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Quality assessment and antioxidant study of *Pleurotus djamor* (Rumph. ex Fr.) Boedijn

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ABSTRACT

Pleurotus djamor is an important oyster mushroom that has attracted much attention due to its high nutritional value and diverse therapeutic properties. However, quality standards of this species remain unexplored so far. For this purpose, microscopic features of powder such as physical characters, spore measurement, type of hyphae were examined and described. Physico-chemical parameters like organoleptic features and fluorescent behaviour against 16 reagents were also determined. In addition, an alcoholic extract was prepared using methanol as solvent to characterize the mushroom chemically and biologically. Preliminary mycochemical analysis indicated presence of major bioactive components in the following order of phenol> ascorbic acid> flavonoid> β-carotene> lycopene. To determine phenolic fingerprint of the extract, an HPLC profile was recorded. The chromatogram indicated existence of at least nine phenolic components in the extract. Moreover, antioxidant activity of the macrofungus was also evaluated using *in vitro* assays like DPPH radical scavenging activity (EC_{50} value 0.653 mg/ml) and total antioxidant capacity (16.67 µg AAE/mg of extract). In summary, the data as described in this study are significant towards future identification and authentication of genuine mushroom material establishing the pharmacognostic standards. Furthermore, results of antioxidant study indicate that the mature fruiting bodies could be valuable for defending radical induced ailments.

INTRODUCTION

Pharmacognosy is considered as one of the oldest modern science and derived from two Greek words namely “pharmakon” (drug) and “gnosis” (knowledge). About 200 years ago, this subject area was introduced for the study of products and crude drugs of natural origin to authenticate such drugs. Over the course of history, pharmacognosy has expanded considerably beyond traditional techniques such as macroscopic and microscopic identification due to advancement in screening methods. In recent years, it includes study of ethnomedicine, ethnopharmacology, phytochemical analysis, isolation of active principles and more recently, the investigation of biological activity (Sarkar 2012; Jones *et al*., 2006; Orhan *et al*., 2014).

For maintaining biological processes, oxidation is essential for production of energy in many living organisms. However, gradually the mechanism of oxidation becomes unbalanced, as a result free radicals are produced causing cancer, rheumatoid arthritis, atherosclerosis etc. (Valko *et al*., 2007). Antioxidants can scavenge free radicals and could be used to help human body from oxidative damage. Several commercial synthetic antioxidants are available in market which are widely used in food industry such as butylatedhyroxyanisole (BHA), butylatedhydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ). Though, these synthetic antioxidants have been restricted for use due to their toxicity and carcinogenicity effects to liver. Therefore, investigation and production of effective natural antioxidants is necessary to reduce oxidative damage without any interference (Khatua *et al*., 2013; Ahmad *et al*., 2014). Mushrooms have been used as therapeutic aid for centuries and recently have gained popularity as they are scientifically proved to be a source of physiological beneficial medicine.

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Recent studies have established multi-purpose application of several mushrooms including *Astraeus hygrometricus* (Pers.) Morgan (Mallick *et al*., 2015); *Entoloma lividoalbum* (Kühner & Romagn.) Kubička (Maity *et al*., 2014; Maity *et al*., 2015); *Lentinula edodes* (Berk.) Pegler (Acharya *et al*., 2015); *Pleurotus flabellatus* Sacc. (Dasgupta *et al*., 2014); *Pleurotus florida* (Mont.) Singer (Saha *et al*., 2013); *Pleurotus ostreatus* (Jacq.) P. Kumm. (Mitra *et al*., 2013); *Pleurotus sajor-caju* (Fr.) Singer (Giri *et al*., 2012); *Ramaria aurea* (Schaeff.) Quél. (Khatua *et al*., 2015); *Macrocybe crassa* (Sacc.) Pegler & Lodge (Acharya *et al*., 2015); *Russula albonigra* (Krombh.) Fr. (Dasgupta *et al*., 2014); *Russula senceis* S. Imai (Khatua *et al*., 2015); *Termitomyces clypeatus* R. Heim (Pattanayak *et al*., 2015); *Termitomyces eurrhizus* (Berk.) R. Heim (Chatterjee *et al*., 2013); *Termitomyces medius* R. Heim & Grassé (Mitra *et al*., 2014) etc. However it has been noticed that many investigations have been conducted on highly nutritious *Pleurotus* mushrooms confirming their medicinal importance (Khan *et al*., 2012). One such popular oyster mushroom is *Pleurotus djamor* (Rumph. ex Fr.) Boedijn that is recognized worldwide as food which in turn have encouraged for artificial cultivation contributing high economic importance. Researchers are interested with this species as it possesses phytochemical constituents similar with some mushrooms that are already proved to be medicinal such as *P. florida*, *P. ostreatus*, *P. sajor-caju* and *P. pulmonarious* (Guo *et al*., 2007; Suseem *et al*., 2011). In earlier experimental studies, *P. djamor* has been found to possess strong analgesic, anti-inflammatory and antipyretic activity. Crude extracts of the macrofungus has also exhibited significant *in vitro* free radical scavenging property, antimicrobial and antiplatelet potentiality (Susem *et al*., 2013). However literature survey revealed that its quality assessment study is still lacking. Hence, the present work was conducted to evaluate the pharmacognostic parameters and chemical analysis of *P. djamor* collected from West Bengal, India, with a view to establish standards for its identity, quality, purity and molecular composition. In addition, antioxidant activity of the macrofungus was also investigated for future application.

MATERIALS AND METHODS

Collection

Basidiocarps of *P. djamor* were collected from Narendrapur Ramkrishna Mission Mushroom Cultivation Unit. A representative specimen (Accession no: CUH AM032) was deposited at CUH herbarium following the method of Pradhan *et al*., (2015). For powder analysis basidiocarps were dried properly by a field drier at 40°C for 1 day 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 results various stains (Congo red, Melzer's reagent) were used to distinguish different cellular structure. 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 samples were evaluated.

Fluorescence analysis

Fluorescence analysis was determined using standard Pharmacopoeial method (2007). 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 were 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 powdered 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 the 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, for no more than 1 month. Percentage yield and organoleptic parameters of the extract were recorded.

Quantitative estimation of myco-chemicals

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 (Singleton *et al*., 1965). Total flavonoid content was determined using aluminium nitrate and potassium acetate. Quercetin (5–20 µg) was used to calculate the standard curve. The results were expressed as µg of quercetin equivalents per mg of dry extract (Park *et al*., 1997). β-carotene and lycopene were estimated by measuring absorbance at 453, 505 and 663 nm (Nagata and Yamashita 1992). Ascorbic acid was determined by titration against 2, 6-dichlorophenol indophenol dye (Rekha *et al*., 2012).

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 (Khatua *et al*., 2015).

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 radical. Various concentrations of extract (0.5, 1 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 (Shimada *et al*., 1992). EC_{50} value is the effective concentration at which DPPH radicals were scavenged by 50%. Degree of scavenging was calculated by the following equation:

Scavenging effect (%) = {(A0-A1)/ A0} × 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

The assay was carried out as described by Prieto *et al*., (1999) 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). Blank was prepared by adding 0.3 ml water and 3 ml reagent solution. 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) were used to obtain a standard curve. Total antioxidant activity was expressed as the number of ascorbic acid equivalents (AAE).

RESULTS AND DISCUSSION

After passing through sieve, the dried powder appeared yellowish-brown coloured, pungent in smell, tasteless and granular in texture (Figure 1). After maceration with HNO_3 and KOH separately, micro-morphological observations revealed the following characters: basidiospores (7.87-)8.28-11.18(-12.43) × (3.73-)4.14-4.97(-5.38) µm, oblong, hyaline, inamyloid, thik walled with suprahillar depression; basidia 20.71-21.12 × 4.14-4.56 µm, narrowly clavate, tetrasterigmatic sterigmata 3.31-3.73 × 0.41 µm; pleurocystidia absent; rostrate to lecithiform cystidium like element cystidiole present, 25.78-29.4 × 3.73-5.73 µm; hyphal system dimitic, generative hyphae hyaline to yellowish brown, thin to thick-walled; skeletal hyphae blackish, thin walled; clamp connection present.

Fluorescence is the property of some atoms and molecules to absorb light at a particular wavelength and subsequently emit light of longer wavelength. It is an important parameter for pharmacognostic evaluation by which crude drugs are often assessed qualitatively. The crude drug may consisted of various chemical constituents that exhibit fluorescence in UV light. However, the non-fluorescent compounds often can be converted to fluorescent compounds by applying different reagents. As a result fluorescence analysis displayed an array of colours that could be employed for identification of authentic samples and recognize adulterates (Sonibare *et al*., 2015; Bhattacharya *et al*., 2009). In the present study powder drug was treated with 16 different chemical reagents which gave characteristic colour when observed under UV light (254 nm, 365 nm) and was compared with colour detected under visible light (Table 1).

It has been reported that most of the components in mushroom fruiting bodies have high polarity (Wu and Hansen 2008). In the present study, methanol was chosen for preparation of a fraction where the formulation appeared yellow in colour. The extract was subjected for quantitative analysis of different myochemicals using standard protocol. The fraction was found to contain phenol as much as 7.845 ± 1.22 µg gallic acid equivalent/mg of dry extract. Total flavonoid content was determined by using quercetin as standard. The extract contained flavonoid as 2.876 ± 0.893 µg quercetin equivalent/mg of extract. Very negligible amount of β-carotene and lycopene were found such as 0.045 ± 0.006 µg/mg and 0.033 ± 0.002 µg/mg of the extract respectively. Ascorbic acid was found in the higher amount i.e. 6.94 ± 1.389 µg/mg of extract.

HPLC is an efficient and valuable technology for the preliminary separation and determination of constituents. Figure 2 represents HPLC fingerprint profile of the methanol extract which was found to be consisting of at least nine phenols (Rt 4.583 min, area 10.145; Rt 5.044 min, area 299.758; Rt 5.14 min, area 410.51; Rt 5.685 min, area 42.835; Rt 6.37 min, area 62.236; Rt 9.997 min, area 10.14 397.012; Rt 10.733 min, area 55.696; Rt 11.009 min, area 71.672; Rt 15.972 min, area 227.292).

To assess the antioxidant capacity of an extract, DPPH radicals are most often used as they are extremely stable and easy to use. It is a stable N_2-centered free radical which accepts an electron/hydrogen to gain stability. In methanol solution DPPH radical produces violet colour which is changed to yellow, depending on capacity of the antioxidant tested. The solution loses colour depending upon the number of electron taken up and colour change is determined by decrease in its absorbance at 517 nm (Dash *et al.*, 2005; Waltasinghe and Shahidi 1999). DPPH radical scavenging activity of the methanol extract from *P. djamor* was directly correlated with the concentration of the sample (Figure 3). The extract exhibited radical scavenging activity at the rate of 33.33%, 87.84% and 95.58% at 0.5, 1 and 1.5 mg/ml concentrations respectively. EC_{50} value was found to be at 0.653 ± 0.156 mg/ml which was much higher than that of ascorbic acid, a positive control, (0.004 mg/ml). Recently Jeena *et al* (Jeena *et al*., 2014) have reported the DPPH radical scavenging activity of methanolic extract from three oyster mushrooms viz. *P. ostreatus*, *P. sapidus* and *P. sajor-caju*. Results showed that the lowest EC_{50} value was at 2 mg/ml concentration represented by *P. sajor-caju* which was much higher than that of *P. djamor*. Phosphomolybdenum method is a good technique 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 the fraction was investigated and compared against ascorbic acid. The extract presented 16.67 ± 2.66 µg AAE/ mg of extract total antioxidant activity.

Table 1: Fluorescence analysis of dry powder from *Pleurotus djamor*.

Sl No.	Reagent	Visible	UV	
			Long (365nm)	Short (254nm)
1	Powder as such	Whitish yellow	Deep brown	Whitish brown
2	Hager's	Lemon yellow	Dark golden brown	Butter yellow to maize yellow
3	Mayer's	Birch bark to flesh	Greyish violet	Creamish yellow
4	Dragendroff's	Reddish orange	Persian red to cuba	Light brown
5	Iodine solution	Reddish brown	Purplish brown	Dark brown
6	1(N) HNO_3	Light cream	Light brown	Creamish yellow
7	50% HNO_3	Reddish green	Brownish green	Light brownish green
8	Phloroglucinol	Light yellow	Violet brown	Sap green
9	Barfoed	Olive green	Brownish black	Greyish brown
10	Sodium nitroprusside	Creamish yellow	Reddish brown	Sap green
11	H_2O	Whitish yellow	Deep brown	Light yellow
12	$FeCl_3$	Light brown	Deep brown	Sap green
13	1(N)NaOH	Greyish orange	Dark golden brown	Greenish brown
14	Acetic acid	Straw	Greyish brown	Light yellow to greenish yellow
15	1(N) HCl	Greenish yellow	Brownish grey	Greyish yellow
16	Methanol	Creamish orange	Dark brownish red	Creamish green
17	1(N) NaOH in methanol	Greyish orange	Dark golden brown	Greenish brown

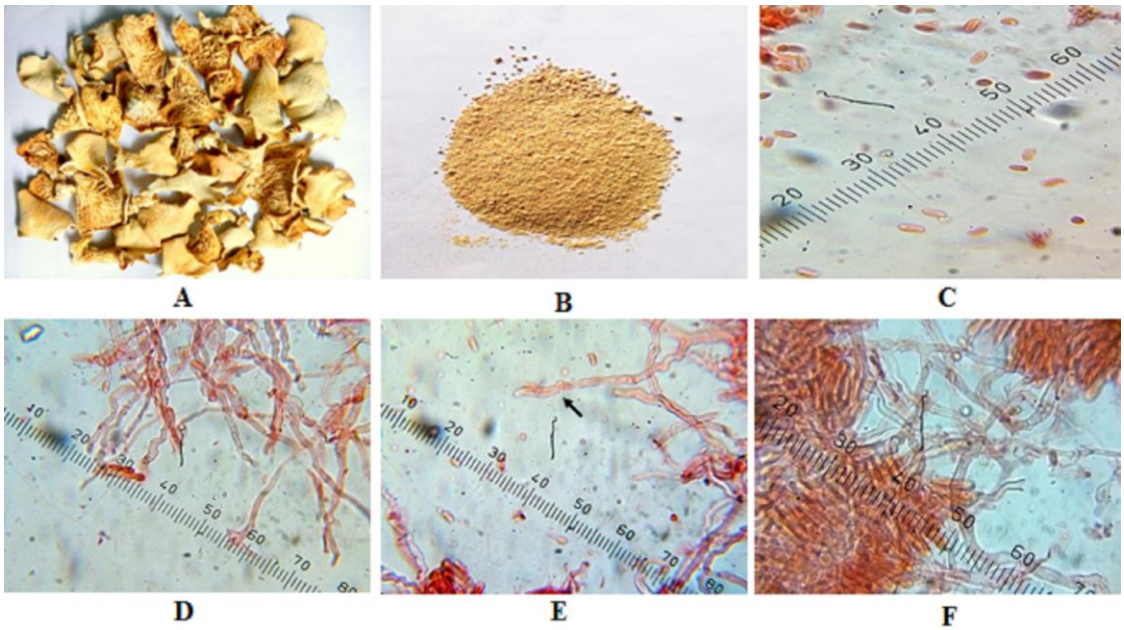

Fig. 1: Macroscopic and microscopic characters of *Pleurotus djamor*.
A: Dried basidiocarps B: Fruit body in powder form B: Spores D-F: Types of hyphae (arrow indicates clamp connection).

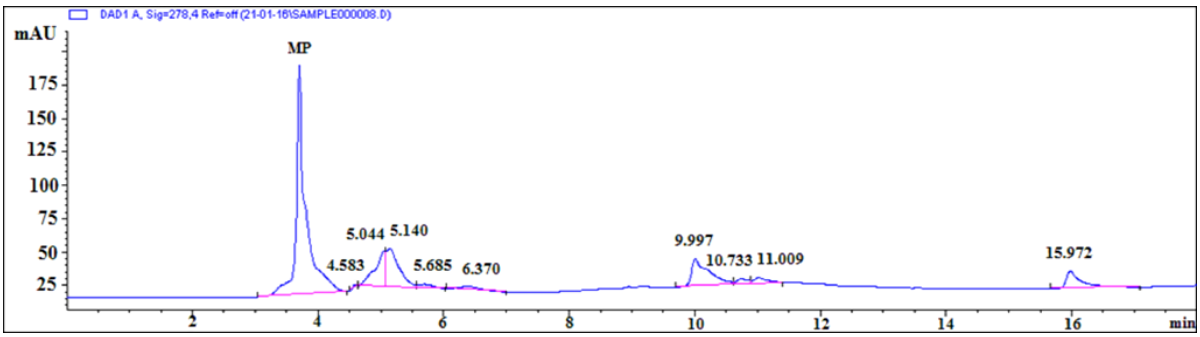

Fig. 2: Enlarged HPLC chromatogram of methanol extract from *Pleurotus djamor* (MP: Mobile phase).

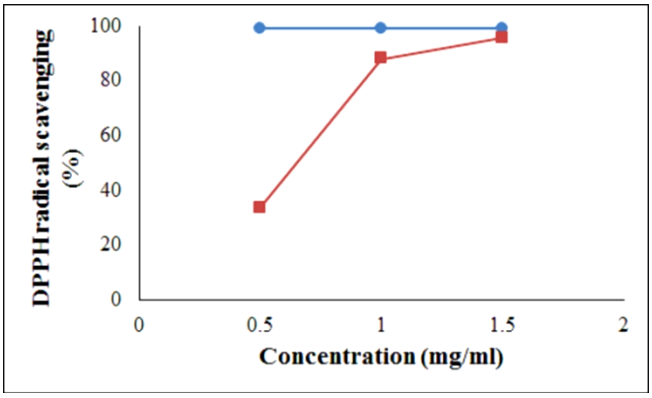

Fig. 3: DPPH radical scavenging activity of methanol extract from *Pleurotus djamor*.

CONCLUSION

The present work provides information for correct identification and standardization of *P. djamor* powder with help of modern techniques and suitable parameters. For quality control studies microscopic features, physico-chemical constants, mycochemical investigation and HPLC profile were evaluated which would be helpful for establishing the pharmacopeia standards. Moreover, antioxidant activity of methanol extract from the mushroom was also investigated. The macrofungus exhibited high DPPH radical inhibitory action and total antioxidant capacity. Thus this study provides information concerning pharmacognosy and bioactivity of *P. djamor* which may be useful for authentication and development of nutraceuticals respectively.

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