molecular structure. Purified polysaccharides from different mushrooms have been reported as strong immunomodulators [9-13]. Even purified active components have shown different medicinal prospects [14-16]. Hence it can be said that they not only exert a number of nutritional properties but are also valuable sources of natural medicines.

Calocybe indica is a wild edible mushroom of Basidiomycota under Agaricales order and belongs to family Lyophyllaceae. It generally grows solitary or sometimes in groups in moist soil or at shade of trees [17] and was first identified in West Bengal and the commercial production of this mushroom started from 1998 followed by its

discovery from Tamil Nadu in 1997 by Krishnamoorthy who also standardized the protocol of its commercial cultivation [18]. This mushroom is widely designated as Milky mushroom due to its milky white colour. The characteristic white colour and robust nature as well as its palatability made this mushroom as a popular of its kind in market. However the medicinal nutritional and other pharmacognosical prospect was gradually revealed by research. Besides being rich in nutritional components like carbohydrate, protein, crude fat, and fibres [19], *Calocybe indica* has been proved to have significant medicinal properties. The effect of ethanolic extract to prevent hepatotoxicity [17] has been elucidated. Not only that, anticancer activity of this mushroom was also reported against human Ewing's sarcoma MHH-ES-1, breast cancer MCF-7 [20] and Dalton's lymphoma Ascites [19]. Medicinally this mushroom is also prospectus because of its antidiabetic properties [22]. It is reported to contain different types of vitamins, minerals, flavored compounds and immunoenhancing abilities too [23].

Hence, the present study was to evaluate the pharmacognostic parameters, chemical analysis and antioxidant capacity of *Calocybe indica* collected from West Bengal with a view to establish standards for its identity, quality, purity and chemical composition.

EXPERIMENTAL SECTION

Materials

Basidiocarps of *Calocybe indica* were collected from West Bengal, India. Basidiocarps were dried properly by a field drier at 40°C for 1 day to make them crispy. A reference specimen was deposited in Calcutta University (CUH AM054). Dried fruit bodies were pulverized using an electric blender, sieved through mesh and stored in air tight containers.

Microscopic evaluation of powdered basidiocarps and organoleptic study

Powdered sample was hydrated and macerated with 10% KOH and mounted on glass slide with glycerol. For effective results various stains (Congo red, Melzer reagent) were used to distinguish different cellular structure. Photomicrographs were taken using compound binocular microscope having sensor aided digital camera and computer attachment. Different organoleptic characters like colour, odour, taste, nature of powdered samples were evaluated during organoleptic study.

Physico-chemical evaluation

Fluorescence analysis

Fluorescence analysis was done according to the methods of Kokashi et al (1958) [24]. A small quantity of dried sieved powder material was placed on grease free clean glass slide, 1-2 drops of freshly prepared reagent solutions (Hager's, Mayer's, Dragendroff's, iodine solution, 1(N) HNO₃, 50% HNO₃, phloroglucinol, Barfoed, sodium nitroprusside, FeCl₃, 1(N) NaOH, acetic acid, 1(N) HCl, methanol, 1(N) NaOH in methanol) were added, mixed by gentle tilting the slides and left for 1-2 mins. Changes in colour due to addition of different reagents were recorded in normal light, as well as short (254nm) and long (365nm) UV light.

Preparation of methanolic extract

5gm of sieved dried sample powder of each sample material were dipped in 100ml of methanol for 2 days at room temperature. Solvent was separated by Whatmann No. 1 filter paper. The residue was re-extracted with same volume of methanol. After filtration, solvent was evaporated. Concentrated extract was stored at 4°C for further analysis.

Quantitative estimation of phytochemicals

Folin-ciocalteu reagent and sodium carbonate was used to measure the total phenolic compounds present in extract, using gallic acid as standard as per the method of Singleton and Rossi, 1965 [25]. The results were expressed as µg of gallic acid equivalents per mg of dry extract. Total flavonoid content was estimated using aluminium nitrate and potassium acetate following method of Park *et. al.*, 1997 [26]. Quercetin (5-20 µg/ml) was used as a standard, expressing results as µg of quercetin equivalents per mg of dry extract. β-carotene and lycopene were estimated by measuring absorbance at 3 different wavelengths viz.; 453, 505 and 663 nm following methods of Nagata and Yamashita (1992) [27]. Ascorbic acid was determined with titration against 2, 6-dichlorophenol indophenol dye in presence of oxalic acid [28].

HPLC profile of methanol extract

The extract was filtered through 0.2 µm syringe filter 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. 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 [29].

Evaluation of Antioxidant Activity

Radical scavenging activity in extract was evaluated using purple coloured DPPH radicals based on the method by Shimada *et. al.* (1992) [30]. 2 ml reaction mixture consisted of methanol solution of DPPH (0.1 mM) and various concentrations of extract. After 30 min incubation absorbance was measured at 517 nm against blank. EC₅₀ value is the effective concentration at which DPPH radicals were scavenged by 50%. Ascorbic acid was used for comparison. Total antioxidant assay was carried out following methods of Prieto *et. al.* (1999) [31] with little modification. 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). Tubes were capped and incubated at 95°C 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) were used to obtain a standard curve. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid.

RESULTS AND DISCUSSION

According to the World Health Organization (WHO, 1998) [33] microscopic description of a plant is the first step to establish the identity and the degree of purity of such materials and should be carried out before any tests are undertaken. The dried basidiocarps (Figure 1a) were powdered which showed light yellow or greyish yellow to blond coloured appearance (Figure 1b), salty in taste and powdery in nature and possessed a milky odour. Studies of this powder under light microscope showed that it was hymenophoraltramaregular, Spores $5.99-8.325 \times 3.66-5.33\mu\text{m}$, ovoid, brownish, smooth, hyaline with an apiculis; basidia $20.65-36.63 \times 6.7-8.67\mu\text{m}$, narrowly clavate, tetrasterigmate. (Figure 1c-f). Mehlzer test gave negative result signifying its inamyloid nature.

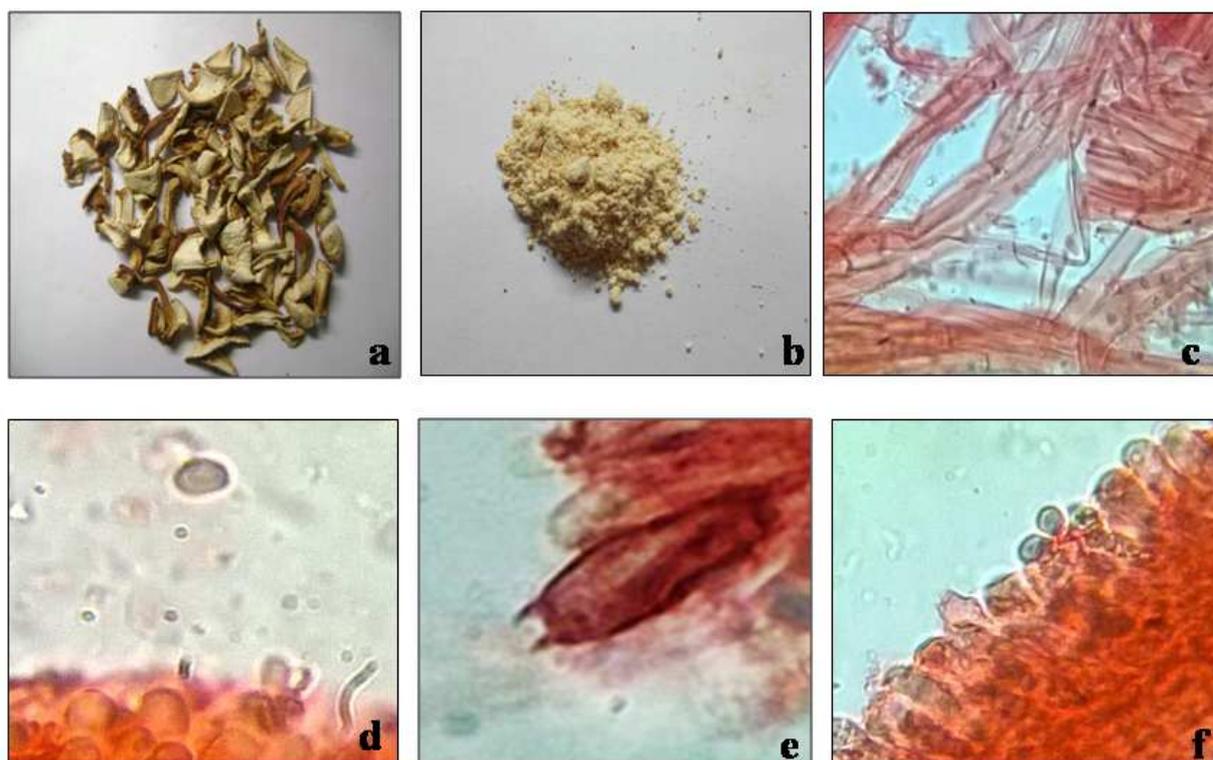


Figure 1: Fruit body of *Calocybe indica* a: Dried fruit body b: Fruit body in powder form c: hyphal cells d: Single spore e: Basidium and f: Spore embedded in Hymenium layer

The fluorescence tests of the powdered drug are an important tool for qualitative analysis by authenticating samples and recognising adulterants. Some components are not visible under day light but fluoresce in ultra violet light. Some on the other hand are non-fluorescent compounds but can be converted to fluorescent ones as they can form fluorescent derivatives in presence of various reagents. The colours produced by them represent the presence of active constituents. As a result fluorescence analysis displayed an array of colours that could be employed for identification of probable classes of compounds in the mushroom [33, 34]. In the present study powder drug was

treated with different chemical reagents which gave characteristics colour when seen under long and short wavelengths of UV light and was compared to that, observed under visible light (Table 1).

Table 1: Fluorescence analysis of dry powder from *Calocybe indica*

Sl No.	Reagent	Visible	UV	
			Long (365nm)	Short (254nm)
	Powder as such	Pale brown	Greyish brown	Greenish brown
1	Hager's	Reddish yellow	Greenish brown	Yellowish brown
2	Mayer's	Pale yellow	Greyish brown	Greenish yellow
3	Dragendroff's	Light orange	Dark brown	Olive green
4	Iodine solution	Brownish red	Dark chocolate brown	Dark greenish brown
5	1(N) HNO ₃	Pale yellow	Light grey	Pale green
6	50% HNO ₃	Pale yellow	Greenish grey	Greenish buff
7	Phloroglucinol	Yellow	Dark grey	Greyish green
8	Barfoed	Sea green	Blackish brown	Bluish green
9	Sodium nitroprusside	Pale yellow	Greyish brown	Pale yellow
10	H ₂ O	Pale brown	Greyish brown	Pale yellow
11.	FeCl ₃	Orangish yellow	Greenish brown	Light green
12.	1(N) NaOH	Buff	Dark grey	Pale green
13	Acetic acid	Light yellow	Light grey	Olive green
14	1(N) HCl	Pale yellow	Greenish brown	Olive green
15	Methanol	Orangish brown	Light brown	Light green
16	1 (N) NaOH in Methanol	Cream	Pale grey	Buff

The powder was subjected to methanolic extraction yielding an orangish brown coloured liquid whose extractive value was 18.5 %. It contained phenol as much as 3.8 ± 0.14 μg gallic acid equivalent/mg. Total flavonoid content was determined by using quercetin as standard. The extract contained flavonoid as 2.5 ± 0.35 μg quercetin equivalent/mg. Very negligible amount of β -carotene and lycopene were found such as 0.234 ± 0.05 $\mu\text{g}/\text{mg}$ and 0.181 ± 0.05 $\mu\text{g}/\text{mg}$ of the extract respectively. All of these mycochemical constituents which help various systems of organisms to work properly, were present in much more quantity in *C. indica* than that of *Pleurotus ostreatus* [35]. Ascorbic acid content was 3.61 ± 0.43 $\mu\text{g}/\text{mg}$ of extract. It was much higher than earlier reports for that of *Ganoderma lucidum* (2.77mg/ml) [36], *Macrocybe crassa* (1.81 mg/ml) [37] and *Grifola frondosa* (0.37 mg/ml) [38].

HPLC chromatogram of the methanolic extract of *Calocybe indica* at 278nm is represented in Figure 2; consisting of 6 peaks in 18 minutes the run. On the other hand their retention times and respective areas are given in Table 2.

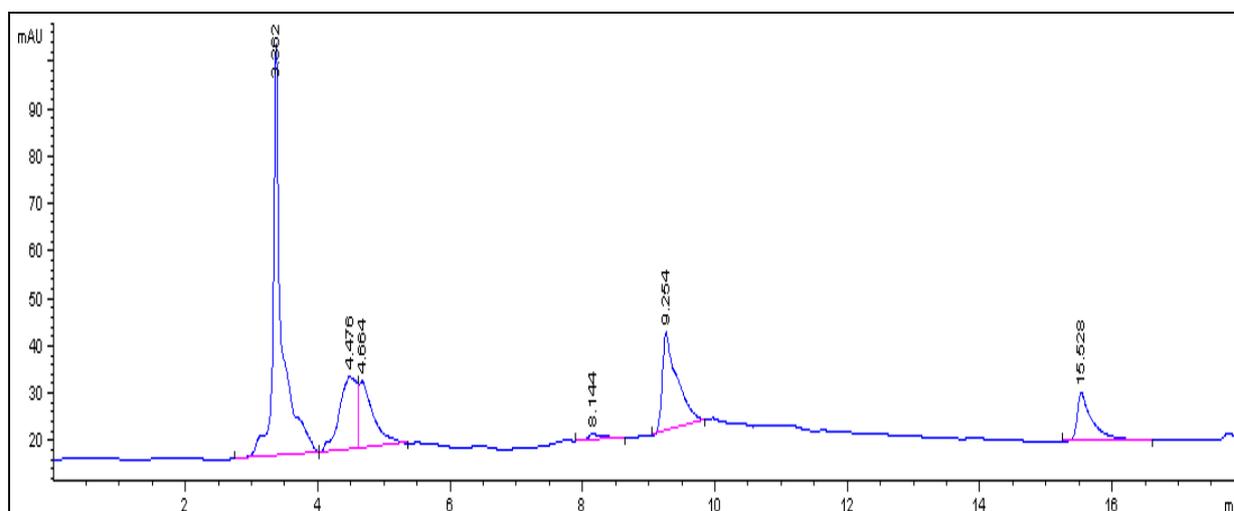
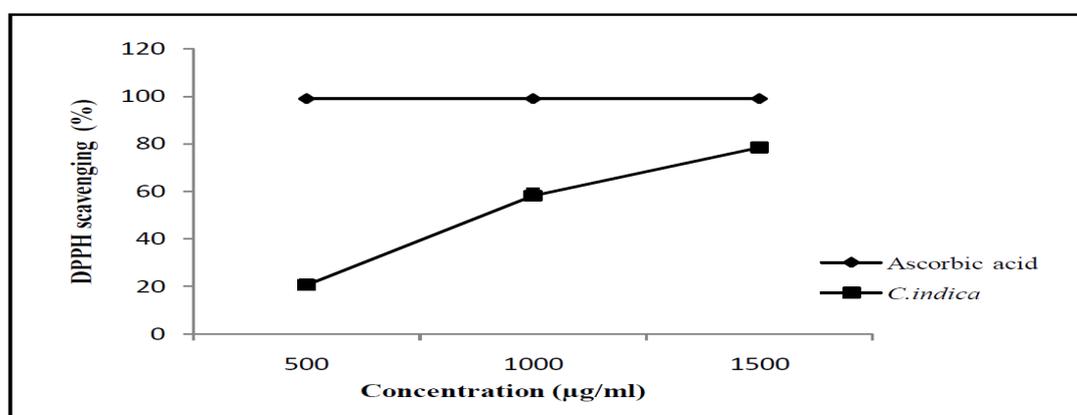


Figure 2: Enlarged HPLC chromatogram of methanol extract from *Calocybe indica*

Table 2: HPLC profile of methanol extract of *Calocybe indica* at 278nm

Peak No.	Retention Time (min.)	Area
1	3.362	43.81
2	4.476	15.84
3	4.664	11.80
4	8.144	1.2005
5	9.254	18.22
6	15.528	9.14

DPPH accepts electron or hydrogen to earn stability. Antioxidants, on the other hand are capable of donating electron or hydrogen atom. A solution containing DPPH and methanol gives violet colour. But when electrons are donated to DPPH, then solution starts losing colour. This change is spectrophotometrically measured at 517nm [30]. As shown in Figure 3, at 0.05, 1.0 and 1.5 mg concentrations extract exhibited radical scavenging activity at the rate of 20.63 %, 58.06 % and 78.39 % respectively. EC₅₀ value was found to be at a concentration of 0.957 mg/ml whereas that of, *Lentinula edodes* (1.25 mg/ml), *P. ostreatus* (1.232mg/ml) as well as *M. crassa* (2.45mg/ml) was much lower [29, 35, 37].

**Figure 3: DPPH radical scavenging activity of methanol extract from *Calocybe indica*.**

Phosphomolybdenum method is used for evaluation of total antioxidant capacity. The method is based on reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphate /Mo (V) complex [31]. Total antioxidant capacity of the methanolic extract of *C. indica* was investigated and compared against ascorbic acid. The extract presented 19.0 ± 1.25 µg AAE/ mg of extract total antioxidant activity which proved that it was more potential than *P. ostreatus* [35].

CONCLUSION

The present study provides standards of *Calocybe indica* powder with help of modern techniques and suitable parameters. Its powder was subjected to microscopic, pharmacognostical, preliminary phytochemical assay, and HPLC. This were first time reported and hence adds to the existing knowledge of *Calocybe indica* so that it can be quite useful for identification, standardization, development and preparation of crude drug formulation as well as inclusion in various pharmacopoeias for treating various ailments. All these data together provides relevant information which may be helpful in authentication of the crude drug and check adulteration for quality control of raw material.

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