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Evaluation of Antioxidant and Nitric Oxide Synthase Activation Properties of *Astraeus hygrometricus* (Pers.) Morg.

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ABSTRACT

The present study was conducted to evaluate the antioxidant activity and nitric oxide synthase (NOS) activation properties of the different extracts of *Astraeus hygrometricus* (Pers.) Morg. Crude, boiled and ethanolic extracts of *A. hygrometricus* were prepared using standard protocol. The antioxidant potential was studied by using different *in vitro* assay systems, viz., lipid peroxidation, carotene linoleic acid assay, DPPH (2, 2-diphenyl-1-picryl hydrazyl), hydroxyl and superoxide radical scavenging activity. Nitric oxide (NO) production was determined spectrophotometrically by conversion of oxyhemoglobin to methemoglobin. IC_{50} values of crude, boiled and ethanolic extracts of *A. hygrometricus* represented 209.3, 289.8 and 81.2 µg/ml, respectively in the case of hydroxyl radical scavenging activity; 502.53, 531.69 and 357.95 µg/ml, respectively for superoxide scavenging activity; 98.02, 119.8 and 94.74 µg/ml, respectively for DPPH radical scavenging activity; 478.65, 632.66 and 377.27 µg/ml, respectively for β-carotene bleaching activity and 99.40, 83.96 and 87.96 µg/ml, respectively for lipid peroxidation inhibition. Furthermore, crude, boiled and ethanolic extracts also increased significantly nitric oxide production (195, 220 and 955 pmol/mg dry wt/h, respectively) over the control. Among the three kinds of extracts, the ethanolic extract was the most effective in relation to antioxidant and NOS activation properties. The present results revealed *A. hygrometricus* as a promising source of therapeutics.

Keywords: carotene, DPPH radical, hydroxyl radical, linoleic acid, lipid peroxidation, mushroom, nitric oxide synthase, reactive oxygen species, superoxide radical

INTRODUCTION

Astraeus hygrometricus is an ectomycorrhizal edible mushroom commonly known as false earthstar belonging to the Family Astraeceae (Roy and Samajpati 1976). This mushroom grows on the floor of sal (*Shorea robusta* G.f.) forest of some dry Eastern Parts of India predominantly having red laterite to sandy loam soil of a pH ranging from 5.5-6.0 (Shajahan and Samajpati 1995). It flourishes abundantly during the period of July to October. It is eaten by the villagers and local people as a healthy food having a belief that consumption of this mushroom could prevent several age-related disorders. A literature survey on *A. hygrometricus* revealed that a water-soluble glucan, fraction I, was isolated from the aqueous extract of the fruit bodies of *A. hygrometricus*, which showed strong splenocyte activation (Chakraborty *et al*. 2004). Dried, powdered spores of *A. hygrometricus* are applied to sores as dusting powders, espe-cially to heal an infant's navel. Poultices or infusions as lotions are also used for treatment of burns or itching from spores of *A. hygrometricus* (Leland and Stuart 1941).

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 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 central nervous system (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). 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.

ROS or 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 (Liu *et al*. 1997). 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 (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, mushroom or their derivatives or extracts occupy an elite position to perform this function (Acharya *et al*. 2004; Acharya *et al*. 2005; Rai *et al*. 2006; Acharya 2007).

The present study was conducted to evaluate the antioxidant activity and NOS activation properties of the different extracts of *A. hygrometricus.*

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MATERIALS AND METHODS

Sample collection and preparation

Basidiocarp of *A. hygrometricus* was collected from local market and from the sal (*Shorea robusta* G.f.) forests of Bankura and West Midnapore, West Bengal, India. It was identified according to Ramsbottom (1965) and Shajahan and Samajpati (1995).

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 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.

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 (Turkoglu *et al*. 2006).

Hydroxyl radical (OH^-)-scavenging activity assay

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 *et al*. 1987). 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), ascorbate (100 µM) and various concentrations of the different extracts. Reaction mixture was incubated at 37°C for 1 h and colour developed as described above. IC_{50} values of deoxyribose degradation by the crude, boiled and ethanolic extracts of *A. hygrometricus* over the control were measured. Catechin (Sigma Chemicals, St. Louis, USA) was used as positive control.

Preparation of human red blood cells

Blood was collected from normal healthy volunteers (n = 3) between the ages of 25-50 years by venipuncture using 19 gauge siliconized needles and anticoagulated by mixing 9.0 vol of blood with 1.0 vol 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).

Lipid peroxidation assay

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). 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 concentrations of extracts of *A. hygrometricus* in final volume of 1 ml. The reaction mixture was incubated at 37°C for 1 h. Lipid peroxidation was measured as MDA equivalent using TBA (trichloroacetic acid)-TCA (thiobarbituric acid) reagent. TBA-TCA reagent was prepared with 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 boiled in a 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.

Superoxide radical (O_2^-)-scavenging activity assay

The method used by Martínez *et al*. (2001) for determination of the superoxide dismutase was followed with modification (Dasgupta and De 2004) in the riboflavin-light-nitrobluetetrazolium (NBT) system (Beauchamp and Fridovich 1971). 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.

DPPH 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 2, 2-diphenyl-1-picryl hydrazyl (DPPH, Sigma Aldrich Inc., St. Louis, MO, USA). 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 Ltd., Mumbai, India) was used as control.

Carotene-linoleic acid assay

In this assay, antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius *et al*. 1998). A stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 ml of chloroform (HPLC grade) and 25 µl linoleic acid and 200 mg Tween 40 were added. Chloroform was completely evaporated using a vacuum evaporator. Then, 100 ml distilled water saturated with oxygen was added with vigorous shaking at a rate of 100 ml/min for 30 min. 4 ml of this reaction mixture were dispensed into test tubes and 200 µl portions of the extracts, prepared at 2 mg/ml concentrations, were added and the emulsion system was incubated for 2 h at 50°C. The same procedure was repeated with TBHQ (*tert*-butyl-hydroquinone; Sigma) as standard positive control as well as a blank. After this incubation period, absorbance of the mixtures was measured at 490 nm. Antioxidative capacities of the extracts were compared with those of TBHQ and blank.

Determination of nitric oxide (NO) synthase activity

NO was determined according to Jia *et al*. (1996) by a 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 *A. hygrometricus* 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 centri-

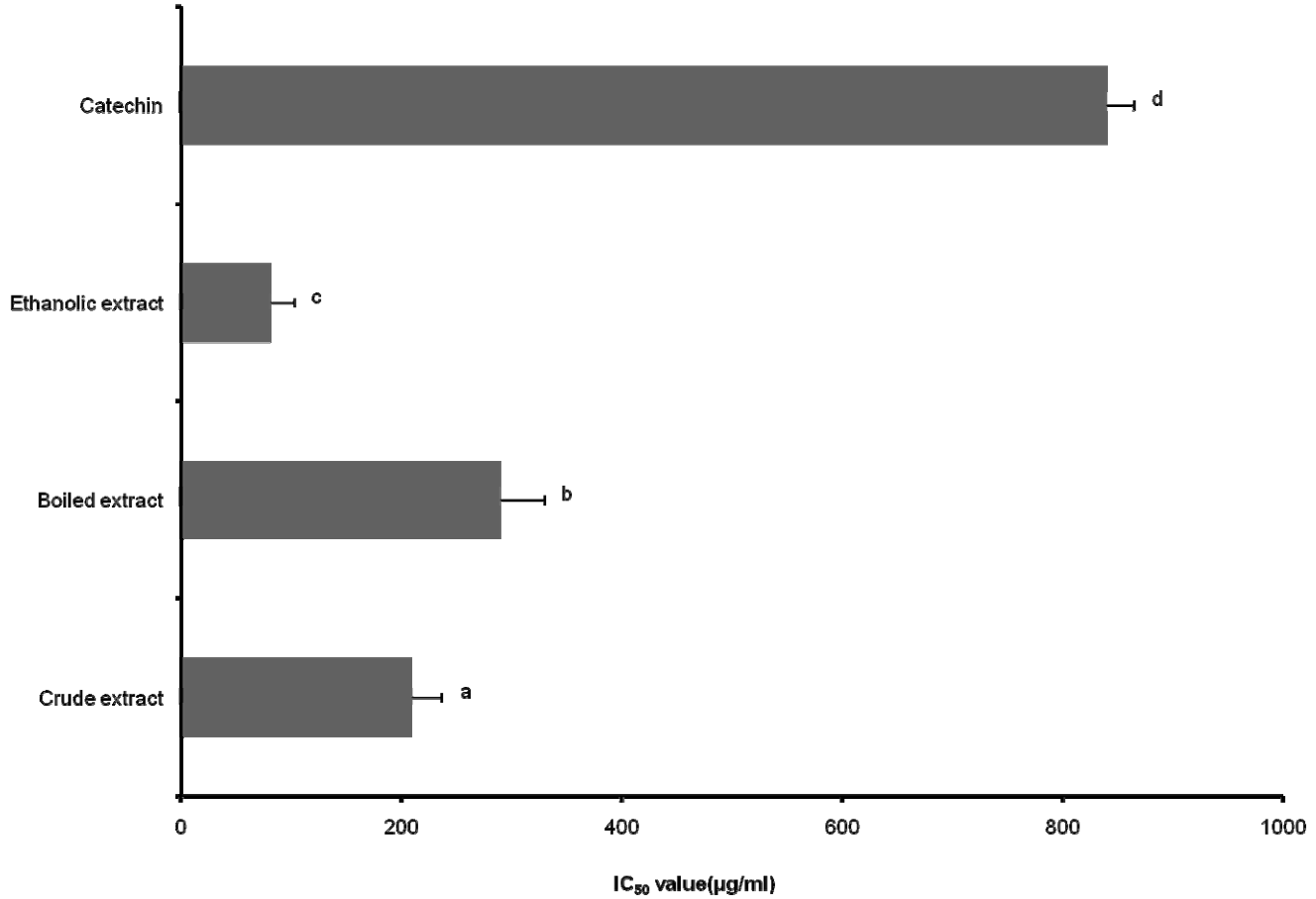

Fig. 1 Inhibitory concentration 50% of hydroxyl radical scavenging activity by *Astraeus hygrometricus* extracts. Results are the mean ± SD of three separate experiments, each in triplicate. Bars showing the same letters do not differ significantly, $P < 0.05$. Catechin is used as standard.

fuged at 8,000 × g for 5 min at 37°C and NO content of the supernatant was compared with an appropriate control set. The changes in absorbance of the reaction mixture at 575 nm were measured and the NO levels were calculated by using an extinction coefficient of 77 nM^{-1} cm^{-1}.

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 (OH^-)-scavenging activity assay

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, i.e., crude, boiled and ethanolic extracts of the mushroom showed potential hydroxyl radical scavenging activity (**Fig. 1**). With regard to the scavenging ability of hydroxyl radicals, various extracts were effective in order of their IC_{50} values: boiled > crude >> ethanolic extracts, which was higher than the catechin (840 µg/ml), a synthetic antioxidant (F = 401.970; df = 3.8; $P < 0.05$).

Lipid peroxidation assay

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 (Coyle and Puttfarcken 1993). The results presented in **Table 1** showed that all the extracts of *A. hygrometricus* inhibit Fe^{2+}-ascorbate-induced lipid peroxidation much better than standard catechin. The 50% of inhibition value of ethanolic extract (87.96 µg/ml) of *A. hygrometricus* seems to be approximately one fifth when compared to standard (IC_{50} = 455 µg/ml for catechin) (F = 209.198; df = 3.8; $P < 0.05$).

Table 1 *In vitro* lipid peroxidation inhibition, superoxide anion scavenging and β-carotene bleaching activity of *Astraeus hygrometricus* extracts (IC_{50} µg/ml). Data with the same letters do not differ significantly, $P < 0.05$.

Antioxidant assays	Extracts			Standard
	Crude	Boiled	Ethanolic	
Lipid peroxidation inhibition activity	99.40 ± 19.2 a	83.96 ± 7.6 b	87.96 ± 21.4 c	455 ± 32 d^+
Superoxide radical scavenging activity	502.53 ± 19.93 e	531.69 ± 37.14 e	357.95 ± 13.08 f	65 ± 3.5 g^*
β-carotene bleaching activity	478.65 ± 75.46 h	632.66 ± 52.08 i	377.27 ± 75.94 i	82.6 ± 3.2 $j^¥$

Note: Values represented as mean ± SD from three independent observations. $^+$ = catechin as standard, * = ascorbic acid as standard, $^¥$ = TBHQ (*tert*-butyl-hydroquinone) as standard.

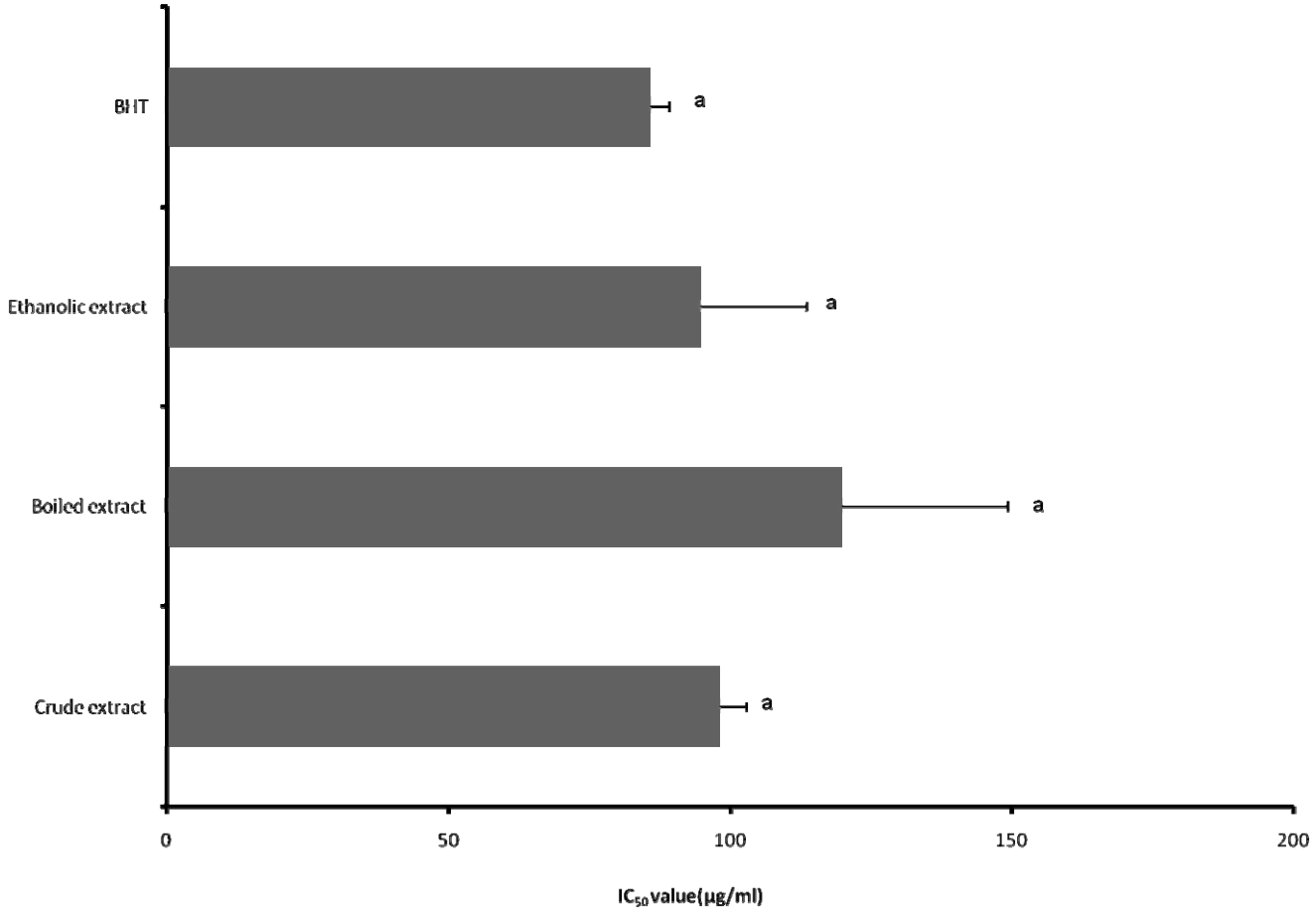

Fig. 2 Free radical scavenging capacities of *Astraeus hygrometricus* extracts measured in DPPH assay. Results are the mean ± SD of three separate experiments, each in triplicate. Bars showing the same letters do not differ significantly, $P < 0.05$. BHT (butylated hydroxylated toluene) was used as standard.

Superoxide radical (O_2^-)-scavenging activity assay

Superoxide radical (O_2^-) is known to be very harmful to cellular components as a precursor of more reactive species (Halliwell and Gutteridge 1989). One risk of the superoxide generation is related to its interaction with nitric oxide to form peroxinitrite (Nishikawa *et al*. 2000; Brownlee 2001) which is a potent oxidant that causes nitrosative stress in the organ systems. Among all the extracts, ethanolic extract showed moderate activity with an IC_{50} = 357.95 µg/ml when compared to that of ascorbic acid (IC_{50} = 65 µg/ml) (**Table 1**) ($F = 279.088$; df = 3.8; $P < 0.05$).

DPPH assay

DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the radical scavenging effects of the extracts of the mushroom. As antioxidants donate protons to these radicals, the absorption decreases. The decrease in absorption is taken as a measure of the extent of radical scavenging. Free radical scavenging capacities of the extracts of *A. hygrometricus*, measured by DPPH assay, are shown in **Fig. 2**. All concentrations studied showed free radical scavenging activity. The 50% of inhibition value for ethanolic extract of *A. hygrometricus* (IC_{50} = 94.74 µg/ml) seems to be fairly comparable to commonly used synthetic antioxidant BHT (IC_{50} = 85.7 µg/ml) ($F = 2.015$; df = 3.8; $P > 0.05$).

Carotene-linoleic acid assay

Table 1 shows the antioxidant activities of mushroom crude, boiled and ethanolic extracts with the coupled oxidation of β-carotene and linoleic acid. The ethanolic extract of *A. hygrometricus* had higher antioxidant activity than the other extracts. The 50% of inhibition value for ethanolic extract of *A. hygrometricus* (IC_{50} = 377.265 µg/ml) seems to be fairly comparable to that of TBHQ standard (IC_{50} = 82.6 µg/ml) ($F = 44.594$; df = 3.8; $P < 0.05$). It is probable that the antioxidative components in the mushroom extracts can reduce the extent of β-carotene destruction by neutralizing the linoleate free radical and other free radicals formed in the system.

Determination of nitric oxide 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 (Schmidt *et al*. 1993). Further study was made to evaluate the nitric oxide synthase activation properties of crude, boiled and ethanolic extracts of *A. hygrometricus*. All the three extracts, i.e., crude, boiled and ethanolic extracts of *A. hygrometricus* showed significant increase in nitric oxide production over control (**Fig. 3**). These were 195 ± 35, 220 ± 30, 955 ± 65 pmol/mg dry wt/h, respectively ($F = 312.474$; df = 3.8; $P < 0.05$). 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. Ethanolic extract showed considerable NOS activation properties when compared to the other extracts.

Literature survey revealed that ethanolic extracts from *Auricularia auricula* (Acharya *et al.* 2004), *Ganoderma applanatum* (Acharya *et al.* 2005), *Polyporus grammocephalus* (Rai *et al.* 2007), *Macrocybe gigantea* (Banerjee *et al.* 2007), *Ramaria botrytis* (Rai *et al.* 2008), *Morchella conica* (Turkoglu *et al.* 2006), *ρ*-terphenil isolated from *Thelephora aurantiotincta*, *Thelephora ganbajum*, *Boletopsis grisea*

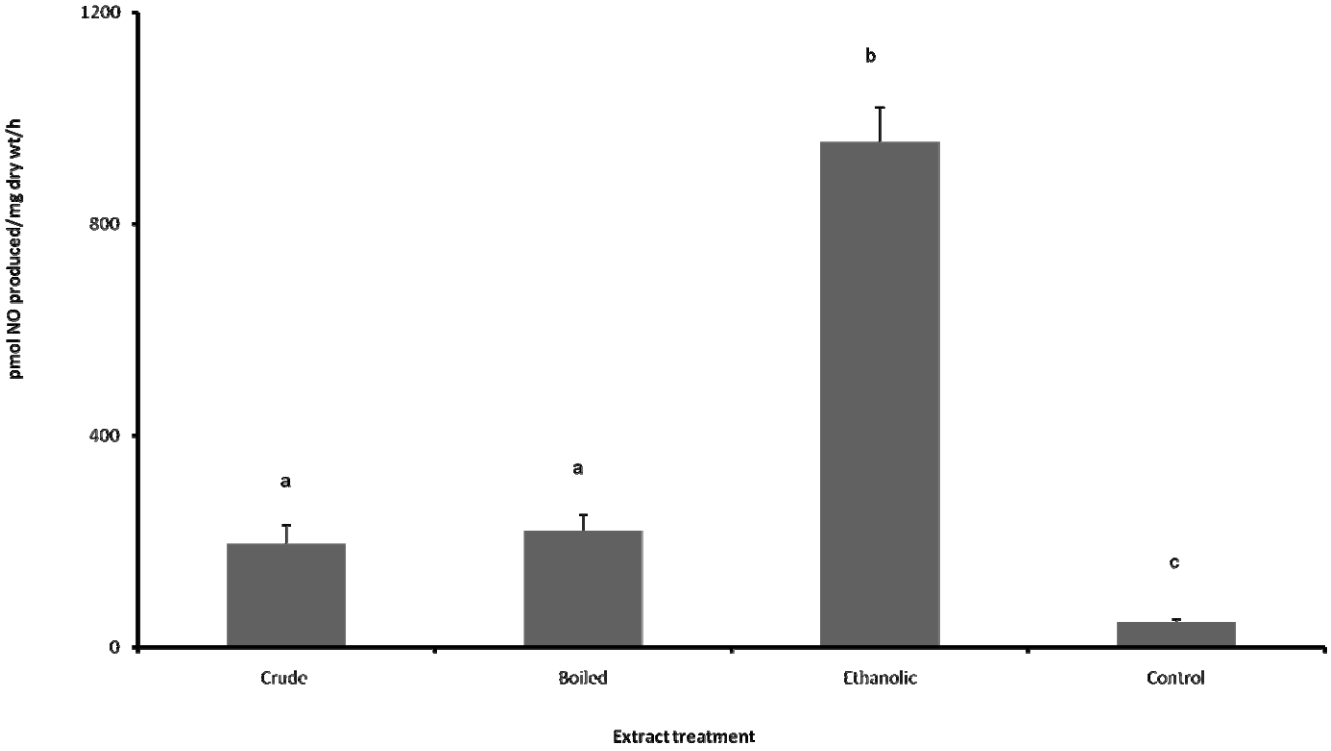

Fig. 3 Production of nitric oxide by different extracts of *Astraeus hygrometricus* with the control. Results are the mean ± SD of three separate experiments, each in triplicate. Bars showing the same letters do not differ significantly at $P < 0.05$.

(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.* 2003), ethanolic extract of *Auricularia auricula* (Acharya *et al.* 2004), *Ganoderma applanatum* (Acharya *et al.* 2005), *Polyporus grammocephalus* (Rai *et al.* 2007), *Macrocybe gigantea* (Banerjee *et al.* 2007) and *Ramaria botrytis* (Rai *et al.* 2008) were capable of inducing significant increase in *in vitro* NO production.

Present findings were compared with earlier works on mushrooms that had been reported from our laboratory. Like other cases, the ethanolic extract showed highest activity among all the extracts namely crude, boiled and ethanolic. Ethanolic extract of *A. hygrometricus* was more potent than *Auricularia auricula* and *Ganoderma applanatum* and less than those of *Polyporus grammocephalus*, *Macrocybe gigantea* and *Ramaria botrytis* in hydroxyl radical scavenging activity. Only the ethanolic extract of *M. gigantea* had higher lipid peroxidation inhibition activity than that of *A. hygrometricus*. Ethanolic extracts of *P. grammocephalus* and *R. botrytis* had lower activity than *A. hygrometricus* in DPPH radical scavenging activity. Ethanolic extract of *M. gigantea* was better than *A. hygrometricus* in superoxide scavenging assay. Nitric oxide production activity of *A. hygrometricus* ethanolic extract was best among all reported mushrooms (Acharya *et al.* 2004, 2005; Banerjee *et al.* 2007; Rai *et al.* 2007, 2008).

It is evident from our work that the ethanolic extract of *A. hygrometricus* 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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