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Research Article

PROXIMATE COMPOSITION, FREE RADICAL SCAVENGING AND NOS ACTIVATION PROPERTIES OF A WILD EDIBLE MUSHROOM

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ABSTRACT

Mushrooms are a high valued source of nutrition and mineral constituents which are of paramount importance in the present age. Here, different nutritional parameters, i.e., protein, carbohydrate, fat, amino acid, crude fiber and mineral contents of *Meripilus giganteus* were evaluated. Results showed that this mushroom had significant amount of carbohydrate, protein, free amino acids and crude fiber where as low amount of fat signifies its importance as diet for the sufferers of diabetes, obesity, atherosclerosis, high blood pressure, etc. Mineral analyses showed that this mushroom was good source of calcium, magnesium, potassium, phosphorous and iron. Cellular damage caused by reactive oxygen species has been implicated in several diseases and hence antioxidants have significant importance in human health. The cold water, hot water and ethanolic extract of *Meripilus giganteus* were analyzed for their antioxidant activities in different systems namely inhibition of lipid peroxidation, DPPH free radical and hydroxyl radical scavenging activities. The antioxidant activities among the three different extracts, ethanolic extract showed the strongest antioxidant activities in all the test systems. Furthermore, crude, boiled and ethanolic extracts also increased significantly nitric oxide production (189.2, 121.6, 279.7 pmol NO produced/mg dry wt/h respectively) over the control. The present results revealed that this mushroom might be utilized as a promising dietary supplement.

Keywords: Antioxidant activity, DPPH radical, Hydroxyl radical, Lipid peroxidation, *Meripilus giganteus*, Nitric oxide.

INTRODUCTION

Consumption of wild growing mushrooms has been preferred to eating of cultivated fungi in the world. Mushrooms are esteemed primarily for their flavor, but they can also be a healthy supplement to the diet. Mushrooms have been considered as rich food because they contain protein, sugar, glycogen, lipid, vitamins, amino acids and crude fibre. They also contain important mineral nutrients which are required for the normal functioning of the body[1,2]. They are also rich in vitamin-B, which act as a protective agent for liver, vitamin-D and vitamin-K. Some are also high in vitamin-A (e.g. *Cantharellus cibarius)* and a few (e.g. *Fistulina hepatica*) contain vitamin-C[3]. The tenet "Let food be thy medicine and medicine be thy food," espoused by Hippocrates nearly 2,500 years ago, is receiving renewed interest. In particular, there has been an explosion of consumer interest in the health enhancing role of specific foods or physiologically-active food components, so-called functional foods. The term functional as it applies to the food has adopted a different connotation – that of providing an additional physiological benefit beyond that of meeting basic nutritional needs. Today fungi as a food and medicine are consumed all over the world in a vast quantity and the commercial production of fungi are becoming a new sector of food and pharmaceutical industries. Their products have been called variously: vitamins, dietary supplement, functional food, neutraceuticals[4], nutriceuticals[5].

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase (SOD) and catalase or chemical compounds such as ascorbic acid, α-tocopherol, carotenoids, polyphenol compounds and glutathione[6]. But the uncontrolled productions of reactive oxygen species (ROS) were responsible for several pathophysiological processes[7]. The cause of a majority of disease conditions like atherosclerosis, hypertension, ischaemic disease, alzheimer,s disease, parkinsonism, cancer, diabetes mellitus and inflammatory conditions are being considered to be preliminary due to the imbalance between prooxidant and antioxidant homeostasis[8].

Nitric oxide (NO) produced at the cellular level from L-arginine catalyzed by nitric oxide synthase (NOS) is a very important signaling molecule[9,10]. It is well studied in mammalian system and has been found to have numerous roles in pathophysiology[11,12]. Cellular production of NO below physiologic level causes initiation of different diseases like hypertension, atherosclerosis, diabetes mellitus, ischemia, stroke, myocardial infarction, heart failure, hypoxia, Alzheimer's disease, fibrosis, cancer, renal failure, etc[13]. Activation of NOS enzyme to elevate NO production could protect the body from these killer diseases. Thus, NOS activation by supplementation of food would find a new route of therapy.

In recent years, mushrooms have become attractive as functional foods and as a source of physiologically beneficial medicines, while being devoid of undesirable side effects[14]. Edible mushrooms are highly nutritious and having therapeutic potentiality for the treatment of cancer, heart ailments, diabetes, inflammation, hepatic damage, high blood pressure, microbial pathogens etc[15-24].

The present study was focused on the evaluation of proximate compositions, antioxidant and NOS activation properties of a wild edible mushroom, *Meripilus giganteus.*

MATERIALS AND METHODS

Collection and Identification

Fruit bodies of an edible mushroom *Meripilus giganteus* (Pers.) P.Karst., commonly called "*Kukra chau* (Nepali)", were collected from local market of Darjeeling, West Bengal, India. They were saprophyte and growing in clusters on the logs and dead tree trunk. The voucher specimen was deposited at the Mycological Herbarium of Department of Botany, University of Calcutta, Kolkata, West Bengal, India.

Analyses of proximate composition

Preparation of tissue homogenate

1 g of fresh tissue was crushed and homogenized with liquid nitrogen. The resultant powder was extracted with 10 ml of 1 M phosphate buffer (pH 7.4). The homogenate was centrifuged at 16,000 × g for 30 min at 4˚C and the supernatant was collected, kept in -20˚C until further work.

Estimation of protein and soluble carbohydrate

Protein and soluble carbohydrate was estimated from the supernatant. Protein content was determined following the method of Lowry et al.[25] (1951) and soluble carbohydrate by dinitrosalicylic acid (DNS) method[26].

Estimation of total carbohydrate

For estimation of total carbohydrate, fresh thallus sample (1 g) was ground in mortar with liquid nitrogen. Then 5 ml of 2.5 (N) HCl was added to it and hydrolysed by keeping it in water bath for 3 h, cooled and neutralized with solid sodium carbonate[27]. The quantity of carbohydrate was determined after centrifugation according to DNSa method[26].

Estimation of fat

Fat was estimated by homogenizing tissue in 20 ml chloroform: methanol (2:1 v/v) mixture for 10 min in a tissue homogenizer. After vigorous shaking and filtering, the residue was again stripped with 25 ml chloroform: methanol mixture for 30 min. This combined filtrate was then shaken with 0.9 % sodium chloride to remove not fat contaminant[28]. The solvent layer was dried in vacuum and the total amount of fat was weighed according to the method of Itoch and Koneko[29] (1974).

Estimation of crude fibre

For estimation of crude fibre, 2gm of dried tissue was boiled in 200ml of sulphuric acid (1.25% w/v) for 30 min. Then it was filtered through muslin and washed with boiling water until the filtrate was no longer acidic, further boiled with 200 ml of sodium hydroxide (1.25% w/v) solution for 30 min, filtered through muslin, washed with 25 ml of boiled 1.25% w/v sulphuric acid, then washed thrice with water and finally with 25 ml absolute alcohol. The residue was then transferred into pre weighed ashing dish and dried for 2 hours at 130±2˚C. The dry weight was taken and the residue was ignited for 30 min at 660±15˚C cooled in a dissector and reweighed. The crude fibre was calculated according to the method of Maynard[30] (1970).

Estimation of moisture

Initially an amount (10 gm) of fresh tissue sample was taken. The amount of moisture in the tissue material was taken determined by drying the tissue in an over drier at about 60oC for 72 hours. The dried sample was weighed again after 72 h and the moisture percentage (M%) was calculated as following way.

M% = (Dry wt /Fresh wt) x 100

Determination of mineral elements

0.5 g. of dried specimens of each sample were used. Phosphorus was measured by Olsen method according to the Black[31] (1965). Ca, Mg, K, and Fe were determined by Varian Spectra AA55[32] following the extraction by 1 N CH3 COONH4 (pH 7).

Sample preparation for free radical scavenging and NOS assay

Fresh fruit bodies of *Meripilus giganteus* were freeze-dried. For each of ethanolic, cold water, hot water extractions, mushroom samples were randomly selected and prepared for analysis. A coarse powder (20 mesh) was obtained using a mill. For ethanolic extraction, a sub sample (10 g) was extracted by stirring at 100 rpm with 100 ml of methanol at 25˚C for 24 hr and filtering through Whatman No. 1 filter paper. The residue was then extracted with two additional 100 ml portions of methanol, as described above. The combined ethanolic extracts were then rotary evaporated at 40˚C to dryness. For cold water extraction a sub sample (10 g) was extracted by stirring at 100 rpm with 100 ml of cold water at 25˚C for 24 hr, centrifuging at 5000 g for 15 min and then filtering through Whatman No. 1 filter paper. The residue was then extracted with two additional 100 ml portions of cold water, as described above. For hot water extraction a sub sample (10 g) was extracted by stirring at 100 rpm with 100 ml of boiling water at 100˚C for 24 hr, centrifuging at 5000 g for 15 min and then filtering through Whatman No. 1 filter paper. The residue was then extracted with two additional 100 ml portions of boiling water, as described above. The cold and hot water extract was freeze-dried. The dried extracts were redissolved in water or methanol (For DPPH radical assay) to a concentration of 10 mg/ml and stored at 4˚C for further used[33].

Assay of hydroxyl radical

Hydroxyl radicals (OH^-) are generated from Fe^{2+}- ascorbate- EDTA-H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set off a series of reactions that eventually result in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535 nm[34]. Reaction mixture (1 ml) contained deoxyribose (2.8 mM), KH_2PO_4- KOH (20 mM; pH 7.4), $FeCl_3$ (100 mM), EDTA (104 µM), H_2O_2 (1 mM) and ascorbate (100 µM). Reaction mixture was incubated at 37˚C for 1 h and colour developed as described above. IC_{50} value of deoxyribose degradation by the cold water, hot water and ethanolic extracts of *Meripilus giganteus* over the control was measured. Catechin was used as positive control.

DPPH radical scavenging assay

The hydrogen atom or electron donation abilities of the corresponding extracts and a pure compound were measured from the bleaching of the purple colour methanol solution of 1, 1-diphenyl-2- picryl hydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent[35,36]. 200-600 µl of various concentrations of the extracts in methanol were added to 2ml of 0.004 % methanol solution of DPPH. After 30 min. incubation period at room temperature in dark, the absorbance was read against a methanol blank at 517 nm. Inhibition of free radical of DPPH in percent (I %) was calculated in the following way

$$I\ \% = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the plot of inhibition (%) against extract concentration. BHT (Butylated hydroxylated toluene; 2, 6- di-tertiary- butyl- 4-methyl phenol; Merk) was used as control.

Assay of lipid peroxidation

Lipid peroxidation was induced by Fe^{2+}ascorbate system in human red blood cells (RBC) and estimated as thiobarbituric acid reacting substances (TBARS) by the method of Buege and Aust (1978)[37]. The reaction mixture contained RBC- packed cell (10^8cells/ ml) in Tris- HCl buffer (20 mM; pH 7.0) with $CuCl_2$ (2 mM), ascorbic acid (10 mM) and different extracts of *Meripilus giganteus* in final volume of 1 ml. The reaction mixture was incubated at 37^0C for 1 h. Lipid peroxidation was measured as malondialdehyde (MDA) equivalent using trichloroacetic acid (TCA), thiobarbituric acid (TBA) and HCl (TBA-TCA reagent: 0.375 % w/v TBA; 15 % w/v TCA and 0.25 N HCl). The incubated reaction mixture was mixed with 2ml of TBA-TCA reagent and heated in a boiling water bath for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at 10,000 g for 5 min. Finally malondialdehyde concentration in the supernatant fraction was determined spectrophotometrically at 535 nm. The concentrations of cold water, hot water and ethanolic extracts that would inhibit by 50%, the production of thiobarbituric acid reactive substances, i.e., IC_{50} values, were calculated. Catechin was used as control.

Determination of nitric oxide (NO) synthase activity

NO was determined according to Jia *et al.* (1996) by using scanning Elico BL 198 Bio spectrophotometer [38]. Typically, NO content was determined by conversion of oxyhemoglobin to methemoglobin. The reaction mixture containing RBC (10^8 cells) was incubated with L-arginine (10 µM), hemoglobin (30 µM) with different concentrations of cold water, hot water and ethanolic extracts of *Meripilus giganteus*; in a total volume of 2.5 ml for different time periods at 37˚C. After each incubation period, a portion of reaction mixture was centrifuged at 8,000 g for 5 min at 37˚C, and NO content of the supernatant was compared with an appropriate control set.

Statistical Analysis

Results were subjected to statistical analysis using MS Excel software (CORREL, Statistical function). All data presented are means of three separate experiments, each in triplicate along with standard deviations (SD).

RESULTS AND DISCUSSION

Proximate composition of *Meripilus giganteus*

Protein is the most critical component contributing nutritional value of food. Since fats and carbohydrate are rarely lacking in diet, they are not generally considered in nutritional evaluation. Proteins constitute more than half of total nitrogen. The protein content depends on the composition of the substratum, size of the pileus, harvest time and the species of mushrooms. The protein content of the fully grown basidiocarp of *Meripilus giganteus* was 21.2 g / 100 g of dry weight (n=3) (Fig. 1). Based on this value, this mushroom is grouped between low-grade vegetable and high-grade meat. The mushroom protein is known to contain almost all the essential amino acids. Apart from essential amino acids, considerable amount of alanine, arginine, glycine, histidine, glutamic acid, aspartic acid, proline and serine can be found in mushroom.

Of the dry matter constituent of mushroom, carbohydrates were found in the greatest amounts[39]. The carbohydrate of mushrooms as studied by some workers was present as trehalose, which gets hydrolyzed and later gives rise to mannitol[40]. In edible mushrooms the dominant sugar is mannitol. Apart from mannitol, mushrooms also contain glucose, galactose, trehalose, mannose and fructose[41]. The carbohydrate of *M. giganteus* was estimated to contain 18.5 g (n=3) of soluble carbohydrate / 100 g of dry tissue and 53.7 g (n=3) of total carbohydrate / 100 g of dry tissue respectively (Table 1). A considerable portion of the carbohydrate compounds occurs in the form of polysaccharides with particles of different sizes. Fungal polysaccharides are represented by glycogen and such indigestible form as dietary fibre of cellulose, chitin, mannose and glucans[42,43].

The content of crude fibre in 100 g of dry tissue were 23.46 g / 100 g of dry weight in the basidiocarp of *M. giganteus* (n=3) (Fig. 1). The dietary fibre was declared a nutrient by the Nutrition Labelling and Education Act of 1993[44]. According to Gordon (2002)[44], there is a "dietary fibre hypothesis" which suggests that fibre helps to prevent many diseases prevalent in affluent societies. Fresh mushrooms contain both soluble and insoluble fibre. The soluble fibres are mainly beta-glucan polysaccharide and chitosans, which are components of the cell walls[42]. Soluble fibre has been shown to help prevent and manage cardiovascular disease by lowering total and low-density lipoprotein (LDL) cholesterol levels. It also helps regulate blood sugar levels[45]. The main role of the insoluble fibre found in fresh mushrooms is to ensure the peristaltic regularity and good bowel health. It also helps slow digestion and adds satiety or staying power to foods. When fibre-rich foods are chosen, the diet is lower in energy density and has more volume than a low-fibre diet[46]. More fibre means less room for high fat, high-calorie choices, which can translate into weight loss and healthy weight maintenance. Hence, foods that are good sources of dietary fibre are appealing to many consumers.

The content of the fats in mushroom is low ranging from 1.1 to 8.3% on dry weight basis with an average content 4.0% [47]. It was estimated that the basidiocarp of *M. giganteus* contain 2.2 g / 100 g dry tissue respectively (n=3) (Fig. 1). The moisture content of the fresh basidiocarp was 89%.

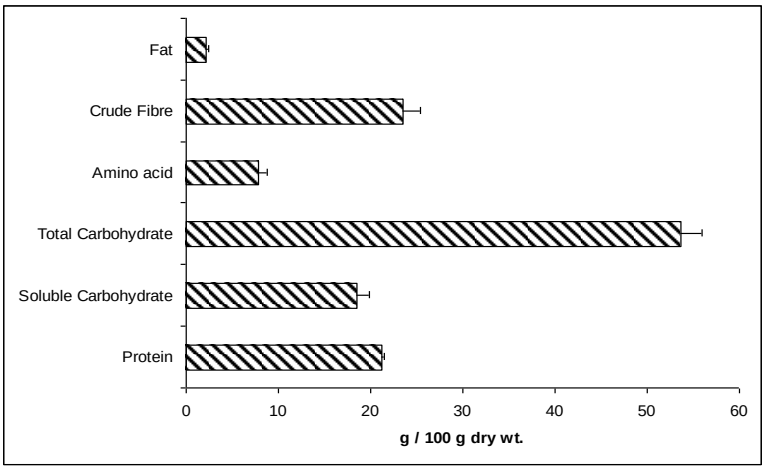

Fig. 1: The proximate composition of *Meripilus giganteus*. Results are the mean ± SD of three separate experiments, each in triplicate.

The fruit body of mushrooms is characterized by a high level of well assimilability mineral constituent[48]. Results in the Fig. 2 showed that *M. giganteus* basidiocarps were rich in different macro and micro mineral composition.

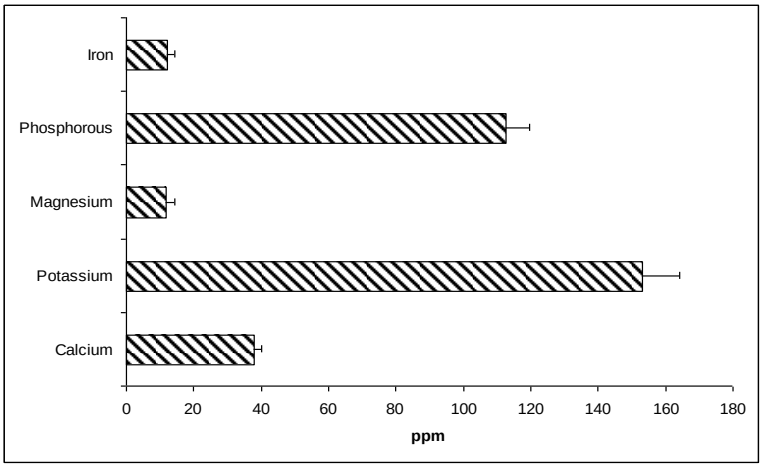

Fig. 2: Mineral composition of *Meripilus giganteus*. Results are the mean ± SD of three separate experiments, each in triplicate.

Free radical scavenging activities of different extracts

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage[49]. Ferric – EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and were detected by their ability to degrade 2-deoxy-2-ribose into fragments that formed a pink chromogen upon heating with TBA at low pH[50]. When the test extracts were added to the reaction mixture, they removed hydroxyl radicals from the sugar and prevented their degradation. All the extracts showed potential hydroxyl radical scavenging activity (Fig. 3). The concentration of the ethanolic extract needed for 50% inhibition was 70.3 µg/ml, comparable to that of catechin (840 µg/ml).

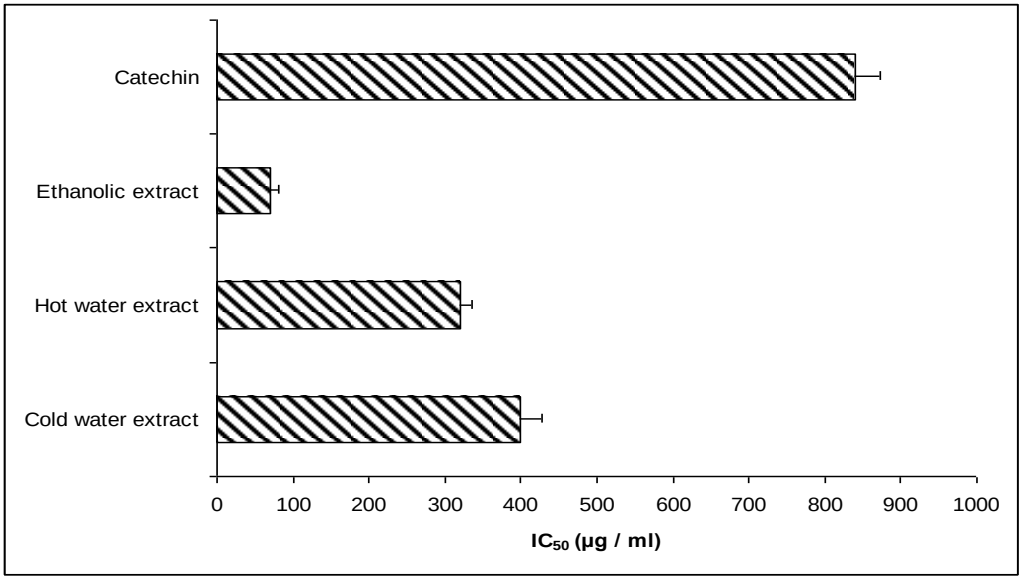

Fig. 3: *In vitro* hydroxyl radical scavenging activity of *Meripilus giganteus* extracts. Results are the mean ± SD of three separate experiments, each in triplicate.

DPPH radical is a stable free radical and possess a characteristic absorbance at 517 nm, which decreases significantly on exposure to radical scavengers by providing hydrogen atom or electron to become a stable diamagnetic molecule[49]. The use of stable DPPH radical has the advantage of being unaffected by side reactions, such as enzyme inhibition and metal chelation[51]. Such reactivity has been widely used to test the ability of the compound or extracts to act as free radical scavengers. Reduction of the DPPH radicals can be observed by the decrease in absorbance at 517 nm. The results presented in (Fig. 4) indicated all the extracts have significant DPPH radical scavenging activity and the 50% of inhibition value seems to be fairly close when compared to commonly used synthetic antioxidant BHT (85.7 µg/ml).

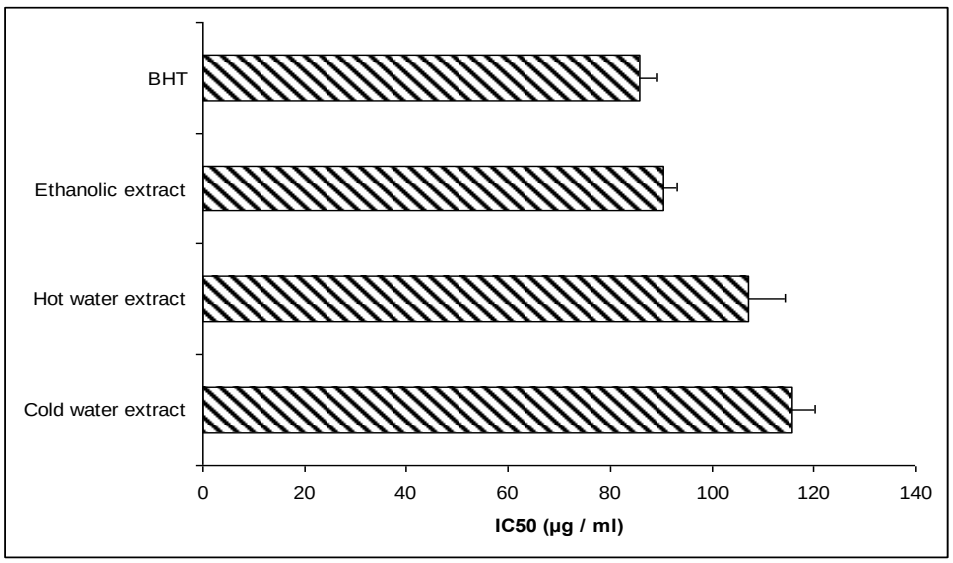

Fig. 4: *In vitro* DPPH radical scavenging activity of *Meripilus giganteus* extracts. Results are the mean ± SD of three separate experiments, each in triplicate.

A free radical prefers to steal electrons from the lipid membrane of the cell, initiating a free radical attack on the cell induced lipid peroxidation in polyunsaturated lipid rich areas like brain and liver[52]. The results presented in Fig. 5 showed that all the extracts of *M. giganteus* inhibit Fe^{2+}- ascorbate induced lipid peroxidation much better than standard catechin. The 50% of inhibition value of ethanolic extract (132.2 µg/ml) of *M. giganteus* seems to be less than one third when compared to standard (IC_{50} = 455 µg/ml for catechin).

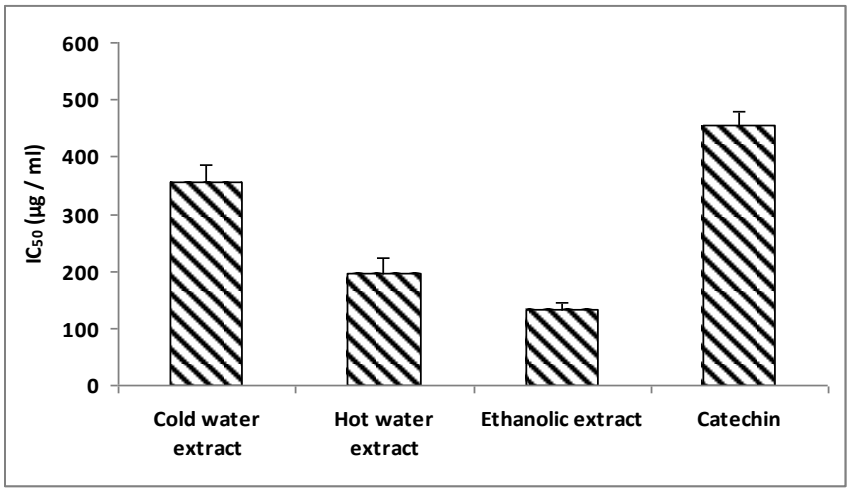

Fig. 5: Inhibitory concentration 50% of lipid peroxidation by *Meripilus giganteus* extracts. Results are the mean ± SD of three separate experiments, each in triplicate.

Determination of nitric oxide (NO) synthase activity

Nitric oxide is recognized to be an inter- and intra- cellular mediator of several cell functions. It acts as a signal molecule in immune, nervous and vascular systems[53]. Further study was made to evaluate the nitric oxide synthase activation properties of cold water, hot water and ethanolic extracts of *M. giganteus*. All the three extracts, of *M. giganteus* showed significant increase in nitric oxide production over control (Fig. 6). Use of 10 µM N^G methyl - L - arginine acetate ester (NAME), a competitive inhibitor of nitric oxide synthase (NOS)[54], in the reaction mixture showed complete inhibition of NO production in all cases, indicating the increased production of NO was due to the activation of NOS. Ethanolic extract showed considerable NOS activation properties when compared to the other extracts.

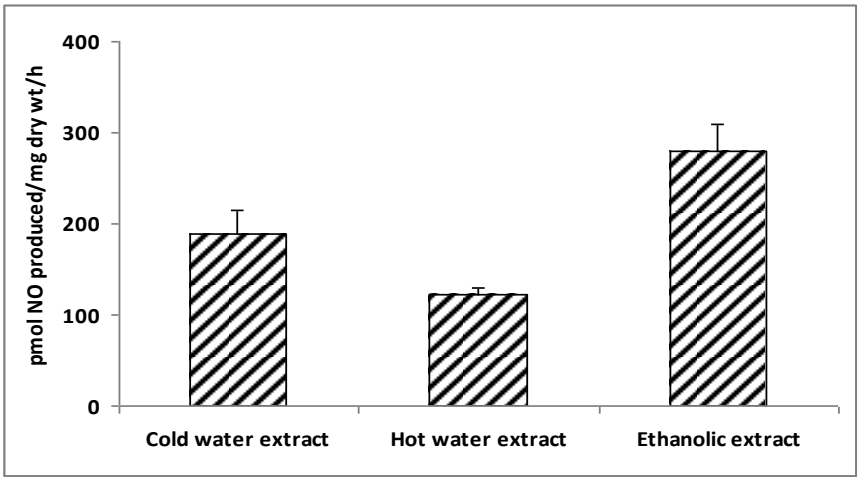

Fig. 6: Production of nitric oxide by different extracts of *Meripilus giganteus* over control. Results are the mean ± SD of three separate experiments, each in triplicate.

Cellular damage caused by reactive oxygen species has been implicated in several diseases and hence, antioxidants have significant importance in human health. Peroxide radicals in biological system are regarded to be associated with a number of pathological complications. Lipid peroxidation *in vivo* destroys biological membranes leading to change in fluidity and permeability[55]. The beneficial role of NO in different pathophysiologic condition is well documented. From the above investigation, it could be concluded that the ethanolic extract of *M. giganteus* possessed significant antioxidant activity and NOS activation properties, thus suggesting the therapeutic value of this mushroom, which might be used as medicine for several killer diseases. In conclusion, all these results should encourage further *in vivo* studies, which could ultimately lead to an inclusion of this mushroom in different pharmaceutical formulations and functional food formulations.

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