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Pharmacognostic standardization of *Grifola frondosa*: A well-studied medicinal mushroom

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ABSTRACT

Grifola frondosa, commonly known as 'Maitake', has been used traditionally by Chinese and Japanese in their curative herbal medication. The present study was undertaken to provide quality control standardization based on microscopic, physico-chemical and chromatographic features and to evaluate antioxidant activity. The physico-chemical parameters such as loss on drying, ash value, fluorescence analysis and methanol soluble extractive were determined by standard methods. HPTLC profile of methanolic extract was carried out by scanning along the range of 200 to 400 nm. The R_f values were noted as an important parameter for identification of the mushroom. HPLC profile of the extract detected presence of 38 peaks. Preliminary mycochemical analysis indicated presence of cardiac glycoside, flavonoid, phenol, saponin and terpenoids. Quantitatively the major bioactive components were present in following order of phenol> flavonoid> ascorbic acid> β-carotene> lycopene in methanolic extract. Along with, DPPH radical scavenging activity (EC_{50} value 666 µg) and total antioxidant capacity (18.64 µg AAE/mg of extract) were determined. The distinctive features established in this work are steps towards identification, standardization and quality control of the medicinal mushroom.

Keywords: Antioxidant property, HPLC, HPTLC, Medicinal mushroom, Physico-chemical evaluation, Quality control

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INTRODUCTION

Since antiquity wild edible mushrooms are a perennial component of human diet in many countries of Asia and Europe. They exert a number of nutritional properties and a valuable source of natural medicines [1]. Recent investigations reveal that more than 100 medicinal functions are proved to be exerted by the medicinal mushrooms including antioxidant [2, 3], anti-diabetic [4], hepato-protective [5-7], antimicrobial [8, 9], immune-modulatory [10-14], anticancer [15, 16], anti-ulcer [17] etc.

Grifola frondosa is a very precious mushroom, commonly known as, 'Maitake', 'hen-of-the-woods', 'sheep's head or the 'ram's head'. In Japan, Maitake is referred as the 'king of mushroom' because of its size. Maitake has long been included by the Chinese and Japanese in their curative herbal medication. The main research on *G. frondosa* has been conducted in anti-HIV, antidiabetes, antitumour, antihyperlipemia and antiobesity where the results demonstrated that *G. frondosa* is one of the most bioactive, safe mushrooms [18]. Several glucans isolated from *G. frondosa* have been patented in Japan as potential anticancer and immune-modulating agents [19].

Because of the tremendous medicinal importance of this mushroom, pharmacognostical studies are very important to assure the purity of drug and to avoid the adulterations. In the present study, pharmacognostic standardization of basidiocarp of *G. frondosa* were carried out to establish its macro and microscopical standards, physico-chemical parameters and preliminary phytochemical investigation to develop a marker tool for identification of the genuine material.

MATERIALS AND METHODS

Mushroom sample

Basidiocarps of *G. frondosa* were purchased from the market of Darjeeling, West Bengal, India. Identity of this mushroom was authenticated by comparing with voucher specimen present in Calcutta University herbarium (CUH) and in consultation with standard literature [20]. A reference specimen was also kept in the herbarium following the process of Prakash *et al* (2015) [21]. Basidiocarps were dried properly by a field drier at 40°C for 24 hour to make them crispy and pulverized using an electric blender and sieved through 160 mesh and stored in an air-tight container.

Microscopic evaluation of the powdered basidiocarp

Powdered sample was hydrated and macerated with 10% KOH and mounted on glass slide with glycerol. For effective results various stains were used to distinguish different cellular structures. The slide was then viewed under Leica DMLS microscope and images were captured at desire magnification.

Organoleptic evaluation

Different characters like color, odor, taste, nature of powdered samples were evaluated during organoleptic study.

Physico-chemical parameters of powdered materials

Physico-chemical constants such as loss on drying, total ash [22], water holding capacity, swelling capacity [23] and methanol soluble extractive were evaluated.

Fluorescence analysis

A small quantity of dried sieved mushroom powder was placed on a grease free clean microscopic slide and added 1-2 drop of freshly prepared reagent solution, mixed by gentle tilting the slide, waited 1-2 minutes. Then the slide was placed inside the UV viewer chamber and viewed in daylight, short (254 nm) and long (365 nm) UV radiations. The color observed by application of different reagents in different radiations were recorded.

Qualitative phytochemical screening of methanol soluble extractive

The methanol soluble extractive was subjected to various qualitative tests to detect presence of organic constituents like alkaloids, glycosides, saponins, steroids, terpenoids, phenolic and flavonoids using standard protocols [24].

Quantitative estimation of some important phytochemicals from methanol soluble extractive

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 [25].

HPTLC profile of methanol soluble extract

A densitometric HPTLC analysis was performed to generate a characteristic fingerprinting profile of the extract. 2 µl of the sample solution was loaded on pre-coated TLC plate (Silica gel 60 F254 sheets 20 X 20 cm, 0.5 mm thickness, Merck Darmstadt, Germany) analysed using CAMAG HPTLC system (Muttenz, Switzerland) comprising Linomat-5 automated sample applicator equipped with a 100 µl syringe, CAMAG TLC scanner with winCATS software (version: 1.4.6). Plate was developed up to a distance of 80 mm in CAMAG twin trough glass chamber pre-saturated with the mobile phase ethyl acetate: toluene (1:1 v/v) for 30 min. The plate was fixed in scanner stage and scanning was done at 200-450 nm.

HPLC of methanol soluble extract

The methanolic 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. 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 [25].

Antioxidant assay

Total antioxidant capacity and DPPH radicals scavenging activity of methanolic extract were analysed as described by Mitra *et al* (2014) [26].

RESULTS AND DISCUSSION

Macerate showed many fragmented mycelium and spores (Figure 1). Two different types of mycelium were observed under microscope *i.e.* hyaline, thin walled, camped, septated generative hyphae and thick walled skeletal hyphae. Spores were ovoid to ellipsoid, 6-7×4-5 µm in size, hyaline, smooth walled. Melzer reaction showed negative result signifying its inamyloid nature. The microscopic data coincided with data present in the standard literature [20].

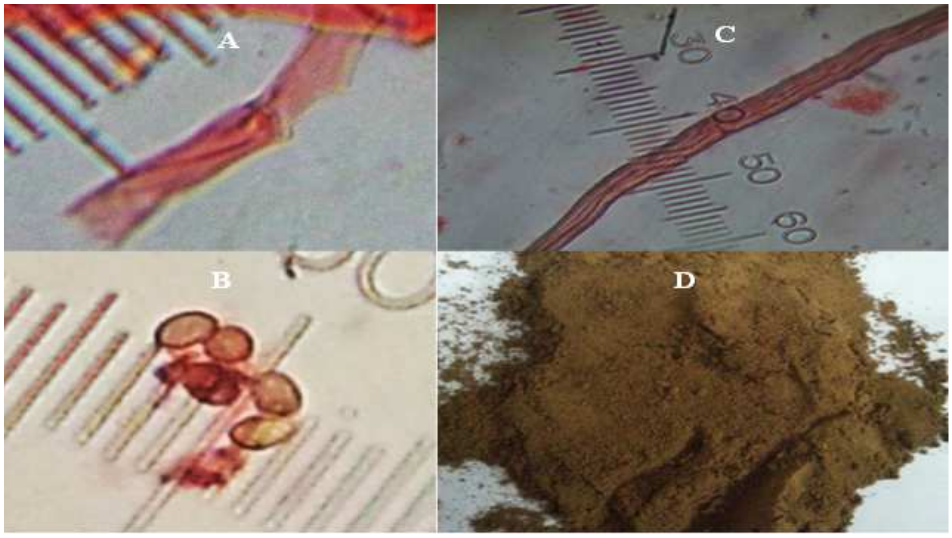

Figure 1: Fruit body of *Grifola frondosa*. A: Generative hyphae B: Spores C: Skeletal hyphae D: Sieved powder

Organoleptic study of the sieved powder showed yellowish-brown color, sweet odor, salty taste and granular texture. Physico-chemical parameters of powder were evaluated as they are important for identifying adulterants and improper handling of drugs. The percentage of water and volatile matter in crude drug were determined by loss on drying test. Result showed presence of low amount of moisture content, thus it could prevent microbial growth. Moisture content of the powder was 4.38 ± 0.58%. Determination of total ash is important in evaluation of purity of drug as it confirms the presence or absence of foreign inorganic matter. The total ash was found to be 2.33 ± 0.02% w/w. The hydration properties of mushroom powder, including the solubility, swelling capacity are desirable to get beneficial physiological functional characteristics. Water Solubility Index, swelling capacity and water holding capacity of the powder were 21.55 ± 2.52%, 200 ± 10 ml/g and 197.62 ± 11.90 g/g respectively. The methanol soluble extractive was 11.18 ± 0.82%. The extract was brown in color and sticky in nature.

Fluorescence is useful in identification of authentic samples and recognizing adulterates and thus is an important pharmacognostic character. The drug was allowed to react with different reagents which resulted in fluorescence by various chemical constituents present in UV light. As a result fluorescence analysis displayed an array of colors that could be employed for identification of probable classes of compounds in the mushroom [27, 28]. In the present

study, powder drug was treated with 16 different chemical reagents which gave characteristics color when seen under UV light (365 nm) and was compared with color observed under ordinary light (Table 1).

Table 1: Fluorescence analysis of dry powder of *Grifola frondosa*

Sl No.	Reagent	Visible	UV	
			Long (365nm)	Short (254nm)
1	Blank	Dark brown	Black	Black
2	Hager's	Reddish brown	Black	Black
3	Mayer's	Brown	Black	Black
4	Dragendroff's	Reddish brown	Black	Light brown
5	Iodine solution	Light brown	Black	Black
6	1(N) HNO_3	Light brown	Dark brown	Dark brown
7	50% HNO_3	Dark brown	Black	Brown
8	Phloroglucinol	Dark brown	Black	Black
9	Barfoed reagent	Dark brown	Black	Black
10	Sodium nitroprusside	Reddish brown	Black	Dark brown
11	Ninhydrin	Light brown	Black	Black
12	$FeCl_3$	Light brown	Black	Black
13	1(N) NaOH	Dark brown	Black	Black
14	Acetic acid	Black	Black	Black
15	1(N) HCl	Light brown	Black	Black
16	Methanol	Reddish brown	Black	Black
17	1(N) NaOH in methanol	Black	Black	Black

The methanol soluble extract was prepared to determine the presence of some important bio-organic groups which confirms the medicinal properties of this mushroom. The methanolic extract of the powder showed positive tests for saponins, glycoside, terpenoids, phenolics and flavonoids (Table 2).

Table 2: Qualitative chemical screening of methanol extract from *Grifola frondosa*

Type of metabolites	Name of tested chemical	Reagent/Test	Nature of change	Result
Secondary	Cardiac glycoside	Keller-Killani	Reddish brown color at the junction of the two liquid layers	++
	Flavonoid	Ferric chloride	Green coloration	++
	Phenol	Folin-ciocalteu	Bluish black coloration	++
	Saponin	Foam	Appearance of foam and its persistence for 10 minutes	+
	Terpenoids	Acetic anhydride with sulphuric acid	Reddish color	+

Note: + = Present in low amount, ++ = Present in moderate amount

The extract was found to contain phenol as much as 8.57 ± 0.53 µg gallic acid equivalent/mg of extract or 960.24 ± 59.38 µg gallic acid equivalent/g of dry weight. Total flavonoid content was determined by using quercetin as standard. The extract contained flavonoid as 1.67 ± 0.26 µg quercetin equivalent/mg of extract or 187.56 ± 29.29 µg quercetin equivalent/g of dry weight. Very negligible amount of β-carotene and lycopene were found such as 0.04 ± 8.26 µg/mg or 4.87 ± 0.92 µg/g of dry weight and 0.03 ± 4.83 µg/mg of the extract or 3.76 ± 0.54 µg/g of dry weight respectively. Ascorbic acid was also found in minor amount (0.37 ± 0.01 µg/mg of extract or 41.76 ± 0.01 µg/g of dry weight).

HPTLC is an efficient and valuable technology for the preliminary separation and determination of constituents. The plate was scanned from 200 nm to 450 nm with 50 nm intervals. At 200 nm, 250 nm, 300 nm, 350 nm and 400 nm 6 (R_f 0.07, 0.14, 0.38, 0.56, 0.72, 0.97), 7 (R_f 0.01, 0.05, 0.14, 0.39, 0.58, 0.63, 0.98), 8 (R_f 0.01, 0.08, 0.14, 0.37, 0.58, 0.64, 0.81, 0.98), 5 (R_f 0.01, 0.11, 0.39, 0.9, 1.01) and 1 (R_f 0.39) spots were detected (Figure 2, Table 3).

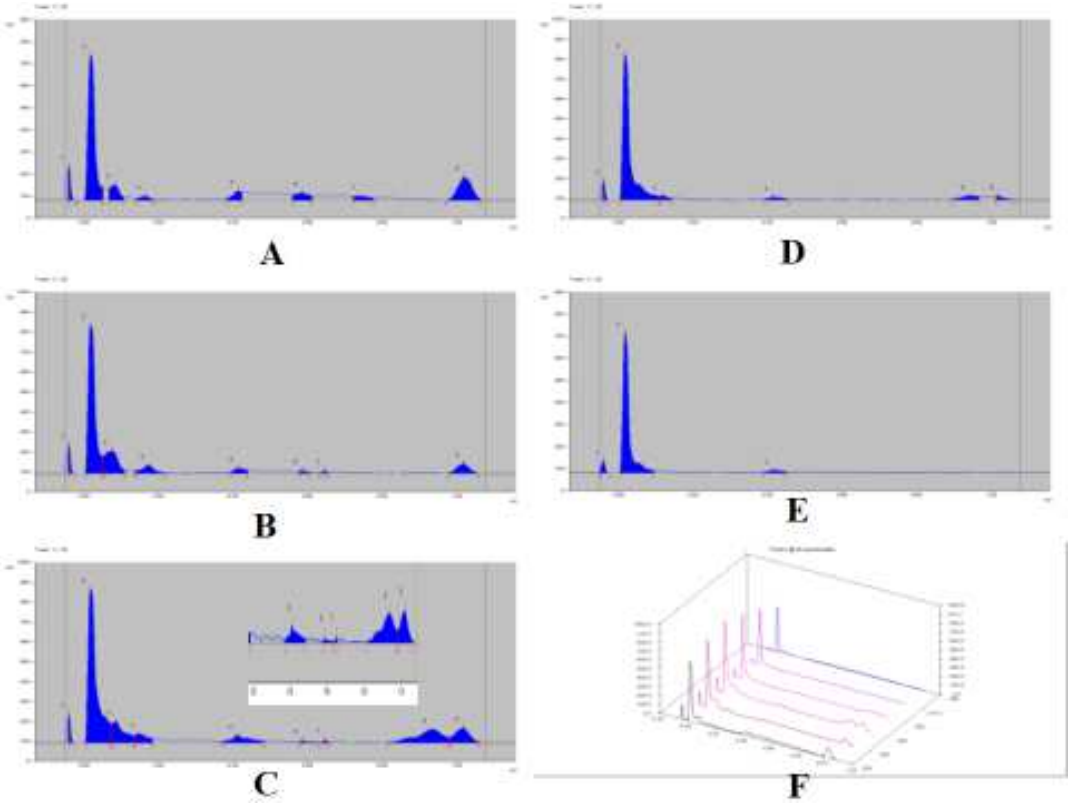

Figure 2: HPTLC fingerprint of methanolic extract from *Grifola frondosa*. A: 200 nm B: 250nm C: 300 nm D: 350 nm E: 400 nm F: Chromatogram in all 5 wavelengths

Table 3: HPTLC Chromatogram at 300 nm of methanol extract from *Grifola frondosa*

Peak no	R_f	Max Height (AU)	Area (AU)
1	0.01	74.5	2732.0
2	0.08	07.9	2462.6
3	0.14	45.8	1105.0
4	0.37	38.6	1315.5
5	0.58	13.4	187.7
6	0.64	15.9	108.5
7	0.81	67.1	3383.3
8	0.98	75.9	2162.9

Figure 3 represents HPLC chromatogram of the extract consisting of 38 phenols. Their retention times and respective area were as follows: 4.32 min (area 848.59), 6.06 min (area 20.45), 6.67 min (area 85.23), 7.2 min (area 48.49), 8.03 min (area 14.49), 8.32 min (area 1828.27), 8.61 min (area 214.09), 8.79 min (area 420.53), 9.07 min (area 227.48), 9.48 min (area 697.96), 9.69 min (area 142.56), 9.98 min (area 1796.39), 10.46 min (area 200.31), 10.69 min (area 21.18), 10.8 min (area 57.62), 11.25 min (area 46.93), 11.62 min (area 29.53), 11.95 min (area 26.23), 12.08 min (area 19.78), 12.39 min (area 20.7), 12.89 min (area 30.53), 13.04 min (area 19.79), 13.47 min (area 1042.85), 13.76 min (area 35.23), 13.92 min (area 32.02), 14.29 min (area 32.16), 14.58 min (area 21.83), 15.08 min (area 30.63), 15.23 min (area 20.46), 15.32 min (area 16.53), 15.83 min (area 446.47), 16.11 min (area 85.42), 16.32 min (area 15.39), 16.55 min (area 14.44), 16.9 min (area 11.23), 17.05 min (area 12.65), 17.35 min (area 60.37) and 17.75 min (area 106.89).

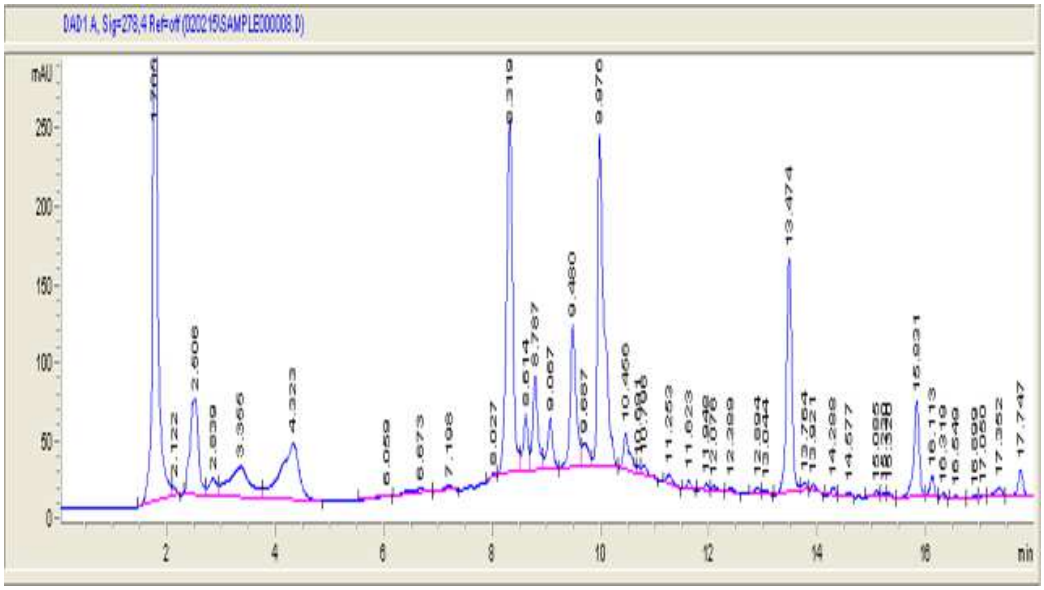

Figure 3: Enlarged HPLC chromatogram of methanolic extract from *Grifola frondosa*

DPPH assay has been widely used to test free radical scavenging ability of various food samples. In methanol solution DPPH produces violet color. Suitable reducing agent donates electron to DPPH and the solution loses color depending upon the number of electron taken up. Color changes from purple to yellow and the reduction capacity of $DPPH^{\cdot}$ is determined by decrease in its absorbance at 517 nm. Extract exhibited radical scavenging activity at the rate of 12.27%, 22.97% and 50% at 133.2, 333 and 666 µg concentrations respectively. EC_{50} value was found to be at 666 µg which was much higher than that of ascorbic acid, a positive control.

Phosphomolybdenum method is a good method 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 at acidic pH. Total antioxidant capacity of methanol fraction was investigated and compared against ascorbic acid. The extract presented 18.64 ± 5.28 µg ascorbic acid equivalent (AAE)/ mg of extract total antioxidant activity.

CONCLUSION

The study presents important diagnostic characters of *G. frondosa* that may be employed in correct identification of the mushroom. Various standardization parameters such as microscopy, physico-chemical constants, preliminary mycochemical investigation, HPTLC, HPLC and antioxidant activity were studied, which are being reported for first time in this mushroom. The results may suggest that the mushroom is a source of pharmacologically active substances having therapeutic applications.

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