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PHENOLIC QUANTIFICATION AND ANTI-OXIDANT ACTIVITY OF *MORCHELLA ESCULENTA*

Corresponding Author

JAVED AHMAD WAGAY

Lab of Microbial Technology and Plant Pathology, Department of Botany, Dr. H. S. Gour Central University, Sagar (M.P)- 470003, India

Co Authors

DEEPAK VYAS.

Lab of Microbial Technology and Plant Pathology, Department of Botany, Dr. H. S. Gour Central University, Sagar (M.P)- 470003, India

ABSTRACT

The present investigation was designed for the analysis to determine the *in vitro* antioxidant potential of a pharmaceutically important mushroom (*Morchella esculenta*). Four basic test systems viz., DPPH (1,1-diphenyl-2-picrylhydrazyl radical) free radical scavenging, nitric-oxide scavenging activity, super-oxide scavenging activity and total phenolic compounds were used. Among all the scavenging tests a significant correlation existed between concentrations of the extract and percent (%) inhibition of free radicals. Measurement of total phenolic content of the extract was achieved using Folin-Ciocalteau reagent containing 23.42±0.89mg g^{-1} of phenolic content, which was found significantly higher when compared to reference standard gallic acid. DPPH scavenging activity of methanolic mushroom extract showed highest % inhibition value, which was found to be 70.8±0.89, followed by super oxide scavenging activity (52.9±0.76). The least value was found to be that of nitric oxide i.e. 49.04±0.23.

KEY WORDS

DPPH, Phenols, Gallic acid, Antioxidant,*Morchella esculenta*

INTRODUCTION

Morchella esculenta (Guchi or sponge mushroom) belongs to family Helvellaceae. The most therapeutic and highly priced mushroom found in Himalayan ranges of extreme north western extremity of Indian subcontinent –Kashmir (J&K).The state is located between 32^0-17″ and 37^0-5″ North latitude and 73^0-26″ and 80^0-30″ East longitude and its average height from the mean sea level varies from 300 to 7200 meters with average rain fall 150 cm .The temperature range is between -5 ^{0}C to 28^{0}C as minimum and maximum in January and June . Due to suitable environmental conditions, the region is rich in mushroom diversity particularly different species of *Morchella*. Its growing season is usually from 15-March to July and is mostly used as food and ethenomedicine after sun dried by the tribals of Kashmir.

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radical formation (Gutteridgde, 1995). Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Tiwari, 2001). The most common reactive oxygen species (ROS) include superoxide, anion, hydrogen peroxide (H202), peroxyl (ROO-) radicals, and reactive hydroxyl (OH-) radicals. The nitrogen derived free radicals are nitric oxide (NO2) and peroxynitrite anion (ONOO). ROS have been implicated in over a hundred of diseases which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (Joyce, 1987). In treatment of these diseases, antioxidant therapy has gained an immense importance. Current research is now directed towards finding naturally occurring antioxidants of plant origin. Antioxidants have been reported to prevent oxidative damages by quenching free radical formation, and may prevent the occurrence of diseases such as, cancer and aging which are directly related to reactive free radical production . It can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers (Buyukokuroglu, 2001).Mushrooms are being used as a source of medicine since long. The medicinal properties of mushrooms have been investigated in the recent scientific developments throughout the world, due to their potent antioxidant activities, no side effects and have economic viability (Auudy, 2003). Phenolic compounds widely distributed in mushrooms which have been reported to exert multiple biological effect, including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic etc (Miller, 1996). They were also suggested to be a potential iron chelators(Boyer, 1998; Harsteen, 1983). The antioxidative and free radical scavenging properties of the phenolic content of mushroom methanolic extracts have been reported, suggesting possible protective roles of these compounds, due to their ability to capture metals, inhibit lipoxygenase and scavenge free radicals (Mau, Chang, Huang, & Chen, 2004). Recently,Valentao et al. (2005) identified the presence of six phenolic compounds (3-, 4- and 5-O-caffeoylquinic acid, caffeic acid, p-coumaric acid and rutin) and five organic acids (citric, ascorbic, malic, shikimic and fumaric acids) in wild edible mushroom *Cantharellus cibarius* Fr., kept under four different conditions (dried, frozen, preserved in olive oil and in vinegar). The organic acids citric, ketoglutaric, malic, succinic, oxalic, ascorbic, quinic, shikimic and fumaric were also found in the edible mushrooms viz, *Amanita caesarea* (Scop.) Pers., *Boletus*

edulis Bull., *Gyroporus castaneus* (Bull.) Que´l., *Lactarius deliciosus* (L.) Gray, *Suillus collinitus* (Fr.) Kuntze and *Xerocomus chrysenteron* (Bull.) Que´l. (Valentao et al., 2005b).

Kashmir being the temperate region with suitable climatic conditions which favours luxuriant growth of different mushroom species including different species of *Morchella*.The aim of present work is to study and analyse the anti-oxidant capacity of one of the dominantly found mushroom species, *Morchella esculenta* Var., as little or no anti-oxidant work has been done of this mushroom in Indian sub –continent so far. It is mostly used by the tribals of the Kashmir region to treat different diseases, particularly for chronic arthritis, general weakness and feed *Morchella esculenta* to women along with milk during and after pregnancy and claims that it provides quick recovery and resistence to the women after birth.

2. MATERIAL AND METHODS

2.1. Preperation of Mushroom extract

Morchella esculenta fruiting bodies were collected in March- May -2009 from coniferous forests of Kashmir(J&K) .The fruiting bodies were dried in an oven at 40 ^{0}C for 4 hours.The specimens were taken to Mushroom Culture Collection Centre –Sherie Kashmir Agriculture University of Science and Technology (SKAUST) Srinagar (Kashmir) for identification .The dried fruiting bodies were crushed to powder by using electronic blender.About 50g of powder were taken in 500ml of methanol in soxhlet extraction unit for extraction at 30^{0}C for 16-18 hours and filtered through Whatman No. 4 filter paper. The residue was then extracted with two additional 500 ml of methanol as described above. The combined methanolic extract was then rotary evaporated at 40 C^0 to dryness, redissolved in methanol to a concentration of 10 mg/ml and stored at 4 ^{0}C for further use.

2.2. Antioxidant activity

2.2.1. Chemicals

1,1 Diphenly -2 picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT),L-ascorbic acid , Trolox (6-hydroxy-2-5-8-tetra methyl chroman-2-carboxilic acid), Gallic acid, Tween-20, Folin ciocalteu's phenol reagent (FCR), Sodium carbonate, Sodium nitroprusside, methanol, chloroform and the other chemicals and reagents purchased from Biochem Pharmaceutical Industries (Mumbay). All other unlabeled chemicals and reagents were of analytical grade.

2.2.2. Scavenging effect on 1,1-Diphenly-2-picrylhydrazyl (DPPH)

The hydrogen atom or electron donating ability of the methanolic mushroom extract was measured from the bleaching of the purple-coloured methanol solution of 1,1-Diphenly-2-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent (Burits M and Bucar F.,2000). 1000µl of various concentrations (20-200µg/ml) of the extract were added to 4 ml of 0.004% methanol solution of DPPH. After 20-30 min incubation period at room temperature, the absorbance was read against a blank at λ = 517 nm. Inhibition of free radical by DPPH in percent (%I) was calculated by the following formula.

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

where A_{blank} is the absorbance of the control (Solvent), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration. Tests were carried out in triplicate.

2.2.3. Total phenolic compounds determination

Total soluble phenolics in the mushroom methanolic extract was determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) by using gallic acid as a standard. 1.0 ml mushroom extract solution was taken in a volumetric flask and was diluted with 45ml of distilled water. 1ml

Folin-Ciocalteu reagent was added and mixed thoroughly. After 5 min, 2ml of Na_2CO_3 (2%) was added and the mixture was allowed to stand for 2½ hours with intermittent shaking. The absorbance of developed blue colour was measured at 760 nm.The concentration of total phenols was expressed as mg/g of the dry extract(Kim et al., 2003). The concentration of total phenolic compounds in the mushroom methanolic extract was determined as µg of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph .
Absorbance = 0.0009 × gallic acid (µg)

2.2.4. Nitric oxide scavenging activity assay

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions.This can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture was incubated at $25C^0$ for 2½ hours. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthyl ethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 546 nm was measured by uv-vis spectrophotometer (fig. 1). The nitric oxide radicals scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = (A_0-A_1) / A_0 \times 100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of test sample.

2.2.5. Superoxide radical scavenging activity assay

This activity was measured using NBT (nitroblue tetrazolium reagent) method as described by Sabu and Ramadasan (2002). The method is based on generation of superoxide radical (O^-_2) by auto-oxidation of hydroxylamine hydrochloride in presence of NBT, which gets reduced to nitrite. Nitrite in presence of EDTA gives a color that was measured at 560 nm. Test solutions of extract (20–200 µg/ml) were taken in a test tube. To this, reaction mixture consisting of 1 ml of (50 mM) sodium carbonate, 0.4 ml of (24 mM) NBT and 0.2 ml of 0.1 mM EDTA solutions were added to the test tube and immediate reading was taken at 560 nm. About 0.4 ml of (1 mM) of hydroxylamine hydrochloride was added to initiate the reaction then reaction mixture was incubated at 25^0C for 15 min and reduction of NBT was measured at 560 nm. Ascorbic acid was used as the reference compound. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. All the extracts of mushroom extract were treated in the similar manner, absorbance was recorded and the percentage of inhibition was calculated according to the following equation:

$$\% \text{ Inhibition} = (A_0-A_1) / A_0 \times 100$$

where A_0 is the absorbance of the control (blank) and A_1 is the absorbance of test samples . All the tests were performed in triplicate and the graph was plotted with the mean values.

Statistical analysis

Data are expressed as mean ± SD. Means of triplicate analyses were calculated. A difference was considered to be statistically significant when $p < 0.05$.

3 .RESULTS AND DISCUSSION

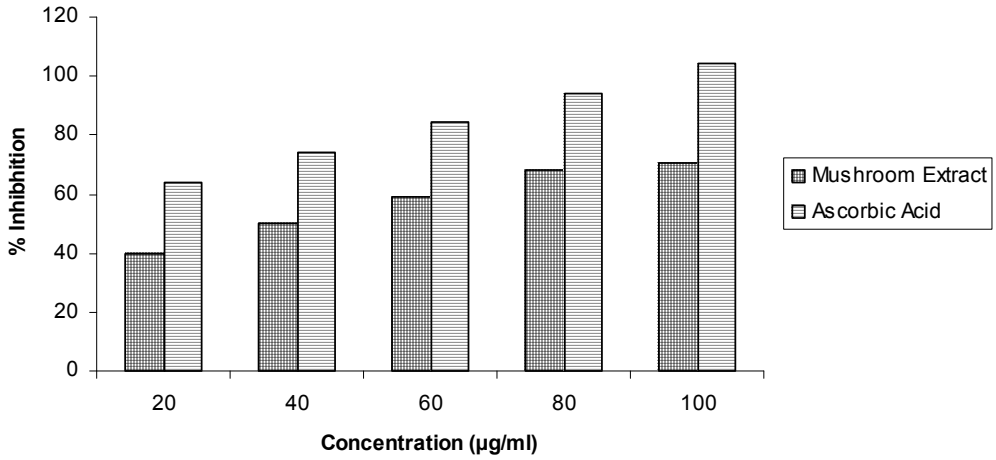
3.1. Antioxidant activity of methanolic mushroom extract

3.1.1. Inhibition of DPPH radical

DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The reduction capability of DPPH was determined by the decrease in its absorbance at λ=517 nm, which is induced by

antioxidants. Positive DPPH test suggests that the methanolic extract of the mushroom was free radical scavenger.The scavenging effect of mushroom extract and ascorbic acid on DPPH radical was compared. On the DPPH radical, mushroom extract had significant scavenging effects with increasing concentrations in the range of 20–100 µg/ml when compared with that of ascorbic acid (fig.1) . 100 µg /ml of mushroom extract and ascorbic acid exhibited 79.8% and 99.8% inhibition respectively (Table.1). The IC50 values were found to be 67.12 and 28.42 µg /ml for mushroom and ascorbic acid, respectively (Table 2). The different concentrations of mushroom extract (20, 40, 60, 80 and 100 µg /ml) showed antioxidant activities in a dose dependent manner on DPPH radical.

Fig. 1
Shows comparative scavenging activity of DPPH radicals of methanolic extract of Morchella esculenta at different concentrations.



3.1.2. Determination of total phenolic content

The total amount of phenolic content present in the investigated mushroom extract is shown in table.2, which was found to be 47.01 µg/mg gallic acid equivalent of phenols .The 20 µg/mg of phenols in any mushroom is considered to be sufficient for antioxidant activity ,thereore, our results are much better as it contains adequate proportion of phenols (47.01 µg/mg gallic acid equivalent of phenols) . These results clearly suggest that the higher levels of antioxidant activity of this mushroom is due to the presence of phenolic components. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano et al., 1989). Duh et al., (1999) showed that the phenolic compounds in mushrooms may contribute directly to antioxidative action.

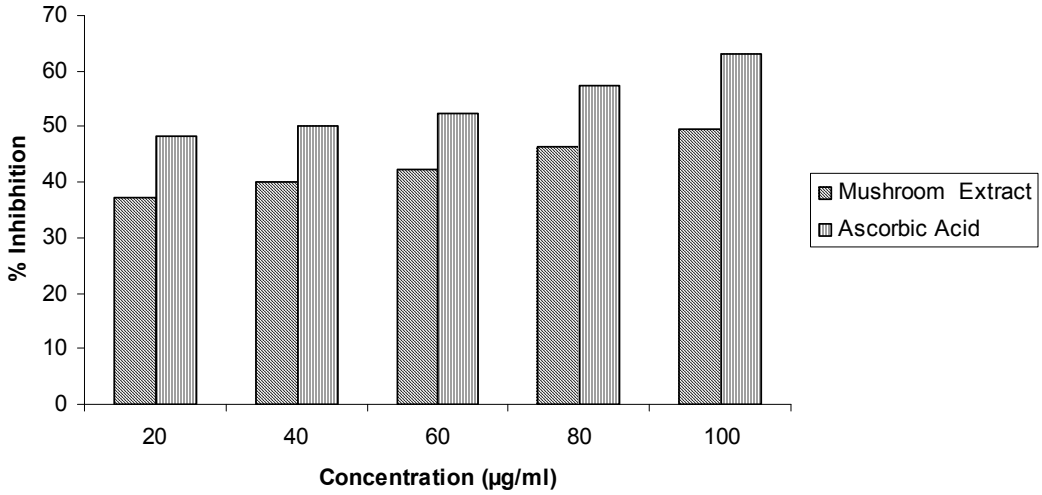
3.1.3.Nitric-oxide radical scavenging assay

Nitric oxide plays an important role in various types of inflammatory processes in the animal body. In this study, the methanolic mushroom extract of *M.esculenta* was checked for its inhibitory effect on nitric oxide production. Nitric oxide radicals generated from sodium nitroprusside at a physiological PH, was found to be inhibited by the mushroom extract. The various concentrations of the extract (20–100 µg/ml) showed 37.2%, 40.01%, 42.04%, 46.02% and 49.04% inhibition, respectively. Results showed the percentage of inhibition in a dose dependent manner (Fig. 3). A 100µ g/ml of the extract and ascorbic acid exhibited 49.04% and 63.02% inhibition, respectively.

The concentration of the extract needed for 50% inhibition (IC_{50}) was found to be 114.34µg/ml, whereas 67.13 µg/ml was needed for ascorbic acid (Table 2). These results were found to be statistically significant ($P < 0.05$).

Fig-2

Shows increasing scavenging activity of nitric oxide raical of the methanolic extract of Morchella esculenta along increasing concentrations.



3.1.4. Superoxide radical scavenging assay

Superoxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases. It was therefore proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical. Several in vitro methods are available for generation of superoxide radicals (Vani et al., 1997). In our study, superoxide radicals were generated by auto-oxidation of hydroxylamine in presence of NBT (nitroblue tetrazolium). The reduction of NBT in presence of antioxidants was measured. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. Different concentrations of methanolic mushroom extract (20–100 µg/ml) had strong superoxide scavenging activity (36.12%, 40.10%, 44.9%, 48.10% and 52.9% inhibition, respectively). A 100 µg/ml of methanolic mushroom extract and ascorbic acid exhibited 52.9% and 64.9 % inhibition, respectively (Fig. 4). IC_{50} value of methanolic mushroom extract on superoxide radical scavenging activity was found to be 102.14 µ g/ml, whereas the IC_{50} value of ascorbic acid was found to be 36.69 µg/ml (Table 2). All of the extracts had a scavenging activity on the superoxide radicals in a dose dependent manner (20–100 µg/ml in the reaction mixture). Nonetheless, when compared to ascorbic acid, the superoxide scavenging activity of the extract was found to be low. This could be due to the presence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract. Results were found statistically significant ($P < 0.05$).

Fig - 3

Shows comparative scavenging activity of Super-oxide radical of the methanolic extract of Morchella esculenta at different concentrations.

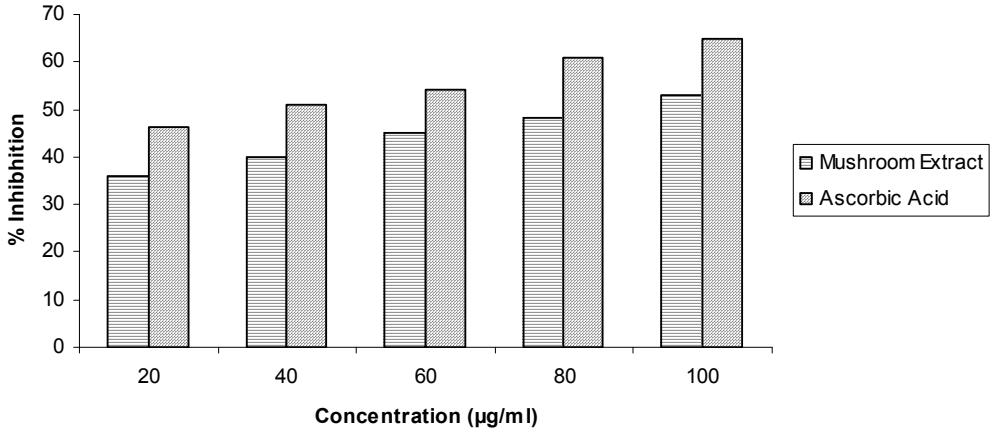


Fig.4

The graph represents comparative study of % inhibhition of different free radical formation by different concentrations of Morchella esculenta extract and standard (ascorbic acid).

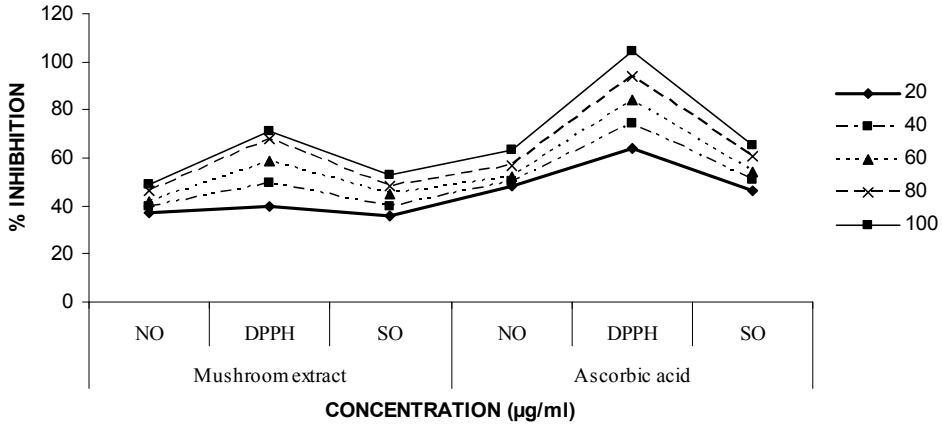


Table 1

Shows anti-oxidant activity of Morchella esculenta extract and Ascorbic acid (Standard) at different concentrations by % inhibhition on different free radical formation.

Group	Concentration (µg/ml)	% Inhibition		
		Nitric –oxide (NO)	DPPH	Super-oxide (SO)
Methanolic extract of mushroom	20	37.2 ± 0.45	39.7 ± 0.87	36.12 ± 0.77
	40	40.01 ± 0.36	49.8 ± 0.81	40.10 ± 0.87
	60	42.04 ± 0.53	58.9 ± 0.96	44.9 ± 0.34
	80	46.02 ± 0.65	67.9 ± 0.94	48.10 ± 0.65
	100	49.04 ± 0.23	70.8 ± 0.89	52.9 ± 0.76
Ascorbic acid (Standard)	20	48.3 ± 0.56	64.2 ± 0.65	46.10 ± 0.98
	40	50.02 ± 0.76	74.2 ± 0.77	50.9 ± 0.88
	60	52.04 ± 0.87	84.2 ± 0.98	54.10 ± 0.79
	80	57.03 ± 0.74	94.2 ± 0.86	60.8 ± 0.87
	100	63.02 ± 0.84	104.3 ± 0.87	64.9 ± 0.86

***Values are** : Mean ± Standard Deviation (n =3). p<0.05*

***Values are** : NO = Nitric oxide, DPPH = 1,1-Diphenly-2-picrylhydrazyl, SO = Superoxide*

Table 2

Shows total phenolic content (µg GAEs/mg extract) and IC50 (µg/ml) values of DPPH, Super- oxide and Nitric-oxide of methanolic extract of Morchella esculenta and ascorbic acid (Standard)

Morchella esculenta	Total Phenolic Compounds (µg GAEs/ mg extract)	IC50 Values (µg/ml)	Mushroom methanolic extract	Ascorbic acid
Methanolic Extract	23.42±0.89	DPPH	67.12	28.42
		Super -oxide	102.14	36.23
		Nitric -oxide	114.34	67.13

***Values** : Mean ± Standard Deviaton, (n = 3). p< 0.05*

Oxidative stress has been implicated in the pathology of many diseases , such as Parkinson disease, Alzheimer, diabetes, cardio vascular disoders, aging and inflammatory c onditions etc. The results obtained in the present studies may be attributed to several

reasons ie, scavenging of superoxide radicals, nitric oxide radicals and DPPH radicals generated in the mammalian cells,involved in the regulation of various physiological processes. However, excess production of these radicals are associated with several diseases (Ialenti, 1993). In the present study the nitrite produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25 ^{0}C was reduced by the methanolic extract of the investigated mushroom. This may be due to the antioxidant active principle in the extract which compete with oxygen to react with nitric oxide thereby, inhibiting the generation of these free radicals. Superoxide dismutase catalyses the dismutation of the highly reactive superoxide anion to oxygen and hydrogen peroxide (Kamalakkannan, 2003). Superoxide anion is the first reduction product of oxygen (Ray, 2002), which is measured in terms of inhibition of generation of O_2.

According to the several references which reported anti-inflammatory,antibacterial and antitumoral activities of this mushroom have previously been investigated (Mau et al., 2004; Elmasta et al., 2006; Nitha et al., 2007; Ramirez-Anguiano et al., 2007).Based on a detailed report on the antioxidant activity of *M. esculenta*, it has exhibited excellent activity patterns in beta-carotene/linoleic acid, reducing power, and metal chelating effect systems (Mau et al., 2004). In the light of these reports,our results are parallel and more excellent as investigated the antioxidant activity of the test mushrooms in more advanced scavenging test systems viz,nitric oxide,super oxide and DPPH systems and find best positive results.

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