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Original Article

DETECTION OF ANTIOXIDANT ACTIVITY AND BIOACTIVE CONSTITUENTS IN THE FRUITING BODIES OF *HERICIUM ERINACEUS PERS*-AN EDIBLE MUSHROOM

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ABSTRACT

Objectives: *Hericium* is a genus of mushrooms in the *Hericiaceae* family. *Hericium erinaceus* has been used for the treatment of various diseases for over 2000 y in China. The study investigated the bioactive components and antioxidant properties of the hot water extract from the fruiting body of *H. erinaceus*.

Methods: The hot water extract of *H. erinaceus* was estimated for antioxidant potency using different analysis, such as 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay, ferric reducing antioxidant power (FRAP) assay, cupric ions reducing antioxidant capacity (CUPRAC) assay, metal ion chelation, erythrocyte hemolysis inhibition assay and compared with the reference standards. Total phenolic, flavonoid contents, and total antioxidant capacity were also determined using spectrophotometric methods.

Results: *H. erinaceus* showed concentration dependent activity in all the assays with following EC_{50} values. DPPH (EC_{50}= 2.13 mg/ml), FRAP (EC_{50}= 7.57 mg/ml), CUPRAC (EC_{50}= 0.97 mg/ml), ferrous ion chelation (EC_{50}= 2.31 mg/ml) and erythrocytes hemolysis inhibition (EC_{50}= 4.88 mg/ml). The total phenolic and flavonoid content in *H. erinaceus* was found to be 8.77 mg GAE/g and 5.62 mg RE/g respectively.

Conclusion: It was concluded that the hot water extract of the *H. erinaceus* exhibit potent free radical scavenging activity and can be used as natural antioxidants owing to their significant antioxidant activity.

Keywords: Antioxidant, Scavenging activity, Free radicals, Reducing power, *Hericium erinaceus pers*, Total flavonoids, Total phenolics

INTRODUCTION

Oxidation is an essential biological process in all living organisms for the production of energy. Production of reactive Oxygen species (ROS) is the end product of this process. An excessive amount of ROS can damage many bio-molecules such as lipids, proteins, and DNA, inducing many diseases such as cancer, inflammation, aging, diabetes and atherosclerosis [1]. ROS is constantly formed in the human body and removed from protective antioxidant systems in the body. Many synthetic drugs give protection against this oxidative injury, but show some side effects or adverse effects. Natural antioxidants derived from herbs and plants extract are the indigenous sources to minimize the level of ROS.

Edible Mushrooms consist of high contents of nutritive substances such as qualitative protein, fibers, vitamins and minerals which can be eaten or taken in the oral form unlike administrating through cells. Mushrooms are also the sources of bioactive substances that promote good health. A wide range of secondary metabolites are being produced by mushrooms with high therapeutic values. Both fruiting bodies and mycelium of the mushroom contain bioactive compounds with a wide range of antioxidant and antimicrobial activities, which is entirely a natural source of medicine [32].

Mushrooms are very commonly preferred as an excellent food choice as it has a high nutritional composition and also low calories, poor in fat [2, 3]. Several bioactive compounds from mushrooms have exhibited strong antioxidant effects that are more relevant to their health-protecting and promoting functions [4].

H. erinaceus is a well-known edible mushroom, also called as Lion's mane mushroom or Monkey head mushroom, which has been widely used as a traditional medicine in most of the Asian countries to cure various vulnerable diseases. It is also used as a food and flavoring agent in Chinese and Japanese cuisines. It is called "Houtou" in Chinese and "Yamabushitake" in Japanese [5] and this mushroom grows on old or dead broadleaf trees. The constituents of *H. erinaceus* have been previously investigated and shown to contain aromatic compounds, fatty acids, sterols, and polysaccharides. In recent years, *H. erinaceus* have attracted an attention of many biological researchers because of its beneficial medical functions such as anticancer [6], antimicrobial [7], anti-ulcer [8], anti-inflammation, antiaging [9], hypoglycemic and hypolipidemic [10], increasing cardiac blood output and improving the blood circulation. The goal of the study was to analyze the antioxidant activities of hot water extract from the fruiting bodies of *H. erinaceus* using the different standard chemical *in vitro* assays.

MATERIALS AND METHODS

Chemicals and reagents

1,1-diphenyl-2-picryl-hydrazyl (DPPH), rutin, 2, 2-azobis (2-amidino-propane) dihydrochloride (AAPH) and 2, 4, 6-tripyridyl-s-triazine (TPTZ) were obtained from Sigma-Aldrich (Bangalore, India). Rutin, EDTA, BHA, ascorbic acid and gallic acid were obtained from Himedia (Mumbai, India). All other chemicals were of analytical grade.

Mushroom samples

The dried fruiting bodies of *Hericium erinaceus pers (Haudan-1)* were obtained from Hangzhou Haudan Agri-food mushroom farms, Zhejiang Province, China. The dried fruiting bodies were powdered (20 mesh) and stored in airtight plastic bags for further analysis.

Preparation of the extracts

The mushroom powder (10 g) was extracted by stirring with 100 ml of boiling water at 100 °C for 6 h. After centrifugation at 5000 rpm for 20 min, the residues were re-extracted twice with the boiling water. The supernatants were collected together, and the combined extracts were evaporated under reduced pressure at 45 °C for 30 min using a rotary vacuum evaporator. The obtained extract was then dissolved in distilled water at 100 mg/ml (stock solution) and stored at 4 °C for further use. From the stock solution, successive

dilutions were made to analyze the antioxidant activity of the samples in triplicates.

DPPH free radical scavenging activity

The scavenging effect of the mushroom extract on DPPH radicals was determined according to the method of [11]. Various concentrations of the sample (4 ml) were mixed with 1 ml of a sample 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: [(A0-A1)/A0] × 100, where A0 was the absorbance of the control and A1 was the absorbance of the sample. BHA was used as a standard.

FRAP assay

In this assay, Ferric iron (Fe^{3+}) is initially reduced to ferrous form (Fe^{2+}) by donating antioxidants in the form of electrons present within the sample by developing a complex with dark blue color. The FRAP assay was used to determine the reducing capacity of mushroom extracts, according to the method of [12]. The FRAP reagent contained 2.5 ml of a 10 mM TPTZ in 40 mM HCl, 2.5 ml of a 20 mM $FeCl_3.6H_2O$ and 25 ml of 300 mM acetate buffer (pH 3.6). It was freshly prepared and warmed at 37 °C. 900 µl FRAP reagent was mixed with 90 µl water and 30 µl of the extract. The reaction mixture was then incubated at 37 °C for 30 min and the absorbance was recorded at 595 nm. BHA was used as a standard.

Cupric ions (Cu^{2+}) reducing power-CUPRAC assay

CUPRAC assay is advantageous over FRAP assay as it reacts to a broader range of thiol antioxidants and much stable method. The cupric ion (Cu^{2+}) reducing power was determined by the method proposed by [13] with minor modifications of [14]. 0.25 ml of copper (II) chloride solution (0.01 M), 0.25 ml of ethanolic neocuproine solution (7.5 x 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 incubated at room temperature. After 30 min of incubation, the absorbance was measured at 450 nm against a blank. The increase in absorbance of the reaction mixture indicates the increase in reduction capability. BHA was used as a standard.

Chelating effects on ferrous ions

The ability of the mushroom extracts to chelate ferrous ions was estimated by the method of [15]. Briefly, 2 ml of various concentrations of the extract was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 Mm ferrozine (0.2 ml). The mixture was then shaken vigorously and left undisturbed at room temperature for 10 min. The absorbance of the solution was measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as [(A0-A1)/A0] × 100, where A0 was the absorbance of the control and A1 of the mixture containing the extract or the absorbance of a standard solution. EDTA was used as a standard.

Inhibition of erythrocyte hemolysis

The erythrocyte hemolysis inhibition mediated by peroxyl free radicals by the mushroom extract was determined according to the procedure described by [16]. Blood was obtained from the rat and erythrocytes were separated from the plasma and the buffy coat were washed three times with 10 ml of 10 mM PBS, pH 7.4 and centrifuged at 1500 rpm for 5 min. During the last washing, the erythrocytes were obtained by centrifugation at 1500 rpm for 10 min. A suspension of erythrocytes in PBS (20%, 0.1 ml) was added to 0.2 ml of 200 mM AAPH solution in PBS, and 0.1 ml of various concentrations of the sample. The reaction mixture was shaken gently (30 rpm) while being incubated at 37 °C for 3 h. The reaction mixture was diluted with PBS (8 ml) and centrifuged at 3000 g for 10 min; the absorbance of the supernatant was then read at 540 nm. The percentage hemolysis inhibition was calculated by the equation: [(AAAPH-AS)/AAAPH] × 100, where AS was the absorbance of the sample and AAAPH was the absorbance of the control sample. Ascorbic acid was used as a standard.

Total antioxidant activity assay (Phospho molybdenum assay)

The antioxidant activity of the sample was evaluated by the phospho molybdenum method according to the procedure of [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, 4 mM ammonium molybdate and 28 mM sodium phosphate). The tubes were capped with silver foil and kept at 95 °C for 90 min. The tubes were cooled to room temperature, and the absorbance was measured at 695 nm against a blank. Ascorbic acid was used as a standard and total antioxidant capacity was expressed as milligrams of ascorbic acid equivalents (AAE) per gram of extract.

Total phenol estimation

The total phenolic in the mushroom extract was measured according to the method of [18] with some modifications. 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 deionized water. The mixture was kept for 90 min at room temperature in the dark. The absorbance was measured at 725 nm against the blank. Gallic acid was used as the reference standard. The total phenol content was expressed as milligrams of gallic acid equivalents (GAE) per gram of extract.

Total flavonoid estimation

Total flavonoid content was determined as described by [19]. 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 min, 150 µl of a 10% aluminum 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. Rutin was used as the reference standard. The total flavonoid content was expressed as milligrams of rutin equivalents (RE) per gram of extract.

Statistical analysis

All assays were carried out in triplicates, and the results are expressed as mean±Standard Deviation (SD). The data were analyzed using SPSS software. Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was used to analyze the differences between scavenging activity with a least significance difference (LSD) of $P<0.05$ as the level of significance.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

The principle of DPPH method is based on the reduction of DPPH compound in the presence of a hydrogen-donating antioxidant.

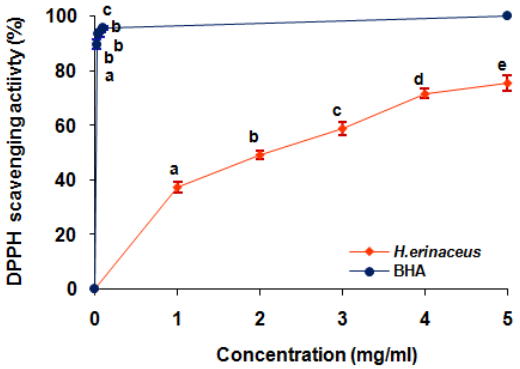

Fig. 1: DPPH radical scavenging activity of hot water extract of *H. erinaceus* and standard BHA at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

At 517 nm, it reduces the colour of DPPH due to the hydrogen donating ability of DPPH and produces a strong purple color in return [20, 21]. The scavenging activity of *H. erinaceus* hot water

extract was shown in fig. 1. The DPPH scavenging activity of hot water extract increased with increase in concentration with an inhibition percentage of 37.44-75.63% in 1-5 mg/ml, but lower than BHA (95.72%, 0.1 mg/ml). EC_{50} values of DPPH radical scavenging activity were found to be in the order: BHA (0.029 mg/ml)>*H. erinaceus* (EC_{50} = 2.13 mg/ml) and the difference was statistically significant between various concentrations of *H. erinaceus* ($P<0.05$). The better scavenging ability of the mushroom extract might be due to the presence of more hydrogen donating components. According to Ferreira *et al.* [22], the EC_{50} values for *L. deliciosus* and *T. portentosum* to scavenge DPPH free radicals are 8.52 mg/ml and 22.9 mg/ml respectively. The methanolic extract from *Agrocybe cylindracea* strain B scavenged DPPH radicals by 93.8% at 5 mg/ml. However, *H. Marmoreus* scavenged DPPH radicals by 59.7% at 5 mg/ml and 93.2% at 20 mg/ml [23].

Ferric reducing antioxidant power (FRAP) assay

This assay depends upon the reduction of Fe (III)-TPTZ to Fe (II)-TPTZ by reactants at low pH and develops an intense blue color which can be measured at 595 nm [24, 25]. Reducing the power of a compound acts as a significant indicator of its potent antioxidant activity. Ferric reducing the power of *H. erinaceus* increased from 0.482 to 1.169 at 4 to 20 mg/ml, but lower than BHA (0.987 at 0.1 mg/ml) (fig. 2). Based on EC_{50} value, FRAP was found to be in the order: BHA (0.057 mg/ml)>*H. erinaceus* (7.57 mg/ml). A significant difference ($P<0.05$) was observed in the concentration tested for *H. erinaceus.* An increase in absorbance of the reaction mixture indicated an increase in reducing capacity due to an increase in the complex formation. The FRAP assay was based on an electron transfer reaction in which a ferric salt was used as an oxidizing agent. Shwetha and Sudha reported that the aqueous extract from the mycelia of *V. Volvacea* showed EC_{50} value of 0.61 mg/ml for FRAP assay [25].

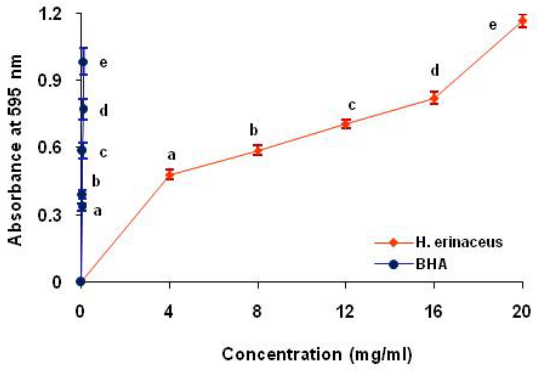

Fig. 2: Ferric reducing antioxidant power (FRAP) of hot water extract of *H. erinaceus* and standard BHA at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

CUPRAC assay

The CUPRAC assay is based on the reduction of $Cu^{2+}$to $Cu^{+}$by antioxidants in the extract. This method is cost-effective, stable, and more suitable for mushroom samples. This redox reaction was performed at a pH (7.0) close to the physiological pH [26] and this method was found to be capable of analyzing non-protein thiol group [27, 28]. Cu^{2+} reducing capability of *H. erinaceus* was found to be concentration dependent (fig. 3). At 0.5-2.5 mg/ml, cupric ion (Cu^{2+}) reducing the ability of hot water extract from *H. erinaceus* was found between 0.411-0.924, but lower than BHA (2.353 at 0.1 mg/ml). *H. erinaceus* extract exhibited EC_{50} value of 0.97 mg/ml and significant difference ($P<0.05$) was found between the different concentrations tested. Abdullah *et al.* reported that *G. lucidum* exhibited higher CUPRAC of 1.058 than other mushroom species at 0.50 mg/ml [29].

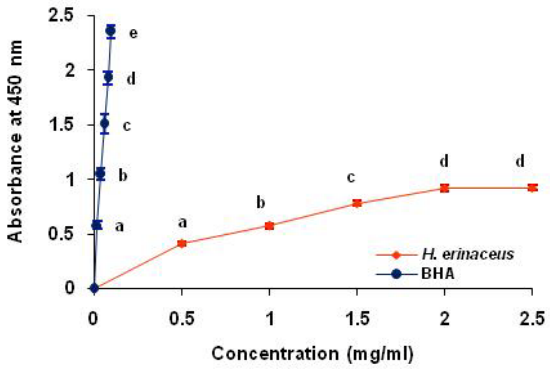

Fig. 3: CUPRAC assay of hot water extract of *H. erinaceus* and standard BHA at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

Chelating ability on ferrous ions

Chelation of transition metal ions, such as $Fe^{2+}$and Cu^{2+}, which could trigger a process of free radical reaction and resulted in lipid peroxidation and DNA damage. $Fe^{2+}$is one of the most powerful pro-oxidant that can quantitatively form red complexes with ferrozine [30]. So the Fe^{2+}chelating activity was considered as an important antioxidant property. The red color was quantitatively formed by the reaction of ferrozine with Fe^{2+}which was measured at 562 nm. The results of chelating abilities of *H. erinaceus* on ferrous ions were given in fig. 4. The chelating ability on ferrous ions of hot water extract exhibited good chelating activity in a dose-dependent manner. At the concentrations ranging from 1 to 5 mg/ml, the Fe^{2+}chelating activity of hot water extract of *H. erinaceus* showed 40.11-71.46%, but lower than EDTA (86.82% at 0.1 mg/ml). EDTA showed an excellent chelating ability (EC_{50} = 0.024 mg/ml) than *H. erinaceus* (EC_{50} = 2.31 mg/ml). A statistically significant difference ($P<0.05$) was observed with various concentrations of *H. erinaceus* extract tested. At 0.2-1 mg/ml, the different extracts of *Boletus edulis* exhibited chelating effect in the following order: ethanolic extract (18.16–65%)>methanolic extract (11.67–49.9%)>hot water extract (12.17–46.96%)>cold water extract (2.99–41.11%) [31].

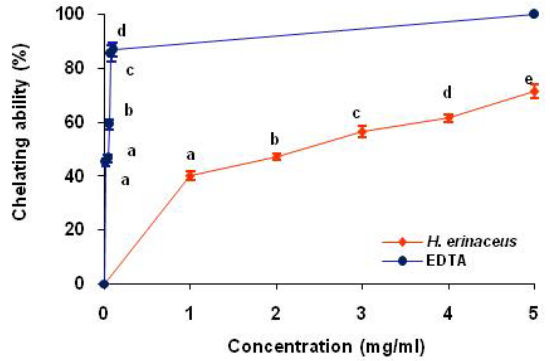

Fig. 4: Chelating ability of hot water extract of *H. erinaceus* and standard EDTA at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

Erythrocyte hemolysis inhibition assay

To explain the biological relevance of the free radical scavenging capacity of hot water extract of *H. erinaceus*, the human erythrocyte

was studied here as a cell-based model system. The AAPH induced oxidative damage on human erythrocytes has been most extensively studied as a model for the peroxidative injury in biological membranes [32-34]. The erythrocyte membrane is rich in PUFA which are very susceptible to free radical mediated lipid peroxidation and eventually leading to hemolysis. *H. erinaceus* extract showed hemolysis inhibition in a concentration dependent manner (fig. 5). The inhibition of erythrocyte hemolysis by hot water extracts of *H. erinaceus* was between 11.52-38.94% at 0.8-4.0 mg/ml, but lower than ascorbic acid (46.27% at 0.2 mg/ml, EC_{50} = 0.17 mg/ml). The hot water extract revealed moderate hemolysis inhibition (EC_{50} = 4.88 mg/ml). This study proved that *H. erinaceus* extract could effectively inhibit erythrocyte hemolysis under *in vitro* condition. The inhibition of erythrocyte hemolysis by *A. auricula* was between 6.67-59.37% at 2-10 mg/ml with an EC_{50} value of 9.01 mg/ml [35].

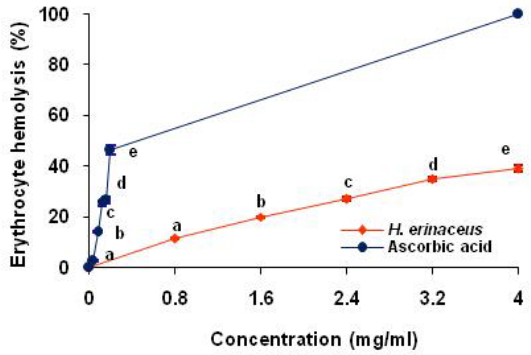

Fig. 5: Erythrocyte hemolysis inhibition of hot water extract of *H. erinaceus* and standard ascorbic acid at different concentrations. Results are expressed as mean±SD (n = 3). Different letters (a-e) indicate a significant difference between the concentrations of the same extract (P<0.05, ANOVA, DMRT)

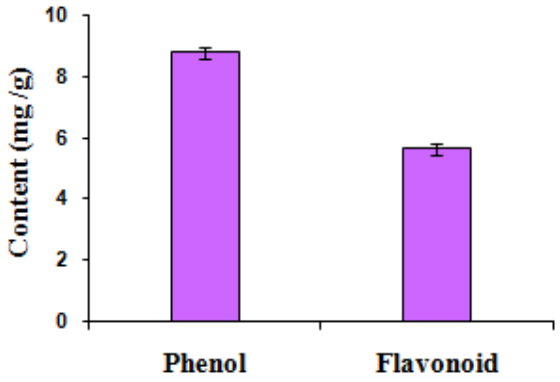

Fig. 6: Total phenol and flavonoid content of *H. erinaceus*

Total antioxidant capacity (TAC) assay

TAC assay was based on the reduction of Mo (VI) to Mo (V) by free radical scavengers in the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH. The total antioxidant capacity of *H. erinaceus* hot water extract was found to be 11.93 mg AAE/g. The TAC of aqueous extract of *V. Volvacea* mycelia was 231.49 nM GAE/g extract [25].

Determination of total phenol and flavonoid content

Phenolic compounds, ubiquitous in plants, are an essential part of the human diet and are of considerable interest due to their powerful antioxidant properties, anti-inflammatory and anticancer activities [36]. Fig.6 represents the total phenol and flavonoid content in hot water extract of *H. erinaceus*. The total phenol and flavonoid content were found to be 8.77 mg GAE/g and 5.62 mg RE/g respectively. Phenolic compounds have free radical scavenging ability facilitated by their hydroxyl groups. Flavonoids, on the other hand, suppress the reactive oxygen formation, chelate trace elements involved in the free-radical production, scavenge reactive species and up-regulate antioxidant defenses [37]. Flavonoids are the most common plant phenolic compounds which are very effective and more potent antioxidants. According to Keles *et al.* [23], the phenolic contents in the methanolic extracts of *Hydnum repandum* and *Boletus edulis* was 4020.0 g of GAE/kg of dry mushroom and 12775.33 mg of GAE/kg of dry mushroom respectively. The total flavonoid content found in the acetone extract of *Boletus edulis* and *Boletus aestivalis* was 4.93 µg of rutin equivalent and 3.20 µg of rutin equivalent respectively [38].

CONCLUSION

The results obtained in the present study indicate that hot water extract of *H. erinaceus* exhibited potent free radical scavenging and antioxidant activity in a dose-dependent manner. The overall antioxidant activity might be attributed to its polyphenolic content and other photochemical constituents. Thus, the findings suggest that *H. erinaceus* could be a potential source of natural antioxidant to prevent oxidative stress and cellular damage. Further, *in vivo* studies are in progress to evaluate its therapeutical potential against various diseases.

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CONFLICT OF INTERESTS

Declare none

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