

BIOACTIVE CONSTITUENTS AND ANTIOXIDANT EFFICACY OF *AURICULARIA POLYTRICHA*Packialakshmi B<sup>1\*</sup>, Sudha G<sup>2</sup>, Charumathy M<sup>1</sup><sup>1</sup>Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India. <sup>2</sup>Department of Biochemistry, School of Biosciences, Periyar University, Salem, Tamil Nadu, India. Email: biochem\_bagya@yahoo.co.in

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## ABSTRACT

**Objective:** *Auricularia polytricha* is a most popular edible and medicinal fungus in China and other Asian countries because of its high nutritional value and good taste. The objective of this study was to evaluate the antioxidant potency, total phenol, and flavonoid content in the hot water extract of *A. polytricha* fruiting bodies.

**Methods:** The antioxidant property was investigated using various *in vitro* chemical and biological models such as 2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid], dimethyl-4-phenylenediamine radical scavenging assay, cupric reducing antioxidant capacity assay, phosphomolybdenum reducing antioxidant power assay,  $\beta$ -carotene bleaching and lipid peroxidation inhibition assays.

**Results:** *A. polytricha* exhibited antioxidant activity in a dose-dependent manner with lower EC<sub>50</sub> values in all scavenging assays. A considerable amount of secondary metabolites such as phenol and flavonoids were observed in the extract.

**Conclusion:** The results demonstrated that *A. polytricha* has a potential significance for seeking new natural antioxidant agents against oxidative stress.

**Keywords:**  $\beta$ -carotene bleaching, Free radicals scavenging, Lipid peroxidation, Total phenols, Total flavonoids, Wood ear mushrooms.

## INTRODUCTION

Oxidative stress can be associated with an increased rate of reactive oxygen species or free radicals generation. Free radicals can cause damage to a wide range of essential biomolecules and which have been associated with many lifestyle-related diseases such as atherosclerosis, arthritis, cancer, aging, emphysema, and many other health problems [1]. Antioxidant refers to a compound that can inhibit the oxidation of lipids and other molecules by inhibiting the initiation of oxidative chain reactions and thus prevent cellular damage by oxygen [2]. If the antioxidant protection system becomes unbalanced by free radicals, impaired physiological functioning may occur, resulting in diseases [3]. The search and research for natural antioxidants have received much attention recently [4]. Therefore, dietary or pharmacological intake of antioxidants that can scavenge free radicals may be considered as one of the therapeutic strategies to treat diseases.

Since ancient times, many species of mushrooms have been widely used as medicine, food, and functional resources in Asian countries because of their diverse biological activities [5]. Edible mushrooms provide a nutritionally significant content of Vitamins (B1, B2, B12, C, D, and E), polysaccharides, polyphenol, tannins, terpenoids, fatty acids, proteins, glycoproteins, proteoglycans, and lectins [6-8]. *Pleurotus*, *Agaricus*, *Ganoderma*, *Auricularia*, *Volvariella*, and *Tremella* mushroom species have become an attractive source for the development of drugs and nutraceuticals [9].

*Auricularia polytricha* is an edible jelly fungus, belongs to *Auriculariaceae* and *Basidiomycota*, and its Chinese name is "Mu Er" or "Wood ear." It acts as a popular ingredient in Chinese dishes (hot and sour soup) and also as a medicine for many centuries in China [10,11]. *A. polytricha* has recently been reported to possess several medicinal functions such as moistening lung, enriching the blood, decreasing blood sugar and having anti-tumor, hypoglycemic, anti-ulcer, and hemostatic properties [12,13]. In the present study, we have focused on evaluating the antioxidant properties of *A. polytricha* hot water extract using different *in vitro* free radical scavenging assays with an objective to determine bioactive constituents.

## METHODS

## Chemicals and reagents

2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS), dimethyl-4-phenylenediamine (DMPD), and  $\beta$ -carotene were obtained from Sigma-Aldrich (Bangalore, India). Butylated hydroxytoluene, gallic acid, rutin, pyrocatechol, linoleic acid, and thiobarbituric acid (TBA) were obtained from Himedia (Mumbai, India). All other chemicals are of analytical grade.

## Mushroom samples

The dried fruiting bodies of *A. polytricha* (Huadan AU781) were obtained from Hangzhou Haudan Agri-food mushroom farm, Hangzhou City, Zhejiang Province, China. The dried fruiting bodies were powdered (20 mesh) and stored in air-tight plastic bags for further analysis.

## Preparation of the extract

Mushroom powder (10 g) was extracted by stirring with 100 ml of boiling water at 100°C for 6 hrs. After centrifugation at 5000 g for 20 minutes, the residues were re-extracted twice with the boiling water. The supernatants were pooled together, and the combined extracts were evaporated under reduced pressure at 45°C for 30 minutes using a rotary vacuum evaporator. The extract obtained was dissolved in hot water at 100 mg/ml and stored at 4°C for further use. From the stock solution, successive dilutions were made and used for various *in vitro* assays to analyze the antioxidant activity of the samples. Analyses were carried out in triplicates.

## Estimation of total phenol

The total phenol in the mushroom extract was measured according to the method of [14] with some modifications. 1.0 ml of the sample was mixed with 1.0 ml of Folin-Ciocalteu's phenol reagent. After 3 minutes, 1.0 ml of saturated sodium carbonate (35%) was added to the mixture, and it was made up to 10 ml by adding deionized water. The mixture was kept for 90 minutes at room temperature in the dark. The absorbance was measured at 725 nm against the blank. Pyrocatechol was used as the reference standard. The total phenol content is expressed as milligrams of catechol equivalents (CE) per gram of extract.

### Estimation of total flavonoid

Total flavonoid content was determined as described by [15]. 0.25 ml of mushroom extract was diluted with 1.25 ml of distilled water. 75 µl of a 5% sodium nitrite were added and after 6 minutes, 150 µl of a 10% aluminum chloride were added, and mixed. After 5 minutes, 0.5 ml of 1 M sodium hydroxide was added. The absorbance was measured immediately against the prepared blank at 510 nm. Rutin was used as the reference standard. The total flavonoid content is expressed as milligrams of rutin equivalents (RE) per gram of extract.

### Total antioxidant capacity by phosphomolybdenum assay

The antioxidant activity of the sample was evaluated by the phosphomolybdenum method according to the procedure of [16]. An aliquot of 0.1 ml of sample solution was mixed with 1.0 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped with silver foil and incubated at 95°C for 90 minutes. The tubes were cooled to room temperature, and the absorbance of the sample was measured at 695 nm against a blank. Gallic acid was used as a standard, and total antioxidant capacity was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

### ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was performed with slight modifications described by [17]. The ABTS<sup>+</sup> cation radicals were produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 hrs. Prior to use, the solution was diluted with ethanol to get an absorbance of 0.700±0.025 at 734 nm. Free radical scavenging activity was assessed by mixing 10 µl of the test sample with 1.0 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly after 6 minutes. The percentage inhibition was calculated according to the formula:  $[(A_0 - A_1)/A_0] \times 100$ , where  $A_0$  was the absorbance of the control, and  $A_1$  was the absorbance of the sample.

### DMPD radical scavenging activity

The principle of DMPD<sup>+</sup> assay is that at acidic pH and in the presence of a suitable oxidant solution, DMPD can form a stable and colored radical cation (DMPD<sup>+</sup>). The assay was performed according to the method of [18,19]. DMPD<sup>+</sup> was obtained by adding 0.2 ml ferric chloride (0.05 M) to DMPD in acetate buffer. 0.5 ml of various concentrations of the extract and 1 ml of DMPD<sup>+</sup> solution were vortexed and incubated in dark at room temperature for 10 minutes. The absorbance was measured at 505 nm. The buffer solution was used as a blank sample. The scavenging activity was calculated using the following equation:  $(A_0 - A_1/A_0) \times 100$ , where  $A_0$  was the absorbance of the control and  $A_1$  was the absorbance of the sample.

### Cupric ions (Cu<sup>2+</sup>) reducing power - cupric reducing antioxidant capacity (CUPRAC) assay

The cupric ion (Cu<sup>2+</sup>) reducing power was determined by the method proposed by [20] with minor modifications of [21]. 0.25 ml of copper (II) chloride solution (0.01 M), 0.25 ml of ethanolic neocuproine solution ( $7.5 \times 10^{-3}$  M), and 0.25 ml ammonium acetate buffer solution (1 M) were added to a test tube, followed by mixing with different concentrations of the sample. The total volume was adjusted to 2.0 ml with distilled water, and the reaction was mixed well. The tubes were kept at room temperature. After 30 minutes of incubation, the absorbance was measured at 450 nm against a blank. Increased absorbance of the reaction mixture indicates increased reduction capability.

### β-Carotene bleaching assay

The antioxidant activity of the extract was evaluated by the β-carotene linoleate model system according to the method of [22]. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. 2 ml of this solution were pipetted into a 100 ml round-bottom flask. After the chloroform was removed at 40°C under vacuum, 40 mg of linoleic acid, 400 mg of Tween 80 emulsifier, and 100 ml of distilled water were added to the flask with vigorous shaking. 4.8 ml

of this emulsion were transferred into different test tubes containing 0.2 ml of different concentrations of the mushroom extract. The tubes were shaken and incubated at 50°C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. A blank, devoid of β-carotene, was prepared for background subtraction. β-carotene bleaching inhibition was calculated using the following equation:  $(\beta\text{-carotene content after 2 hrs of assay}/\text{initial } \beta\text{-carotene content}) \times 100$ .

### Phosphomolybdenum reducing antioxidant power (PRAP) assay

The method is used to determine the reducing power of antioxidant compounds [23]. The sample is treated with phosphomolybdic acid to produce a greenish blue and the absorbance measured at 600 nm. To 300 µl of extract, add 10 ml of 10% phosphomolybdic acid solution in ethanol (w/v). The solution was incubated at 80°C for 30 minutes, and the absorbance was measured at 600 nm.

### Lipid peroxidation (LPO) inhibition assay

A modified TBA-reactive species (TBARS) assay [24,25] was used to measure the lipid peroxide formed, using rat liver homogenate. Malondialdehyde has been identified as the product of LPO that reacts with TBA to give a red absorbing at 535 nm. To 1.0 ml of extract, add 1.0 ml of 1% liver homogenate, then 0.05 ml of 0.5 mM FeCl<sub>2</sub> and 0.5 mM H<sub>2</sub>O<sub>2</sub> were added to initiate LPO. After incubation at 37°C for 60 minutes, 1.5 ml of 20% TCA and 1.5 ml of 0.8% TBA solution (0.8%, w/v) were added to quench the reaction. The resulting mixture was heated at 100°C for 15 minutes and then centrifuged at 4000 rpm for 10 minutes. The absorbance of the upper layer was measured at 532 nm. The inhibition effect on LPO was calculated as follows: Inhibition effect (%) =  $[1 - (A_1 - A_2)/A_0] \times 100$ , where  $A_0$  was the absorbance of the control (water instead of sample),  $A_1$  is the absorbance of the sample, and  $A_2$  was the absorbance of the sample only (water instead of liver homogenate).

### Statistical analysis

All experiments were carried out in triplicates, and results are expressed as mean±standard deviation. The data were analyzed using SPSS software. Analysis of variance and Duncan's multiple range test were used to analyze the differences among scavenging activity and EC<sub>50</sub> of various extracts for different antioxidant assays with least significance difference  $p < 0.05$  as a level of significance.

## RESULTS AND DISCUSSION

### Total phenol, flavonoid content, and total antioxidant capacity assay

Mushrooms accumulate a variety of secondary metabolites such as phenolic compounds, terpenes, polyketides, and steroids. Among these secondary metabolites, phenolic compounds such as flavonoids, phenolic acids, and tannins are well-correlated with free radical scavenging activity, LPO inhibition, reducing power, and metal chelation, probably due to their hydroxyl groups [26]. Table 1 represents the total phenol and flavonoid content of *A. polytricha* extract. The total phenolic and flavonoid content of the sample was found to be 7.20 mg CE/g and 3.10 mg RE/g, respectively. The presence of these natural antioxidant components explains its antioxidant efficiency. Gursoy *et al.* found that the methanolic extract of *Ramaria flava*, *Rhizopogon roseolus*, and *Russula delica* were found to have

**Table 1: Total phenol, flavonoid content, and total antioxidant capacity of *Auricularia polytricha* hot water extract<sup>#</sup>**

Sample	Total phenols (mg CE/g) <sup>A</sup>	Total flavonoids (mg RE/g) <sup>B</sup>	Total antioxidant capacity (mg GAE/g) <sup>C</sup>
<i>Auricularia polytricha</i>	7.20±0.30	3.10±0.17	17.93±0.64

<sup>#</sup>Values are expressed as mean±SD (n=3). <sup>A</sup>CE: Catechol equivalents, <sup>B</sup>RE: Rutin equivalents, <sup>C</sup>GAE: Gallic acid equivalents, SD: Standard deviation

the phenolic content of 10.50, 6.65, and 2.09  $\mu\text{g}$  GAE/mg extract, respectively, and the flavonoid content of 0.50, 0.48, and 0.16  $\mu\text{g}$  QE/mg extract, respectively [27].

The total antioxidant capacity (TAC) assay are based on the reduction of Mo (V) to Mo (IV) by the antioxidants in the sample and subsequent formation of a green phosphate/Mo (V) complex at acidic pH with an absorbance maximum at 695 nm [28]. The total antioxidant capacity of *A. polytricha* hot water extract were 17.93 mg GAE/g (Table 1) which was observed to be higher than TAC of *Pleurotus eous* hot water extract (1.98 mg GAE/g) [29].

#### ABTS radical scavenging assay

ABTS<sup>•+</sup> radicals are more reactive radicals and scavenged by antioxidants by the mechanism of electron-hydrogen donation [30]. ABTS<sup>•+</sup>, the oxidant, was generated by potassium persulfate oxidation of ABTS<sup>2-</sup>, and the radical cation is measured spectrophotometrically at 734 nm. This is a direct generation of a stable form of radical to create a blue-green ABTS<sup>•+</sup> chromophore prior to the reaction with antioxidants. This decolorization assay measures the total antioxidant capacity in both lipophilic and hydrophilic substances [31]. Depending on this mechanism, the ABTS radical inhibition effect of *A. polytricha* hot water extract is measured and the result is given in Fig. 1. At 4-20 mg/ml, the ABTS radical scavenging activities of *A. polytricha* increased with increase in concentration with an inhibition percentage of 51.15-80.16%, respectively. A significant difference ( $p < 0.05$ ) was found between the different concentrations tested, and the EC<sub>50</sub> value was found to be 4.63 mg/ml. Thetsrimuang et al. reported that EC<sub>50</sub> value of the crude polysaccharides from *Lentinus* sp. was  $< 0.5$  mg/ml [32]. These results indicate its strong scavenging power for the ABTS radical due to its electron donating ability.

#### DMPD radical scavenging assay

The DMPD radical cation decolorization method has been developed for the measurement of the antioxidant activity in food and biological samples. This assay is based on the reduction of buffered solution of colored DMPD in acetate buffer and ferric chloride. The procedure involves measurement of the decrease in absorbance of DMPD in the presence of scavengers at its absorption maximum of 505 nm. The activity was expressed as the percentage reduction of DMPD [33]. *A. polytricha* extract were shown to scavenge DMPD radicals (Fig. 2) to a different extent over a concentration range of 2-10 mg/ml with an inhibition percentage of 38.01-73.17%. A significant difference ( $p < 0.05$ ) was found between the different concentrations tested. The extract exhibited an EC<sub>50</sub> value of 3.27 mg/ml. However, the aqueous

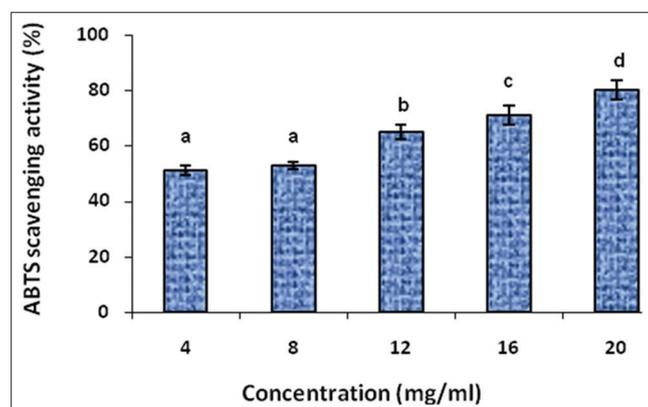


Fig. 1: 2, 2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] radical scavenging activity of *Auricularia polytricha* hot water extract. Values are expressed as mean  $\pm$  standard deviation (n=3). Different letters (a-b) indicate a significant difference between the concentrations of the same extract ( $p < 0.05$ , analysis of variance, Duncan's multiple range test)

extract of *V. volvacea* showed the EC<sub>50</sub> value of 0.61 mg/ml [34] which was remarkably higher than the extract studied here. Therefore, the DMPD scavenging activity of *A. polytricha* extract indicates its ability to scavenge free radicals, thereby preventing lipid oxidation via chain-breaking reaction.

#### CUPRAC assay

The CUPRAC assay is an excellent tool to determine the reducing power of antioxidant compounds [35]. This assay is based on the reduction of Cu (II)- neocuproine to Cu (I)- neocuproine by antioxidants and the absorbance can be measured at 450 nm [36]. In this method, a higher absorbance indicates higher Cu<sup>2+</sup> reducing potency. Increasing cupric ions reducing ability was seen with increasing concentration of *A. polytricha* extract. At 0.5-2.5 mg/ml, Cu<sup>2+</sup> reducing the capability of *A. polytricha* hot water extract were between 0.243 and 0.761 (Fig. 3). A significant difference ( $p < 0.05$ ) was found between the different concentrations tested and the EC<sub>50</sub> value was found to be 1.14 mg/ml. At the concentration of 0.5 mg/ml, CUPRAC of *G. lucidum* exhibited significantly higher CUPRAC of 1.058 which was higher than CUPRAC of hot water extract of *Auricularia polytricha* studied here [37]. This CUPRAC redox reaction is carried out at a pH (7.0) close to the physiological pH, and the assay is capable of measuring thiol type antioxidants such as glutathione and non-protein thiols, unlike the mostly applied FRAP test, which is non-responsive to -SH group antioxidants [20].

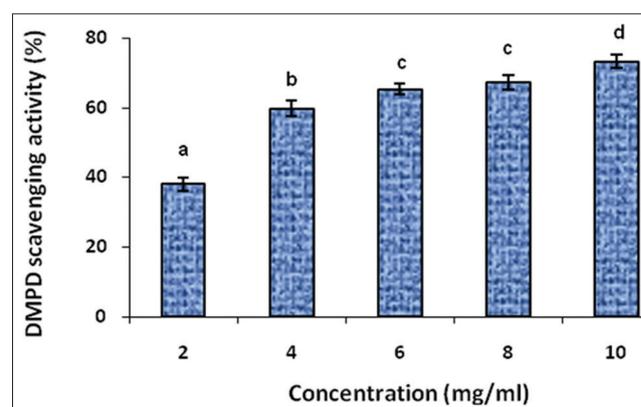


Fig. 2: Dimethyl-4-phenylenediamine radical scavenging activity of *Auricularia polytricha* hot water extract. Values are expressed as mean  $\pm$  standard deviation (n=3). Different letters (a-d) indicate a significant difference between the concentrations of the same extract ( $p < 0.05$ , analysis of variance, Duncan's multiple range test)

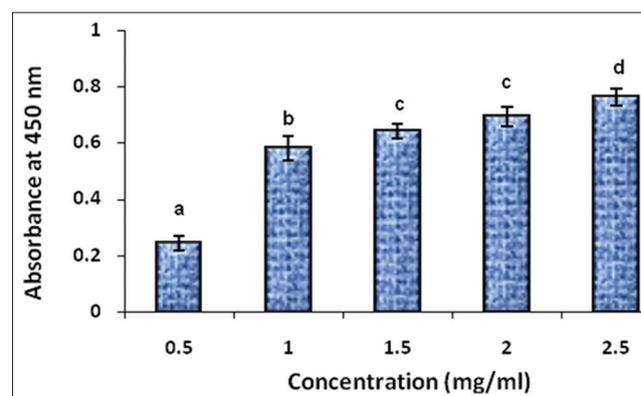


Fig. 3: Cupric reducing antioxidant capacity of *Auricularia polytricha* hot water extract. Values are expressed as mean  $\pm$  standard deviation (n=3). Different letters (a-b) indicate a significant difference between the concentrations of the same extract ( $p < 0.05$ , analysis of variance, Duncan's multiple range test)

### $\beta$ -carotene bleaching assay

$\beta$ -carotene is a fat-soluble pigment and natural antioxidant found mostly in plants, which is well-known for its immunomodulatory effect. In this assay, the linoleic acid-free radical attacks the highly unsaturated  $\beta$ -carotene by oxidation, and the presence of different antioxidants in the extract can prevent the extent of  $\beta$ -carotene bleaching by neutralizing the linoleate free radical and other free radicals developed in the system [38,39]. The change in absorbance can be monitored spectrophotometrically at 470 nm. Fig. 4 represents the antioxidant activity of *A. polytricha* hot water extract as measured by the inhibition of bleaching the  $\beta$ -carotene linoleate system. At 0.8-4.0 mg/ml,  $\beta$ -carotene bleaching inhibition of *A. polytricha* was found to be 14.35-40.43%. A significant difference ( $p < 0.05$ ) was found between the different concentrations tested and the  $EC_{50}$  value was found to be 5.19 mg/ml. It was reported that the  $EC_{50}$  value for  $\beta$ -carotene bleaching activity of hot water extract from *S. commune* was 2.21 mg/ml which was more potent than *A. polytricha* [37].

### PRAP assay

The sample is treated with phosphomolybdic acid to produce a greenish blue and the absorbance measured at 600 nm [23]. The reduction of absorbance is proportional to the antioxidant content. PRAP of *A. polytricha* hot water extract is shown in Fig. 5. The reducing power increased with increase in concentration. The PRAP of the various concentrations of hot water extract were 0.152-0.504 at 0.6-3 mg/ml, and the  $EC_{50}$  value was found to be 2.74 mg/ml. A significant difference ( $p < 0.05$ ) in ferric reducing antioxidant power was observed between various concentrations of extract.

### LPO inhibition assay

LPO, a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids. LPO inactivates cellular components and there in plays a key role in oxidative stress in biological systems. Several toxic byproducts of LPO can damage various biological macromolecules [40]. The LPO of rat liver was triggered by  $Fe^{2+}$  or ascorbate, and the end products of the process were measured in terms of TBARS formed. The hot water extract of *A. polytricha* inhibited LPO in a concentration dependent manner (Fig. 6). The LPO inhibition of hot water extract of *A. polytricha* was between 16.67-36.93% at 1-5 mg/ml, respectively. An  $EC_{50}$  value of the LPO inhibition of the extract was observed to be 6.77 mg/ml. No statistically significant difference ( $p < 0.05$ ) in LPO inhibition was observed with 3-5 mg/ml sample concentrations. The ability of the hot water extract of *P. eous* to inhibit LPO on rat liver homogenate ( $EC_{50} = 0.58$  mg/ml) was reported by Sudha et al. [29] which was approximately 10 fold higher than that of *A. polytricha*. The extract could inhibit LPO by scavenging the  $OH^{\cdot}$  or  $O_2^{\cdot-}$  radicals or by chelating the iron itself.

### CONCLUSION

In conclusion, the results obtained from this study clearly indicated that the hot water extract of *A. polytricha* mushroom species had more potent antioxidant activity and was found to be dose-dependent. The extract was shown to be excellent scavengers of ABTS, DMPD radicals, reducer of cupric ions and ferric ions, an inhibitor of  $\beta$ -carotene bleaching and LPO. This report reveals their medicinal and therapeutic value and could be used as a promising source of natural antioxidants.

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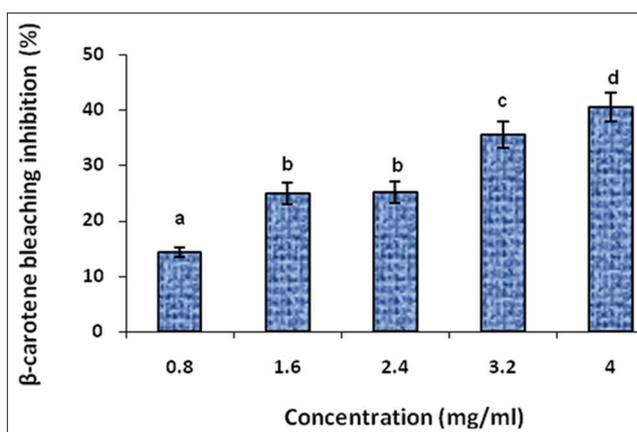


Fig. 4:  $\beta$ -carotene bleaching inhibition of *Auricularia polytricha* hot water extract. Values are expressed as mean  $\pm$  standard deviation ( $n=3$ ). Different letters (a-b) indicate a significant difference between the concentrations of the same extract ( $p < 0.05$ , analysis of variance, Duncan's multiple range test)

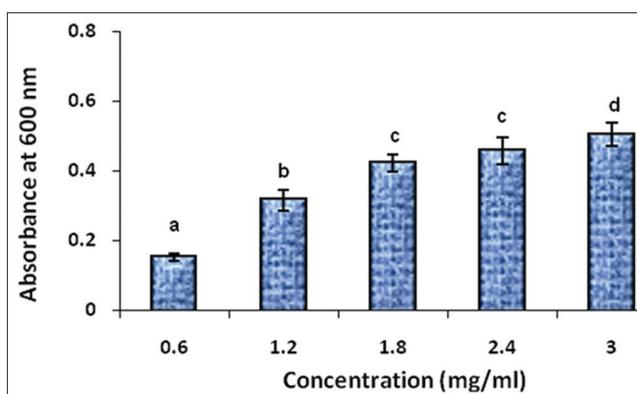


Fig. 5: Phosphomolybdenum reducing antioxidant power of *Auricularia polytricha* hot water extract. Values are expressed as mean  $\pm$  standard deviation ( $n=3$ ). Different letters (a-b) indicate a significant difference between the concentrations of the same extract ( $p < 0.05$ , analysis of variance, Duncan's multiple range test)

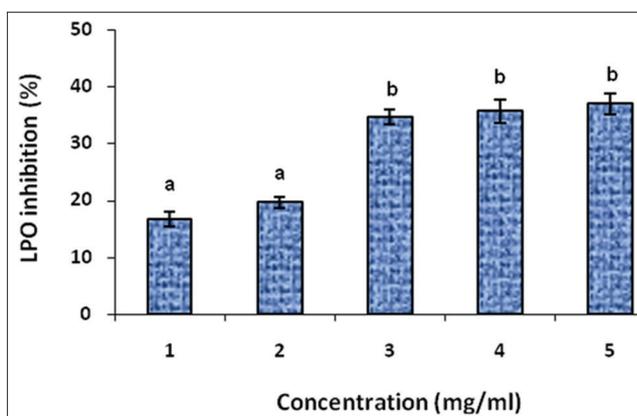


Fig. 6: Lipid peroxidation inhibition of *Auricularia polytricha* hot water extract. Values are expressed as mean  $\pm$  standard deviation ( $n=3$ ). Different letters (a-b) indicate a significant difference between the concentrations of the same extract ( $p < 0.05$ , analysis of variance, Duncan's multiple range test)

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