International Journal of Biomedical and Pharmaceutical Sciences ©2007 Global Science Books

Antioxidant and Nitric Oxide Synthase Activation Properties of *Polyporus grammocephalus* Berk.

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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 *Polyporus grammocephalus* Berk. showed significant inhibition of lipid peroxidation, potent hydroxyl and 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) radical scavenging activity when compared with a standard drug. IC_{50} values of crude, boiled and ethanolic extracts of *P. grammocephalus* represented 393.6 ± 24.3, 300.4 ± 25.43 and 62.25 ± 9.67 µg/ml in the case of hydroxyl radical scavenging activity, 125.10 ± 34.61, 92.31 ± 34.49 and 129.52 ± 12.09 µg/ml in DPPH radical scavenging activity, and 362.13 ± 22.98, 179.33 ± 27.44 and 137.2 ± 22.13 µg/ml for lipid peroxidation, respectively. Furthermore, crude, boiled and ethanolic extracts also increased nitric oxide production significantly, one of the important multifunctional molecules that mediate a number of diverse physiological functions (198.2 ± 22.6, 112.6 ± 6.70, 297.7 ± 19.2 pmol/mg dry wt/h, respectively) over the control. Although all the three extracts showed antioxidant activity and NOS activation properties, the ethanolic extract was the most effective when compared with crude and boiled extracts. The present results revealed that *P. grammocephalus* has potent therapeutic use.

Keywords: DPPH radical, hydroxyl radical, lipid peroxidation, mushroom, reactive oxygen species, superoxide radical

INTRODUCTION

Reactive oxygen species produced by sunlight, ultraviolet, ionizing radiation, chemical reactions and metabolic processes are considered to be important factors in the etiology of several pathological conditions such as cardiovascular diseases, diabetes, inflammation, cancer, etc. (Josh and Janardhanan 2000; Sorg and Kaya 2007). Synthetic compounds are found to be strong radical scavengers but usually they have side effects (Zhou and Zheng 1991). 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, mushrooms or their derivatives or extracts occupy an elite position to perform this function (Acharya *et al*. 2004, 2005; Rai *et al*. 2006; Acharya 2007).

Likewise, nitric oxide (NO) produced at the cellular level from L-arginine catalyzed by nitric oxide synthase (NOS) is a very important signaling molecule (Ignaro 1996; Kahn *et al*. 2000) from the ground of pathophysiologic condition of living entities. It is well studied in the mammalian system and has been found to have numerous roles in pathophysiology including vasodialation (Ignaro 1996), regulation of blood pressure (Fledman *et al*. 1993), inhibition of platelet aggregation and adhesion (McDonald *et al*. 1993), inhibition of neutrophil adhesion (Ignaro 1996), neuromodulation in the CNS (Ignaro 1996), antioxidant (Beckman 1996), antithrombotic (Sinha *et al*. 1998) and as second messenger of insulin (Kahn *et al*. 2000). 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. (Malinski 2005; Villalobo 2007). 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.

The genus *Polyporus* is an assemblage of white-rotting lignicolous basidiomycetes. *Polyporus grammocephalus*, a wild edible mushroom, is common in India where it normally occurs on dead hardwood causing white rot and occasionally growing as a parasite on living trees (De and Roy 1981).

A literature survey revealed that the active component, xyloglucan derived from *Polyporus confluence* is said to have an antitumor property (Mizuno *et al*. 1992). *P. umbellatus* has been found to be effective against *Plasmodium falciparum*, the pyremethamine-resistant malaria parasite (Lovy *et al*. 1992) and it also inhibits platelet aggregation (Lu *et al*. 1985). Extracts of *P. badidus* inhibit *in vitro* binding of lipopolysacharides to the receptor and therefore prevent LPS-mediated septic shock (Koch *et al*. 2002). Yuan *et al*. (2004) reported the anti-aldosteronic dieuretic effect of *P. sclerotium*. The present study was conducted to evaluate the antioxidant activity and NOS activation properties of different *P. grammocephalus* extracts.

MATERIALS AND METHODS

Sample collection and preparation

Basidiocarps of *P. grammocephalus* were purchased from the Darjeeling market, which was collected by the local people from a high altitudinal zone of Sikkim Himalaya like the Sandakhfu region during the month of June and brought to the laboratory for detailed studies. Identification was done with the aid of a standard monograph (Roy and De 1996).

Crude extract was prepared from fresh tissue after thorough washing by homogenization in distilled water and centrifugation at 15,000 × *g* for 30 min at 4°C. Supernatant was lyophilized (Lyolab BII LSL Secfroid Lyophilizer) and then stored at –20°C for further use.

Boiled extract was also prepared from fresh fruit body (100 g/100 ml) after thorough washing and boiled in a water bath for 1 h, then homogenized and centrifuged at 15,000 × *g* for 30 min at room temperature. Supernatant was lyophilized and stored at –20°C for further use.

Received: 13 September, 2007. Accepted: 21 October, 2007.

Original Research Paper

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 by 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 (Turkoglu *et al*. 2006).

Assay of hydroxyl radical

Hydroxyl radicals (OH^-) are generated from the 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 (Halliwell 1987). The 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). This mixture was incubated at 37°C for 1 h and the colour was developed as described above. The IC_{50} value of deoxyribose degradation by the crude, boiled and ethanolic extracts of *P. grammocephalus* over the control was measured. Catechin (Sigma Chemicals, St. Louis, USA) was used as a 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 (Cuendet *et al*. 1997; Burits and Bucar 2000). 200-600 µl of various concentrations of the extracts in ethanol were added to 2 ml of 0.004% methanol solution of DPPH. After 30 min incubation period at room temperature in the dark, the absorbance was read against a methanol blank at 517 nm. Inhibition of free radical of DPPH in percent (I%) was calculated as follows:

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

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; Merck Limited, Mumbai, India) was used as control.

Preparation of human red blood cells

Blood was collected from normal healthy volunteers between the ages of 25-50 years by venipuncture using 19 gauge siliconized needles and anticoagulated by mixing 9.0 volume of blood with I volume of 130 mM sodium citrate (13 mM final). None of the volunteers had taken any medication at least for 14 days prior to the donation of blood and none of them had systemic hypertension or diabetes mellitus. Blood was centrifuged at 2,500 rpm in an eppendorf centrifuge (5415 C table top centrifuge) for 15 min. The plasma and buffy coat was removed by aspiration. The packed RBCs were resuspended and washed 3 times in isotonic saline (Reitz *et al*. 1989).

Assay of lipid peroxidation

Lipid peroxidation was induced by the 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). The reaction mixture contained an 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 *P. grammocephalus* in a final volume of 1 ml. The reaction mixture was incubated at 37°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 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 were inhibited 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 scavenging activity

Superoxide radical (O_2^-) was generated from the autooxidation of haematoxylin and was detected by an increase in absorbance at 560 nm in a Hitachi 330 spectrophotometer (Martin *et al*. 1987). The reaction mixture contains 0.1 M phosphate buffer (pH 7.4), 0.1 mM EDTA, 50 µM haematoxylin and incubated at 25°C for different time periods. The inhibition of autooxidation of haematoxylin by the crude, boiled and ethanolic extracts over the control was measured.

Determination of NOS activity

NO was determined according to Jia *et al*. (1996) by using a scanning Hitachi 330 spectrophotometer. Typically, NO content is 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 *P. grammocephalus* 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.

Analysis of data

Statistical analyses were performed by one way ANOVA followed by Tukey's test as applicable (Zar 1999). In all the cases results are the mean ± SD (standard deviation) of at least three individual experimental data.

RESULTS AND DISCUSSION

Hydroxyl radical scavenging activity

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage (Dash *et al*. 2005). 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 (Aruoma *et al*. 1989). 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 62.25 µg/ml, much less than the standard catechin (840 µg/ml) ($F = 649.661$; $df = 3.8$; $P < 0.05$).

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. The 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 (Dash *et al*. 2005). 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** indicate that all the extracts have a significant DPPH radical scavenging activity. The

Table 1 *In vitro* hydroxyl radical and DPPH radical scavenging activity of *Polyporus grammocephalus* extracts (IC_{50} µg/ml). Data sharing the same letters do not differ significantly, $P < 0.05$.

	Extracts			Standard
	Crude	Boiled	Ethanolic	
Hydroxyl radical scavenging activity	393.60 ± 24.30 a	300.40 ± 25.43 b	62.25 ± 9.67 c	840.0 ± 25.0 d +
DPPH radical scavenging activity	125.10 ± 34.61 e	92.31 ± 34.49 e	129.52 ± 12.09 e	85.7 ± 3.4 e *

Note: Values represented as mean ± SD from three independent observations. + = catechin as standard, * = BHT as standard.

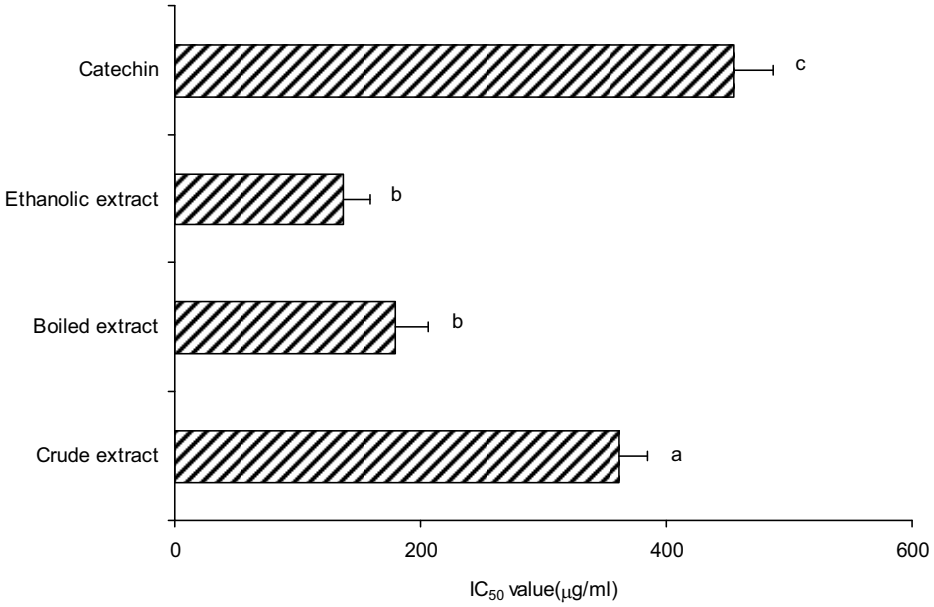

Fig. 1 Inhibition of lipid peroxidation by extracts of *Polyporus grammocephalus*. Results are the mean ± SD of three separate experiments, each in triplicate. Bars showing the same letters do not differ significantly at $P < 0.05$.

50% of inhibition value for boiled extract (92.31 µg/ml) of *P. grammocephalus* seems to be fairly close when compared to commonly used synthetic antioxidant BHT (85.7 µg/ml) ($F = 2.356$; $df = 3.8$; $P > 0.05$).

Superoxide radical scavenging activity

Superoxide radical is a highly toxic species which is generated by numerous biological and phytochemical reactions. Inhibition of autooxidation of haematoxylin was not observed by any extract of *P. grammocephalus*. The reason for the no superoxide scavenging activity of the extract is unknown.

Assay of lipid peroxidation

A free radical prefers to remove electrons from the lipid membrane of the cell, initiating a free radical attack on the cell inducing lipid peroxidation in polyunsaturated lipid-rich areas like the brain and liver (Coyle and Puttfarcken 1993). The results presented in **Fig. 1** show that all the extracts of *P. grammocephalus* inhibit Fe^{2+}-ascorbate–induced lipid peroxidation much better than standard catechin. The 50% inhibition value of the ethanolic extract (137.2 µg/ml) of *P. grammocephalus* is approximately one fourth when compared to standard (IC_{50} = 455 µg/ml for catechin) ($F = 97.11$; $df = 3.8$; $P < 0.05$).

Determination of nitric oxide (NO) synthase activity

NO is recognized as an important messenger molecule having a broad spectrum of functions in many biological systems ranging from physiological control to pathological cytotoxic effect (Bredt and Snyder 1992; Lowenstein and Snyder 1992; Snyder and Bredt 1992; Villalobo 2007). We conducted a further study to evaluate the NOS activation properties of crude, boiled and ethanolic extracts of *P. grammocephalus*. All three extracts showed a significant increase in nitric oxide production over the control (**Fig. 2**); these were 198.2 ± 22.6, 112.6 ± 6.70, 297.7 ± 19.2 pmol NO produced/mg dry wt of the extract/h respectively ($F =$

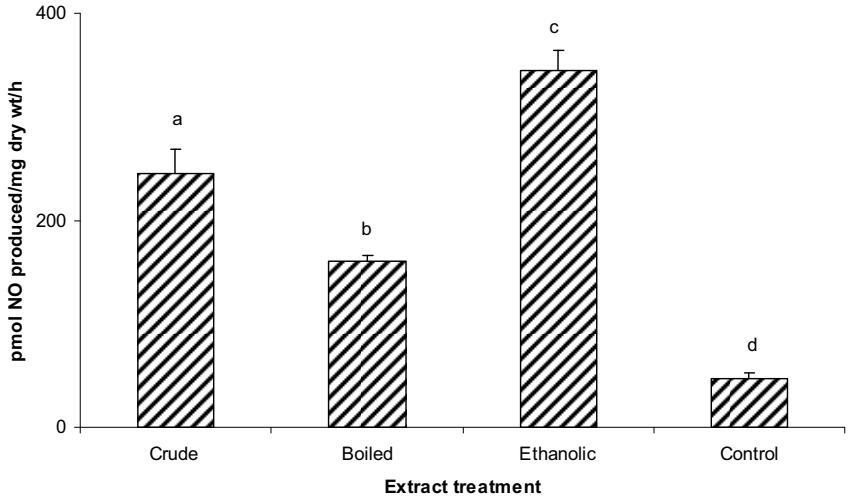

Fig. 2 Production of nitric oxide by different extracts of *Polyporus grammocephalus* with the control. Values are the mean ± SD of three separate experiments each in triplicate. Bars showing the same letters do not differ significantly at $P < 0.001$.

200.99; df = 3.8; P < 0.001). Use of 10 μM N^G methyl-L-arginine acetate ester (NAME), a competitive inhibitor of NOS (Sprague *et al*. 1994), 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. The ethanolic extract showed significant NOS activation properties when compared to the other extracts.

A literature survey revealed that ethanolic extracts from *Auricularia auricula* (Acharya *et al*. 2004), *Ganoderma applanatum* (Acharya *et al*. 2005), *Morchella conica* (Turkoglu *et al*. 2006), *p*-terphenil isolated from *Thelephora aurantiotincta*, *Thelephora ganbajum*, *Boletopsis grisea* (Liu *et al*. 2004), *Paxillus curtissii* (Yun *et al*. 2000), betulinan A from *Lenzites betulinus* (Lee *et al*. 1996) and ethyl acetate extract from *Pleurotus florida* (Josh and Janardhanan 2000) showed potent antioxidant properties. A few experiments have been performed which show NOS activation and NO production by mushroom products. The acidic polysaccharide from *Phellinus linteus* (Han *et al*. 1999), fucogalactan from *Sarcodon aspratus* (Mizuno *et al*. 2000), ubiquitin-like peptide from *Agrocybe cylindracea* (Ngai *et al*. 2003), methanolic extracts from *Cordyceps pruinosa* (Kim *et al*. 2003c), ethanolic extract of *Auricularia auricula* (Acharya *et al*. 2004) and *Ganoderma applanatum* (Acharya *et al*. 2005) were capable of inducing significant increase in *in vitro* NO production. Our results include a new addition to the list and also provide information that the ethanolic extract of *P. grammocephalus* possesses significant antioxidant activity and NOS activation properties, thus suggesting the therapeutic value of this mushroom, which could be used as medicine for several fatal diseases. These results should encourage further *in vivo* studies for inclusion of this medicinal mushroom in different pharmaceutical formulations.

ACKNOWLEDGEMENTS

We are grateful to Prof. Anjali Roy, Visva Bharati University, West Bengal, for identification of the specimen.

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