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Antioxidant and Nitric Oxide Synthase Activitation Properties of *Macrocybe gigantea* (Massee) Pegler and Lodge

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Abstract: Cellular damage caused by reactive oxygen species has been implicated in several diseases and hence antioxidants have significant importance in human health. *In vitro* evaluation of antioxidant activities of *Macrocybe gigantea* showed significant inhibition of lipid peroxidation, potent hydroxyl and superoxide radical scavenging activity when compared with standard drug. IC_{50} values of crude, boiled and ethanolic extracts of the basidiocarp represented 94.25, 81.03 and 74.18 μg ml^{-1} in hydroxyl radical scavenging activity; 471.63, 601.73 and 349.72 μg ml^{-1} in superoxide scavenging activity and 123.3, 105.2 and 79.9 μg ml^{-1} in case of lipid peroxidation inhibition respectively. Furthermore, crude, boiled and ethanolic extracts also increased significantly nitric oxide production (208.33, 300.00 and 612.33 pmol mg^{-1} dry wt/h respectively) over the control. Among different extracts, the ethanolic extract was the most effective in relation to antioxidant and nitric oxide synthase (NOS) activation properties. The present results revealed that *Macrocybe gigantea* as a promising source of therapeutics.

Key words: Antioxidant activity · Hydroxyl radical · Lipid peroxidation · *Macrocybe gigantea* · Nitric oxide synthase · Superoxide radical

INTRODUCTION

Reactive oxygen species produced by sunlight, ultraviolet, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects, such as causing DNA damage, carcinogenesis and cellular degeneration related to aging [1]. Superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, superoxide radical is normally formed first and its effects can be magnified because it produces other kinds of cell-damaging free radicals and oxidizing agents. However, the damaging action of the hydroxyl radical is the strongest among free radicals. Synthetic compounds are found to be strong radical scavengers but usually they have side effects [2]. Neutralization of this radical activity by naturally occurring substances mainly by supplementation of food having antioxidant property is becoming one of the most acceptable modes of modern therapy. Amongst them, mushroom or their derivatives or extracts occupy an elite position to perform this function [3-6].

Likewise, Nitric Oxide (NO) produced at the cellular level from L-arginine catalyzed by Nitric Oxide Synthase (NOS) is a very important signaling molecule [7, 8] from the ground of pathophysiologic condition of living entities. It is well studied in mammalian system and has been found to have numerous roles in pathophysiology including vasodialation [7], regulation of blood pressure [9], inhibition of platelet aggregation and adhesion [10], inhibition of neutrophil adhesion [7], neuromodulation in the CNS [7], antioxidant [11], antithrombotic [12] and as second messenger of insulin [8]. Cellular production of NO below physiologic level causes initiation of different diseases like hypertension, atherosclerosis, diabetes mellitus, ischemia, stroke, myocardial infraction, heart failure, hypoxia, Alzheimer 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.

Macrocybe gigantea of the family *Tricholomataceae* [14], a wild edible mushroom is distributed in outskirts of

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West Bengal, India during the months of July to October. Literature survey revealed that no work has been done relayed to the medicinal importance of this mushroom. Wang *et al.* [15] have shown the immunomodulatory and anticancer activity of a protein bound polysaccharide complex from *M. lobayense*. Liu *et al.* [1] reported the free radical scavenging activities of polysaccharide of *M. lobayense*. The present study was conducted to evaluate the antioxidant activity and NOS activation properties of the different extracts of *M. gigantea*.

MATERIALS AND METHODS

Sample collection and preparation: Basidiocarp of *Macrocybe gigantea* was collected from the forest and local market of Kolkata and adjoining area.

Crude extract was prepared from fresh tissues (100 gm/100 ml of distilled water) after homogenization in distilled water and centrifugation at 15000 g for 30 min at 4°C. Supernatant was lyophilized (Lyolab BII LSL Secfroid lyophilizer) and lyophilized material stored at -20°C for further use.

Boiled extract was also prepared from fresh fruit body (100 gm/100 ml of distilled water) and boiling it in water bath for 1 h, then homogenized and centrifuged at 15000g for 30 min at room temperature. Supernatant was lyophilized and then stored at-20°C for further use.

Fresh mushrooms were randomly selected into three samples of 150 g each and air-dried in an oven at 40°C for 48 h. Dried powdered mushroom sample was extracted by stirring with 200 ml of ethanol at 30°C for 24 h at 150 rpm and filtering through Whatman No. 4 filter paper. The residue was then extracted twice with another 200 ml of ethanol as described above. The total extract was then rotary evaporated to dryness at 40°C and redissolved in ethanol to a concentration of 10 mg ml^{-1} and stored at-20°C for further use [16].

Assay of hydroxyl radical (OH^{-})-scavenging activity: Hydroxyl radical ($OH^{\cdot}$) was generated from Fe^{2+}-ascorbate-EDTA-H_2O_2 system (Fenton's reaction) which attack the deoxyribose and set of a series of reaction that eventually resulted in formation of MDA, measured as a pink MDA-TBA chromogen at 532 nm [17]. Reaction mixture (1 ml) contained deoxyribose (2.8 mM), KH_2PO_4-KOH (20 mM; pH 7.4), $FeCl_3$ (100 mM), EDTA (104 mM), H_2O_2 (1 mM), ascorbate (100 μM) and various concentrations of the different extracts. Reaction mixture was incubated at 37°C for 1h and colour developed as described above. IC_{50} values of deoxyribose degradation by the crude, boiled and ethanolic extracts of *Macrocybe gigantea* over the control were measured. Catechin was used as positive 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, [18]. The reaction mixture contained RBC-packed cell (10^8 cell ml^{-1}) in Tris-HCl buffer (20 mM, pH 7) with $CuCl_2$ (2 mM), ascorbic acid (10 mM) and different concentrations of extracts of *Macrocybe gigantea* in final volume of 1 ml. The reaction mixture was incubated at 37°C for 1h. 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 2 ml 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 crude, boiled and ethanolic extracts that would inhibit by 50% of the production of thiobarbituric acid reactive substances, i.e. IC_{50} values, were calculated. Catechin was used as control.

Assay of superoxide radical (O_2^{-})-scavenging activity: The method used by Martinez *et al.* [19] for determination of the superoxide dismutase was followed with modification [20] in the riboflavin-light-nitrobluetetrazolium (NBT) system [21]. Each 3 ml reaction mixture contained 50 mM sodium phosphate (pH 7.8), 13 mM methionine, 2 μM riboflavin, 100 μM EDTA, 75 μM NBT and 500 μl sample solution of various concentrations of crude, boiled and ethanolic extracts. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination of a fluorescent lamp. Identical tubes with the reaction mixture were kept in the dark and served as blanks.

Determination of nitric oxide (NO) synthase activity: NO was determined according to Jia *et al.* [22] by using scanning Hitachi 330 spectrophotometer. Typically, NO content was determined by conversion of oxyhemoglobin to methemoglobin. The reaction mixture containing RBC (10^6 cells) was incubated with L-arginine (10 μM), hemoglobin (30 μM) with different concentrations of crude, boiled and ethanolic extracts of *Macrocybe gigantea* 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 8,000g for 5 min at 37°C and NO content of the supernatant was compared with an appropriate control set.

Statistical analysis: Statistical analyses was performed by student's 't' test and in all the cases results are mean±SD (standard deviation) of at least three individual experimental data.

RESULTS AND DISCUSSION

Assay of hydroxyl radical (OH^-)-scavenging activity: Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage [23]. 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 [24]. 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 (Table 1). The concentration of the ethanolic extract needed for 50% inhibition was 74.18 μg ml^{-1}, comparable to that of catechin (840 μg ml^{-1}).

Assay of lipid peroxidation: 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 [25]. The results presented in Fig. 1. showed that all the extracts of *Macrocybe gigantea* inhibit Fe^{2+}-ascorbate induced lipid peroxidation much better than standard catechin. The 50% of inhibition value of ethanolic extract (79.9 μg ml^{-1}) of *Macrocybe gigantea* seems to be approximately one fifth when compared to standard (IC_{50} = 455 μg ml^{-1} for catechin).

Table 1: *In vitro* hydroxyl radical and superoxide anion scavenging activity of *Macrocybe gigantea* extracts (IC_{50} μg ml^{-1})

	Extracts			
	Crude	Boiled	Ethanolic	Standard
Hydroxyl radical scavenging activity	94.25±14.96	81.03±12.17	74.18±15.13	840±25[a]
Superoxide radical scavenging activity	471.63±24.4	601.73±52.9	349.72±0.37	65±3.5[b]

Values represented as means±SD from three independent observations, a, catechin as standard; b, ascorbic acid as standard

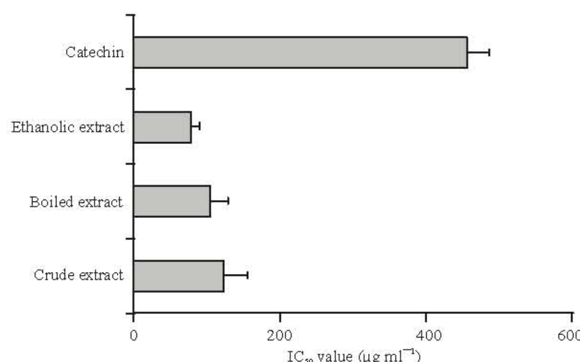

Fig. 1: Inhibitory concentration 50% of lipid peroxidation by *Macrocybe gigantea* extracts. Results are the mean±SD of three separate experiments, each in triplicate

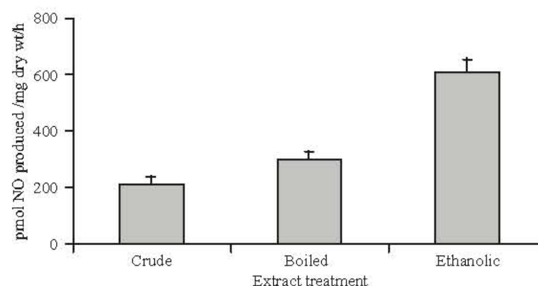

Fig. 2: Production of nitric oxide by different extracts of *Macrocybe gigantea* over control. Results are the mean±SD of three separate experiments, each in triplicate

Assay of superoxide radical (O_2^-)-scavenging activity: Superoxide radical (O_2^-) is known to be very harmful to cellular components as a precursor of more reactive species [26]. Among all the extracts, ethanolic extract showed moderate activity with an IC_{50} = 349.72 μg ml^{-1} when compared to that of ascorbic acid (IC_{50} = 65 μg ml^{-1}) (Table 1).

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 [27]. Further study was made to evaluate the nitric oxide synthase activation properties of crude, boiled and ethanolic extracts of *Macrocybe gigantea.* All the three extracts, i.e., crude, boiled and ethanolic extracts of *Macrocybe gigantea* showed significant increase in nitric oxide production over control (Fig. 2). These were 208.33±31.18, 300±27, 612.33±44.39 pmol mg^{-1} dry wt/h, respectively. Use of 10 μM N^G methyl-L-arginine acetate

ester (NAME), a competitive inhibitor of Nitric Oxide synthase (NOS) [28], 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.

From the above investigation it is evident that the ethanolic extract of *Macrocybe gigantea* possessed significant antioxidant activity and NOS activation properties, thus suggesting the therapeutic value of this mushroom, which could be used as medicine for several killer diseases. These results should encourage further *in vivo* studies which could ultimately lead to an inclusion of this medicinal mushroom in different pharmaceutical formulations.

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