J Food Sci Technol (April 2016) 53(4):1878–1888
DOI 10.1007/s13197-015-2145-0

ORIGINAL ARTICLE

Influence of extraction parameters on physico-chemical characters and antioxidant activity of water soluble polysaccharides from *Macrocybe gigantea* (Massee) Pegler & Lodge

Somanjana Khatua[1] · **Krishnendu Acharya**[1]

Revised: 2 December 2015 / Accepted: 14 December 2015 / Published online: 22 December 2015

Abstract Polysaccharides from mushrooms are potentially active pharmaceutical ingredients and their action is dependent upon conformation, composition, size etc. In this context, three water soluble crude polysaccharide rich fractions viz. hot water extracted polysaccharide (HWP), cold alkaline extracted polysaccharide (CAP) and hot alkaline extracted polysaccharide (HAP) have been isolated using varying extraction parameters from *Macrocybe gigantea*, a well-known edible mushroom collected from Gangetic plain of West Bengal and authenticated by DNA barcoding of nrDNA ITS region. Physico-chemical investigation revealed that the fractions were mainly composed of β-configuration in pyranose form of sugars conjugated with small amount of protein. Further analysis presented that polysaccharides were composed of same monosaccharide even in similar order of ratio (D-glucose > D-galactose > D-mannose > D-xylose). However, D-glucose as well as β-glucan were found to be in the highest amount in CAP. The helical structure was determined by Congo red assay which indicated that polysaccharides were in aggregate forms except HWP which consisted of tertiary structure. These diverse structural features may have imparted effect on free radical scavenging activity of polysaccharides where HWP was the most active in all assays. HWP was proved to be a good scavenger of free radicals, strong chelator of ferrous ion and had high reducing power. Thus it can be inferred that HWP may foster further studies for searching active compound which might be used as ingredients of functional foods, nutraceuticals and pharmaceuticals. Moreover, to the best of our knowledge this is the first report on chemical composition and antioxidant activity of different crude polysaccharides from *M. gigantea*.

Keywords Antioxidant activity · DNA barcoding · Extraction parameters · HPTLC · GC-MS · Wild edible mushroom

Introduction

The growing occurrence of free radical induced diseases is a serious threat to world and create major problem in treatment of human (Khatua et al. 2013). Free radicals especially reactive oxygen species (ROS) can be generated inside human body by many environmental causes (drugs, tobacco smoke, UV radiation, air pollution, pesticide etc.) and endogenous sources (nutrient metabolism, ageing process, inflammation etc.). Biological macromolecules (i.e., proteins, lipids and nucleic acids) can suffer oxidative damage induced by ROS leading to various diseases including diabetes, coronary heart diseases, arthritis and cancer. The molecule which has ability to scavenge free radicals is commonly referred as antioxidants (Valko et al. 2007). Biochemical with property of antioxidant is required to protect human body when endogenous immune system deteriorates and cannot be recovered by synthetic chemicals (Smolskaite et al. 2014). Currently there are numerous synthetic antioxidants in market but they are gradually being restricted due to their carcinogenicity. Thus searching for natural drugs is now a very attractive field of research as nature is the most important source of medicines (Khatua et al. 2013).

For millennia, polysaccharides especially β-glucans from mushroom are being used as medicine in the Far East (Nandi et al. 2014). Among all the properties, antioxidant capacity is

✉ Krishnendu Acharya
krish_paper@yahoo.com

[1] Molecular and Applied Mycology and Plant Pathology Laboratory, Department of Botany, University of Calcutta, 35, Ballygunge Circular Road, Kolkata 700019, West Bengal, India

one of the most important bioactivities of polysaccharides (Lung and Chang 2011). The antioxidant properties of polysaccharides are influenced by chemical characteristics like molecular weight, degree of branching, types of monosaccharides, ratio of monosaccharides, intermolecular associations of polysaccharides, protein/polysaccharide ratio, polysaccharide content, glycosidic branching and modification of polysaccharides such as sulfation, acetylation, phosphorylation (Khatua et al. 2013; Lo et al. 2011; Kozarski et al. 2012). Among different types of polysaccharides, β-(1 → 3) (1 → 6) glucans are the most bioactive among monosaccharides (Lo et al. 2011). Therefore, studies on chemical composition are highly desirable so that the mushroom with bioactive components may be used to reduce damage to human body.

West Bengal, extending from 21°38′ to 27°10′ N latitude and 85°50′ to 89°50′ E longitude, is a phytogeographically unique state in India. Its wide ranges of topographical feature, types of soils and substrata make the state ideal for being treasure house of rich diversity of mushrooms (Dutta and Acharya 2014). Recent studies have proven multi-purpose application of mushrooms collected from the place viz. *Astraeus hygrometricus* (Pers.) Morgan; *Entoloma lividoalbum* (Kühner & Romagn.) Kubička, *Meripilus giganteus* (Pers.) P. Karst., *Pleurotus flabellatus* Sacc., *Ramaria aurea* (Schaeff.) Quél.; *Pleurotus florida* (Mont.) Singer; *Macrocybe crassa* (Sacc.) Pegler & Lodge; *Russula albonigra* (Krombh.) Fr.; *Russula senceis* S. Imai; *Termitomyces eurrhizus* (Berk.) R. Heim; *Termitomyces medius* R. Heim & Grassé etc. (Khatua et al. 2013, 2015; Chatterjee and Acharya 2015; Patra et al. 2012). *Macrocybe gigantea* (Massee) Pegler & Lodge is also a medicinally potent edible mushroom which is common to West Bengal in rainy season. In regards to its bioactivity, there are some studies reporting antitumor, antioxidant, antimicrobial and anticancer activities (Banerjee et al. 2007; Pushpa et al. 2014; Chatterjee et al. 2011). Mizuno et al. (1995) isolated and analysed structure of four pure heteroglycans from this white coloured macrofungi reporting antitumor properties. However, to the best of our knowledge, there is no documentation related to the structural characterization and bioactivity of crude polysaccharide. Thus in the course of seeking bioactive polysaccharides from nature and also to highlight effect of extraction parameters on structure as well as activity, the fruit bodies of *M. gigantea* were investigated chemically and biologically. The present paper deals with the isolation, structural elucidation and antioxidant activity of three water-soluble polysaccharide rich fractions such as HWP, CAP and HAP prepared by hot water, cold alkaline and hot alkaline methods respectively from the fruit bodies of *M. gigantea*, confirmed through BLASTn searches of nrDNA ITS sequence in NCBI database.

Materials and method

Chemicals

All chemicals used were of analytical grade and freshly prepared before use. 2-Deoxy-D-ribose, ferric chloride, hydrogen peroxide (H_2O_2), thiobarbituric acid (TBA), L methionine, nitroblue tetrazolium (NBT), riboflavin, ferrozine, potassium ferricyanide, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), sodium persulfate, Folin-Ciocalteu reagent, sodium borohydride, pyridine, toluene, dichloromethane (DCM) and standards such as ascorbic acid, ethylene diaminetetraacetic acid (EDTA), butylated hydroxylanisole (BHA), ascorbic acid, Trolox, gallic acid, bovine serum albumin (BSA) and monosaccharides were purchased from Sigma chemicals Co. (St. Louis, MO, USA). DCM, toluene, chloroform were HPLC grade and monosaccharides were of extra pure form. A fungal gDNA mini kit (Xcelris Genomics, Ahmedabad, India), QIAquick gel extraction kit (QIAGEN, Germany) and mushroom β glucan kit (Megazyme Institute Wicklow, Ireland) were also used.

Collection and deposition of specimen

Fresh basidiocarps of *M. gigantea* were collected from coastal region of West Bengal, India. The collected materials were preserved based on the protocol as described by Pradhan et al. (2015) and deposited in the Calcutta University Herbarium (CUH) with accession number CUH AM115.

DNA extraction, polymerase chain reaction and sequencing

Genomic DNA was extracted from dried (50 °C) basidiocarps (10–50 mg) using the fungal gDNA mini kit. PCR amplification was done of the region spanning nuclear ribosomal internal transcribed spacer region 1, 5.8S and spacer region 2 (nrITS), using primer pair ITS1 and ITS4 (White et al. 1990). The DNA fragments were amplified on Applied Biosystems® 2720 automated thermal cycler. A hot start of 4 min at 94 °C was followed by 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C, and a final elongation step of 7 min at 72 °C. PCR products were purified using a QIAquick gel extraction kit and were subjected to automated DNA sequencing on an ABI3730xl DNA Analyzer (Applied Biosystems, USA) using primers identical with amplification for the ITS rDNA region. The newly generated sequences were then deposited in GenBank (www.ncbi.nlm.nih.gov) with an accession number HM120872.

Extraction of polysaccharide

The extraction procedure was followed according to Palacios et al. (2012) with modifications. Dried and powdered fruit bodies were steeped with 10 volume of 99 % ethanol for 2 days at room temperature to extract the alcohol soluble constituents. Residue was filtered and re-extracted with ethanol. The air dried filtrate was suspended and refluxed with distilled water at boiling condition for 7 h. The extract was filtered through nylon cloth and concentrated to one-tenth of volume with a rotary evaporator at 80 °C under vacuum (Rotavapor R3, Butchi, Switzerland). Four volumes of absolute ethanol was added to precipitate polysaccharide and left at 4 °C overnight. After centrifugation (11,000 rpm for 10 min at 4 °C), pellet was washed with ethanol and acetone twice to acquire hot water extracted polysaccharide (HWP). The residue was then immersed in 10 % NaOH solution at 4 °C for 24 h to extract cold alkaline soluble polysaccharide (CAP). After filtration, residue was extracted with 10 % NaOH at 65 °C for 1 h to obtain hot alkaline soluble polysaccharide (HAP). Both alkaline extracts were neutralized by glacial acetic acid and were precipitated by addition of four volume of absolute alcohol. After centrifugation, pellets were dissolved in water to obtain water soluble crude polysaccharide. Alcohol precipitated pellets were recovered by centrifugation followed by washing with ethanol and acetone respectively. The polysaccharides were kept in amber containers under cold and dry condition until analysed.

Characterization of crude polysaccharide

Determination of total carbohydrate, protein and phenol

Total sugar content was measured by phenol sulphuric acid method at 490 nm using glucose as standard (Dubois et al. 1956). The protein content of protein bound polysaccharide was determined by Bradford reagent using BSA as standard (Bradford 1976). Gallic acid was used as a standard to quantify total phenolic compounds present in the crude polysaccharide using Folin–Ciocalteau reagent (Ziestin and Ben-Zaker 1993). All values were expressed as gram of standard equivalents per 100 g of crude dry polysaccharide.

Determination of glucan content

α-Glucan, β-glucan and total glucan of the polysaccharide were estimated using mushroom and yeast β-glucan assay kit (Megazyme Int.) as per the kit's manual. Briefly, polysaccharide was hydrolyzed with HCl and a mixture of exo-β-(1–3)–β-glucanase plus β-glucosidase was added to break β-1, 3 as well as β-1, 4-glucans. Finally, glucose oxidase/peroxidase reagent was added to oxidize glucose which can be visualized spectrophotometrically. α-glucan content was estimated using the same reagent after enzymatic hydrolysis with amyloglucosidase plus invertase. The β-glucan content was calculated by subtracting α-glucan from total glucan content. All values of glucan contained were expressed as gram of glucose equivalents per 100 g of crude dry polysaccharide.

Congo red reaction

The helical structure of polysaccharides was analysed by characterizing Congo red-polysaccharide reaction according to the method described by Qiu et al. (2013). Different sets of solutions were prepared containing HWP, CAP and HAP (0.5 mg/ml) in 0–0.5 M NaOH (increasing stepwise by 0.05 M increments) and 91 μM of Congo red. These were analysed in the range of 400–700 nm with a UV–VIS spectrophotometer and the maximum absorption wavelengths were recorded as a function of NaOH concentration. Congo red in NaOH served as the negative control.

Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR spectra were recorded on Perkin Elmer Precirety Spectrum 100 Model. The crude polysaccharide were ground with potassium bromide powder and then pressed into pellets for FT-IR measurements in frequency range 400–4000 cm^{-1}.

Determination of monosaccharide composition

Crude polysaccharides (10 mg) were hydrolysed with 2 M TFA (5 ml) at 100 °C for 2 h in a screw cap vial. TFA was removed by evaporation at 55 °C under reduced pressure. One ml ethanol (50 %) was added to dissolve hydrolysate followed by centrifugation (12,000 rpm, 5 min) to remove non-hydrolysed polysaccharide and subjected for monosaccharide composition analysis by high performance thin layer chromatography (HPTLC) and gas chromatography mass spectrometry (GC-MS).

HPTLC

HPTLC was performed according to Yang et al. (2010) with some modifications. Standards (1 μl from 1 mg/ml) and sample (3 μl) were applied on TLC aluminium sheet pre-coated with silica (Silica gel 60 F254 sheets 20 × 20 cm, 0.5 mm thickness, Merck Darmstadt, Germany) using CAMAG HPTLC system (Muttenz, Switzerland) comprising Linomat-5 automated sample applicator equipped with a 100 μl syringe with winCATS software (version: 1.4.6). The developing agent was chloroform: *n*-butanol: methanol: water: acetic acid (4.5: 12.5: 5: 1.5: 1.5) (v/v) which was applied in a glass flat bottomed chamber for 30 min to equilibrate mobile phase vapour. The monosaccharides were visualized on the plate after spraying with orcinol solution (5 % H_2SO_4 in ethanolic

solution containing 0.1 % orcinol) and heating at 80 °C for 10 min. L-arabinose, D-glucose, D-galactose, D-fructose, D-fucose, D-mannose, D-rhamnose and D-xylose were used as standard monosaccharides.

GC-MS

Monosaccharides were derivatized to alditol-acetates followed by the method of Leung et al. (2004) with modifications. 50 mg sodium borohydrate ($NaBH_4$) and 5 ml of 50 % ethanol were added to concentrated TFA hydrolysate. Mixture was stirred at room temperature overnight followed by concentration at 55 °C under reduced pressure. Dried residue was mixed with 3 ml pyridine and 3 ml of acetic anhydride. Mixture was incubated at 80 °C for 5 h and co-evaporated with toluene (3 × 10 ml) at 55 °C under reduced pressure. Dried residue was dissolved in 5 ml water and 10 ml DCM and poured in separating funnel. DCM layer was collected and concentrated to 1 ml for GC-MS analysis with analyser (Agilent technologies, USA). GC column was HP5MS (dimension 30 m × 0.25 mm × 0.25 μm) + 10 m Duraguard (Agilent technologies, USA). The injector temperature was 260 °C. GC was carried out under defined conditions (80 °C for 2 min, then 15 °C/min to 200 °C for 2 min, then 4 °C/min to 240 °C for 2 min, then 15 °C/min to 280 °C for 5 min), pressure: 9.4 psi, at a constant flow rate mode with 1 ml/min, carrier gas: He). Final chromatograms of the samples were evaluated by comparing results with standard carbohydrates as well as using a database (NIST) stored in the system.

Evaluation of antioxidant activity

Radical scavenging activity of polysaccharides was evaluated using riboflavin-light-NBT system based on the method of Martinez et al. (2001) where ascorbic acid was used as a standard. The method described by Halliwell et al. (1987) was followed for determination of hydroxyl radical scavenging activity. The radicals were generated by Fenton's reaction in presence of variable concentrations (0.1–1 mg/ml) of polysaccharides and BHA was used as a positive control. In addition, the antioxidant activity was also evaluated using DPPH radicals based on modified method by Gismondi et al. (2013a) where ascorbic acid was treated as standard. DMSO solution of the radical (0.1 mM) was tested against various concentrations of polysaccharides and absorbance was measured at 517 nm. The ability of investigated extract to chelate ferrous ion was determined as described by Dinis et al. (1994). In this assay different concentrations of polysaccharides (0.01–0.1 mg/ml) were compared with EDTA, a positive control. A modified method of reducing power described by Oyaizu (1986) was considered. Various concentrations of polysaccharides (1–3 mg/ml) were mixed in 1.5 ml reaction mixture and the absorbance was measured at 700 nm. Ascorbic acid was used for comparison. The sample concentrations providing 50 % of antioxidant activity or 0.5 of absorbance were calculated from the graphs of antioxidant activity percentages and regarded as EC_{50} value. Furthermore, total antioxidant capacity of the extracts were determined by two different assays such as phosphomolybdenum method (Prieto et al. 1999) and Trolox equivalent antioxidant capacity (TEAC) using ABTS as free radical (Re et al. 1999). The activities were expressed as μg of ascorbic acid equivalent/mg of extract and μg of Trolox equivalent/mg of extract respectively.

Statistical analysis

The assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). Results were compared by means of one-way analysis of variance (ANOVA) 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

Identification by DNA Barcoding

DNA barcoding is a revolutionizing molecular technique for identification of species. Recently scientists have rely on this approach for the documentation and taxonomic revision of specimen (Dentinger et al. 2011; Gismondi et al. 2013b). In the present study, this DNA sequence based method has been applied to verify the species identified morphologically. In this context, DNA from the specimen was subjected to PCR amplification with the combination of primer set ITS1 and ITS4 resulted in 588 bp long stretch. The generated sequence was submitted to GenBank with accession number KT025844. The closest hit of KT025844 was *Macrocybe gigantea* (GenBank HM120872; sequence identity = 512/518 (99 %); gaps = 5/518 (0 %).

Extraction parameters and yield

In order to obtain neutral and water-soluble polysaccharides from the fruiting bodies of *M. gigantea* mushroom, an improved extraction procedure has been followed (Fig. 1). The process included a previous step consisting of an ethanolic extraction in order to remove phenolic compounds and other related molecules. The residue was further subjected to hot water, cold alkaline and hot alkaline methods to prepare three crude polysaccharides namely HWP (whitish), CAP (dark brown) and HAP (light brown) respectively. In general, extraction conditions including time, temperature and solvent

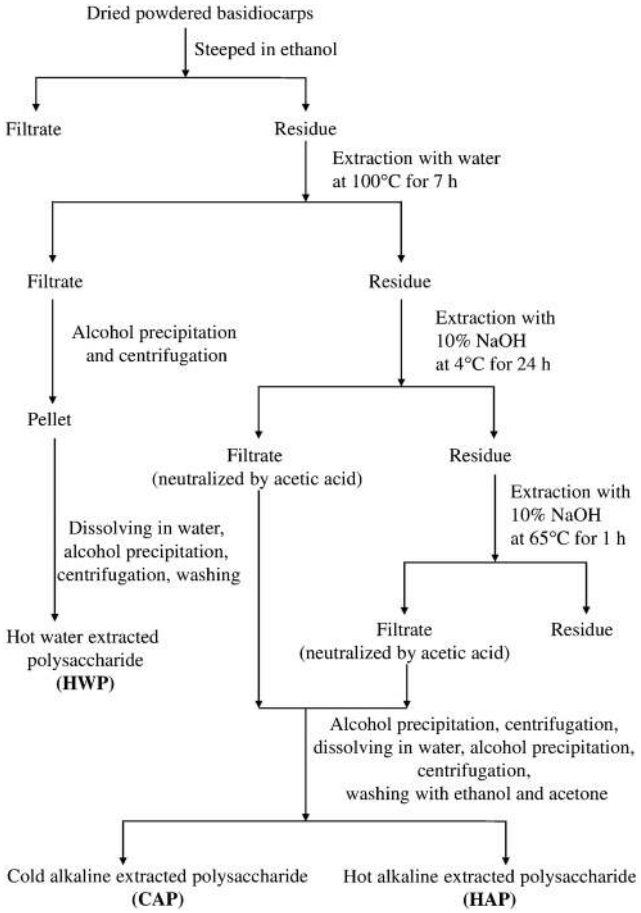

Fig. 1 Diagrammatic representation of extraction method for polysaccharides from *Macrocybe gigantea*

are important for production of a fraction with adequate yield, phytochemicals and high antioxidant activity. Usually hot water extraction method is used for preparation of polysaccharide but it is often associated with lower leaching efficiency, higher extraction temperature and more time-consuming. In contrary, alkaline treatment results in higher precipitation as high pH favours more extraction of polysaccharides from wall complex than neutral pH (Klaus et al. 2011). The same phenomenon was observed in our study where yield was the highest when cold alkaline solution for 24 h was used as extractant, followed by hot water and hot alkaline conditions (Table 1).

Physico-chemical characterization of polysaccharides

Total polysaccharides, protein and phenol contents in the polysaccharides were measured and are presented in Table 1. Total polysaccharide content of extracts varied considerably, i.e., from 21.75 to 41 %, whereas protein content was pretty similar ranging from 7.75 to 10.88 %. CAP consisted of the highest amount of carbohydrate and protein, whereas phenol was only present in HWP. Glucan content was also variable in these polysaccharides where total glucan content ranged from 16.9 to 44.45 %. β-glucan content was determined by subtracting α-glucan content from total glucan content and it ranged from 16.14 to 44.32 %. Total glucan and β-glucan content were in the order of CAP > HAP > HWP, while α-glucan content was in the order of HWP > CAP > HAP (Table 1).

It is well established that glucans with β-(1–6)-glycosidic bonds arrange usually into a triple helix as their tertiary structure. Congo red has ability to react with such structure but does not react with other polysaccharides. The complex is characterized by the shift of the absorption maximum of the Congo red solutions to longer wavelengths (Villares et al. 2012). Figure 2 shows that incorporation of Congo red into HWP caused a bathocromic shift and absorption maximum is moved from 480 to 493 nm indicating triple helix of polysaccharide. CAP and HAP were extracted by using alkaline solution, so the helical structure had already been broken and they appeared to be in aggregate forms.

FT-IR spectroscopy measurements were carried out to identify structure of polysaccharide such as monosaccharide types, glucosidic bonds and functional groups. The absorption spectra showed typical carbohydrate pattern (Fig. 3). A strong band near the region of 3429 cm^{-1} (HWP: 3413.9 cm^{-1}, CAP: 3413.65 cm^{-1}, HAP: 3418 cm^{-1}) was characteristic of carbohydrate ring. Absorption at 2922 cm^{-1} (HWP: 2918.1 cm^{-1}, CAP: 2927.79 cm^{-1}, HAP: 2928.2 cm^{-1}) indicated CH_2 stretching. Peak of amide band at 1631 cm^{-1} (HWP: 1635.4 cm^{-1}, CAP: 1650.09 cm^{-1}, HAP: 1634.48 cm^{-1}) indicated presence of some residual protein in crude polysaccharide. Band at 1410 cm^{-1} (HWP: 1410.7 cm^{-1}, CAP: 1403.43 cm^{-1}, HAP: 1407.79 cm^{-1}) corresponds to OH group of phenolic compound. Absorption peaks between 1250 and 950 cm^{-1} (HWP: 934.2 cm^{-1}, 1026.9 cm^{-1}, 1154.9 cm^{-1}; CAP: 1042.43 cm^{-1}, 1151.81 cm^{-1} 1243.65 cm^{-1}; HAP: 1041.52 cm^{-1}, 1155.82 cm^{-1}, 1201.94 cm^{-1}) indicated that sugar rings were pyranose rings. Absorption region at 1076 (HWP: 1073.3 cm^{-1}, CAP: 1077.29 cm^{-1}, HAP: 1078.39 cm^{-1}) was characteristic of presence of β-glucan due to O-substituted glucose residue. The bands visible in between 500 and 749 cm^{-1} (HWP: 577.5 cm^{-1}, 771 cm^{-1}; CAP: 558.57 cm^{-1}, 620.43 cm^{-1}; HAP: 580.33 cm^{-1}, 608.4 cm^{-1}) signified the presence of R–CH group. Weak bands at around 800 cm^{-1} (CAP: 897.15 cm^{-1}) specified the presence of β-glycosidic bonds and therefore indicated the existence of β-glucan (Klaus et al. 2011; Lim et al. 2005; Synytsya et al. 2009). Thus, on the basis of the aforementioned results it can be concluded that the polysaccharides were mainly composed of β-configuration in pyranose form of sugars. Small amount of lipid and protein were also extracted together with polysaccharide.

Monosaccharide composition of extracts were analysed by HPTLC and the results are shown in Fig. 4. The glucans in these polysaccharides contained mainly glucose

Table 1 Characterization of three polysaccharides of *Macrocybe gigantea*

Characters	HWP	CAP	HAP
Yield of polysaccharide gm/ 100 g of dry fruit body	2.985 ± 0.985^{a}	3.873 ± 0.127^{a}	1.95 ± 0.05^{b}
Total carbohydrate gm/ 100 g of crude polysaccharide	21.75 ± 3.25^{a}	41 ± 2^{b}	37.2 ± 5.8^{b}
Total protein gm/ 100 g of crude polysaccharide	7.75 ± 0.75^{a}	10.88 ± 1.13^{b}	10.75 ± 0.25^{b}
Total phenol gm/ 100 g of crude polysaccharide	1.15 ± 0.85	0	0
Total glucan gm/ 100 g of crude polysaccharide	16.9 ± 1.1^{a}	44.45 ± 1.75^{b}	28 ± 1.5^{c}
α glucan gm/ 100 g of crude polysaccharide	0.67 ± 0.16^{a}	0.105 ± 0.05^{a}	0
β glucan gm/ 100 g of crude polysaccharide	16.14 ± 1.26^{a}	44.32 ± 1.68^{b}	28 ± 1.5^{c}
Congo red reaction	Triple helix	Aggregates	Aggregates
FT-IR peaks	3413.9, 2918.1, 2119.2, 1635.4, 1410.7, 11549, 1073.3, 1026.9, 934.2, 771, 577.5	3413.65, 2927.79, 2112.44, 1650.09, 1544.82, 1403.43, 1243.65, 1151.81, 1077.29, 1042.43, 897.15, 620.43, 558.57	3418, 2928.2, 2124.49, 1634.48, 1407.79, 1201.94, 1155.82, 1078.39, 1041.52, 921.21, 608.4, 580.33
Monosaccharide composition	Xyl: Man: Glc: Gal (0.6: 20: 41: 33)	Xyl: Man: Glc: Gal (3: 3: 89.5: 4)	Xyl: Man: Glc: Gal (4: 5: 84: 7)

Within the same row, means followed by different letters are significantly different at $p \leq 0.05$

with considerable amounts of galactose, mannose and minor proportion of xylose. Other four monosaccharides viz. arabinose, fructose, fucose and rhamnose were not detected. Thus HWP, CAP and HAP appeared to be complex glycan (Table 1). The composition of monosaccharides were further verified by GC-MS (Fig. 5). Each monosaccharide peak in the order of increasing retention time was identified as xylose, mannose, glucose and galactose. The corresponding retention times were 13.219, 16.57, 16.72 and 16.85 min, respectively. The molar ratio of xylose: mannose: glucose: galactose depicts that all three polysaccharides were mainly composed of glucose whereas other monosaccharides were present in the following order of galactose > mannose > xylose (Table 1). Our study coincides with Mizuno et al. who have reported xylose, mannose, glucose and galactose monosaccharides to be present in *M. giagntea* fruit body (Mizuno et al. 1995).

Evaluation of antioxidant activity

To determine free radical scavenging activity the polysaccharides were subjected to five in vitro antioxidant methods. One of widely used assay is superoxide radical scavenging. This primary reactive oxygen species (ROS) can be produced inside human body by auto-oxidative processes. Although it is a weak oxidant, it gives rise to generation of powerful and secondary ROS such as hydroxyl radicals, singlet oxygen; both of which contribute to the oxidative stress (Liu et al. 2013). Therefore, it is important to characterize scavenging ability of superoxide radical of antioxidants. The method used herein is based on generation of superoxide radical by auto-oxidation of riboflavin which in turn reduces yellow dye NBT to produce blue formazon in presence of light. Decrease in absorbance at 560 nm with antioxidants designates the consumption of superoxide

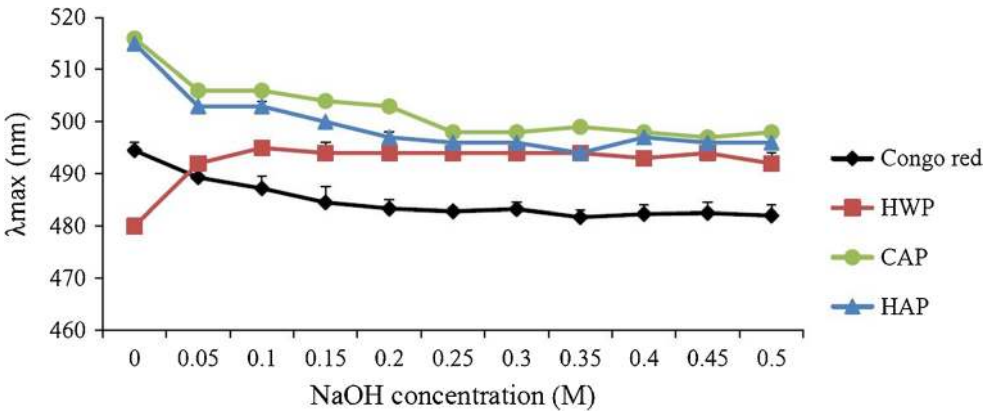

Fig. 2 Changes in absorption maximum of Congo red-polysaccharide complex at various concentrations of sodium hydroxide solution

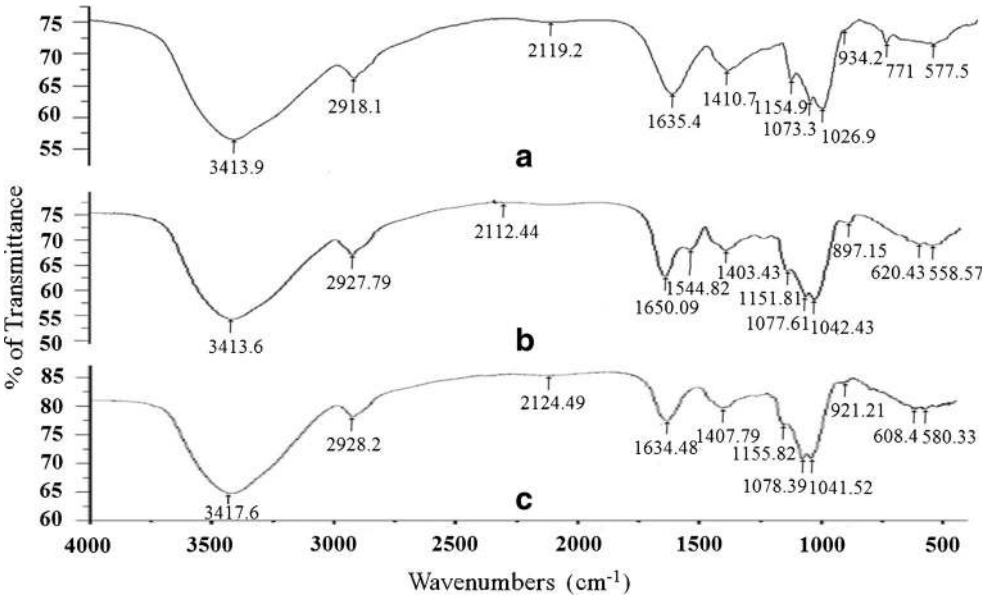

Fig. 3 FT-IR of polysaccahrides from *Macrocybe gigantea*. **a**: Hot water polysaccharide (HWP), **b**: Cold alkaline polysaccharide (CAP), **c**: Hot alkaline polysaccharide (HAP)

anion. Table 2 summarizes the superoxide radical scavenging activity of HWP, CAP and HAP. HWP presented highest potentiality among all fractions as evident by its lowest EC_{50} value. At 0.2 mg/ml concentration HWP, CAP and HAP scavenged superoxide radicals 77.8, 39 and 23 % respectively. The superoxide radical scavenging activity of other fractions was in the order of HWP > CAP > HAP (Fig. 6a). All three polysaccharides exhibited better superoxide radical scavenging activity than that of crude polysaccharidic fraction from *Auricularia auricula* (L.) Underw. as evident by its high EC_{50} value (>2 mg/ml) (He et al. 2012).

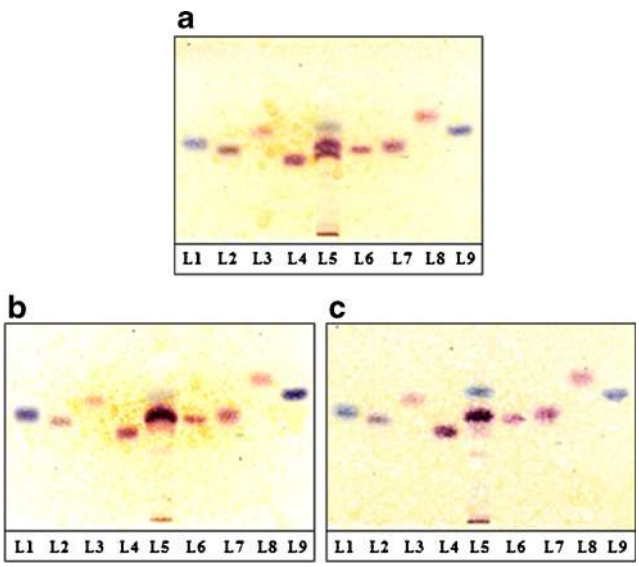

Fig. 4 Identification of monosaccharides in hydrolysed polysaccharides of *Macrocybe gigantea* by HPTLC; **a**: Hot water polysaccharide (HWP) **b**: Cold alkaline polysaccharide (CAP) **c**: Hot alkaline polysaccharide (HAP); Lanes: 1: L-arabinose, 2: D-fructose, 3: D-fucose, 4: D-galactose, 5: sample, 6: D-glucose, 7: D-mannose, 8: D-rhamnose, 9: D-xylose

Hydroxyl radical (OH), the most toxic among all reactive oxygen species, are mainly responsible for the oxidative injury of biomolecules. The radical can damage DNA by attacking purines, pyrimidines and deoxyribose. This free radical can result in hydroxycyclohexadienyl radical which again can give rise to generation of peroxyl radical and phenoxyl type radicals. OH has very short life (half-life of approx. 10^{-9} s) (Valko et al. 2007), thus the antioxidant should have ability to scavenge OH immediately after formation. Principle of the method followed here was based on generation of OH by a reaction of iron-EDTA complex with H_2O_2. Ascorbic acid favours the reaction as it reduces Fe(III) to Fe(II). The radical attacks deoxyribose to form products that upon heating with TBA under acid conditions yields a pink tint. When antioxidant is added to reaction mixture, they compete with deoxyribose for the resulted hydroxyl radical and diminish pink tint formation. The hydroxyl radical scavenging activities of different extracts were concentration dependent. HWP exhibited strongest activity among three polysaccharides (Fig. 6b). At 0.1 to 1 mg/ml concentration HWP, CAP and HAP scavenged hydroxyl radical in the range of 18.72 to 75.59 %, 14.14 to 54.13 % and 20.4 to 55.2 % respectively. The EC_{50} value of these polysaccharides are presented in Table 2 which demonstrated their higher activity than that of crude polysaccharide from *Flammulina velutipes* (Curtis) Singer (He et al. 2012).

The model of scavenging DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. DPPH· is a stable organic nitrogen radical and accepts an electron or hydrogen to become stable diamagnetic radical. Upon reduction, solution

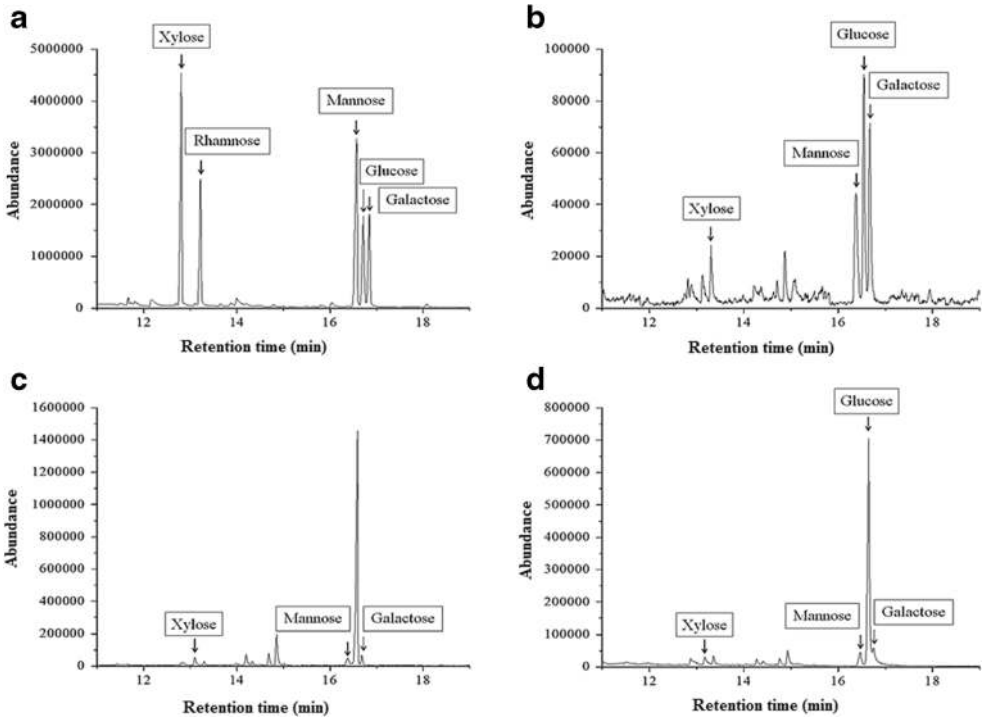

Fig. 5 The GC-MS chromatogram of standards and polysaccharides of *Macrocybe gigantea* **a**: Standards **b**: Hot water polysaccharide (HWP) **c**: Cold alkaline polysaccharide (CAP) **d**: Hot alkaline polysaccharide (HAP) (Retention time of D-xylose: 12.8 min, D-rhamnose: 13.2 min, D-mannose: 16.6 min, D-glucose: 16.7 min, D-galactose: 16.8 min)

of DPPH fades from purple to yellow. Thus a lower absorbance at 517 nm indicates a higher radical scavenging activity of extract (Elmastas et al. 2007). As shown in Fig. 6c, HWP exhibited the best radical scavenging activity at the rate of 21.6, 40 and 56 % at 0.5, 1 and 1.5 mg /ml concentrations respectively. While CAP and HAP presented similar extent of potentiality ranging from 12 to 58 % at 0.5 to 2 mg/ml concentrations respectively. However, EC_{50} value of crude polysaccharides from *Pleurotus ostreatus* (Jacq.) P. Kumm. were very high i.e., >3 mg/ml concentration (Vamanu 2012).

Ferrous iron, a transition metal, has been reported to reduce oxygen to superoxide radical. It helps in Fenton's reaction to generate hydroxyl radical from hydrogen peroxide. Chelating agents have ability stabilize transition metals and inhibit generation of radicals; as a result reduce free radical induced damage in living system (Hasnat et al. 2014). Development of potential chelating agents from natural agents thus may protect human beings from free radical damage. Regarding chelating ability of crude polysaccharides, an experiment was conducted that deals with binding capacity of antioxidant with ferrous ions. At 0.01, 0.05 and 0.1 mg/ml concentrations HWP presented chelation ability of 12.1, 58.5 and 78.2 % respectively. At 0.05 mg/ml concentration CAP and HAP showed chelation ability of 29.2 and 26 % respectively (Fig. 6d). Table 2 summarizes EC_{50} values of HWP, CAP and HAP. HWP presented highest potentiality among all fractions as evident by its lowest EC_{50} value. Vamanu

Table 2 Antioxidant property of three polysaccharides from *Macrocybe gigantea*

Antioxidant activity		HWP	CAP	HAP
EC_{50} value (mg /ml)	Superoxide radical scavenging activity	0.0825 ± 0.003^{a}	0.4125 ± 0.03^{b}	0.69 ± 0.07^{b}
	Hydroxyl radical scavenging activity	0.693 ± 0.11^{a}	0.903 ± 0.1^{a}	0.862 ± 0.04^{a}
	DPPH radical scavenging activity	1.33 ± 0.03^{a}	1.87 ± 0.02^{b}	1.95 ± 0.1^{b}
	Chelating ability of ferrous ion	0.043 ± 0.01^{a}	0.073 ± 0.01^{b}	0.088 ± 0.003^{b}
	Reducing power	1.63 ± 0.13^{a}	2.58 ± 0.08^{b}	2.73 ± 0.13^{b}
Total antioxidant activity (μg ascorbic acid equivalent/mg of dry polysaccharide)		17.39 ± 1.13^{a}	5.96 ± 2.56^{b}	9.19 ± 1.73^{c}
Trolox equivalent antioxidant capacity (μg trolox equivalent/mg of dry polysaccharide)		65.64 ± 3.14^{a}	22.02 ± 0.22^{b}	60.32 ± 1.51^{c}

Each value is expressed as mean ± standard deviation ($n = 3$). Within the same row, means followed by different letters are significantly different at $p \leq 0.05$

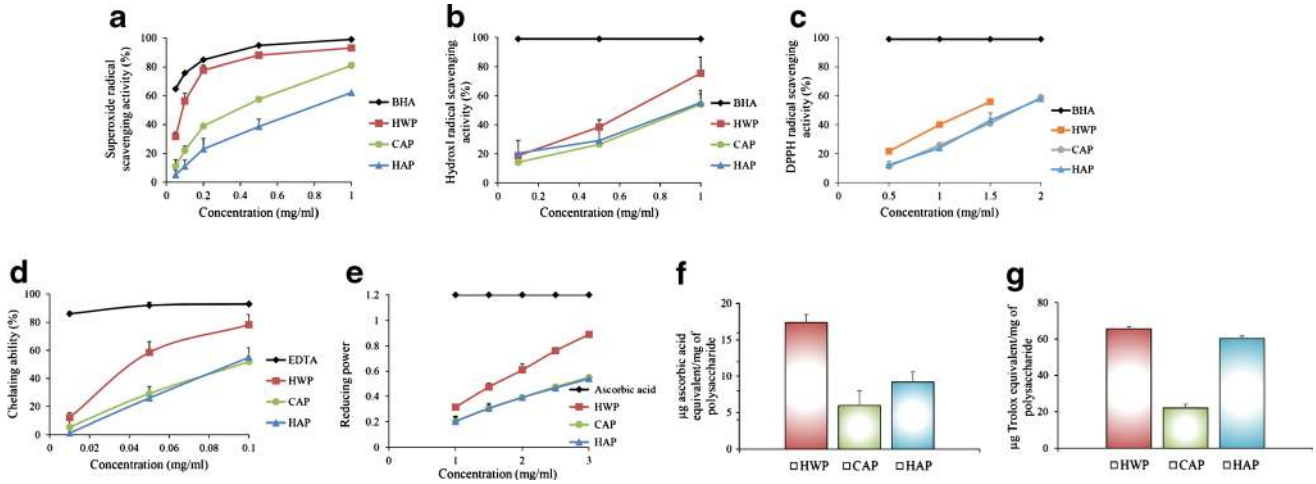

Fig. 6 Comparative antioxidant activity of three polysaccharides of *Macrocybe gigantea* **a**: superoxide radical scavenging activity **b**: hydroxyl radical scavenging activity **c**: DPPH radical scavenging activity **d**: chelating ability **e**: reducing power **f**: total antioxidant activity by phosphomolybdenum method **g**: Trolox equivalent antioxidant activity (Data represent mean ± standard deviation $n = 3$)

(2012) reported chelating ability of crude polysaccharide from *P. ostreatus* which performed 50 % chelating ability at >1 mg/ml concentration.

Previous report had indicated that the reducing power of any bioactive compound is directly related with the electron donation capacity. Antioxidant as a reducer can be described as redox reaction in which one reaction species is reduced at the expanse of the oxidation of the other. The increasing absorbance at 700 nm by measuring the formation of Perl's Prussion Blue indicate increase in reducing capacity (Zeković et al. 2010). HWP showed the best activity among three polysaccharides. At 1.5 mg/ml concentration HWP, CAP and HAP exhibited reducing power of 0.474, 0.304 and 0.306 respectively (Fig. 6e). Our observation coincides with Klaus et al. (2011) where they have isolated two polysaccharides from *Schizophyllum commune* Fr. and showed that hot water extracted polysaccharide (($EC_{50} = 7.9$ mg/ml) possessed higher reducing power than alkaline extracted polysaccharide ($EC_{50} = 12.5$ mg/ml).

Phosphomolybdenum method is an easy 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 all five fractions was investigated and compared against ascorbic acid. HWP showed highest antioxidant capacity followed by HAP > CAP (Fig. 6f).

The scavenging ability of polysaccharide fractions of an organic radical, $ABTS^{\cdot+}$, was measured by TEAC assay. The radical is a blue-green chromophore with characteristic absorption at 734 nm. Principal of the method relies on quenching ability of antioxidants to the preformed radical cation resulting decolourization. The activity is further compared with that of Trolox, a water soluble vitamin E analog. (Pellegrini et al. 2003). Results of the assay using HWP, CAP and HAP extracts are presented in Table 2 and Fig. 6g. All the extracts showed good activity. However, TEAC value of HWP was found to be higher than that of other two fractions indicating its higher potentiality. Li et al. (2012) have reported the $ABTS^{+}$ radical scavenging activities of crude polysaccharides from *Pleurotus eryngii* (DC.) Quél. Results showed that administration of acid extract exhibited higher activity than water fraction, while alkaline extract was found to possess lower potentiality.

Conclusion

Three diverse water soluble crude polysaccharide rich fractions (HWP, CAP and HAP) have been successfully isolated from *M. gigantea*. The identity of macrofungi was confirmed through DNA barcoding of nrDNA ITS region. Results showed that cold NaOH solution was capable of extracting more polysaccharide indicating that the extraction parameters were suitable for producing a fraction with higher yield. FT-IR and spectrophotometric data indicated that polysaccharides were mainly composed of β-configuration in pyranose form of sugars with small amount of conjugated protein. Further analysis by HPTLC and GC-MS presented that all three polysaccharides were composed of identical monosaccharides in the similar order of glucose > galactose > mannose > xylose. However, alkaline extracted polysaccharides consisted of higher proportion of glucose especially cold alkaline method resulted in a crude polysaccharide rich in β-glucan as well as D-glucose. Helical structure was determined by Congo red assay where polysaccharides were found to be in aggregate form except HWP which was consisted of tertiary structure. Beside physico-chemical characterization, bio-activity all three fractions were also evaluated. The results of antioxidant assays showed that HWP demonstrated best activity on

superoxide radical, hydroxyl radical, DPPH radical, chelating ability, reducing power and total antioxidant capacity by phophomolybdenum as well as Trolox equivalent antioxidant capacity methods. However, alkaline extracted polysaccharide also showed their potentiality where CAP was better than HAP in some assays. Thus it can be inferred that HWP may be treated as a potential antioxidant agent for functional food and medicine. In future work it would be of interest to analyze the effect of these physico-chemical parameters on other bio-activities such as immune-modulation, as is currently underway in our laboratory.

Acknowledgments The author, S. K., is grateful to University Grant Commission, Government of India for a grant (UGC/1145/Jr. Fellow (SC) Dated 4.10.2012).

References

Banerjee A, Biswas G, Rai M, Saha GK, Acharya K (2007) Antioxidant and nitric oxide synthase activation properties of *Macrocybe gigantea* (Massee) Pegler and Lodge. Glob J Biotech Biochem 2(2):40–44

Bradford MM (1976) A refined sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248–255

Chatterjee A, Acharya K (2015) Include mushroom in daily diet – a strategy for better hepatic health. Food Rev Int. doi:10.1080/87559129.2015.1057839

Chatterjee S, Saha GK, Acharya K (2011) Antioxidant activities of extracts obtained by different fractionation from *Tricholoma giganteum* basidiocarps. Pharmacologyonline 3:88–97

Dentinger BTM, Didukh MY, Moncalvo J (2011) Comparing COI and ITS as DNA barcode markers for mushrooms and allies (Agaricomycotina). PLoS ONE 6(9), e25081

Dinis TCP, Mudaira VMC, Alnicida LM (1994) Action of phenolic derivatives (acetaminophen, salicylate, and 5-amino salicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys 315:161–169

Dubois M, Gilles A, Hamitton JK, Reders PA, Smith F (1956) Colorimetric method for determination of sugars related substances. Anal Chem 28:350–356

Dutta AK, Acharya K (2014) Traditional and ethno-medicinal knowledge of mushrooms in West Bengal, India. Asian J Pharm Clin Res 7(4): 36–41

Elmastas M, Isildak O, Turkekul I, Temur N (2007) Determination of antioxidant activity and antioxidant compounds in wild edible mushrooms. J Food Comp Anal 20:337–345

Gismondi A, Canuti L, Impei S, Marco GD, Kenzo M, Colizzi V, Canini A (2013a) Antioxidant extracts of African medicinal plants induce cell cycle arrest and differentiation in B16F10 melanoma cells. Int J Oncol 43:956–964

Gismondi A, Fanali F, Labarga JMM, Caiola MG, Canini A (2013b) *Crocus sativus* L. genomics and different DNA barcode applications. Plant Syst Evol 299:1859–1863

Halliwell B, Gutteridge JMC, Arumo OI (1987) The deoxyribose method: a simple test tube assay for determination of rate constants for reactions of hydroxyl radicals. Anal Biochem 165:215–219

Hasnat MA, Pervin M, Debnath T, Lim BO (2014) DNA protection, total phe nolics and antioxidant potential of the mushroom *Russula virescens*. J Food Biochem 38:6–17

He J, Ru Q, Dong D, Sun P (2012) Chemical characteristics and antioxidant properties of crude water soluble polysaccharides from four common edible mushrooms. Molecules 17:4373–4387

Khatua S, Paul S, Acharya K (2013) Mushroom as the potential source of new generation of antioxidant: a review. Res J Pharm Technol 6(5): 496–505

Khatua S, Dutta AK, Acharya K (2015) Prospecting *Russula senecis*: a delicacy among the tribes of West Bengal. PeerJ 3:e810. doi:10.7717/peerj.810

Klaus A, Kozarski M, Niksic M, Jakovljevic D, Todorovic N, Griensven LJLDV (2011) Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. LWT - Food Sci Technol 44: 2005–2011

Kozarski M, Klaus A, Nikšić M, Vrvić MM, Todorović N, Jakovljević D, Griensvenic LJLDV (2012) Antioxidative activities and chemical characterization of polysaccharide extracts from the widely used mushrooms *Ganoderma applanatum*, *Ganoderma lucidum*, *Lentinus edodes* and *Trametes versicolor*. J Food Compos Anal 26:144–153

Leung MYK, Liu C, Zhu LF, Hui YZ, Yu B, Fung KP (2004) Chemical and biological characterization of a polysaccharide biological response modifier from *Aloe vera* L. var. *chinensis* (Haw.) Berg. Glycobiology 14(6):501–510

Li X, Wang Z, Wang L, Walid E, Zhang H (2012) In vitro antioxidant and anti-proliferation activities of polysaccharides from various extracts of different mushrooms. Int J Mol Sci 13:5801–5817

Lim JM, Joo JH, Kim HO, Kim HM, Kim SW, Hwang HJ, Yun JW (2005) Structural and molecular characterization of exopolysaccharides produced by submerged mycelial culture of *Collybia maculata*. Carbohydr Polym 61:296–303

Liu J, Jia L, Kan J, Jin C (2013) In vitro and in vivo antioxidant activity of ethanolic extract of white button mushroom (*Agaricus bisporus*). Food Chem Toxicol 51:310–316

Lo TC-T, Chang CA, Chiu K-H, Tsay P-K, Jen J-F (2011) Correlation evaluation of antioxidant properties on the monosaccharide components and glycosyl linkages of polysaccharide with different measuring methods. Carbohydr Polym 86:320–327

Lung M-Y, Chang Y-C (2011) In vitro antioxidant properties of polysaccharides from *Armillaria mellea* in batch fermentation. Afr J Biotechnol 10(36):7048–7057

Martinez AC, Marcelo EL, Marco AO, Moacyr M (2001) Differential responses of superoxide dismutase in freezing resistant *Solanum curtibolum* and freezing sensitive *Solanum tuberosum* subjected to oxidative and water stress. Plant Sci 160:505–515

Mizuno T, Kinoshita T, Zhuang C, Ito H, Mayuzum Y (1995) Antitumor-active heteroglycans from niohshimeji mushroom, *Tricholoma giganteum*. Biosci Biotech Biochem 59(4):568–571

Nandi AK, Samanta S, Maity S, Sen IK, Khatua S, Devi KSP et al (2014) Antioxidant and immunostimulant β-glucan from edible mushroom *Russula albonigra* (Krombh.) Fr. Carbohydr Polym 99:774–782

Oyaizu M (1986) Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J Nutr Diet 44(6):307–315

Palacios I, García-Lafuente A, Guillamón E, Villares A (2012) Novel isolation of water-soluble polysaccharides from the fruiting bodies of *Pleurotus ostreatus* mushrooms. Carbohydr Res 358:72–77

Patra P, Bhanja SK, Sen IK, Nandi AK, Samanta S, Das D et al (2012) Structural and immunological studies of hetero polysaccharide isolated from the alkaline extract of *Tricholoma crassum* (Berk.) Sacc. Carbohydr Res 362:1–7

Pellegrini N, Serafini M, Colombi B, Del Rio D, Salvatore S, Bianchi M, Brighenti F (2003) Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different in vitro assays. J Nutr 133(9):2812–2819

Pradhan P, Dutta AK, Acharya K (2015) A low cost long term preservation of macromycetes for fungarium. Protocol Exch. doi:10.1038/protex.2015.026

Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of phosphomolybdenum complex: Specific application to the determination of vitamin E. Anal Biochem 269:337–334

Pushpa H, Anand M, Kasimaiah P, Pradeep PJVS, Purushothama KB (2014) Antioxidant and anticancer activity of *Tricholoma giganteum* Massee an edible wild mushroom. Acad J Can Res 7(2):146–151

Qiu T, Ma X, Ye M, Yuan R, Wu Y (2013) Purification, structure, lipid lowering and liver protecting effects of polysaccharide from *Lachnum* YM281. Carbohydr Polym 98(1):922–930

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237

Smolskaite L, Venskutonis PR, Talou T (2014) Comprehensive evaluation of antioxidant and antimicrobial properties of different mushroom species. LWT - Food Sci Technol 60(1):1–10

Synytsya A, Míčková K, Synytsya A, Jablonský I, Spěváček J, Erban V et al (2009) Glucans from fruit bodies of cultivated mushrooms *Pleurotus ostreatus* and *Pleurotus eryngii*: Structure and potential prebiotic activity. Carbohydr Polym 76(4):548–556

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84

Vamanu E (2012) Biological activities of the polysaccharides produced in submerged culture of two edible *Pleurotus ostreatus* mushrooms. J Biomed Biotechnol Article ID 565974, 8 pages, doi: 10.1155/2012/565974

Villares A, Mateo-Vivaracho L, Guillamón E (2012) Structural features and healthy properties of polysaccharides occurring in mushrooms. Agriculture 2:452–471. doi:10.3390/agriculture2040452

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, London, pp 315–322

Yang C, Guan J, Zhang J, Li S (2010) Use of HPTLC to differentiate among the crude polysaccharides in six traditional Chinese medicines. J Planar Chromatorg 23:46–49

Zeković Z, Vidović S, Mujić I (2010) Selenium and Zinc content and radical scavenging capacity of edible mushrooms *Armilaria mellea* and *Lycoperdon saccatum*. Croat J Food Sci Technol 2(2):16–21

Ziestin N, Ben-Zaker R (1993) Peroxidase activity presence of phenolic substance in peduncles of rose flowers. Plant Physiol Biochem 31: 333–339