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Research Article

ANTIOXIDANT POTENTIAL AND CURRENT CULTIVATION ASPECTS OF AN EDIBLE MILKY MUSHROOM-*CALOCYBE INDICA*

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ABSTRACT

Purpose: The aim of present study was to investigate the cultivation aspects and the antioxidant activities of *Calocybe indica* to establish their various health promoting activities.

Material and Method: *Calocybe indica* was cultivated during the period (winter season of 2010-2011) for high yield of growth the temperature 30-35°c and relative humidity of 80-90% can be maintained. The cultivated of *C. indica* mushroom were further investigated for its antioxidative activity by using various *in-vitro* antioxidant assay such as ABTS, DPPH, Hydroxyl radical scavenging activity, superoxide scavenging activity, reducing power and nitric oxide scavenging activity.

Result: Our result showed that the ethanolic extract of *C. indica* exhibited the potent scavenging activity there by posses increased antioxidant capacity which is compared to that of the standard antioxidant gallic acid.

Conclusion: *Calocybe indica* was well cultivated by using paddy straw within a short period of time of about 13-15 days and potent antioxidant activity may overcome free radicals mediated diseases. So intake of the *C. indica* may prevent oxidative stress diseases.

Keyword: *Calocybe indica,* Antioxidants, Cultivation and free-radicals.

INTRODUCTION

Medicinal plants have served through the ages, as a constant source of medicaments for the exposure of variety of diseases [1]. Mushrooms are the member of higher fungi which either hypogeous or epigeous large enough to be seen with the naked eye and usually picked by hands. They produced fleshy fruit bodies are belongs to Basidiomycotina and Ascomycotina [2].Since ancient times, higher basidiomycetes mushrooms have been used in folk medicine throughout the world [3]. It is the fruiting body of a macro-fungus. Mushrooms are seen growing above the soil or on decaying logs of wood and tree stumps [4]. In many countries mushrooms have long been valued as delicious and nutrional food [5]. Howover, dietary mushrooms provide a wide variety of medicinal properties and they are effective against certain life-threatening diseases [6, 7]. Mushrooms are part of the human diet for thousands of year, involving a large number of edible species [8]. In most countries, there is a well established consumer's acceptance for cultivated mushrooms, probably not only due to their unique flavor and texture but also for their physicochemical properties and nutrional characteristics [9, 10]. These are considered as valuable health foods since they are known for rich proteinacious food, it consist of about 75% proteins and are low in calories, fat, fatty acids, vitamins and minerals [8].

Edible mushrooms such as *Volvariella volvacea* (Fam: *Pluteaceae,* Ver: Paddy straw mushroom), *Agaricusbisporus* (Fam: *Agaricaceae,* Ver: Button mushroom) and *Calocybe indica* (Fam: *Tricholomataceae*,Ver: Milky mushroom) are abundant in India [5]. Among the various edible mushroom *calocybe* genus consists of about 20 species of mushroom, including *calocybe indica*, which can be cultivated throughout the year in the entire of India even in hot humid climate. It is a fleshy, milky white, umbrella like mushroom [11, 12]. *C. indica* is an indigenous popular edible mushroom, possessing a variety of secondary metabolites such as phenolic compounds, terpenes and steroids possibly involved in their medicinal effects and nutritive values in range/100grams are energy - 27kcal, moisture - 90.67%, carbohydrate - 6.3~7.3 g, fat - 0.1g, protein - 2.6~2.9 g, lipids - 0.6~0.7g, fiber- 1.5~1.8 g, thiamine (vit B1) - 0.1mg(8%), Riboflavin (Vit B_2) - 0.5 mg (33%), Niacin (Vit B_3) - 3.8 mg (25%), Pantothenic acid (B_5) - 1.5 mg (30%), Calcium - 18 mg (2%), Phosphorous - 448 mg (10%), Sodium -6 mg (0%), Zinc - 1.1 mg (11%) [13]. It is important to note that the accumulation of these compounds depends on management, processing and maturity at the time of harvest. Though a revolution in mushroom cultivation has been witnessed, serious effects are needed to perfect the production technologies of newer edible mushroom including *C. indica* [14]. Recently, *C. indica* have become an attractive functional food mainly because of their chemical composition, and this can be explained by the antioxidant capacity of mushrooms. As far as milky mushroom is concern there is a lack of scientific investigations. Therefore, we have carried out a preliminary study on the cultivation aspects and antioxidant activity of *C. indica* to establish their health promoting properties.

Antioxidants have been reported to prevent oxidative damage by free radical and ROS may prevent the occurrence of disease, cancer and aging. It can interfere with the oxidation process by reacting with free radicals, chelating and catalytic metals and also by acting as oxygen scavengers [15, 16]. Current research is now directed towards finding antioxidants from natural sources. Although often grouped with plants and vegetables for treating various ailments, mushroom a macro fungus is used in scientific investigation to establish their health promoting benefits against various dreadful diseases.

Majority of the diseases/disorders are mainly linked to oxidative stress due to free radicals [17]. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism [18, 19]. The most common reactive oxygen species (ROS) include superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2) peroxyl radicals (ROO^-). The nitrogen derived free radicals are nitric oxide anion (NO^-) and peroxynitrite anion ($ONOO^-$) [20]. ROS have been implicated in over a hundreds of disease states which range from arthritis and connective tissue disorders to carcinogenesis, ageing, physical injury, infection and cardiovascular disease [21, 22]. In treatments of these diseases, antioxidant therapy has gained an immense importance.

MATERIALS AND METHOD

Chemicals

2,2′-azinobis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Phenazine methosulphate (PMS), Nicotinamide adenine dinucleotide (NADH) from Sigma Aldrich (MO,USA). Butylated hydroxyl tolune (BHT) from SD Fine Chemicals (India). All other chemicals and reagents were of analytical grade.

Cultivation of Calocybe Indica

Milky mushroom (*Calocybe indica*) can be grown on wide range of substrates as in case of oyster mushroom. It can be grown on substrates containing lignin, cellulose and hemicelluloses. Substrate should be fresh and dry. Substrates exposed to rain or harvested premature (green colour) are prone to various weed moulds which may result in failure of the crop. It can be grown on straw of paddy, wheat, ragi, maize/bajra/cotton stalks and leaves, sugarcane bagasse, cotton and jute wastes, dehulled maize cobs, tea/coffee waste etc., However cereal straw (paddy/wheat) easily available in abundance, is being widely used.

Straw is chopped in small pieces (2-4cm size) and soaked in fresh water for 8-16 hours. This period can be reduced when pasteurization is to be done by steam. Main purpose of soaking is to saturate the substrate with water. It is easier to soak if straw is filled in gunny bag and dipped in water. The purpose of pasteurization is to kill harmful microbes. This can be achieved in two ways. Water is boiled in wide mouth container and chopped wet straw filled in gunny bag is submersed in hot water for 40 minutes at 80-90^{0}C to achieve pasteurization. This is very popular method particularly with small growers. Wet straw is filled inside insulated room either in perforated shelves or in wooden trays. Steam is released under pressure from a boiler and temperature inside substrate is raised to 65^{0}C and maintained for 5-6 hours. Air inside the room should be circulated to have uniform temperature in the substrate. Substrate is filled in polypropylene bags (35x45cm, holding 2-3 kg wet substrate) and sterilized at 15 lb psi for 1 hour. Once pasteurization/sterilization is over straw is shifted to spawning room for cooling, bag filling and spawning.

Casing means covering the top surface of bags after spawn run is over with pasteurized casing material in thickness of about 2-3cm. Casing provides physical support, moisture and allows gases to escape from the substrate. Casing material (soil 75% + sand 25%) with pH adjusted to 7.8-7.9 with chalk powder is pasteurized in autoclave at 151b psi for one hour or chemically treated with formaldehyde solution (4%) about a week in advance of casing. Solution should be enough to saturate the soil. It is covered with polythene sheet to avoid escape of chemical and at a interval of 2 days soil is turned so that at the time of casing soil is free from formalin fumes. Bag's top is made uniform by ruffling top surface of the substrate and sprayed with solution of carbendazim (0.1%) + formaldehyde (0.5%).

Casing material is spread in uniform layer of 2-3 cm thickness and sprayed with solution of carbendazim and formaldehyde to saturation level. Temperature 30-35^{0}C and R.H. 80-90% are maintained. It takes about 10 days for mycelium to reach on top of casing layer when fresh air is introduced while maintaining temperature and R.H. as above. Light should be provided in long time. The changes thus made in environment, result in the initiation of fruiting bodies within 3-5 days in the form of needle shape which mature in about a week. Mushrooms 7-8cm diam. are harvested by twisting, cleaned and packed in perforated polythene/polypropylene bags for marketing. Mushrooms can also be wrapped in klin film for longer storage.

The mushroom requires high temperature and high humidity along with good light and aeration. Yield is adversely affected when these conditions are not provided. While good mycelial growth occurs between 20-37°C. For fruiting the temperature requirement is from 25-35°C. The pH of the casing material should be around 7.0 so that competitor moulds do not attack the beds.

Sample Preparation

The harvested mushrooms were air–dried in an oven at 40°C and then dried mushroom sample (50g) was extracted by stirring with 500ml of ethanol and filtering through Whatman No.1 filter paper. The residue was again extracted with two additional 200ml ethanol as described above. The combined ethanolic extract of Milky mushroom (*C. indica*) was then rotary evaporated at 40°C to dryness. The dried extract was stored at 4°C until further used.

Antioxidant Analysis

ABTS radical scavenging activity

The gallic acid equivalent antioxidant capacity was estimated using ABTS method for total antioxidant activity (Re *et al*., 1999) [23]. In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\bullet+}$, which is intensely colored and AOC (antioxidant capacity) is measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\bullet+}$ radical. Results of test compounds are expressed relative to gallic acid. The stock solution included 7.4 mM ABTS solution and 140 mM potassium per-sulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in dark. The solution was then diluted by mixing 1 ml ABTS solution with ethanol to obtain an absorbance of 0.7 ± 0.02 units at 734 nm using UV-1601 spectrophotometer. The antioxidant property was determined by reduction in the O.D. compared with the standard gallic acid. Inhibition of free radical by $ABTS^{\bullet+}$ in percent (I %) was calculated in following way

$$\% \text{ of inhibition} = [(A_{blank} - A_{sample})/A_{blank}] \text{ X } 100$$

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. The values of inhibition were calculated for the various concentrations of ethanol extracts. Tests were carried out in triplicates.

DPPH Free Radical Scavenging Activity

The free radical scavenging activity of the ethanolic extract of *C. indica* was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using the method described by Shimada *et al.,* [24]. Briefly 0.1 mM solution of DPPH in ethanol was prepared; 1ml of the solution was added to 3 ml of extract in water at different concentrations (25-50 µg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm by using a UV-Visible Spectrophotometer. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation:

DPPH Scavenging effect (%) = [(A0-A1)/A0] x 100

Where A0 was the absorbance of the control reaction and A1 was the absorbance in the presence of the standard sample or extract.

Hydroxyl Radical Scavenging Activity

The capacity to scavenge hydroxyl radicals was measured according to a modification of the method of Halliwell, Gutteridge, and Arnoma [25]. Stock solutions of EDTA (1 mM) were prepared in DMSO and $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding, in sequence, 0.1 ml EDTA, 0.01 ml of $FeCl_3$, 0.1 ml of H_2O_2, 0.36 ml of deoxyribose, 1.0 ml of the extract (2 to10 mg/ml) dissolved in distilled water, 0.33 ml of phosphate buffer (50 mM, pH 7.4) and 0.1 ml of ascorbic acid. This mixture was then incubated at 37 °C for 1 hr. A 1.0 ml portion of the incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% butylated hydroxyanisole) to develop a pink chromogen that was measured at 532 nm. The hydroxyl radical-scavenging activity of the extract was reported as % inhibition of deoxyribose degradation and was calculated by the formula

$$OH^{\bullet}\text{- scavenged (\%)} = \frac{(A_{cont} - A_{test}) \times 100}{A_{cont}}$$

Where A_{cont} was the absorbance of the control reaction and A_{test} was the absorbance of the mixture containing the extract or the absorbance of a standard solution.

Superoxide Anion Radical Scavenging Activity

Measurement of the superoxide anion radical-scavenging capacity of the mushroom extract was essential according to the method described by Liu et al., [26] using a minor modification proposed by

Rajeshwar, Senthil Kumar, Gupta and Upal Kanti (2005). The principle of this method is that superoxide radicals are generated in phenazine methosulphate (PMS) – nicotinamide adenine dinucleotide (NADH) systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide radicals were generated in 3.0 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1.0 ml of NBT (50 µM) solution, 1.0 ml NADH (78 µM) solution and samples of the extracts (2 to 10 mg/ml) in water. The reaction was initiated by adding 1.0 ml of phenazine methosulphate (PMS) solution (10 µM) to the mixture. The reaction mixture was incubated at 100°C for 5 min, and the absorbance at 560 nm was measured against a blank. Gallic acid was used as a standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion-scavenging activity. The % inhibition of superoxide anion generation was calculated using the formula as described in hydroxyl radical-scavenging activity.

Reducing Power

The reducing power of the *C. indica* extract was determined by the method of Oyaizu [27]. Various concentrations of the extract (2 to10 mg/ml) in 1.0 ml of deionized water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixture was incubated at 50 °C for 20 min. Aliquots of trichloroacetic acid (2.5 ml, 10%) were added to the mixture, which was then centrifuged at 1036×g for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared FeCl3 solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. An increased absorbance of the reaction mixture was taken to mean an increased reducing power. The standard gallic acid was also processed by the same procedures.

Nitric Oxide Radical Inhibition Assay

Nitric oxide radical scavenging can be estimated by the use of Griess Illosvoy reaction (Garrat 1964) [28]. In this study, the Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *C. indica* extract (10 µg to 160 µg) or standard solution (BHT, 0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Gallic acid was used as positive controls.

Statistical Analysis

All the analysis was performed in triplicate. Statistical analyses were performed using a one-way analysis of variance ANOVA, and the significance of the difference between means was determined by Duncan's multiple range test. Differences at $P < 0.05$ were considered statistically significant. The results were presented as mean values ± SD (standard deviations).

RESULTS

On casing the spawn bed in to the soil, small mushroom has been developed which are shown [Figure 1]. After 3 days the mushroom growth is increased to 4-5cm in length [Figure 2] and it is ready to harvest. Now the harvested mushrooms [Figure 3] are used to carry out further antioxidant activity – *in vitro*

The free radical scavenging activity of the extract was tested through ABTS method and the results were depicted [Figure 4]. The various concentrations of *C. indica* (10-50 µg/ml) showed maximum inhibition of 63% at 50µg/ml. Results showed the percentage of inhibition in a dose dependent manner. The IC_{50} value of *C. indica* was found to be 16.2µg/ml. As positive controls, 50% inhibitory concentrations of gallic acid (IC_{50}=33.4 µg/ml).

Fig. 1: Initial Stage of mushroom (After 8 – 10 days)

Fig. 2: Harvesting stage (After 13 days)

Fig. 3: After Harvesting

The antioxidant capacity of *C. indica* extract was determined by the DPPH method and the results are presented [Figure 5]. Different concentrations of *C. indica* (10-50 µg/ml) showed maximum 58% inhibition at 50 µg/ml. Results showed the percentage of inhibition in a dose dependent manner. The IC_{50} value of *C .indica* was found to be 20µg/ml. As positive controls, 50% inhibitory concentrations of gallic acid (IC_{50}=26.3µg/ml).

Whereas [Figure 6] summarizes the hydroxyl radical scavenging activities of the extract using the deoxyribose assay. The various concentrations of *C. indica* (10-50 µg/ml) showed 51% maximum inhibition. The IC_{50} value of *C. indica* was found to be 27.2µg/ml. As positive controls, 50% inhibitory concentrations of gallic acid (IC_{50}=40.1µg/ml).[Figure 7] depicts the superoxide anion scavenging activity of the extract. Different concentrations of *C. indica* (10-50 µg/ml) showed 55% inhibition at the concentration of 50µg/ml. The extract showed less scavenging activity (IC_{50}=26.4 µg/ml) than the synthetic antioxidant gallic acid (IC_{50}=17.8 µg/ml).

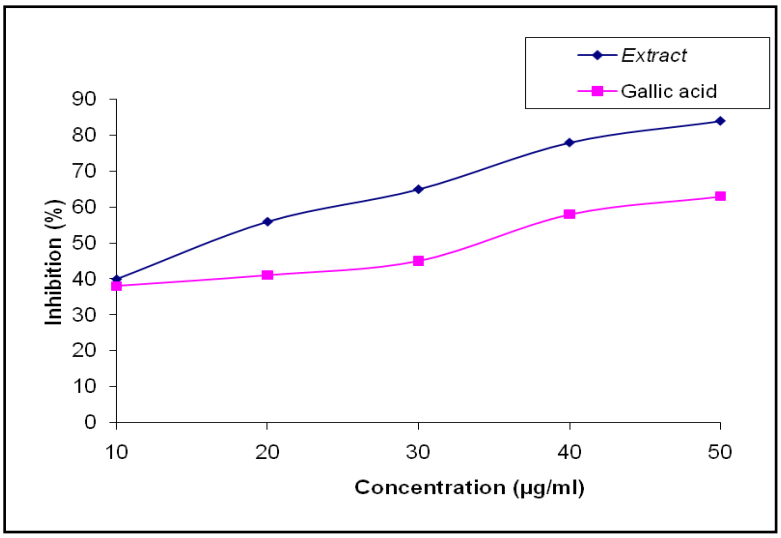

Fig. 4: ABTS Radical Scavenging Activity of ethanolic extract of *C. indica* and Standard

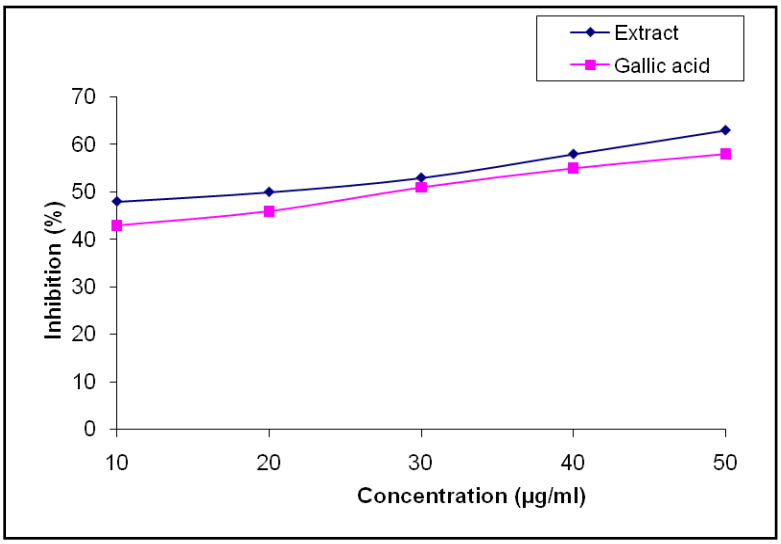

Fig. 5: DPPH Radical Scavenging Activity of ethanolic extract of *C. indica* and Standard

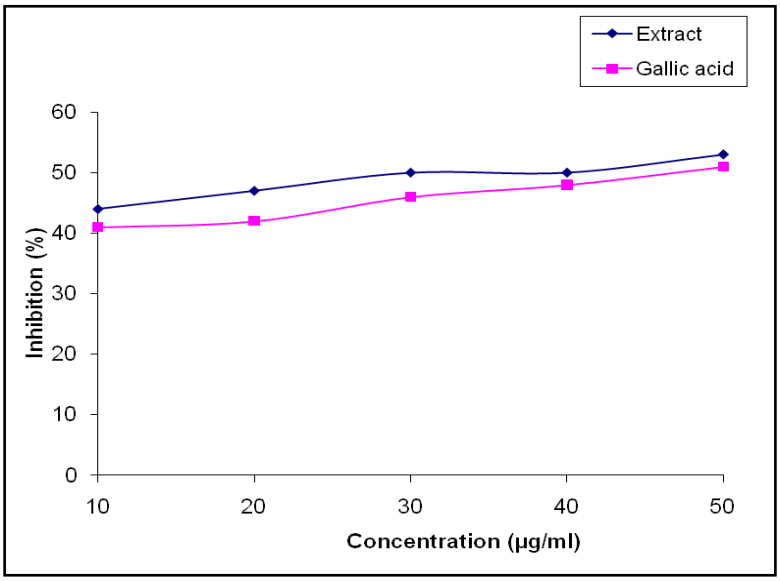

Fig. 6: Hydroxy Radical Scavenging Activity of ethanolic extract of *C. indica* and Standard

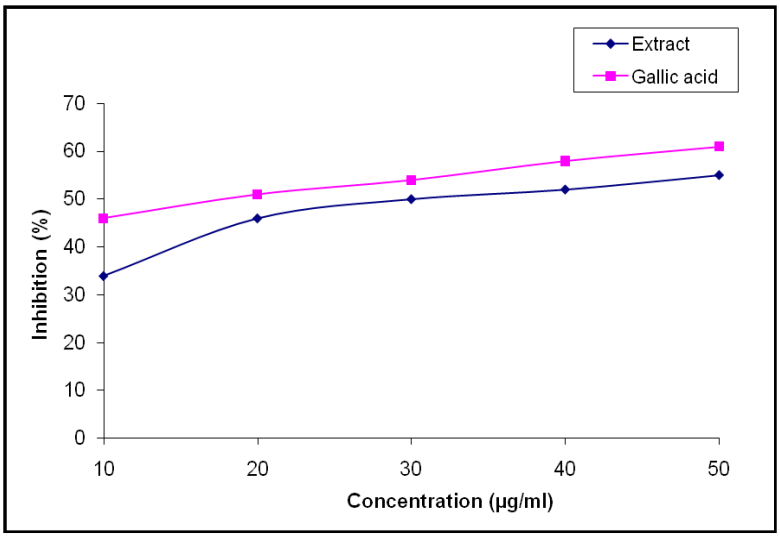

Fig. 7: Superoxide Anion Scavenging Activity of ethanolic extract of *C. indica* and Standard

The reducing power of *C. indica* was shown [figure 8]. The reducing power of *C. indica* increased with increasing concentration when compared with gallic acid. The nitric oxide scavenging activity of the extract was tested and summarized [Figure 9]. Various concentrations of *C. indica* (10-50 µg/ml) showed 55% inhibition. The extract showed more scavenging activity (IC_{50}=15.1 µg/ml) than the synthetic antioxidant gallic acid (IC_{50}=27.3 µg/ml).

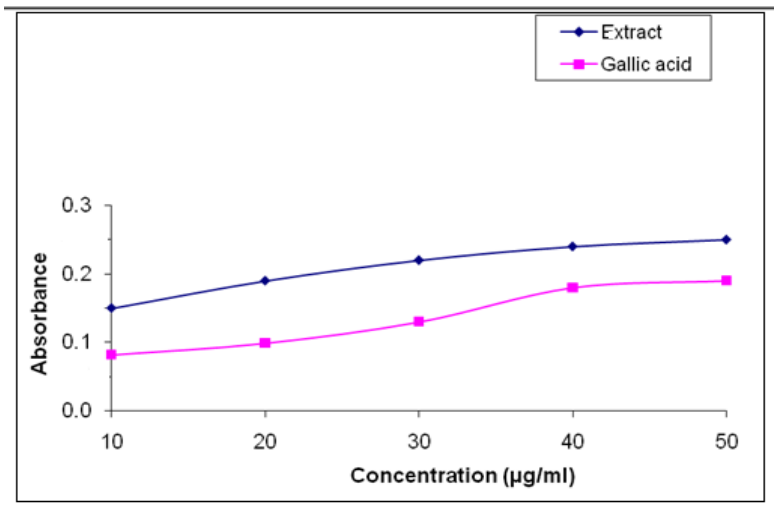

Fig. 8: Reducing Power of ethanolic extract of C. *indica* and Standard

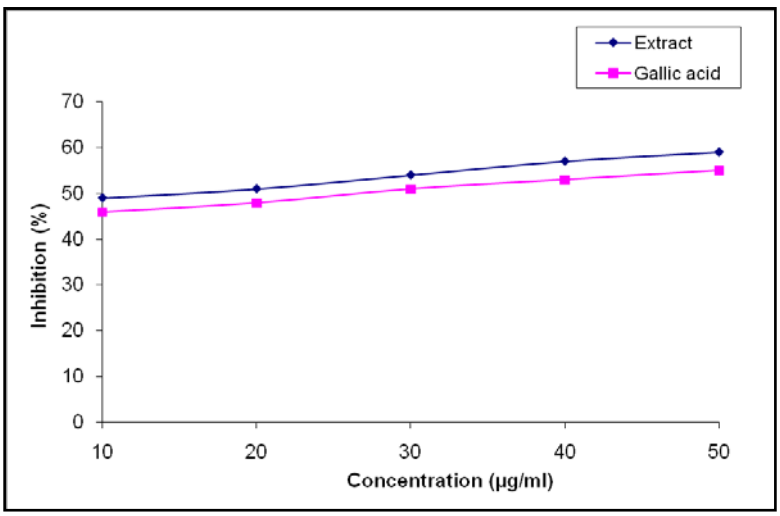

Fig. 9: Nitric Oxide Scavenging Activity of ethanolic extract of *C. indica* and Standard

DISCUSSION

ABTS, a stable free radical with a characteristic absorption at 734 nm, was used to study the radical-scavenging effects of extracts. $ABTS^{+}$ reacts rapidly with antioxidants, and it can be used over a wide pH range to study the effects of pH on antioxidant mechanisms [29]. In the present study it is noticed that *C. indica* extract showed potent antioxidant activity as revealed by scavenging the ABTS free radical. DPPH method is based on the reduction of DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption. The present investigation suggested that *C. indica* possess scavenging of stable radical species of DPPH by antioxidants. The values are also compared with commercial antioxidant gallic acid [30]. Hydroxyl radical is the most reactive among reactive oxygen species (ROS) and it bears the shortest half-life compared with other ROS. The ability of extracts to quench hydroxyl radicals seems to be good scavenger of active oxygen species, thus reducing the rate of the chain reaction [31]. According to our investigation, it is clear that the extract has the ability to decrease the absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The reducing power of the mushroom methanolic extracts increased with concentration. In the present study *C. indica* extracts exhibited the reducing activity indicating that some antioxidant constituents may be involved in the ferricyanide reduction [32]. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as NO_2, N_2O_4, N_3O_4, NO_3^- and NO_2^- are very reactive. It was also observed that all the extracts are likely to have the nitric oxide radical scavengers and primary antioxidants which react with free radicals.

C. indica, a common edible mushroom possess many nutritive ingredients such as vitamins, proteins, minerals, amino acids, polyphenols such as flavonoids, alkaloid. triterpenoids. These active compounds may be involved in scavenging processess and thereby enhancing antioxidant capacity. Moreover our results also collabarates with previous reports, in which Agaricus sp. edible mushrooms exhibit the highest ‘‘antioxidant power” in there chemical and biochemical assays [33].

CONCLUSION

Dietary supplementation with antioxidants may provide great relief to the problem caused by ROS. *C. indica* extract has scavenging effect that might be contributed to the presence of antioxidants. Further research should be focused to isolate the active compounds from *C. indica* mushrooms having nutraceutical and medicinal properties and to commercialize their production and marketing.

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