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Physiochemical characterization and antioxidant activity of methanol extract from an edible mushroom *Agrocybe pediades*

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Abstract : *Agrocybe pediades*, an edible macrofungus well recognized by the name 'Common Field cap', is inhabitant of grassy fields, lawns, and pasture lands. Literature review suggest that to date no detailed standardized work has been reported for this macrofungus. The present study focuses a step towards unveiling the quality control standardization of *Agrocybe pediades* by various qualitative and quantitative parameters like microscopic, physio-chemical properties, chromatographic features, antioxidant activity. Physio-chemical parameters such as organoleptic features and fluorescent behaviour against seven different reagents were also examined. Chromatographic parameters entailed the HPLC profiles of methanolic extract of *A. pediades* supports the data of phenol estimation. Quantitative measurement of the major bioactive components in the extract confirmed the presence of flavonoid, ascorbic acid, phenol, β-carotene and lycopene in descending order. Antioxidant potentiality of the extract also screened by DPPH radical scavenging activity (EC_{50} value 1.03 mg/ml), total antioxidant capacity (11.71 µg AAE) and ABTS assay (0.02807 TAE/mg of extract). On the other hand, molecular studies including DNA extraction and sequencing of the internal transcribed spacer region of the genomic DNA serve as the molecular standard. The distinctive features established in this work could be considered to be a major step in identification, standardization and quality control of this edible mushroom.

Keywords: antioxidant activity, HPLC, physico-chemical evaluation, pharmacognostic standardization, molecular standardization.

Introduction

West Bengal lies between 22º58′–43.0464″ N latitude and 87º44′–52.0908″ E longitude and is situated in the Eastern part of India having diversified geomorphology[1]. At the north surrounded by the Himalayas to the extreme south encompassed by the Bay of Bengal and gangetic plain in between. This diversified geography and there by various climatic condition creates ample of opportunity for the luxuriant growth of various types of mushroom. To date, our research group have collected and contributed in reporting several mushrooms from different regions of the state. Among these reported mushrooms, there are assorted category of edible, inedible and poisonous mushroom as well. Wild edible mushrooms have long been considered as a part of human diet because of having medicinal and nutritional prospects in many Asian and European countries since the ancient times. Mushrooms are considered to be a rich source of biologically active compounds that possess antifungal, antibacterial, antioxidant, hepatoprotective, immunomodulation, apoptogenic properties[2-14].

In the context of the world, *Agrocybe pediades* (Jungh.) Höhn (family - *Strophariaceae*) is highly prized due to its edibility. This mushroom is commonly known as 'Common Field cap' that prefers to grow among grassy fields. This mushroom has been recently explored for its medically active bio-components that are responsible for antimicrobial and antiviral activities[15]. However, literature survey revealed that phytochemical, antioxidant as well as molecular standardization of *A. pediades* is still unreported and there lies a scope for its proper investigation. The present study focuses on documenting *Agrocybe pediades* with regard to its morphological characters, physicochemical and antioxidative properties. As *Agrocybe pediades* is often confused with other 'little brown mushrooms' because of its similar morphology, proper identification has been done in aspect of its morphological as well as molecular standardization tool.

Materials and Methods

Collection

Basidiocarps of *A. pediades* were collected from Murshidabad district, West Bengal. A representative specimen was deposited at the Calcutta University Herbarium (CUH) in the dried form following the method of Pradhan et al[16]. For powder analysis basidiocarps were dried properly by a field drier at 40 °C for 24 hours to make them crispy. Dried fruit bodies were pulverized using an electric blender, sieved through 160 mesh and stored in an air tight container.

Microscopic evaluation of powdered basidiocarps

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

Fluorescence analysis

Fluorescence analysis was determined using standard Pharmacopoeial method[17]. A small quantity of dried sieved mushroom powder was placed on a grease free clean microscopic slide and 1–2 drop of freshly prepared reagent solutions was added, mixed and waited for 1–2 minutes. The slide was further placed inside the UV chamber and viewed under daylight, short (254 nm) and long (365 nm) UV radiations. The colour observed by application of different reagents in different radiations were recorded.

Preparation of methanol extract

Dried powder of fruiting bodies (5 g) were extracted by stirring with 100 ml of methanol for overnight and subsequently separated by Whatman filter paper. The residue was then re-extracted with 30 ml of methanol and then combined methanolic extracts were evaporated at 40 °C (Rotavapor R3 Büchi, Switzerland) to reduce volume. The methanolic fraction was stored at –20 °C in dark bottle until analysis. Percentage yield and organoleptic parameters of the extract were recorded.

Quantitative estimation of myco-chemicals

The content of total phenolic compounds in the 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[18]. 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[19]. β-carotene and lycopene were estimated by measuring absorbance at 453, 505 and 663 nm[20]. Ascorbic acid was determined by titration against 2, 6-dichlorophenol indophenol dye[21].

High performance thin layer chromatography (HPLC) fingerprinting

The extract was filtered through 0.2 µm filter paper and 20 µl filtrate was loaded on 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[22].

DPPH radical scavenging assay

Hydrogen atom or electron donation abilities of the methanol extract and a pure compound, ascorbic acid, were measured by bleaching of purple coloured methanol solution of DPPH. Various concentrations of extract (0.5, 1.0 and 1.5 mg/ml) were added to 2 ml of 0.004% methanol solution of DPPH (w/v). After 30 min incubation period at room temperature in dark, the absorbance was read against a methanol blank at 517 nm[23]. EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50%. Percentage of scavenging activity was calculated by the following equation:

$$\text{Scavenging effect (\%)} = \{(A_0\text{-}A_1)/ A_0\} \times 100$$

Where A_0 and A_1 were the absorbance of control and absorbance in presence of sample respectively.

Determination of total antioxidant capacity by phosphomolybdenum method

In this assay, 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). Blank was prepared by adding 0.3 ml water and 3 ml reagent solution. Tubes were capped and incubated at 95 ºC for 90 mins. Finally, samples were cooled at room temperature and absorbance was measured at 695 nm against blank[24]. Concentrations of ascorbic acid (1–30 µg) were used to obtain a standard curve. Total antioxidant activity was expressed as the number of ascorbic acid equivalents (AAE).

ABTS assay

ABTS [2, 2′ - azinobis-(3-ethyl-benzo-thiazoline-6 sulfonic acid)] is a peroxidase substrate which when oxidized generates a metastable radical cation. The ability to inhibit the accumulation of ABTS radical cation by methanol extract and antioxidant standard, Trolox, were measured spectrophotometrically[25]. ABTS was dissolved in methanol. ABTS radical solution was prepared by reacting ABTS (7.4 mM) with 2.6 mM potassium persulfate solution and allowing the mixture to stand in dark for 12–16 h at room temperature before use. The mother stock was diluted to achieve an absorbance of 0.7 ± 0.02 at 734 nm. Methanol extract at 1 concentration was allowed to react with the ABTS working solution for 5 minutes and the absorbance was measured at 734 nm.

DNA extraction, PCR and cycle sequencing

Genomic DNA of the specimen was extracted from the dried powdered sample following the method of Dutta et al[26]. For amplification of the nuclear ribosomal internal transcribed spacer region (nrDNA ITS), recognised as the universal barcode region for fungi (Schoch *et al.*, 2012), the pair of primer ITS1 and ITS4 were used[27]. The thermal cycler protocol which used to amplify the ITS region of the specimen follows a hot start of 4 min at 94 °C, followed by 30 cycles consisting of 30 secs at 94 °C, 30 secs at 55 °C, 1 min at 72 °C, and a final elongation step of 5 min at 72 °C. The DNA fragments were amplified on Applied Biosystems® 2720 automated thermal cycler and purified by QIAquick® Gel Extraction Kit (QIAGEN, Germany). The purified products were then subjected to automated DNA sequencing on ABI3730xl DNA Analyzer (Applied Biosystems, USA) using primers identical with amplification for nrITS region. The newly generated sequence was then edited manually using BioEdit sequence alignment editor version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, USA) and deposited in GenBank (www.ncbi.nlm.nih.gov).

Results and Discussion

Microscopic and organoleptic standardization

Dried powder passed through sieve and macerated with KOH showed many fragmented of mycelia and basidiospores (Figure 1). **Hyphal system** was found to be monomitic in nature. **Generative hyphae** 2–3 µm broad, thin-walled, septate, branched, hyaline, clamp-connections absent. **Basidia** 21–32 µm × 7–10 µm,

clavate, tetra-sterigmatic with basal clamp. **Basidiospores** (10.7)11.1–11.43–11.5(–12.5) µm × 7.2–6.8–7.5 µm, Q = 1.4–1.5–1.8, ellipsoid with a germ-pore and small hilar appendage, rusty brown coloured, inamyloid, smooth, thin-walled. The microscopic data coincides with data available in the literature.

Organoleptic characterization was conducted with the dried sieved mushroom powder. The powder was light brown coloured, with no distinctive odour, tasteless and of fibrous texture.

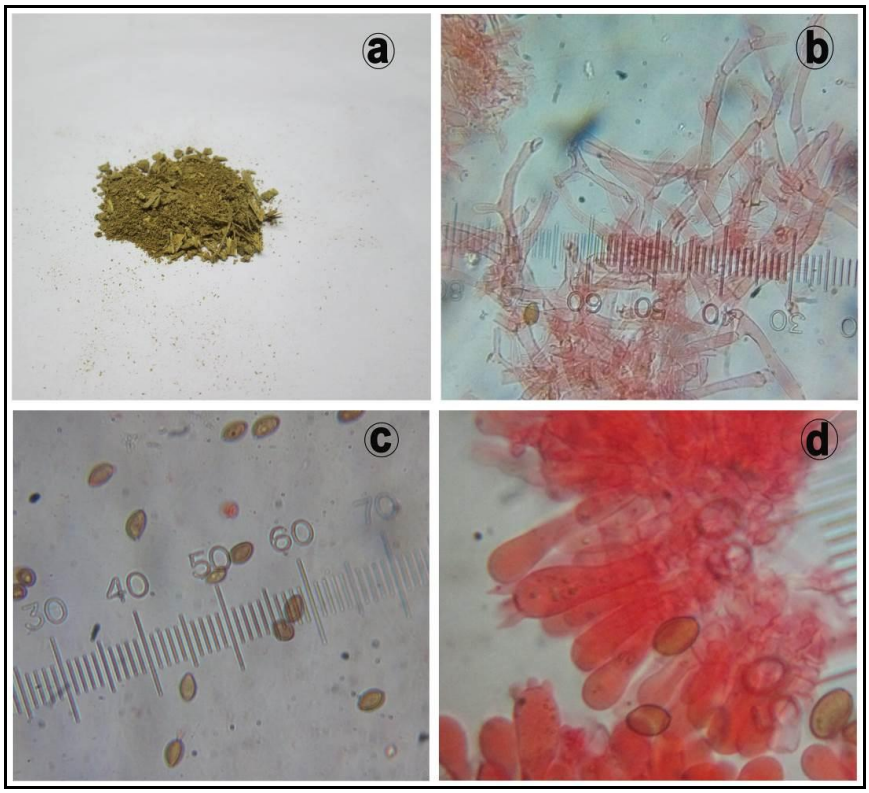

Figure 1. Macroscopic and microscopic characters of the powdered basidiocarps of *Agrocybe pediades* (a) macerated powder (b) hyphae under light microscope (c) basidiospores (d) basidium

Fluorescence analysis

The fluorescence tests of the powdered drug are an important pharmacognostic evaluation as it helps in identification of purity and authenticity of the samples and recognizing sample depreciate quality. Various organic and chemical constituents present in drug exhibits fluorescence in UV light but not visible in day light. By treating samples with certain reagents convert some non-fluorescent compounds to fluorescent compounds. As a result, fluorescence analysis displayed an array of colours that could be employed for identification of probable classes of compounds present in the drug[28,29]. In the present study, powder drug was treated with 7 different chemical reagents which gave characteristics colour when viewed under long (365 nm) and short (254 nm) wavelength of the UV light and was compared with colour observed under ordinary visible light (Table 1).

Table 1. Fluorescence analysis of dry powder of *Agrocybe pediades*

Sl. No.	Reagent	Visible	UV	
			Long (365 nm)	Short (254 nm)
1.	Powder as such	Light brown (7D6)	Deep brown (7F4)	Brown(7E8)
2.	Hager's	Brownish yellow (5B8)	Brown (7F5)	Brown (6E6)
3.	Mayer's	Light brown (7D6)	Deep brown (7F4)	Olive green (1D6)
4.	Dragendroff's	Reddish orange (7B7)	Dark brown (7F6)	Light brown (74D)
5.	Phloroglucinol	Brownish orange (5C4)	Violet brown (10F5)	Reddish brown (9D5)
6.	Barfoed	Reddish brown (9E4)	Dark brown (7F5)	Violet brown (10F5)
7.	H_2O	Light brown (6D6)	Deep brown (7F4)	Light brown (5D5)

Molecular analysis

The agarose gel electrophoresis (2%) of the amplified sequence showed single band of ca. 600 bp in length. After sequencing the generated sequence was edited manually using BioEdit sequence alignment editor version 7.0.9.0 (Tom Hall, Ibis Biosciences, Carlsbad, USA). The edited DNA fragment of the collected specimen produced 604 bp long stretches containing internal transcribed spacer 1(ITS1), 5.8s and ITS2 region. This newly generated sequence of our collected *A. pediades* sequence was then deposited in the GenBank (www.ncbi.nlm.nih.gov) with the accession number KA767081 that could be utilized as a signature sequence to identify the mushroom specimen.

Quantitative estimation

Methanolic extract was prepared from *Agrocybe pediades* and the fraction was yellowish brown in colour. The extract was found to contain phenol as much as 2.9 ± 0.62 µg gallic acid equivalent/mg of extract. Total flavonoid content was determined by using quercetin as standard. The extract contained flavonoid as 3.8 ± 0.66 µg quercetin equivalent/mg of extract. Very negligible amount of β-carotene and lycopene were found such as 0.01359 ± 0.002 µg/mg of the extract and 0.0098869 ± 0.003 µg/mg of the extract respectively. Extract also contained ascorbic acid at a concentration of 2.93 ± 0.41 µg/mg of the extract.

Chromatographic fingerprinting

High performance liquid chromatographic (HPLC) is considered as one of the efficient chromatographic technology for the preliminary determination of chemical constituents present in the sample. For this purpose, HPLC was carried out using the methanolic extract of *Agrocybe pediades*. UV spectrum analysis of the chromatogram at 278 nm depicts presence of 12 peaks excluding the solvent peaks. This chromatographic data may act as a specific fingerprint towards authentication of the crude samples. Represented peaks may serve as a phenolic fingerprint of this mushroom which helps to authenticate the crude samples. Chromatographic profile along with the retention time of each peak was presented in Figure 2. Respective areas of each peak were documented in Table 2.

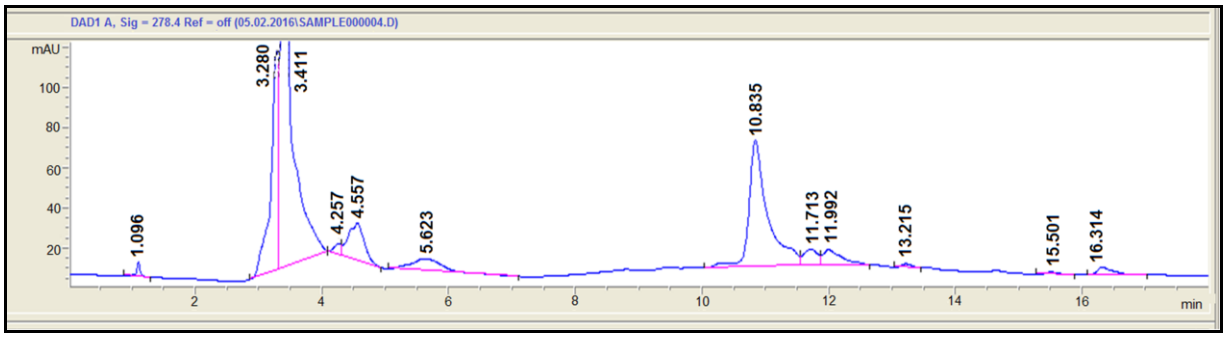

Figure 2. Enlarged HPLC chromatogram of methanol extract from *Agrocybe pediades* (MP: Mobile phase)

Table 2. HPLC profile at 278 nm of methanol extract from *Agrocybe pediades*

R_f	Area(AU)	Max Height(AU)
1.096	26.98286	6.96942
3.280	919.32196	108.45036
3.411	2775.33472	310.56534
4.257	43.79739	5.41201
4.557	317.46606	18.87804
5.623	214.52724	6.14379
10.835	1312.42957	62.90694
11.713	130.71944	8.32491
11.992	165.64780	7.97901
13.215	19.58560	1.85178
15.501	14.71525	1.14274
16.314	67.43873	3.92020

Antioxidant activity

For determination of antioxidant potentiality of the methanolic extract of *Agrocybe pediades*, three in vitro assays were carried out such as DPPH radical scavenging potentiality, total antioxidant activity and ABTS radical scavenging assay. The DPPH assay constitutes a quick and simple method to test free radical scavenging ability of various natural products. DPPH is a stable N_2-centered free radical which accepts an electron/ hydrogen to gain stability. In methanol solution DPPH produces purple colour. But when any suitable reducing agent donates electron to DPPH then the methanol solution loses colour changing from purple to yellow and the reduction capacity of DPPH is determined by decrease in its absorbance at 517 nm[30]. The reduction capacity of the solution is represented in terms of EC_{50} value. With regard to scavenging ability of DPPH radicals, methaolic extract of *A. pediades* performed well as evidenced by its low EC_{50} value 1.03 ± 0.04 mg/ml. Recent investigation on EC_{50} values of DPPH radical scavenging activity by the methanolic extracts of *Laetiporus sulphureus*[31], *Grifola frondosa*[32], *Lentinula edodes*[33], *Macrocybe crassa*[34], *Pleurotus ostreatus*[35] were 0.11 mg/ml, 0.66 mg/ml, 1.25 mg/ml, 2.45 mg/ml and 1.232 mg/ml respectively.

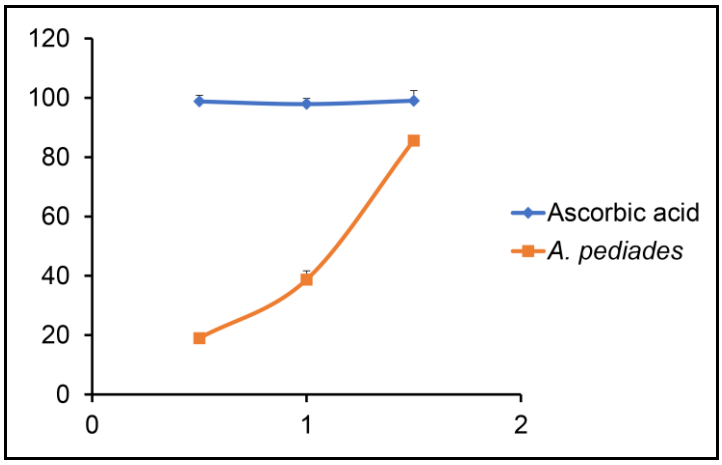

Figure 3. DPPH radical scavenging activity of methanol extract from *Agrocybe pediades*

Total antioxidant capacity of the methanolic extract of *Agrocybe pediades* was evaluated by phosphomolybdenum method where reduction of Mo(VI) to Mo(V) takes place by the antioxidant compounds present in the extract and the formation of green phosphate/Mo(V) complex at acidic pH. The result was compared against ascorbic acid. The extract showed 11.71 ± 0.32 µg/mg ascorbic acid equivalent antioxidant capacity. The ABTS assay is based on the inhibition of the formation of $ABTS^{\cdot+}$, a stable radical cation by one electron oxidants. The methanolic extract of the sample showed 0.02807 ± 0.0 µM Trolox equivalents (TE)/mg of extract antioxidant activity.

Conclusion

In the present study, an attempt has been taken to standardize *A. pediades* powder with the help of pharmacognostic, molecular and chemical characters. Various standardization parameters such as microscopy, physio-chemical constants, preliminary mycochemical quantification, HPLC and antioxidant activity are reported herein for the first time for this mushroom that could be quite useful for identification, standardization, development and preparation of crude drug's formulation. All these aggregated data together provide relevant information to identify the genuine species and purity of the powder which may be helpful in authentication of the crude drug and check adulteration for quality control of raw material of *Agrocybe pediades*.

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