

**Free radical scavenging and antioxidant efficacy of ethanolic fractions from an edible medicinal mushroom - *Auricularia polytricha***B. Packialakshmi^{1*}, G. Sudha², M. Charumathy¹¹Department of Biochemistry, Kongunadu Arts and Science College, Coimbatore - 641029, Tamil Nadu, India²Department of Biochemistry, School of Biosciences, Periyar University, Salem - 636011, Tamil Nadu, India***Corresponding author e-mail:** biochem_bagya@yahoo.co.in*Received on: 06-02-2017; Revised on: 24-03-2017; Accepted on: 28-03-2017***ABSTRACT**

The main aim of this research was to analyse the free radical scavenging potency and antioxidant capacity of ethanolic fractions from *Auricularia polytricha* (APEF) fruiting bodies. The free radical scavenging property was evaluated using DPPH (1,1-diphenyl-2-picryl-hydrazyl), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging assay, reducing power, phenanthroline and lipid peroxidation (LPO) inhibition assays and the activity was compared with standard antioxidants. The EC₅₀ values were observed to be 3.15 mg/ml, 3.90 mg/ml, 2.07 mg/ml, 3.12 mg/ml and 6.32 mg/ml respectively. The content of total phenol and flavonoid in APEF were 17.60 mg/g as Ferulic Acid Equivalents (FAE) and 4.96 mg/g as Catechin Equivalents (CAE) respectively. The present study reveals the potential of APEF to scavenge free radicals and the activity was found to be higher in all used *in vitro* methods. Thus, *Auricularia polytricha* mushrooms could be used as a potent therapeutic agent and a food supplement.

Keywords: *Auricularia polytricha*, Free radicals scavenging, Flavonoids, Lipid peroxidation, Phenols.**INTRODUCTION**

Free radicals normally acquired from the environment and also generated during the normal body metabolism [1]. The uncontrolled reactive oxygen species generation is associated with protein and lipid peroxidation and leads to cell structural damage, gene mutation, tissue injury which ultimately leads to the development of health disorders like, cancer, diabetes, ageing, alzheimer's disease, hypertension and atherosclerosis [2]. Thus, in recent years, identifying and searching safe and natural antioxidants, especially from plant origin has notably increase to protect the human body from several oxidative damages [3].

For thousands of years, mushrooms have been valued as an edible and medicinal resource and a number of

bioactive biomolecules have been isolated and identified from mushrooms [4]. Among 1, 40,000 mushrooms on earth, only 10% are known. It comprise a vast and yet largely untapped source of new pharmaceutical products [5,6]. Several mushroom compounds have been shown to potentiate the host's innate and acquired immune responses and activate many kinds of immune cells that are important for the maintenance of homeostasis [7,8].

Auricularia polytricha, one of edible mushrooms, has been widely used as a healthy food in East Asian countries, especially in China and Korea [9]. Their fruiting bodies are a kind of black-brown mushroom with high content of heteropolysaccharides which consists of D-glucose residues with various chains of β -1,3-branch residues, such as xylose, mannose, glucose, and glucuronic acid [10]. Its nutritional

value and taste components have been investigated. Few studies have reported its biological activity and active substances [11,12]. Many researches have been performed to study its pharmaceutical effects such as, decreasing liver damage, lipid peroxidation inhibition, metal ion chelation, hypolipidemic and cardioprotective properties. But no attempt has been made to study its antioxidant capacity in ethanolic fractions. Thus, the objective of the present study was to explore the free radicals scavenging activity of ethanolic fractions of *Auricularia polytricha* (APEF) using various *invitro* assays.

MATERIAL AND METHODS

Chemicals and reagents: 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were obtained from Sigma-Aldrich (Bangalore, India). BHT (Butylated hydroxy toluene), quercetin (QUE), ferulic acid, catechin, gallic acid, linoleic acid, and thiobarbituric acid (TBA) were obtained from Himedia (Mumbai, India). All other chemicals are of analytical grade.

Preparation of the extracts Mushroom samples:

The dried fruiting bodies of *Auricularia polytricha* (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 extracts: Mushroom powder (10 g) was extracted by stirring with 100 ml of boiling water at 100°C for 6 h. After centrifugation at 5000 g for 20 min, the residues were re-extracted twice with the boiling water. The concentrated supernatants were then precipitated with three volumes of absolute ethanol (95%) and polysaccharides were precipitated overnight at 4°C. The ethanolic supernatant was evaporated at 40°C under reduced pressure (rotary evaporator), giving the ethanolic fraction. The extract obtained was dissolved in ethanol at 100 mg/ml and stored at 4°C for further use. Analyses were carried out in triplicates.

Estimation of total phenol: The total phenol in mushroom extracts was measured according to the method of with some modifications [13]. 1.0 ml of the sample was mixed with 1.0 ml of Folin-Ciocalteu's phenol reagent. After 3 min, 1.0 ml of saturated sodium carbonate (35%) was added to the mixture and it was made up to 10 ml by adding deionised water. The mixture was kept for 90 min at room temperature in the dark. The absorbance was measured at 725 nm against the blank. Ferulic acid

was used as the reference standard. The total phenol content is expressed as milligrams of ferulic acid equivalents (FAE) per gram of extract.

Estimation of total flavonoid: Total flavonoid content was determined as described by [14]. 0.25 ml of mushroom extracts was diluted with 1.25 ml of distilled water. 75 µl of a 5% sodium nitrite were added and after 6 min 150 µl of a 10% aluminium chloride were added and mixed. After 5 min, 0.5 ml of 1 M sodium hydroxide was added. The absorbance was measured immediately against the prepared blank at 510 nm. Catechin was used as the reference standard. The total flavonoid content is expressed as milligrams of catechin equivalents (CAE) per gram of extract.

Phosphomolybdenum assay: The antioxidant activity of the sample was evaluated by the phosphomolybdenum method according to the procedure of [15]. An aliquot of 0.1 ml of sample solution was mixed with 1.0 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped with silver foil and incubated at 95°C for 90 min. The tubes were cooled to room temperature and the absorbance of 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.

DPPH radical scavenging activity: The scavenging effect of mushroom extract on DPPH radicals was determined according to the method of Shimada et al. [16]. Various concentrations of sample (4.0 ml) were mixed with 1.0 ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517 nm. 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. Quercetin (QUE) was used as a positive control.

ABTS radical cation scavenging activity: The ABTS radical cation scavenging activity was performed with slight modifications described by [17]. The ABTS^{•+} 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 h. 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 test sample

with 1.0 ml of ABTS working standard in a microcuvette. The decrease in absorbance was measured exactly after 6 min. The percentage inhibition was calculated according to the formula: $[(A0-A1)/A0] \times 100$, where A0 was the absorbance of the control, and A1 was the absorbance of the sample. Quercetin was used as a positive control.

Determination of reducing power: The reducing power of polysaccharide extracts were measured according to the method of Oyaizu [18]. The reaction mixture contained 2.5 ml of various concentrations of the extracts, 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide were mixed and incubated at 50°C for 20 min and centrifuged for 10 min at 5000 g after addition of 2.5 ml of 10% TCA. To 2.5 ml aliquot of supernatant, 2.5 ml of deionised water and 0.5 ml of 0.1% ferric chloride were added and mixed well. After 10 min of incubation, the absorbance was measured at 700 nm against a blank. Quercetin was used as a positive control.

Phenanthroline assay: The Phenanthroline assay was used to determine the reducing capacity of mushroom extract, according to the method of Szydłowska-Czerwińska et al. [19]. Various concentrations of the sample (0.10 ml), ferric chloride (0.50 ml, 0.2%) and 0.5% 1, 10-phenanthroline solution (0.25 ml) were mixed and made up the volume to 5 ml with methanol. The reaction mixture was then incubated at 30°C in dark for 20 min and the absorbance orange red solutions was measured at 510 nm against a reagent blank. BHT was used as a positive control.

Lipid peroxidation inhibition assay: The lipid peroxide formed was estimated by measuring TBA reacting substances (TBARS) by a modified procedure of [20] using rat liver homogenate. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour 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 FeCl₂ and 0.5 mM H₂O₂ were added to initiate lipid peroxidation. After incubation at 37°C for 60 min, 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 min and then centrifuged at 4000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm. The inhibition effect on lipid peroxidation was calculated as follows: Inhibition effect (%) = $[1-(A1-A2)/A0] \times 100$, where A0 was the absorbance of the control (water instead of sample), A1 is the absorbance of the sample, and

A2 was the absorbance of the sample only (water instead of liver homogenate). Quercetin was used as a positive control.

Statistical analysis: All experiments were carried out in triplicates and results are expressed as mean±SD. The data were analyzed using SPSS software. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to analyze the differences among scavenging activity and EC₅₀ of various extracts for different antioxidant assays with least significance difference (LSD), $P < 0.05$ as a level of significance.

RESULTS AND DISCUSSION

Total phenol, flavonoid content and total antioxidant capacity assay: Phenolic compounds are one of the nonessential dietary components that have been associated with the inhibition of cancer, diabetes, and atherosclerosis [21]. Phenolic compounds such as flavonoids, tannins and phenolic acids are the important contributors of antioxidant efficiency of plants [22]. Flavonoids have been proven to display a wide range of pharmacological and biochemical actions like, antimutagenic, anticarcinogenic, antithrombotic and antimicrobial activities [23,24]. Thus the phenolics may contribute directly to antioxidative action of mushrooms. Table 1 shows the total phenol, flavonoid content of APEF extracts. The total phenolic and flavonoid content was found to be 17.60 mg FAE/g, 4.96 mg CAE/g respectively. Phenolic compounds such as flavonoids, tannins and phenolic acids are the important contributors of antioxidant efficiency of mushroom extracts.

The total antioxidant capacity (TAC) assay is 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 and the absorbance maximum at 695 nm [25]. The total antioxidant capacity APEF was found to be 29.26 mg GAE/g (Table 1).

DPPH radical scavenging assay: The principle of this assay is that, the antioxidant reacts with the stable free radical DPPH and converts it to 1, 1-diphenyl- 2-picryl hydrazine [26]. DPPH radical has certain advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation. DPPH is a stable free radical and possesses a characteristic absorbance at 517 nm, which decreases significantly on exposure to radical scavengers by donating a hydrogen atom to become a stable diamagnetic molecule. At 1-5 mg/ml, the DPPH radical scavenging ability of APEF was 40.56-72.43%, and it was found to be concentration

dependent (Fig. 1). The statistically significant difference was found between the concentrations tested ($P < 0.05$) and the DPPH radical scavenging activity decreased in the following order: QUE ($EC_{50}=0.031$ mg/ml) > APEF ($EC_{50}=3.15$ mg/ml). The DPPH radical scavenging effect of APEF was found to be better than *L. edodes* water extract which was measured as 55.4% at 6 mg/mL [27]. Sushila et al. [28] reported that the ethanolic extract of *L. conatus* exhibited EC_{50} value of 2.367 mg/ml which was found to be better than APEF. These results suggest that APEF with its proton donating ability could act as primary antioxidant to scavenge DPPH radical and could help to ameliorate cellular damage.

ABTS radical scavenging assay: ABTS^{•+} radical is a quite stable radical with a maximum absorption at 734 nm [29]. ABTS radicals are scavenged by antioxidants by the mechanism of electron-hydrogen donation. This decolorization technique is a widely used assay to evaluate the antioxidant capacity of the all types of samples [30]. APEF showed ABTS radical scavenging effect in a concentration dependent manner (Fig. 2). The ABTS radical scavenging ability was between 52.52-85.55% at 4-20 mg/ml. A significant difference ($P < 0.05$) was found between the different concentrations tested and the EC_{50} value of ABTS radical scavenging activity was found to be 3.90 mg/ml (EC_{50} of QUE= 0.024 mg/ml). Thus the extract showed ABTS^{•+} radical scavenging activity by preventing lipid oxidation via a chain breaking reaction. According to Kalyoncu et al. [31], the water extract of *Postia stiptica* exhibited 4.97% ABTS^{•+} radical scavenging activity at 1 mg/ml, which was found to be less than APEF in the present study.

Reducing power: Reducing power assay by Oyaizu method, is based on the measurement of the reductive ability of the mushroom extracts to transform Fe^{3+} to Fe^{2+} and this evaluation may serve as indicator of their potential antioxidant activity [32]. The reducing power of APEF increased as the concentration increased from 1 to 5 mg/ml. The reducing powers of APEF were 0.325-1.125 (Fig. 3). The reducing ability of APEF and QUE based on EC_{50} values exhibited the following order: QUE (0.259 mg/ml) > APEF (2.07 mg/ml). The statistically significant difference was found between the concentrations tested ($P < 0.05$). Extracts possess the ability to reduce iron (III) by its capability to donate electrons and exhibited in a dose dependent manner. The reducing power of methanolic extract of *P. florida* was found to be 0.911 at 500 μ g/ml concentration [33]. This property proves that it can act as free radical chain terminators, transforming more reactive free radicals

into stable nonradical molecules.

Phenanthroline assay: Ortho substituted phenolic compounds are very active and exert prooxidant effects by forming complexes with Fe^{2+} , which gets disrupted in the presence of chelators. This change in absorbance can be measured at 510 nm. The metal chelating function is important; since it reduces concentration of the catalyzing transition metal in lipid peroxidation processes [34]. The antioxidants in the extract interfered with ferrous-o-phenanthroline complex formation, thus suggesting that the extract has metal chelating capacity. The reducing ability of AAME was found to be dose dependent (Fig. 4). At the concentration of 2-10 mg/ml, the reducing power was between 0.439-0.863 and the EC_{50} value was observed to be 3.12 mg/ml (EC_{50} of BHT=0.151 mg/ml). A statistically significant difference ($P < 0.05$) in reducing capability was observed with the different concentrations tested.

Lipid peroxidation inhibition assay: Cellular damage is closely related to lipid peroxidation and responsible for many diseases. Initiation of lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex or through hydroxyl radical by Fenton's reaction. The hydroxyl radical is highly reactive when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids by producing lipid hydroperoxides [35]. Lipid hydroperoxide can be decomposed to produce alkoxy and peroxy radical and yield numerous carbonyl products such as malondialdehyde (MDA), which can cause damage to DNA, proteins and leads to the generation of cancer and aging related diseases [36]. Inhibition of lipid peroxidation was assessed by the amount of MDA produced. The production of MDA was inhibited by APEF in a dose-dependent manner (Fig. 5). At 1-5 mg/ml, LPO inhibition of APEF was found to be 8.17%-43.14%. A significant difference ($P < 0.05$) was found between the different concentrations tested and the EC_{50} value was found to be 6.32 mg/ml (EC_{50} of QUE=0.046 mg/ml). Selvi et al. [37] reported that aqueous extract of *Calocybe indica* showed 58.85% LPO inhibition. Thus the decrease in the MDA level with increase in the concentration of the extracts indicates the role of APEF as an effective antioxidant.

CONCLUSION

In conclusion, our data strongly indicate the various extent of antioxidant properties and phytochemical components of *Auricularia polytricha* ethanolic extracts. These antioxidant activities found to be increased significantly with an increase in sample concentration. Thus, the potent free radical

scavenging activity of *Auricularia polytricha* clearly proving its beneficial effects as antioxidants and further studies are in progress to identify the active principles of this mushroom and its biological functions.

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Table 1. Total phenol, flavonoid content and TAC of APEF

Sample	Total Phenols (mg FAE/g) ^A	Total Flavonoids (mg CAE/g) ^B	TAC (mg GAE/g) ^C
APEF	17.60±0.83	4.96±0.22	29.26±1.32

Values are expressed as mean ± SD (n = 3). ^AFAE = ferulic acid equivalents; ^BCAE = catechin equivalents; ^CGAE = gallic acid equivalents.

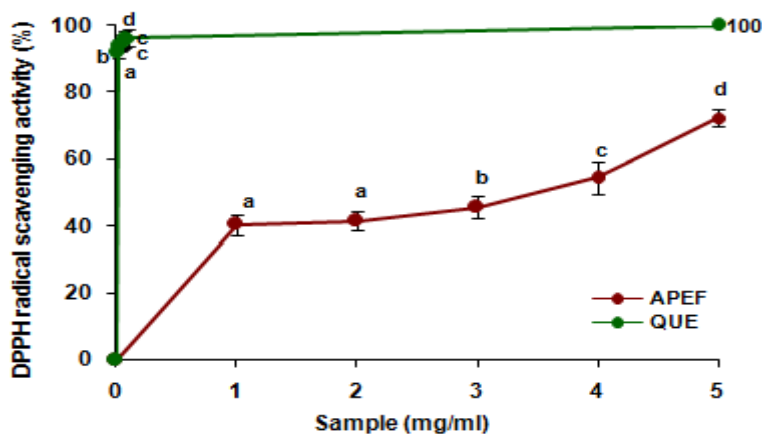


Figure 1. DPPH radical scavenging activity of APEF and QUE.

Results are expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentration of the same extract (P < 0.05, ANOVA, DMRT).

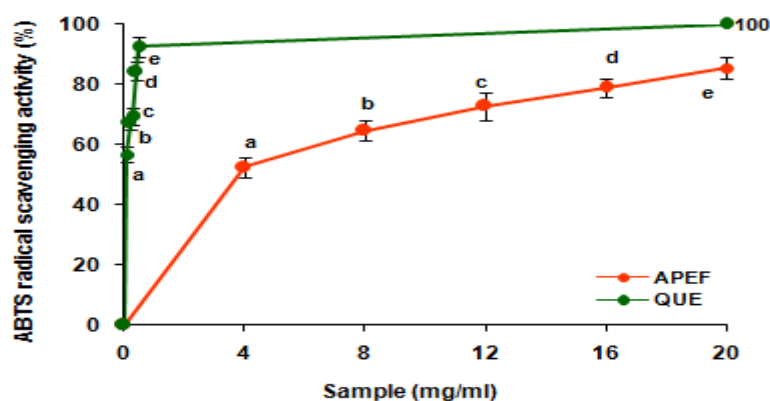


Figure 2. ABTS radical scavenging activity of APEF and QUE.

Results are expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentration of the same extract (P < 0.05, ANOVA, DMRT).

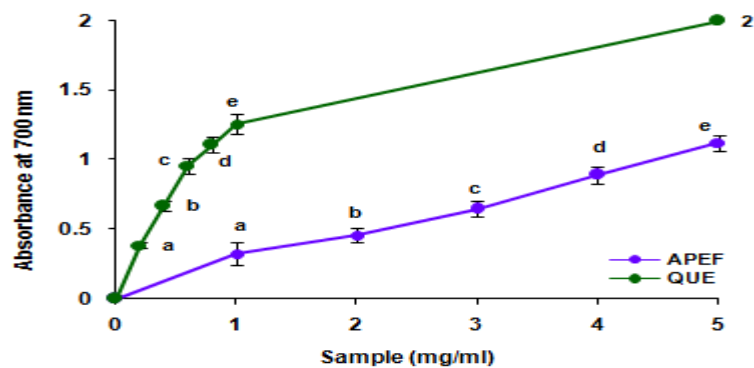


Figure 3. Reducing power assay of APEF and QUE.

Results are expressed as mean \pm SD (n=3). Different letters (a-e) indicate a significant difference between the concentration of the same extract (P < 0.05, ANOVA, DMRT).

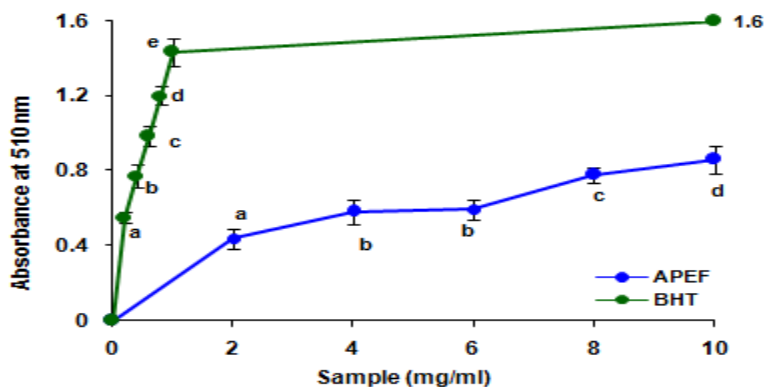


Figure 4. Phenanthroline assay of APEF and BHT.

Results are expressed as mean \pm SD (n=3). Different letters (a-e) indicate a significant difference between the concentration of the same extract (P < 0.05, ANOVA, DMRT).

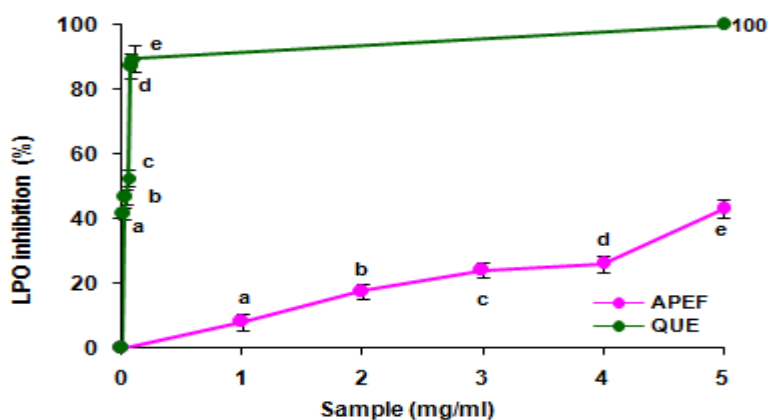


Figure 5. Lipid peroxide inhibition assay of APEF and QUE.

Results are expressed as mean \pm SD (n=3). Different letters (a-e) indicate a significant difference between the concentration of the same extract (P < 0.05, ANOVA, DMRT).

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