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Research Article

BIOCOMPONENTS AND BIOPROSPECTS OF ETHANOLIC EXTRACT OF *TERMITOMYCES HEIMII*

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ABSTRACT

Objective: Wild edible mushroom, *Termitomyces heimii*, was obtained from lateritic zone of West Bengal to determine presence of phytochemicals and anti-oxidative properties. **Methods:** Ethanolic extract of *Termitomyces heimii* was prepared and analyzed for free radical scavenging activity in different test systems, namely 2, 2 -diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, total antioxidant capacity (TAC) and reducing power determining assays. **Results:** It showed best reducing ability with effective concentration 50 value at only 0.575 mg/ml concentration. It also indicated presence of DPPH radical scavenging activity and gave good results for the TAC assay. Estimated phytochemical component analysis were found to be in an order of total phenol > total flavonoids > ascorbic acid > β carotene > lycopene. Among phenolic compounds presence of pyrogallol and vanillic acid were detected with high performance liquid chromatography. **Conclusion:** Hence, the study supports use of *T. heimii* as a therapeutic agent.

Keywords: Antioxidants, Ethanolic extract, High performance liquid chromatography, Phytochemicals.

INTRODUCTION

Maintenance of equilibrium between free radical production and antioxidant defenses is an essential condition for proper biological functioning. When this equilibrium has a tendency to get misbalanced we say that the organism is in oxidative stress. In this situation, excess free radicals may damage cellular lipids, proteins and DNA, affecting normal function and leading to various diseases. The free radicals are constantly produced during the normal cellular metabolism, mainly in the form of reactive oxygen species and reactive nitrogen species, which is nullified normally by the endogenous defense mechanisms. When over production of free radicals take place may be as a consequence of exposure to harmful exogenous sources, then comes in the need to use antioxidant supplements. Natural products with antioxidant activity may help the endogenous defense system. In this perspective the antioxidants present in the diet assume a major importance as possible protector agents reducing oxidative damage. Particularly, the antioxidant properties of wild mushrooms have been extensively studied [1]. Furthermore, extract/bioactive components of mushrooms also showed strong protection against different killer diseases [2,3], where free radical generation is an important causal factor [4].

Wild edible mushrooms might be used directly in diet and promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present. Here an attempt is made for a study on ethanolic extract of *Termitomyces heimii* Natarajan for quantitative analysis of biocomponents present and antioxidant properties.

METHODS

Material collection and sample preparation

The mushroom *T. heimii* was collected from Midnapore district of West Bengal. With proper scientific measures they were brought to the laboratory and cleaned well. Macroscopic and microscopic studies were done for identification.

Fruit bodies of *T. heimii* were dried in the oven and powdered in a mixer and grinder. 5 g of the amorphous form of fruit bodies were extracted with 200 ml of 99% ethanol for 2 days at room temperature to remove phenolic compounds and lipid. Residue was filtered and re-extracted with ethanol. The solvent was separated through Whatman No. 1 filter paper. After filtration, solvent was evaporated by a rotary evaporator under vacuum and stored at −20°C until further analysis. Extraction was done according to the method of Dasgupta *et al.*, 2013 [5].

Chemicals

L-ascorbic acid, quercetin, gallic acid, potassium ferricyanide, ferric chloride, Folin-Ciocalteu reagent, 2, 2 -diphenyl-1-picrylhydrazyl (DPPH), sodium carbonate, trichlroacetic acid (TCA), ammonium molybdate, sodium sulfate, sulfuric acid, acetone, n-hexane, sodium acetate, aluminum nitrate, sodium bicarbonate, dichlorophenol indophenols and oxalic acid were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All chemicals and reagents used were of analytical grade.

Assays

Reducing power

Determination of reducing power of ThEe was done following the method of Oyaizu (1986) [6]. Variable concentrations (0.5-1.5 mg/ml) of ThEe were added to 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. After incubation of 20 minutes at room temperature 2.5 ml of 10% TCA was added to the mixture. It was then centrifuged for 10 minutes at 12,000 rpm. 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride. Absorbance was noted at 700 nm. An increase in absorbance of the reaction mixture signified increase in reducing power of the sample.

Total antioxidant capacity (TAC) assay

According to Prieto *et al.* (1999) [7] the assay is based on the reduction of Mo (VI) to Mo (V) by ThEe. When acidic pH is maintained then subsequent formation of a green phosphate/Mo (V) complex is seen. 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 minutes. After cooling, absorbance for each solution was recorded spectrophotometrically at 695 nm against blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

DPPH radical-scavenging activity

As per the method of Shimada *et al.* (1992) [8] 0.004% methanolic solution of DPPH was prepared and concentrations (0.5-1.5 mg/ml) of ThEe were added to it. The mixture was shaken vigorously and left to stand for 30 minutes in the dark. Gradual fading of purple color against

various concentrations were measured at 517 nm against a blank. Effective concentration 50 (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 following equation:

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

Where A_0 was the absorbance of the control and A_1 was the absorbance if sample is present.

Phytochemical analysis

Determination of ascorbic acid content

Ascorbic acid content was determined following method of Rekha *et al*. (2012) [9] but few modifications were undertaken. Standard used contained ascorbic acid (100 µg/ml) made up to 10 ml with 0.6% oxalic acid. It was titrated with 2, 6- dichlorophenol indophenol dye. The amount of dye consumed (V_1 ml) is equivalent to the amount of ascorbic acid. Similarly, the sample (w µg/ml) was titrated with the dye (V_2 ml). The amount of ascorbic acid was calculated using the formula,

$$\text{Ascorbic acid } (\mu g/mg) = [\{(10\ \mu g/V_1 ml) \times V_2 ml\} \times w\ \mu g] \times 1000$$

Determination of total β -carotene and lycopene content

Method of Nagata and Yamashita (1992) [10] was followed to determine β-carotene and lycopene. 100 mg of mushroom extract (10 mg/ml) was shaken with 10 ml of acetone-hexane mixture (4:6) for 1 minutes and absorbance measured at 453, 505 and 663 nm, immediately. β-carotene and lycopene contents were calculated according to the following equations:

$$\text{Lycopene (mg/100 mg)} = 0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$$

$$\beta\text{-carotene (mg/100 mg)} = 0.216\ A_{663} - 0.304\ A_{505} + 0.452\ A_{453}$$

Determination of total flavonoid content

Flavonoid concentration was determined in accordance with the method as described by Park *et al.* (1997) [11]. 1 ml of mushroom extract (100 mg/ml) was diluted with 4.3 ml of 80% aqueous methanol, 0.1 ml of 10% aluminum nitrate and 0.1 ml of 1 M aqueous potassium acetate. Mixture was incubated for 40 minutes. The absorbance was noted at 415. Quercetin (5-20 µg) was used as standard.

Determination of total phenols

Total phenol content of the extract was determined following the method of Singleton and Rossi (1965) [12]. 1 ml of ethanolic extract (100 mg/ml) was mixed with 1 ml Folin–Ciocalteu reagent. Then incubation for 3 minutes at room temperature. 1 ml of 35% saturated Na_2CO_3 solution was added to the reaction mixture. Volume was adjusted to 10 ml with distilled water and incubated for 90 minutes in dark. Absorbance was measured at 725 nm. Gallic acid (10-40 µg) was used as standard. Total phenol content of the sample was expressed as mg of gallic acid equivalents (GAE) per gram of extract.

Detection of phenols and flavonoids by high performance liquid chromatography (HPLC)

11 standards, gallic acid, chlorogenic acid, vanillic acid, *p*-coumaric acid, ferulic acid, myricetin, salicylic acid, quercetin, cinnamic acid, pyrogallol and kaemphferol were purchased from Sigma Aldrich (MO, USA). For quantitative analysis, a calibration curve (10-50 µg/ml) for each phenolic standard was constructed (gallic acid: y=34.773x −9.2238, R^2=0.9991; chlorogenic acid: y=13.776x −2.9025, R^2=0.9993; vanillic acid: y=19.225x +0.2588, R^2=0.9994; *p*-coumaric acid: y=49.773x −10.541, R^2=0.9994; ferulic acid: y=30.425x −2.8188, R^2=0.9995; myricetin: y=5.0676x −6.0375, R^2=0.9937; salicylic acid: y=4.4974x −0.4763, R^2=0.9994; quercetin: y=5.2478x −5.9763, R^2=0.9954; cinnamic acid: y=108.07x −111.55, R^2=0.9979; pyrogallol: y=1.9986x −1.32,R^2=0.9957; kaemphferol: y=17.01x −16.505, R^2=0.9909). 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 ThEe 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). Separation was achieved on a Agilent Eclipse Plus C18 column (100 mm × 4.6 mm, 3.5 µm) using a flow rate of 0.8 ml/minutes 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 minutes, 5% A; 2-5 minutes, 15% A; 5-10 minutes, 40% A; 10-15 minutes, 60% A; 15-18 minutes, 90% A. The absorbance of standard and sample solution was measured at 280 nm.

RESULTS

Reducing power

Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity [6]. In determination of reducing power, the reducers (i.e., antioxidant) causes oxidized form of Fe^{3+} in ferric chloride to ferrous (Fe^{2+}). The absorbance increases at 700 nm due to formation of Perl's Prussian Blue, which indicates an increase in reductive ability. Results (Fig. 1) showed that EC_{50} for the reducing power of ThEe was of 0.575±0.02 mg/ml.

TAC

TAC of ThEe was determined by the formation of green phosphomolybdenum complex. The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of green phosphate/Mo (V) complex with the maximal absorption at 695 nm. Total antioxidant activity of the extract was estimated, using ascorbic acid as standard. Analyzing the data, it was found that 1 mg of extract is as functional as approximately 0.01±0.005 mg of ascorbic acid (expressed as 100 µg AAE).

DPPH assay

DPPH is a stable free radical that has a characteristic absorption at 517 nm. The use of stable DPPH radical has the advantage of being unaffected by side reactions such as enzyme inhibition and metal chelation [13,14]. ThEe showed DPPH radical scavenging activity in a dose-dependent manner i.e., a decrease in absorption was observed on treatment with gradual increase in concentration of ThEe (Fig. 2). EC_{50} of DPPH radical scavenging activity was 1.25±0.3 mg/ml.

Phytochemical analysis

Quantitative values of extractive and important phytochemicals present in ThEe were presented in Table 1. Phytochemicals were found

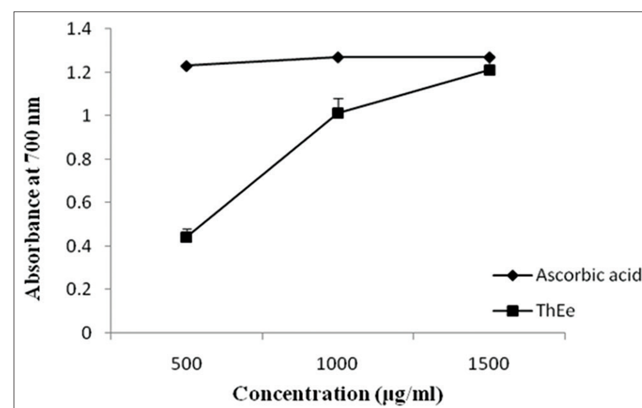

Fig. 1: Reducing power of ethanolic fraction of *Termitomyces heimii* (ThEe). Results are the mean±standard deviation of three separate experiments, each in triplicate

to be present in the order of phenols > flavonoids > ascorbic acid and β-carotene ≈ lycopene.

Detection of phenols and flavonoids by HPLC

HPLC helps to predict phenolic composition of the fraction. As shown in Figs. 3 and 4, eleven phenolic substances were analyzed and two of them were detected in ThEe. Our findings revealed that the dominant phenolic compound in ThEe was pyrogallol (40.704 μg/mg of dry weight of mushroom), followed by vanillic acid (2.66 μg/mg of dry weight of mushroom).

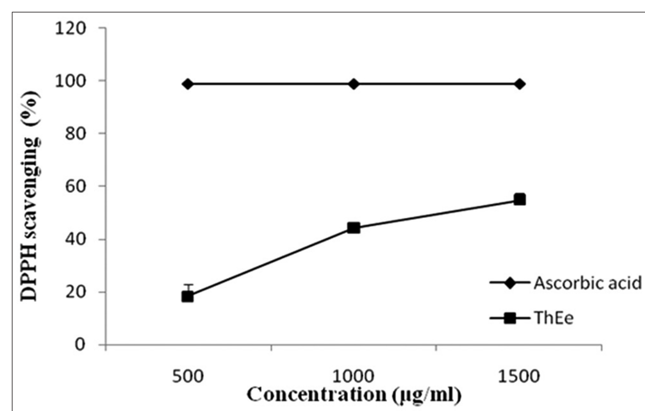

Fig. 2: 2, 2 -diphenyl-1-picrylhydrazyl radical scavenging activity of ethanolic fraction of *Termitomyces heimii* (ThEe). Results are the mean±standard deviation of three separate experiments, each in triplicate

DISCUSSION

Reducing power of any compound can be an identifying character so that it can be considered as an antioxidant. Reducers work by breaking the chain of free radicals by donating hydrogen atoms [15]. This change can be monitored at 700 nm, by measuring the change in the yellow of the test solution to various shades of green and blue. Greater absorbance indicates greater reducing power [16]. The reducing power of ThEe was compared to that of butylated hydroxyanisole, a synthetic antioxidant. Ethanolic extracts of *Termitomyces medius*, *Termitomyces microcarpus*, *Amanita vaginata*, *Pleurotus flabellatus*, *Russula albonigra* gave EC_{50} values at 2.05, 1.98, 0.91, 0.84 and 0.69 mg/ml concentration respectively which were much higher than ThEe, as seen from Fig. 1 [5,17-19].

TAC was measured by the formation of green phosphomolybdenum. High antioxidant activity of ThEe is indicated by high absorbance value. The TAC of ThEe may be attributed to their chemical composition and phenolic acid content. A recent study showed that some bioactive compounds from citrus fruits had strong total antioxidant activity, which was probably due to the presence of flavonoids, carotenoids, and ascorbic acid [20]. Total antioxidant activity suggests that the electron donating capacity of ThEe and thus it may act as radical chain terminator, ultimately transforming reactive free radicals into more stable non-reactive products [18].

In methanol solution DPPH, a stable organic N_2-centered free radical, produces violet color with absorption maxima at 517 nm [21].

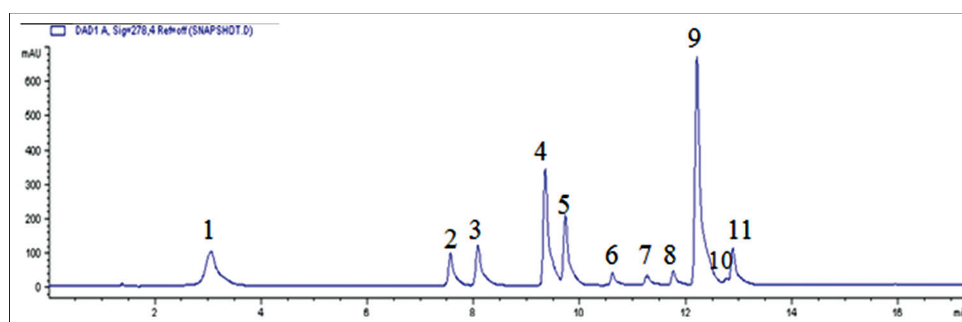

Fig. 3: High performance liquid chromatography chromatogram of standards: (Peaks 1: Gallic acid, 2: Chlorogenic acid, 3: Vanillic acid, 4: *p*-coumaric acid, 5: Ferulic acid, 6: Myricetin, 7: Salicylic acid, 8: Quercetin, 9: Cinnamic acid, 10: Pyrogallol, 11: Kaempferol)

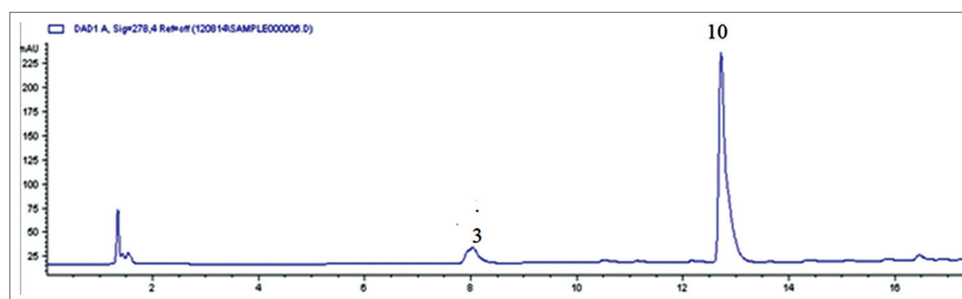

Fig. 4: High performance liquid chromatography chromatogram of ThEe: (3) vanillic acid, (10) pyrogallol

Table 1: Extractive yield, ascorbic acid, β-carotene, lycopene, total phenol, total flavonoid contents of ethanolic extract of *T. heimii* (ThEe)

Extractive yield (%)	Ascorbic acid (μg/mg)	β-carotene (μg/mg)	Lycopene (μg/mg)	Total flavonoids (μg/mg)	Total phenol (μg/mg)
7%	0.259±0.036	0.0071±0.00014	0.0062±0.0004	0.335±0.022	2.768±0.268

Values are mean±SD of three separate experiments each in triplicate. Total phenols are expressed in GAE, and flavonoids as QAE, GAE: Gallic acid equivalent, QAE: Quercetin equivalent, *T. heimii: Termitomyces heimii*

Antioxidant molecules can scavenge DPPH by providing hydrogen atoms or by electron donation. But when electrons are donated to DPPH, then solution starts turning to yellow color. From Fig. 2 EC_{50} value of ThEe with regards to DPPH radical scavenging activity was 1.25±0.3 mg/ml, which was much lower than the EC_{50} value (4.3±0.3 mg/ml) of ascorbic acid, a potent scavenger. In similar studies on ethanolic extract of some mushrooms EC_{50} values were of descending order; *P. flabellatus* (1.8 mg/ml) > *R. albonigra* (1.7 mg/ml) > *T. microcarpus* (1.66 mg/ml) > *Macrocybe crassa* (1.65 mg/ml) > *A. vaginata* (1.48 mg/ml), all of which were higher than that of ThEe [5,19,15,21,18].

Ascorbic acid content of ThEe was also found in minor quantities. Even β-carotene and lycopene were also found in negligible amount. Total flavonoid content was determined by using quercetin as standard (y=0.0094x −0.0106, R^2=0.984). ThEe contained flavonoid as 0.335±0.022 μg quercetin equivalent/mg of extract. This assay was highly sensitive for flavonoids as only flavonoids can form colored complexes with aluminum chloride in alkaline medium. Total phenolic content was evaluated by the Folin–Ciocalteau method and recorded as GAE by reference to standard curve (y=0.0124x −0.0262, R^2=0.997). Transfer of electrons from phenolic compounds and reducing agents present in reaction mixture forms blue complexes, this can be monitored spectrophotometrically at 725 nm. ThEe was found to contain phenol as much as 2.768±0.268 μg GAE to per mg of extract. As bio components contribute to the antioxidant property of ThEe and here pyrogallol is the most dominant phenol present, so it may play the most important role.

CONCLUSION

At the end of this study we may conclude that the ethanolic extract of *T. heimii* (ThEe) is an effective antioxidant for the significant results in various *in-vitro* assays including ferric iron reducing, DPPH free radical scavenging and total antioxidant activity. This may be correlated with the high content of pyrogallol. Earlier reports from our laboratory have showed that mushroom extracts having strong free radical scavenging activity also have potential anticancer [22], hepatoprotective [23], antiulcer [24], cardioprotective [25], anti-inflammatory [26] and antidibetic [27] activities in different *in-vivo* and *ex-vivo* models. As the studied extract has shown potential antioxidant activity, further work is under progress to identify its other medicinal properties.

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