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IN VITRO ANTIOXIDANT ACTIVITY ANALYSIS IN *HERICIUM ERINACEUS PERS* FRUITING BODIES

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Abstract

The aim of this study was to evaluate the antioxidant properties in the fruiting bodies of *Hericium erinaceus pers*, an edible and medicinal mushroom. The hot water extract was prepared from *Hericium erinaceus* fruiting bodies and analyzed for ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) radical scavenging, reducing power, hydroxyl radical scavenging, lipid peroxidation and β-carotene bleaching inhibition assays. In addition, total phenol and flavonoid levels were determined. The extract exhibited good scavenging activity in a concentration-dependent manner in all the tested assays. The EC_{50} values of hot water extract on ABTS radical, reducing power, hydroxyl radical, lipid peroxidation and β-carotene bleaching assay were found to be 1.34, 1.70, 3.58, 5.12 and 2.62 mg/ml, respectively. Thus, the experimental results indicated that *Hericium erinaceus* mushrooms could potentially be utilized in food additives and also for the protective drug formulation.

Key-words: Antioxidant activity, Flavonoids, *Hericium erinaceus pers (Haudan-1)*, Total phenols.

Introduction

Hericium erinaceus is a well-known medicinal and edible mushroom belongs to the Hericium family; widely distributed in Asian counties such as Korea, Japan, and China. Its fruiting bodies and the fungal mycelia exhibit various biological functions, including its hypolipidemic effects, antimicrobial effect[1], anti-tumor activity[2], immunomodulatory effects[3-5]. Inspite of increasing usage and applications of H. erinaceus, most investigations have focused on its functional properties. Oxidative stress is an important mechanism underlying for most of the diseases because of the increased generation of reactive oxygen species (ROS). Antioxidant defence system consists of different enzymes such as catalase, superoxide dismutase and glutathione peroxidase destroys this free radicals[6-7]. Vitamin deficiency together with

overproduction of free radicals and a reduced level of above mentioned enzymes is considered as the main culprit for producing oxidative stress[8]. Mushrooms play a very important role in the treatment of deadly disorders, including infectious diseases. Therefore, some naturally occurring chemical compounds in mushrooms served as sources for clinically proven drugs[9]. Edible mushrooms have multiple functional properties[10], and the chemical analysis of mushrooms not only provides information concerning nutritional value, but also differentiates the species and strains having morphological similarity[11]. Apart from flavour and taste, the fruiting bodies of mushrooms are rich in organic nutrients such as digestible proteins, carbohydrates, fibre and certain vitamins, as well as minerals and antioxidants[12-14]. The present study was carried out to evaluate the antioxidant properties of hot water extract of *H. erinaceus*.

Materials and Methods

Chemicals and reagents: ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) and β-carotene, were obtained from Sigma-Aldrich (Bangalore, India). Ferulic acid, quercetin, gallic acid, 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 *Hericium erinaceus pers* (Haudan-1) 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 3 h. After centrifugation at 5000×*g* for 20 min, 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 min using a vacuum rotary evaporator. The extract obtained was dissolved in hot water at 100 mg/ml. 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 mushroom extract was measured according to the method of Singleton and Rossi with some modifications[15]. The sample (1.0 ml) 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 (FE) per gram of extract.

Estimation of total flavonoid

Total flavonoid content was determined as described by Jia *et al*[16]. The mushroom extract (0.25 ml) was diluted with 1.25 ml of distilled water. Then 75 µl of a 5% sodium nitrite were added into it. 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. Quercetin was used as the reference standard. The total flavonoid content is expressed as milligrams of quercetin equivalents (QE) 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 Prieto *et al*[17]. 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 (GA) per gram of extract.

ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was performed with slight modifications described by Re *et al*[18]. 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] × 100, where A0 was the absorbance of the control, and A1 was the absorbance of the sample.

Determination of reducing power

The reducing power of hot water extract was measured according to the method of Oyaizu[19]. The reaction mixture contained 2.5 ml of various concentrations of the extract, 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.

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of extracts was assayed by the method of Smirnoff and Cumbes[20]. The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM FeSO4, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate and 1.0 ml of varied concentrations of the sample. After incubation for 1 h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The scavenging activity of hydroxyl radical effect was calculated as follows : [1-(A1-A2) / A0] x 100, where A0 is absorbance of the control (without extract) and A1 is the absorbance in the presence of the extract, A2 is the absorbance without sodium salicylate.

Lipid peroxidation inhibition assay

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed, using rat liver homogenate[21]. 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 H_2O_2 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] × 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).

β-carotene bleaching assay

The antioxidant activity of extracts was evaluated by the β-carotene linoleate model system according to the method of Yae *et al*[22]. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two 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. A 4.8 ml of this emulsion were transferred into different test tubes containing 0.2 ml of different

concentrations of the mushroom extracts. 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: (β-carotene content after 2 h of assay/ initial β-carotene content)×100.

Statistical analysis

All assays were carried out in triplicates and results are expressed as mean±SD. The data were analysed using SPSS software. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to analyze the differences among scavenging activity and EC_{50} of various extracts for different antioxidant assays with least significance difference (LSD) $p<0.05$ as a level of significance.

Results and Discussion

Determination of total phenol and flavonoid content

Phenolic compounds are the most important groups of secondary metabolites in medicinal plants and herbs[23]. The total phenolics improve antioxidant activity with different mechanisms such as, singlet oxygen quenching, hydrogen-donation, metal ion-chelation, and acting as substrates for hydroxyl and superoxide radicals[24-26]. Flavonoids have been proven to display a wide range of pharmacological and biochemical actions such as antimutagenic, antimicrobial, anticarcinogenic and antithrombotic activities[27]. In food systems, flavonoids can serve as free radical scavengers and destroy the radical chain reactions that occur during the oxidation of lipids. Total phenolic and flavonoid contents of *H. erinaceus* in hot water extract were found to be 13.74 mg FE/g, 8.63 mg QE/g respectively (Figure 1).

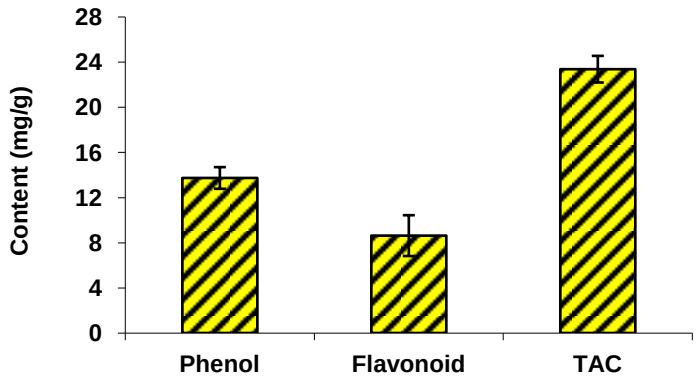

Figure 1: Total phenol (mg FAE/g), flavonoid (mg QE/g) content and total antioxidant capacity (mg GAE/g) of *H. erinaceus*. Each value is expressed as mean±SD (n=3).

Total antioxidant capacity (TAC) assay

The phosphomolybdenum method is based on the reduction of molybdenum by the antioxidants and the formation of a green molybdenum (V) complex, with maximum absorption at 695 nm. The total antioxidant capacity in hot water extract of *H. erinaceus* was observed to be 23.38 mg GAE/g (Figure 1).

ABTS radical scavenging assay

ABTS assay reflect the ability of hydrogen or electron donating activity of tested compound and is based on the inhibition of the absorbance of radical cation ABTS, which has a maximum absorption at 734 nm[18]. The extent of decolorization is determined as percentage inhibition of the ABTS radical cation as a function of concentration. The scavenging effect of *H. erinaceus* on ABTS radical increased with increasing concentrations. At concentrations of 4 to 20 mg/ml, the scavenging ability of *H. erinaceus* hot water extract on ABTS radicals increased from 56.88% to 85.32% (Figure 2A). A significant difference ($p<0.05$) was found between the different concentrations tested. The EC_{50} value was found to be 1.34 mg/ml. The results of the present study suggest that, the hot water extract of *H. erinaceus* exhibits good scavenging activity on ABTS radical.

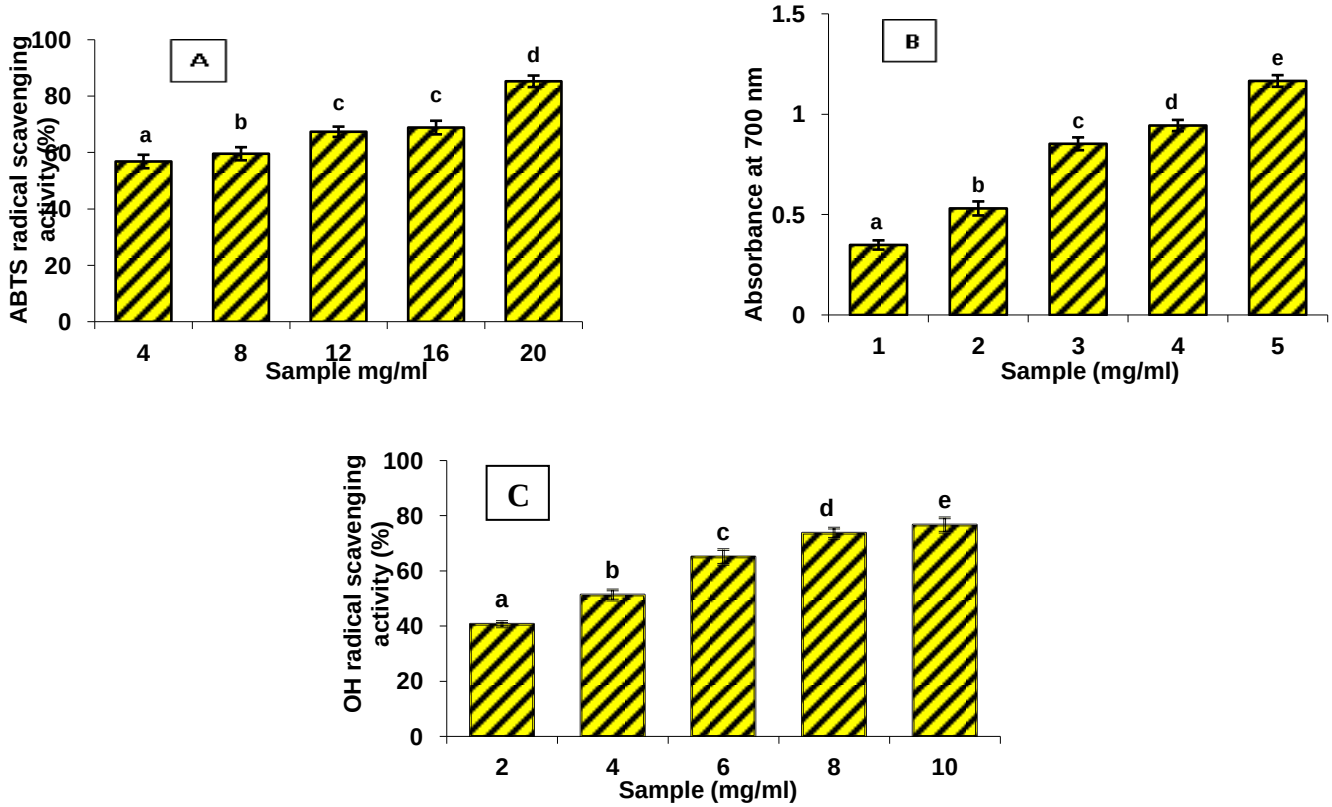

Figure 2: (A) ABTS radical scavenging assay; (B) Reducing power assay; (C) Hydroxyl radical scavenging assay of different extracts of *H. erinaceus*. Each value is expressed as mean±SD (n=3).

Different letters (a-e) indicate a significant difference between the concentrations of the same extract ($p<0.05$, ANOVA, DMRT).

Reducing power

This assay compares antioxidants based on their ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ion through the donation of an electron, with the resulting ferrous ion (Fe^{2+}) formation monitored spectrophotometrically at 700 nm[28]. In reducing power assay, reducing ability of a compound depends on the electron donor and free radical quenching capacity. Reducing agents hinder lipid peroxidation as they donate a hydrogen atom and stop the chain reaction which causes membrane damage[29]. In this study, the scavenging effect of *H. erinaceus* was found to be 0.349% to 1.166% at 1 to 5 mg/ml (Figure 2B). There was a significant difference in reducing power between the concentrations tested ($p<0.05$). The EC_{50} value was found to be 1.70 mg/ml. Hence, the hot water extract of *H. erinaceus* can be considered as a good scavenger of reductones, which could react with free radicals to stop radical chain reactions.

Hydroxyl radical scavenging activity

Hydroxyl radical is one of the most reactive free radical among ROS and it has the shortest half-life compared with other ROS. It induces severe damage to adjacent biomolecules and causes cellular injury[30]. The hot water extract of *H. erinaceus* exhibited hydroxyl radical scavenging activity in a dose-dependent way. At 2 to 10 mg/ml, the activity was found to be 40.64% to 76.60% (Figure 2C) and the EC_{50} value was 3.58 mg/ml. A statistically significant difference was found between the concentrations tested ($p<0.05$). Our results suggest that this mushroom extract could effectively scavenge hydroxyl radical.

Lipid peroxidation inhibition assay

The cell membrane's permeability and fluidity is maintained by phospholipids, fatty acids and glycerides composition. This lipid membrane integrity could be affected by free radicals by inducing lipid peroxidation reactions[31]. The production of lipid peroxides by Fe^{2+}/ascorbate systems in liver homogenate was strongly inhibited by the hot water extract in a concentration-dependent manner. The percentage of lipid peroxidation (LPO) inhibition by *H. erinaceus* is shown in Figure 3A. The LPO inhibition was found to be statistically significant ($p<0.05$) and it ranged from 22.22% to 59.47% at 1 to 5 mg/ml. The EC_{50} value was 5.12 mg/ml. This study suggests that the hot water extract exhibited a scavenging effect on lipid peroxidation.

β-carotene bleaching assay

In this test, β-carotene underwent rapid discoloration in the absence of antioxidants. The pentadienyl free radical molecule attacks the highly unsaturated β-carotene molecules in an effort to reacquire a hydrogen atom. As the β-carotene molecules lost their conjugation, the carotenoids lost their characteristic orange colour[32-33]. In the β-carotene bleaching test, the hot water extract of *H. erinaceus* exhibited a high antioxidant activity over a concentration range of 0.8 to 4 mg/ml with an inhibition percentage of 33.33% to 63.24% (Figure 3B) and the difference was statistically significant ($p<0.05$). The EC_{50} value was found to be 2.62 mg/ml. The antioxidative components in the mushroom extract are responsible to reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system. Thus, it is apparent that hot water extract of *H. erinaceus* have strong effect against the discoloration of β-carotene.

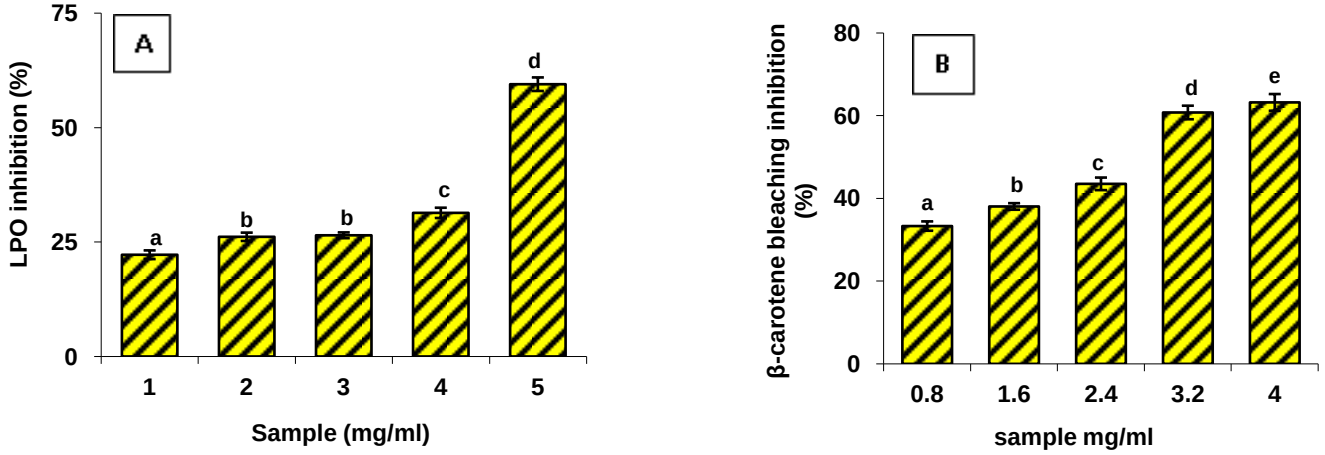

Figure 3: (A) Lipid peroxidation inhibition assay and (B) β-carotene bleaching inhibition assay of different extracts of *H. erinaceus*. Each value is expressed as mean±SD (n=3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract ($p<0.05$, ANOVA, DMRT).

Conclusion

In conclusion, the antioxidant activity of *Hericium erinaceus* was evaluated using several established *in vitro* biochemical assays. It possessed excellent free radical scavenging properties with considerable total phenolic and flavonoid content. Thus, the present study clearly concluded that the *Hericium erinaceus* possesses the ability to control oxidative damage and acts as antioxidant.

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