



## Phytochemical Analysis and Evaluation of Antioxidant Efficacy of Ethanolic Extract of *Termitomyces medius*

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### ABSTRACT

Oxidative stress related diseases are grasping the human race rapidly, hence, the need for antioxidants are increasing. The objective of this study was to investigate the antioxidant activity of ethanolic extract of *Termitomyces medius* using various *in-vitro* assays. Content of phytochemicals, when analysed, were found to be in the following order; total phenol > total flavonoids > ascorbic acid > β carotene > lycopene. DPPH radical scavenging test showed the best results with EC<sub>50</sub> value at concentration of 0.5 mg/ml. Chelation of ferrous ion, superoxide radical scavenging ability and reducing power assays had their 50 % inhibition potential in the range of 0.68 – 2.05 mg/ml. It can be inferred, from the results that, ethanolic extract of *Termitomyces medius* can be used as a potent antioxidant for medication.

**Keywords:** Ethanolic extract, Mushroom, Phytochemicals, Reactive oxygen species.

### INTRODUCTION

The imbalance between pro-oxidants and antioxidants in favour of the oxidants potentially leads to oxidative damage to various essential biomolecules including proteins, lipids, lipoproteins and DNA. This oxidative damage is a critical etiological factor for the development of several pathophysiological conditions in human, such as cancer, diabetes, cardiovascular problem, neuro degenerative diseases and also in the aging process.<sup>1</sup> Antioxidants have been reported to prevent oxidative process by reacting with free radicals, chelating catalytic metals and also by reacting as oxygen scavenger.<sup>2</sup> Therefore research to identify antioxidative compounds is an important issue. Although several synthetic antioxidants such as BHT (butylated hydroxy toluene) and BHA (butylated hydroxy anisole) are available in the market but are quiet unsafe due to their carcinogenic and hepatotoxic effects.<sup>1</sup> As a consequence this evidence accelerated the search for natural source for antioxidants such as plants.

Mushrooms have long been appreciated for their flavour and texture. Now they are recognised as not only a nutritious food but also as an important source of biologically active compounds possessing medicinal value.<sup>3</sup> Mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, terpenes and steroids. Also, a mushroom containing phenolic compounds has been found to be an excellent antioxidant and synergist that is not mutagenic. They may act directly as antioxidant<sup>1</sup> or prevent underlying oxidative stress related pathological conditions such as cancer<sup>4</sup>, heart ailments<sup>5</sup>, diabetes<sup>6</sup>, inflammation<sup>7</sup>, gastric ulcer<sup>8</sup>, hepatic damage<sup>9,10</sup>, microbial pathogens<sup>11,12</sup>, parasites<sup>13</sup> etc. In this study, the antioxidative activity of ethanolic extract of this mushroom has been performed, employing various *in*

*vitro* assays: such as chelating of ferrous ion, reducing power, superoxide radical scavenging, total antioxidant capacity and DPPH radical scavenging tests, along with some phytochemical tests, to estimate the constituent antioxidant components.

### MATERIALS AND METHODS

#### Material collection and sample preparation

The mushroom *Termitomyces medius* was collected from West Midnapore district of West Bengal. With proper scientific measures they were brought to the laboratory and cleaned well. After macroscopic and microscopic observations the mushroom was identified using standard literature.<sup>14, 15</sup>

Known weight of mushrooms were dried in the oven at 50°C and powdered in a mixer and grinder. Amorphous form of fruit bodies of *T. medius* were extracted with ten volumes of 99% ethanol for two days at room temperature, to remove phenolic compounds and lipid. Residue was filtered and re-extracted with ethanol. The solvent was separated by Whatman No. 1 filter paper. After filtration, solvent was evaporated by a rotary evaporator under vacuum and the concentrated extract (EfraTme) was stored at 4°C until further analysis.<sup>16</sup>

#### Chemicals

L-ascorbic acid, quercetin, gallic acid, EDTA (ethylene diamine tetraacetic acid), potassium ferricyanide, ferric chloride, ferrous chloride, ferrozine, Folin-Ciocalteu reagent, NBT (nitroblue tetrazolium), riboflavin, DPPH (2,2-Diphenyl-1-picrylhydrazyl), sodium carbonate, TCA (trichloroacetic acid), ammonium molybdate, acetone, n-hexane, potassium acetate, aluminium nitrate, sodium bicarbonate, dichlorophenol indophenol, sodium phosphate, methionine, oxalic acid, sodium sulphate,



sulphuric acid and BHA (butylated hydroxyanisole) were purchased from Sigma chemicals Co. (St. Louis, MO, USA). All chemicals and reagents used were of analytical grade.

## Assays

### Determination of total phenols

Total phenols in the extract were measured using Folin-Ciocalteu reagent according to the method of Singleton and Rossi (1965).<sup>17</sup> 1ml of ethanolic extract (100 mg/ml) was mixed with 1ml Folin-Ciocalteu reagent and incubated for 3 min at room temperature. Then 1ml of 35 % saturated Na<sub>2</sub>CO<sub>3</sub> solution was added to the reaction mixture. Volume was adjusted to 10 ml with distilled water. Reaction mixture was incubated for 90 min in dark condition. Thereafter 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 per gram of extract.

### Determination of total flavonoid content

Flavonoid concentration was determined by the method as described by Park et al (1997).<sup>18</sup> 1 ml of mushroom extract (100 mg/ml) was diluted with 4.3 ml of 80% aqueous methanol, 0.1 ml of 10% aluminium nitrate and 0.1 ml of 1 M aqueous potassium acetate. After 40 min of incubation at room temperature, the absorbance was recorded at 415 nm. Quercetin (5 – 20 µg) was used as standard.

### Determination of total β -carotene and lycopene content

β -carotene and lycopene were determined by the way as was done by Nagata and Yamashita (1992).<sup>19</sup> In brief, 100 µl of sample extract was vigorously shaken with 10 ml of acetone-hexane mixture (4:6) for 1 min and immediately absorbance of the mixture was measured at 453, 505 and 663 nm. β -carotene and lycopene contents were calculated according to the following equations:

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

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

### Determination of ascorbic acid content

Ascorbic acid content was determined by a method as described by Rekha et al (2012)<sup>20</sup> with few relevant modifications. Standard ascorbic acid (100 µg/ml) was taken in a conical flask and made up to 10 ml by 0.6% oxalic acid. It was titrated with a dye, 2, 6-dichlorophenol indophenol. The amount of dye consumed (V<sub>1</sub> ml) is equivalent to the amount of ascorbic acid. In the same manner the sample (w µg/ml) was titrated with the dye (V<sub>2</sub> ml). The amount of ascorbic acid was calculated using the formula,

$$\text{Ascorbic acid (µg /mg)} = \{[(10 \text{ µg /V}_1\text{ml}) \times V_2\text{ml}] \times w \text{ µg}\} \times 1000.$$

### Chelating effect on ferrous ions

Chelating capacity of EfraTme on ferrous ions was estimated by the method of Dinis et al (1994).<sup>21</sup> Reaction mixture (4 ml) contained different concentrations of EfraTme (300 – 700 µg/ml) mixed with 3.7 ml of water and 0.1 ml of 2 mM ferrous chloride. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine. After 10 min incubation at room temperature, the absorbance was determined at 562 nm against a blank. EDTA was used as positive control. The percentage of inhibition of ferrozine- Fe<sup>2+</sup> complex formation is given by this formula:

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

Where A<sub>0</sub> was the absorbance of the control and A<sub>1</sub> the absorbance in the presence of EfraTme.

### Reducing power

Reducing power of EfraTme was determined following the method of Oyaizu (1986).<sup>22</sup> Variable concentrations (1.5 - 2.5 mg/ml) of EfraTme were added to 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was left for 20 min incubation. After that 2.5 ml of 10% trichloro acetic acid was added to the mixture. It was then centrifuged for 10 min at 12000 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 measured at 700 nm. An increase in absorbance of the reaction mixture signified increase in reducing power of the sample.

### Superoxide radical scavenging assay

The scavenging potential of EfraTme for superoxide radical was analyzed as described by Martinez et al (2001)<sup>23</sup> with some modifications in the riboflavin-light-NBT system. Each 3 ml reaction mixture sequentially contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, different concentrations (0.5-1.5 mg/ml) of EfraTme, 100 µM EDTA, 75 µM NBT and 2 µM riboflavin. Reaction was started by illuminating sample with light for 10 min and the increase in absorbance was measured at 560 nm. Identical tubes with the reaction mixture were kept in dark and served as blank. BHA was used as a positive control. The inhibition percentage was calculated by the following equation:

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

A<sub>0</sub> and A<sub>1</sub> were the absorbance of the control and absorbance in presence of sample respectively.

### Total antioxidant capacity assay

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract; as was done by Prieto et al (1999).<sup>24</sup> 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 sulphate and 4 mM ammonium molybdate) were incubated at 95°C for 90



min. Then the mixture was cooled to room temperature. Absorbance for each solution was recorded spectrophotometrically at 695 nm against blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

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

As per the method of Shimada et al (1992)<sup>25</sup> 0.004% methanolic solution of DPPH was prepared and concentrations (0.2-0.6 mg/ml) of EfraTme were added to it. The mixture was shaken vigorously and left to stand for 30 min in the dark. Gradual fading of purple colour against various concentrations were measured at 517 nm against a blank. EC<sub>50</sub> 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<sub>0</sub> was the absorbance of the control and A<sub>1</sub> was the absorbance if sample is present.

## Statistical Analysis

Results were subjected to statistical analysis using Student's t test. Values are mean ± SD of 3 replications.

## RESULTS AND DISCUSSION

### Chemistry

Here Table 1 shows the results of the phytochemical screening. It depicts that the ethanolic extract of the mushroom has ascorbic acid, β-carotene, lycopene, total flavonoids and total phenol.

**Table 1:** Ascorbic acid, β-carotene, lycopene, total phenol, total flavonoid contents of ethanolic extract of *Termitomyces medius* (EfraTme). Values are mean ± SD of three separate experiments each in triplicate. Total phenols are expressed in gallic acid equivalent (GAE), and flavonoids as quercetin equivalent (QAE).

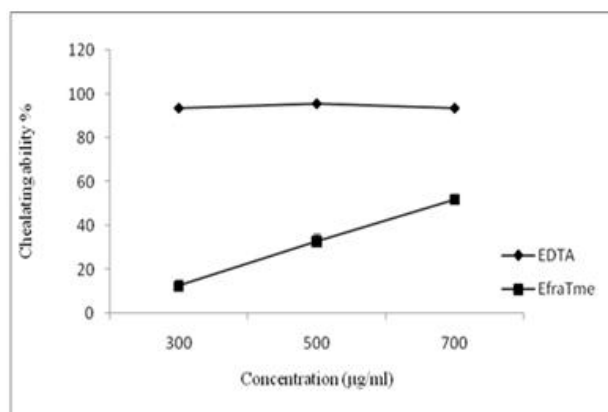
Ascorbic acid (µg/mg)	β-carotene (µg/mg)	Lycopene (µg/mg)	Total flavonoids (µg/mg)	Total phenol (µg/mg)
0.45 ± 0.04	0.00245 ± 0.0001	0.00192 ± 0.00009	1.59 ± 0.13	4.36 ± 0.095

Ascorbic acid is reported to interact directly with radicals such as O<sub>2</sub><sup>-</sup> and OH<sup>-</sup> in plasma, thus preventing damage of cells.<sup>26</sup> In the present study, the ascorbic acid content of EfraTme was found much higher when compared to the values from *Pleurotus ostreatus* (25ng/mg) and *Pleurotus citrinopileatus* (31 ng/mg).<sup>27</sup> β-carotene and lycopene were found in vestigial amounts though they also have antioxidative power. *Amanita vaginata* also contained low amount of β-carotene and lycopene which were 1.7205 µg/mg, 0.637 µg/mg respectively whereas that of *Pleurotus flabellatus* contained 0.00717 and 0.00459 µg/mg of β-carotene and lycopene.<sup>29,16</sup> Flavonoid is a secondary metabolite having more than 10,000 structural forms, which have capacity to inhibit ROS production by absorbing harmful UV rays.<sup>28</sup> In comparison to few referred mushroom extracts the flavonoid content was in the following order *Amanita vaginata* > EfraTme > *Pleurotus flabellatus* > *P.citrinopileatus*.<sup>29,16,27</sup> Phenolic compounds are known to be powerful chain-breaking antioxidants. Their hydroxyl groups provides them scavenging ability. In this study, the total phenolic content of EfraTme (4.36 µg/mg) was found to be comparatively less than that of the ethanolic extract of *Pleurotus flabellatus* (6.875 µg/mg)<sup>16</sup> and *Amanita vaginata* (11.296 µg/mg).<sup>29</sup>

### Chelating effect on ferrous ions

Ferrous ions have the ability to enhance free radical formation, as other transition metals. As per Fenton's reaction ferrous ion is capable of producing hydroxyl radical.<sup>30</sup> A chelating agent can arrest these transition metals and lowers the rate of reactive oxygen species formation. In this way, it can help in protection of human beings. EC<sub>50</sub> value of EfraTme was calculated from the

graph in Figure 1, and it was found to be 0.68 ± 0.02 mg/ml (Table 2). EC<sub>50</sub> value of ethanolic extract of *Russula delica* was 4.0 ± 0.02 mg/ml<sup>31</sup>, *Russula albonigra* was 0.81 mg/ml<sup>32</sup> and that of *Amanita vaginata* was 0.73 mg/ml<sup>29</sup>, values higher than that of EfraTme.

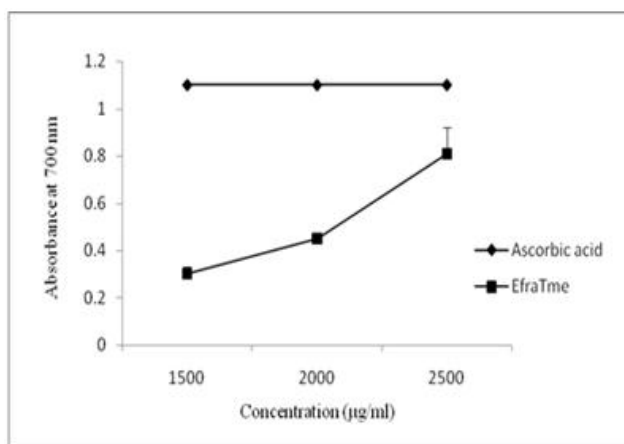


**Figure 1:** Ferrous ion chelating ability of ethanol fraction of *Termitomyces medius* (EfraTme). Results are the mean ± SD of three separate experiments, each in triplicate.

### Reducing power

Reducing power of any compound can be an identifying character so that it can be considered as an antioxidant. Such a compound is EfraTme, as the assay revealed the fact that it could reduce Fe<sup>3+</sup> to Fe<sup>2+</sup>. This change can be monitored at 700nm, by measuring the intensity of the Perl's Prussian blue colour. The reducing power of EfraTme was compared to that of BHA, a synthetic antioxidant. As seen from Figure 2, EC<sub>50</sub> value of EfraTme was 2.05 ± 0.05 mg/ml (Table 2). Ethanolic extracts of *Armillaria mellea*, *Calocybe gambosa*, *Clitocybe odora* and

*Tricholoma giganteum* gave EC<sub>50</sub> values at 17.13, 11.46, 3.63 and 2.2 mg/ml concentration respectively<sup>33, 34</sup> which are much higher than EfraTme.

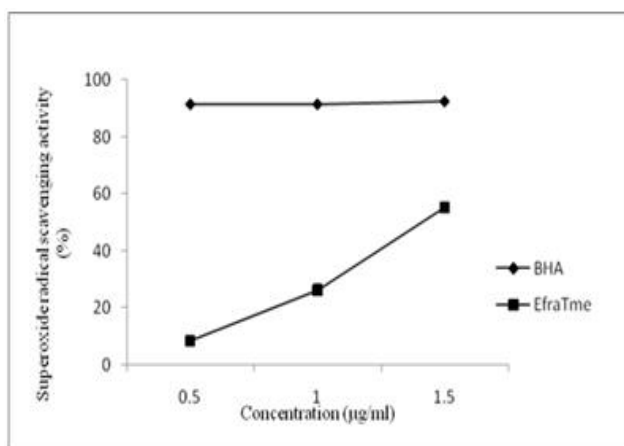


**Figure 2:** Reducing power of ethanol fraction of *Termitomyces medius* (EfraTme)

Results are the mean ± SD of three separate experiments, each in triplicate.

**Superoxide radical scavenging assay**

Superoxide radical is known to be very harmful for cellular components and plays a major role in the formation of other reactive oxygen species such as hydroxyl radical, hydrogen peroxide and singlet oxygen in living system. Regarding data presented in Figure 3, the scavenging activity of EfraTme increased with increasing concentration. The EC<sub>50</sub> value was found to be 1.4 mg/ml for EfraTme (Table 2). In a similar type of experiment EC<sub>50</sub> value for the ethanolic extract of *Russula albonigra* was at 0.74 mg/ml.<sup>32</sup> These results suggested that EfraTme exhibited scavenging effect on superoxide anion radical generation that could help prevent or ameliorate oxidative damage.



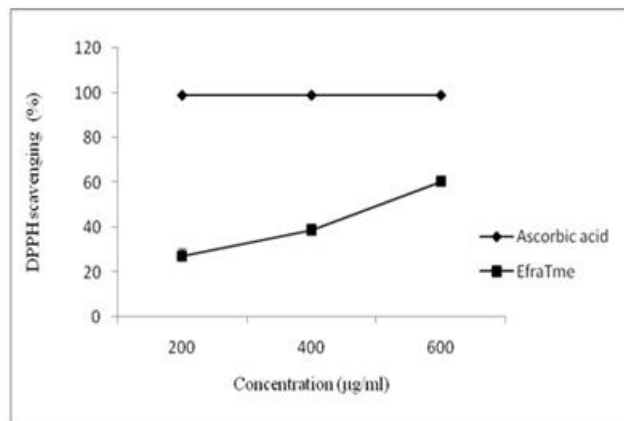
**Figure 3:** Superoxide radical scavenging assay of ethanol fraction of *Termitomyces medius* (EfraTme). Results are the mean ± SD of three separate experiments, each in triplicate.

**Total antioxidant capacity assay**

Total antioxidant capacity was measured by the formation of green phosphomolybdenum complex. The EfraTme resulted 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 was used as standard. The assay conducted gave us the data, that 1mg of extract is as functional as 0.10 ± 0.025 mg of ascorbic acid (expressed as 100 µg AAE). This was more than that of *Pleurotus flabellatus*.<sup>16</sup> *Russula albonigra* was reported to have 17 µg AAE<sup>32</sup>, lower than that of EfraTme.

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

DPPH accepts electron or hydrogen to earn stability. Antioxidants, on the other hand are capable of donating electron or hydrogen atom.<sup>35</sup> A solution containing DPPH and methanol gives violet colour. But when electrons are donated to DPPH, then solution starts losing colour. This change is spectrophotometrically measured at 517nm.<sup>36</sup> From Figure 4 EC<sub>50</sub> value of EfraTme with regards to DPPH radical scavenging activity was recorded to be 0.5 ± 0.04 mg/ml (Table 2), which was much lower than the EC<sub>50</sub> value (4.3 ± 0.3 mg/ml) of ascorbic acid, a potent scavenger. Even EC<sub>50</sub> values of ethanolic extract of *Pleurotus flabellatus* and *Russula albonigra* were 1.8 ± 0.02 mg/ml and 1.7 ± 0.02 mg/ml respectively, that are much higher than EfraTme.<sup>16,32</sup> In similar studies on ethanolic extract of *Amanita vaginata*<sup>29</sup>, *Tricholoma giganteum*<sup>34</sup>, *Volvariella volvacea*<sup>37</sup> and *Ramaria aurea*<sup>38</sup> EC<sub>50</sub> values were at 1.48, 0.75, 0.265 and 0.857 mg/ml respectively.



**Figure 4:** DPPH radical scavenging activity of ethanol fraction from *Termitomyces medius* (EfraTme). Results are the mean ± SD of three separate experiments, each in triplicate.

**Table 2:** EC<sub>50</sub> values (mg/ml) of the extract for different antioxidant properties

Chelating	Reducing power	Superoxide Radical Scavenging	DPPH
0.68 ± 0.02	2.05 ± 0.05	1.40 ± 0.04	0.50 ± 0.05



## CONCLUSION

At the end of this study we may conclude that the ethanolic extract of *Termitomyces medius* (EfraTme) was found to contain many bioactive compounds such as ascorbic acid,  $\beta$  carotene, lycopene, flavonoids and phenols. It can also be claimed as an effective antioxidant for the significant results in various *in vitro* assays including ferrous iron chelating, ferric iron reducing, superoxide radical scavenging, DPPH free radical scavenging and total antioxidant activity. Hence, it can be suggested as not only a natural additive in food but can open a new horizon for the pharmaceutical industries.

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