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Mycochemicals and antioxidant activity of polyphenol-rich fraction of *Termitomyces microcarpus*

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Abstract

Polyphenol-rich fraction from edible mushroom, *Termitomyces microcarpus*, was tested for its antioxidant capacity and estimated for the presence of bioactive components. The extract was found to contain maximum amount of phenols followed by flavonoids, ascorbic acid, β-carotene and lycopene consecutively. HPLC analyses revealed the presence of good amount of pyrogallol (32.07 µg/mg of dry weight of mushroom powder). Findings for *in vitro* antioxidant activities showed that EC_{50} values were below 1mg/ml for DPPH, hydroxyl radical scavenging and superoxide radical scavenging test but reducing ability and chelation of ferrous ion assays showed EC_{50} values at 1.7 mg/ml and 1.3 mg/ml respectively. Results imply that phenol-rich fraction of *T. microcarpus* can be a potential source of natural antioxidant and may be used as food supplement to combat oxidative stress related human disorder.

Introduction

Mushrooms have been a perennial component of the human diet, consumed since antiquity not only as part of the normal diet but also for their good taste, delicacy and aroma (Kalogeropoulos *et al.*, 2013). Research conducted during the last decades has indicated that mushrooms have a number of nutritional and nutraceutical properties and a source of potent bioactive compounds (Ferreira *et al.*, 2009; Yaltirak *et al*., 2009; Jayakumar *et al*., 2009). In spite of the presence of defensive mechanism in the body, at times it becomes insufficient to fight against excessive oxidative damage. In this situation the intake of antioxidants from external sources becomes necessary, which may be in the form of food supplements, where mushrooms have striking significance. Studies on some mushrooms have shown that they possess substantial activity as antioxidant (Khatua *et al*., 2013), anticancer (Chatterjee *et al*., 2014), hepatoprotective (Acharya *et al*., 2012), antiulcer (Chatterjee *et al.*, 2013), cardioprotective (Biswas *et al.*, 2011), antidiabetic (Biswas and Acharya, 2013) and anti-inflamatory (Biswas *et al.*, 2010) agent under different *in-vitro* and *in-vivo* systems.

Termitomyces microcarpus (Berk. & Broome) R. Heim is a small pileate, lamellate and stipitate mushroom of family Lyophyllaceae (Karun and Sridhar, 2013). Ethnomedically this mushroom is used for treatment against gonorrhoea (Oyetayo, 2011). The mushroom is also used as an immunity booster and as an ailment for enhancing recovery of sick people as well as given to lactating mothers (Tibuhwa, 2012). It has been also reported to be enriched with protein, lipid, crude fiber, potassium, calcium and phosphorus by Nabubuya *et al*., (2010). Hence, the antioxidant activity of polyphenol-rich fraction of *T. microcarpus* is examined employing various *in vitro* assay models along with a phytochemical screening for determining the usefulness of this, mushroom as a medicine.

Materials and Methods

Basidiomata sampling and morphological studies

Basidiocarps of *T. microcarpus* were collected from Birbhum district of West Bengal, India during the month of July, 2013. The morphological and ecological features of the collected specimen were noted in the field. The specimen was identified with the help of standard literature (Tibuhwa *et al*., 2010; Karun and Sridhar, 2013) and finally the voucher specimen has been preserved with the accession number AMF 366 (Pradhan *et al.*, 2015).

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Preparation of extract

Polyphenol-rich fraction was extracted according to the method of Dasgupta *et al.* (2014) with slight modification. Dried and powdered basidiocarps of *T. microcarpus* were extracted with ethanol at 25ºC for 2 days. It was then filtered using Whatman No. 1 filter paper. After filtration, the residue was again re-extracted with ethanol. The filtrate was air dried and was boiled with distilled water for 8 hours with continuous stirring. After filtration, 4 volume of ethanol was added to the supernatant and kept undisturbed at 4ºC, for one whole night. After centrifugation the supernatant was concentrated under reduced pressure in a rotary evaporator. This concentrated polyphenol-rich extract of *T. microcarpus* (TmiPre) was stored at -20ºC.

Bioactive compounds

The content of total phenolic compounds in TmiPre was estimated using Folin-ciocalteu reagent. Gallic acid was used as standard (Singleton and Rossi, 1965) and expressed as µg of gallic acid equivalents per mg of dry extract. Aluminium nitrate and potassium acetate were required to determine total flavonoid content (Park *et al*., 1997) and quercetin (5 – 20 µg) was used as standard to express results as µg of quercetin equivalents per mg of dry extract. Quantification of ascorbic acid was done by titration against 2, 6-dichlorophenol indophenol dye using oxalic acid (Rekha et al., 2012). β-carotene and lycopene were estimated by measuring absorbance at 453, 505 and 663 nm (Nagata and Yamashita, 1992).

Detection of phenols and flavonoids by HPLC

Eleven standards of Sigma Aldrich (MO, USA), gallic acid, chlorogenic acid, vanillic acid, p-coumaric acid, ferulic acid, myricetin, salicylic acid, quercetin, cinnamic acid, pyrogallol and kaempferol were used. For quantitative analysis, a calibration curve (10 - 50 µg/ml) for each phenolic standard was constructed (Khatua *et al*., 2015). Sample compounds were identified on the basis of the retention times and absorption spectra of standard materials. Components were quantified by comparing their peak areas with those of standard curves.

Dried TmiPre was dissolved with 1 ml of 50% methanol and diluted to concentration of 0.5 mg/ml. The suspension was filtered through 0.2 µm filter paper. 20 µl filtrate was loaded on the HPLC system (Agilent, USA). Sample with a flow rate of 0.8 ml /min at 25°C was run across Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) for the separation. 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 standard and sample solution was measured at 280 nm.

Hydroxyl radical scavenging activity

The method used was as described by Halliwell *et al.* (1987). Reaction mixture per ml consisted of KH_2PO_4 – KOH buffer (20 mM, pH 7.4), 2-deoxy-D-ribose (2.8 mM), different concentrations (10 – 50 µg/ml) of TmiPre, $FeCl_3$ (100 mM), EDTA (104 µM), ascorbate (100 µM) and H_2O_2 (1 mM) and incubated at 37˚C for 1 hr. The formation of MDA was measured as a pink MDA-TBA chromogen at 535 nm. EC_{50} value of the deoxyribose degradation over the negative control was measured. BHT (butylated hydroxyltoluene) was used as positive control.

Superoxide radical scavenging assay

Superoxide radical scavenging activity was measured following the method of Martinez *et al.* (2001). Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, different concentrations (0.1- 0.5 mg/ml) of TmiPre, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. Reaction was initiated with exposure of sample to light for 10 min. Absorbance was measured at 560 nm. Duplicates were kept in dark and served as blank. BHA was used as a positive control. The the degree of scavenging was calculated by the following equation:

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

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

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity

Various concentrations (0.5- 1.5 mg/ml) of TmiPre were added to 0.004% methanolic solution of DPPH and incubated for 30 min in dark (Shimada *et al*., 1992). Gradual fading of a purple colour were measured at 517 nm against a blank. EC_{50} value is the effective concentration of extract that scavenged DPPH radicals by 50%. Ascorbic acid was used as positive control. The degree of scavenging was calculated by the same equation used in case of superoxide radical scavenging assay.

Total antioxidant capacity assay

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract (Prieto *et al.*, 1999). The tubes

containing extract (1 mg/ml) and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were incubated at 95˚C for 90 min. Then mixture was cooled to room temperature. Absorbance was recorded spectrophotometrically for each solution at 695 nm against blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

Chelating effect on ferrous ions

Different concentrations of TmiPre (0.5-1.5 mg/ml) were mixed with 3.7 ml of water and 0.1 ml of 2 mM ferrous chloride. Again 0.2 ml of 5 mM ferrozine was added. It was incubated for 10 min at room temperature. Absorbance was determined at 562 nm against blank (Dinis *et al*., 1994). EDTA was used as positive control. The percentage of inhibition is given by this formula:

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

Where A_0 was the absorbance of the control and A_1 the absorbance in the presence of TmiPre.

Reducing power

Reaction mixture contained variable concentrations (1.0 -2.0 mg/ml) of TmiPre, 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml 1% potassium ferricyanide and incubated for 20 min. Then 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 12000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml. of 0.1% ferric chloride (Oyaizu 1986). Absorbance was recorded at 700 nm. An increase in absorbance of the reaction mixture signified increase in reducing power of the sample.

Results and Discussion

Bioactive compounds

Extractive yield of TmiPre was 24%. Phytochemicals were found to be present in the order of phenols> flavonoids> ascorbic acid and β-carotene ≈ lycopene. Phenolic compounds are known to be powerful chain-breaking antioxidants. Their hydroxyl groups provide them scavenging ability. The phenolic compounds have antioxidative action. In this study, the total phenolic content of TmiPre was noted to be 7.258 ± 0.323 µg/mg which was much higher than that of *Amanita vaginata* (5.335 µg/mg) (Paloi and Acharya, 2013). The estimated flavonoid content of TmiPre was 1.976 ± 0.041 µg/mg; higher than *Amanita vaginata* (0.81 µg/mg) (Paloi and Acharya, 2013). Ascorbic acid is reported to interact directly

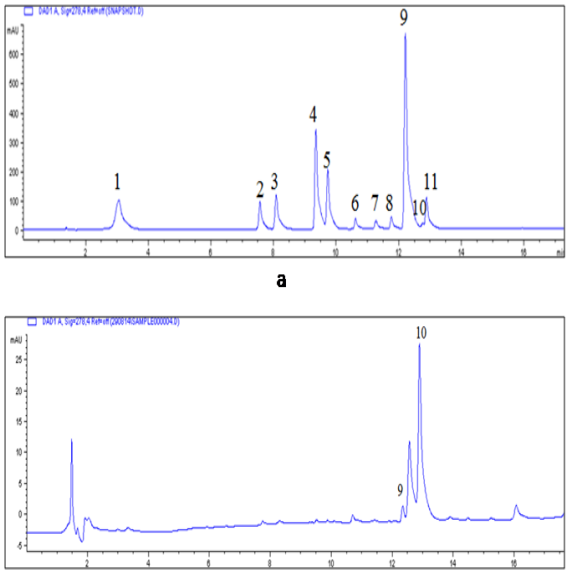

Figure 1. HPLC chromatogram:
a Peaks of standards: 1: gallic acid, 2: chlorogenic acid, 3: vanillic acid, 4: p-coumaric acid, 5: ferulic acid, 6: myricetin, 7: salicylic acid, 8: quercetin, 9: cinnamic acid, 10: pyrogallol, 11: kaempferol.
b Peaks of TmiPre: 9: cinnamic acid, 10: pyrogallol.

with radicals such as O_2^- and OH in plasma, thus preventing damage of cells (Beyer *et al*., 1994). In the present study, the ascorbic acid content of TmiPre (0.457 ± 0 µg/mg) was higher when compared to *Russula albonigra* (0.074 µg/mg) (Dasgupta *et al*., 2014) and *Amanita vaginata* (0.00035 µg/mg) (Paloi and Acharya, 2013). β-carotene protects against cancer and cardiovascular diseases (Mueller and Boehm, 2011). Lycopene, on the other hand is one of the antioxidants that prevents carcinogenesis and atherogenesis (Rao and Agarwal, 2000). In this study β-carotene and lycopene were found in very less amounts i.e., 0.0796 ± 0.008 µg/mg and 0.0721 ± 0.0003 µg/mg respectively.

HPLC has been done to predict phenolic composition of TmiPre. Figure 1a shows eleven phenolic substances which were analysed as standards but two of them were detected in TmiPre (Figure 1b). Our findings revealed that the dominant phenolic compound in TmiPre was pyrogallol (32.07 µg/mg of dry weight of mushroom) followed by cinnamic acid (1.0944 µg/mg of dry weight of mushroom).

Hydroxyl radical scavenging activity

Hydroxyl radicals are the major active oxygen species capable of modifying almost every molecule in the living cells and are toxic too. Furthermore, this radical is also capable of stealing hydrogen atoms from unsaturated fatty acids leading to quick initiation of lipid peroxidation process (Aruoma, 1987; Kappus, 1991). Formation of pink chromogen

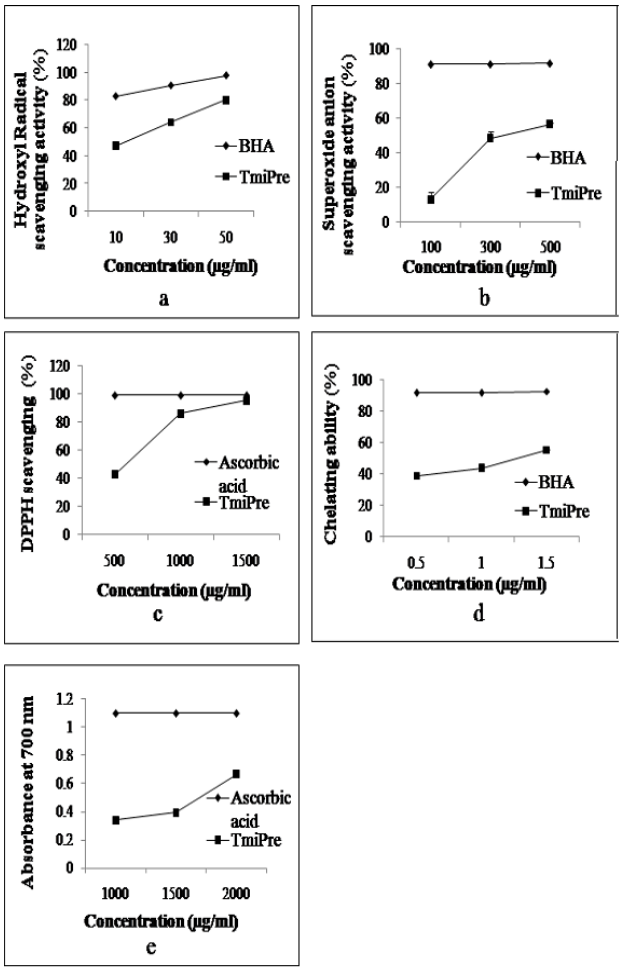

Figure 2. Antioxidative activities of polyphenol-rich fraction of *Termitomyces microcarpus* (TmiPre): a OH radical scavenging activity, b Superoxide radical scavenging activity, c DPPH radical scavenging activity, d Ferrous ion chelating ability and e Reducing power. Results are the mean ± SD of three separate experiments, each in triplicate.

upon heating with TBA at low pH was detected at 535 nm. Data presented in Figure 2a showed that TmiPre was a potent scavenger of hydroxyl radicals which increased in a concentration dependent manner. The EC_{50} value of the TmiPre was 16 µg/ml.

Superoxide radical scavenging assay

Superoxide radical is known to be very harmful to cellular components. As per the data presented in Figure 2b, the scavenging activity of TmiPre increased with increasing concentration of the polyphenol-rich extract. The EC_{50} value was found to be 0.35 ± 0.05 mg/ml for TmiPre. Dasgupta *et al.* (2014) have reported that polyphenol-rich extract of *Russula albonigra* (edible) showed EC_{50} value at 0.74 mg/ml that was higher than TmiPre. These results suggested that TmiPre exhibited scavenging effect on superoxide anion radical generation that could help prevent or ameliorate oxidative damage.

DPPH radical-scavenging activity

Antioxidants are capable of donating electron or hydrogen atom and DPPH has the capacity to accept an electron or hydrogen to earn stability (Anokwuru *et al*., 2011). A solution containing DPPH and methanol gives violet colour. But when electrons are donated to DPPH, then solution gets faded. A lower absorbance at 517 nm (Seal *et al*., 2013) indicates a higher radical-scavenging activity of the extract. From Figure 2c EC_{50} value of TmiPre with regards to DPPH radical scavenging activity was seen to be 0.60 ± 0.01 mg/ml, which was much lower than the EC_{50} value (4.3 ± 0.3 mg/ml) of ascorbic acid, a potent scavenger. Ability to inhibit 50% DPPH radicals of polyphenol-rich extract of *Amanita vaginata* was reported to have EC_{50} at 1.45 mg/ml (Paloi and Acharya, 2013) which was higher than that of TmiPre.

Total antioxidant capacity assay

Total antioxidant capacity is measured by the formation of green phosphomolybdenum complex. The TmiPre results in the reduction of Mo (VI) to Mo (V) and form a green phosphate/Mo (V) complex. The colour intensity is determined with the maximal absorption at 695 nm. Ascorbic acid is used as standard. The assay conducted showed that 1mg of extract is as functional as approximately 0.075 ± 0.025 mg of ascorbic acid. *Russula albonigra* was reported to have 30 µg AAE (Dasgupta *et al.*, 2014).

Chelating effect on ferrous ions

Ferrous ions have the ability to enhance free radical formation, as other transition metals. Undoubtedly, the compounds which interfere with the catalytic activity of metal ions can also affect the preoxidative process. Ferrous ions are most powerful prooxidants among various species of metal ions (Yaltirak *et al.*, 2009). Chelating activity was determined by the ferrozine assay. Ferrozin forms complexes with Fe^{2+}. Lower EC_{50} value indicates higher antioxidant activity. That of TmiPre was 1.3 ± 0.03 mg/ml, calculated from the graph in Figure 2d. EC_{50} values of different polyphenolic extracts were found to be in the descending order of *Amanita vaginata* > TmiPre > *Russula albonigra* (Paloi and Acharya, 2013, Dasgupta *et al*., 2014).

Reducing power

Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. As the data indicates TmiPre, also shows reduction Fe^{3+} to Fe^{2+}. This change can be monitored at 700 nm, by measuring the intensity of the Perl's

Prussian Blue colour. BHA, a synthetic antioxidant was used as standard. As seen from Figure 2e, EC_{50} value of TmiPre was 1.7 ± 0.2 mg/ml. Polyphenol-rich fraction of *Amanita vaginata* had EC_{50} at a concentration of 1.55 mg/ml (Paloi and Acharya, 2013). On the other hand *Russula albonigra* was reported to have EC_{50} at 1.12 mg/ml (Dasgupta *et al.*, 2014).

Conclusion

The polyphenol-rich extract of *Termitomyces microcarpus* contains two dominant phenolic compounds like pyrogallol and cinnamic acid, along with other bioactive compounds such as ascorbic acid, β-carotene and lycopene. Positive results regarding antioxidant activities of the mushroom as evident from various *in vitro* assays which includes ferrous iron chelating, ferric iron reducing, superoxide radical scavenging, DPPH free radical scavenging, hydroxyl radical scavenging, and total antioxidant capacity determining assay could add to the mushroom's usefulness. Hence, it can be suggested that *Termitomyces microcarpus* can serve as functional food and be a boon to the mankind to prevent several hazardous diseases.

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