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Research Article

EVALUATION OF ANTIOXIDANT AND NITRIC OXIDE SYNTHASE ACTIVATION PROPERTIES OF *VOLVARIELLA VOLVACEA*

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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 *Volvariella volvacea* showed significant inhibition of lipid peroxidation, potent hydroxyl and DPPH scavenging activity when compared with standard drug. IC_{50} values of crude, boiled and ethanolic extracts of basidiocarp represented 301.80, 214.50 and 85.70 µg/ml in hydroxyl radical scavenging activity, 607.32, 528.44 and 256.18 µg/ml in DPPH scavenging activity and 459.78, 250.40 and 133.37 µg/ml in case of inhibition of lipid peroxidation respectively. Furthermore, crude, boiled and ethanolic extracts also increased significantly nitric oxide production (186.27, 173.33 and 648.88 pmol/mg dry wt/h respectively) over the control. Among three kinds of extracts, the ethanolic extract was the most effective in relation to antioxidant activity and NOS activation property. The present results revealed that *V. volvacea* is a promising source of therapeutics.

Keywords: Antioxidant activity, Lipid peroxidation, Mushroom, *Volvariella volvacea*

INTRODUCTION

Besides body's normal metabolic processes, xenobiotics, radiation, X-rays, pollution and even stress are considered to be important factors in the etiology of several pathological conditions such as cardiovascular diseases, diabetes, inflammation, cancer etc.[1]. 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.

Nitric oxide (NO) produced at the cellular level from L-arginine catalyzed by nitric oxide synthase (NOS) is a very important signaling molecule[3, 4]. It is well studied in mammalian system and has been found to have numerous roles in pathophysiology[5, 6]. 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[7]. 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[8]. Edible mushrooms are highly nutritious, having strong free radical scavenging activity and therapeutic potentiality for the treatment of cancer, heart ailments, diabetes, inflammation, hepatic damage, high blood pressure, etc[9-19]. *Volvariella volvacea* (Bulliard ex Fries) Singer, commonly known as 'Paddy-straw mushroom', grow abundantly in the rotted paddy straw in the tropical region during rainy session. This species also domesticated almost 300 years ago and produced commercially throughout the Southeast Asia. Consumption of this mushroom is very popular among the people because of their gastronomic and nutritional delicacy. The present study was focused on the evaluation of antioxidant and NOS activation properties of the different extracts of *V. volvacea.*

MATERIALS AND METHODS

Sample collection and preparation

The specimen *V. volvacea* was collected from the village area of Gangetic plane of West Bengal, India. It was identified with the help of "Manual of Indian Edible Mushrooms"[20]. The Voucher specimen has been deposited in Molecular and Applied Mycology and Plant Pathology Laboratory, University of Calcutta, Kolkata, West Bengal.

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 15000*g* 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 and stored at -20°C for further use[21].

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[22]. 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 crude, boiled and ethanolic extracts of *V. volvacea* 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[23, 24]. 200-600 µl of various concentrations of the extracts in ethanol 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)[25]. The reaction mixture contained RBC- packed cell (10^8 cells/ ml) in Tris-HCl buffer (20 mM; pH 7.0) with $CuCl_2$ (2 mM), ascorbic acid (10 mM) and different extracts of *V. volvacea* in final volume of 1 ml. The reaction mixture was incubated at 37^{0}C 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 crude, boiled 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.

Assay of superoxide radical (O_2^-)-scavenging activity

Superoxide radical (O_2) was generated from autooxidation of hematoxilin and was detected by an increase in absorbance at 560 nm, in a Hitachi 330 spectrophotometer[26]. The reaction mixture contains 0.1 M of phosphate buffer (pH 7.4), EDTA (0.1 M), hematoxilin (50 µM) and incubated at 25°C for different time periods. Inhibition of autooxidation of hematoxilin by crude, boiled and ethanolic extracts over the control were measured.

Determination of nitric oxide (NO) synthase activity

NO was determined according to Jia *et al.* (1996) by using scanning Hitachi 330 spectrophotometer[27]. 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 crude, boiled and ethanolic extracts of *V. volvacea*; 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).

RESULT AND DISCUSSION

Assay of hydroxyl radical

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage[28]. 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[29]. 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. With regard to the scavenging ability of hydroxyl radicals, various extracts were effective in order of their IC_{50} value: boiled extract > crude extract >> ethanolic extracts, which was higher than the catechin (838.80 µg/ml), a synthetic antioxidant (Table 1).

DPPH radical scavenging activity

DPPH is a stable free radical which can accept an electron or hydrogen radical to become a stable diamagnetic molecule. Due to its odd electrons, the absorption is at 517 nm. DPPH radical reacts with suitable reducing agents. Then electrons become paired off and the solution loses colour stoichiometrically with the number of electrons taken up[28]. 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 Table 1, indicated all the extracts have significant DPPH radical scavenging activity. The 50% of inhibition value for ethanolic extract (256.18 µg/ml) of *V. volvacea* seems to be comparable to commonly used synthetic antioxidant BHT (86.11 µg/ml).

Table 1: *In vitro* hydroxyl radical and DPPH scavenging activity of *V. volvacea* extracts (IC_{50} µg/ml)

	Extracts			Standard
	Crude	Boiled	Ethanolic	
Hydroxyl radical scavenging activity	301.80 ± 41.85	214.60 ± 20.81	85.70 ±10.58	838.8 ± 28.3[a]
DPPH scavenging activity	607.32 ± 61.11	528.44 ± 37.65	256.18 ± 24.39	86.11 ± 4.32[b]

Note. Values represented as mean ± SD from three independent observations. a= catechin as standard, b= BHT as standard.

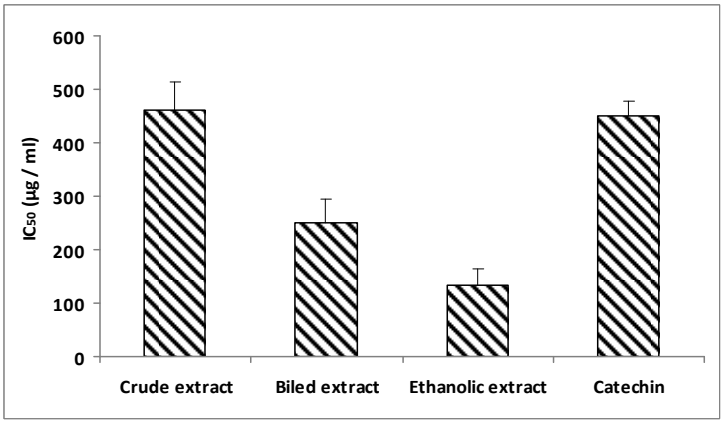

Fig. 1: Inhibitory concentration 50% of lipid peroxidation by *V. volvacea* extracts. Results are the mean ± SD of three separate experiments, each in triplicate.

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[30]. The results presented in Figure 1 showed that all the extracts of *V. volvacea* inhibit Fe^{2+}- ascorbate induced lipid peroxidation much better than standard catechin. The 50% of inhibition value of ethanolic extract (133.37 µg/ml) of *V. volvacea* seems to be fairly high when compared to standard (IC_{50} = 450 µg/ml for catechin).

Assay of superoxide radical scavenging activity

Superoxide radical is highly toxic species which is generated by numerous biological and phytochemical reactions. Inhibition of auto-oxidation of hematoxilin was not observed by any extract of *V. volvacea.* Reason for the no superoxide scavenging activity of the extract was unknown. However, herbs that scavenge superoxide contain a component of flavonoids, which are widely distributed in plants[31].

Determination of nitric oxide synthase activity

Nitric oxide is recognized to be inter- and intra- cellular mediator of several cell functions. It acts as a signal molecule in immune, nervous and vascular systems[32]. Further study was made to evaluate the nitric oxide synthase activation properties of crude, boiled and ethanolic extacts of *V. volvacea*. All the three extracts, i.e., crude, boiled and ethanolic extracts of *V. volvacea* showed significant increase in nitric oxide production over control (Figure 2). Use of 10 µM N^G methyl - L - arginine acetate ester (NAME), a competitive inhibitor of nitric oxide synthase (NOS)[33], 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.

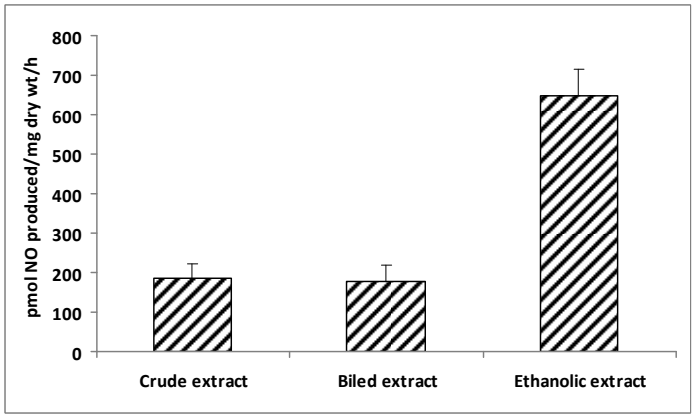

Fig. 2: Production of nitric oxide by different extracts of *V. volvacea* over control. Results are the mean ± SD of three separate experiments, each in triplicate.

CONCLUSION

From the above investigation it is evident that the ethanolic extract of *V. volvacea* 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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