Int J Pharm Bio Sci 2015 April.; 6(2): (P) 485 - 497

Research Article Biotechnology

International Journal of Pharma and Bio Sciences

ISSN
0975-6299

PHYTOCHEMICAL INVESTIGATION AND TOTAL ANTIOXIDANT POTENTIAL OF THE MILKY MUSHROOM, *CALOCYBE INDICA* Var.APK2 CULTIVATED IN TAMILNADU, INDIA

R.SUMATHY*[1], R.KUMUTHAKALAVALLI[1], A.S.KRISHNAMOORTHY[2] AND VENKATESH BALAN[3]

[1]*Department of Biology, The Gandhigram Rural Institute – Deemed University, Dindigul*
[2]*Department of Plant Pathology, Tamilnadu Agricultural University, Coimbatore*
[3]*Department of Chemical Engineering and Material Sciences, Michigan State University,*

ABSTRACT

Phytochemicals of mushrooms are nutritionally functional and a source of physiologically beneficial medicines. In the present study, crude hot water; cold water extracts of *Calocybe indica* was qualitatively screened for the presence of phytochemicals. Hot water extracts showed the presence of many phytochemicals like polysaccharide, phenol, flavonoids, alkaloid, terpenoid and saponin when compared to that of the cold water extract. The hot water extracts were further analysed for their antioxidant activity through *in vitro* studies using reducing power, DPPH, superoxide, hydroxyl, hydrogen per oxide radical scavenging, β-carotene and linoleate leaching and lipid peroxidation assays. All the assays were carried out with the concentration of extracts ranging from 20µg/ml to 100 µg/ml, all the tested assays revealed the significant antioxidant activity from 15-86%. These results provide evidence that the hot water extracts of *C.indica* might indeed be a potential source of natural antioxidant which could be utilized in diet to quench the free radicals produced in the body.

KEYWORDS: **free radicals, reducing power, β-carotene linoleate, lipid peroxidation**

R. SUMATHY
Department of Biology, The Gandhigram Rural Institute – Deemed University, Dindigul

***Corresponding author**

INTRODUCTION

Free radicals are produced in the normal natural metabolism of aerobic cells, mostly in the form of reactive oxygen species (ROS). They are generated by NADPH oxidase during oxidative phosphorylation. ROS are also mediators of the first defensive actions of cells and involved in phagocytosis, apoptosis and detoxification[1,2]. Recently, increasing evidence of overproduction of ROS and oxygen derived free radicals that contribute to a variety of pathological effects and outbreak of many diseases, including diabetes, aging, cancer, atherosclerosis and rheumatoid arthritis are being reported[3]. Therefore, it is necessary to maintain the equilibrium between free radical production and antioxidant defense for normal functioning of organism[4]. There are considerable evidences that antioxidant could help to prevent diseases as they have the capacity to quench free radicals[5]. Synthetic antioxidants such as butylated hydroxyl anisole (BHA), butylated hydroxy toluene (BHT) and tertiary butyl hydroquinone (TBHQ) are being used widely and have been suspected to cause or promote negative health effects[6,7]; toxic to cells and act as carcinogens (Carcinogen association, 1991) that cause liver damage[8]. Hence there is a growing interest in studies of natural potential non toxic antioxidants, to protect human body from free radicals and retard the progress of chronic diseases. Phenolic and Flavonoid compounds from natural origins are found to be good alternatives for synthetic antioxidants. In the search of new natural antioxidants with high phenolic profile, a number of edible and medicinal mushrooms have been explored and recorded as potential natural antioxidants[9, 10]. Mushrooms have been a part of the normal diet for thousands of years and in recent years the amounts consumed have risen greatly involving a large number of species. *Calocybe indica*, commonly known as summer white mushroom or milky mushroom is one of the most economically important edible mushrooms that grow predominantly in hot humid climates in Tamilnadu, India. It is considered as valuable health food with high content of polysaccharides, vitamin B-complex, minerals and aminoacids[11,12.] Moreover, this mushroom has been demonstrated to possess various valuable biological properties including antimicrobial, anti-inflammatory, anti-diabetic, anti-tumor as well as antioxidant activities[1314,15,16]. Till now most studies have been focused on *in vitro* antioxidant activities of *C.indica* concerning reducing power, DPPH and ABTS radical scavenging ability[15,16]. Although a wide range of model systems are available for evaluation of antioxidant activities, the choice mainly depends on the nature of the substances under investigation. There are evidences for discrepancies in antioxidant activities of substances when tested in different *in vitro* model systems. Therefore, in the present study the crude aqueous extract of *Calocybe indica* was subjected to the qualitative analysis of phytochemicals and also for its antioxidant activities using seven different *in vitro* model systems namely, reducing power, DPPH, superoxide, hydroxyl, hydrogen per oxide radical scavenging, β-carotene and linoleate leaching and lipid peroxidation assays.

MATERIALS AND METHODS

Sample collection and processing

Cultivated fruiting bodies of *Calocybe indica* var.APK2 were obtained from the Sujii Mushroom farm, Perundurai, Erode, Tamilnadu, India. The samples were authenticated by Dr.A.S.Krishnamoorthy, Professor, Department of Plant Pathology, Tamilnadu Agricultural University, Coimbatore. A voucher specimen has been deposited at the Mushroom Unit, Tamilnadu Agricultural University, Coimbatore.
All the samples were lyophilized; reduced to a fine dried powder (20 mesh); mixed to obtain homogenous samples and stored in a desiccator, protected from light, until further analysis.

Preparation of aqueous extracts of C.indica

Aqueous (Cold water extract - CWE) extraction

5g of *C.indica* powder was ground and mixed with 50ml of sterile distilled water. Mixture was centrifuged. After centrifugation at 5000g for 10min, the residue was re-extracted twice with 20ml of sterile distilled water as described above. The supernatant were pooled together; concentrated in rotary evaporator at 40°C. The dried extract thus obtained was used directly for the determination of a presence of phytochemicals

Aqueous (Hot water extract - HWE) extraction

5g of *C.indica* powder was extracted by stirring with 50ml of sterile distilled water at 60°C for 1h. Mixture was cooled and centrifuged. After centrifugation at 5000g for 10min, the residue was re-extracted twice with 20ml of sterile distilled water as described above. The supernatant were pooled together; concentrated in rotary evaporator at 60°C and the dried extract thus obtained was used directly for the determination of antioxidant activity and presence of phytochemicals.

Qualitative analysis of Phytochemicals[17]

Test for phenols

Lead acetate test: To 1 ml of the extract, few ml of 1 % lead acetate solution was added and the formation of bluish black precipitate indicated the presence of tannins and phenolic compounds.

Test for Flavonoids

Ferric chloride test: To 1 ml of the extract, 1 ml of ferric chloride solution was added. Appearance of brown color confirmed the presence of Flavonoids.

Test for alkaloids

Dragendroff's test: About 0.2 g of extract was warmed with 1 % of aqueous hydrochloric acid for 2 min. The mixtures were filtered and few drops of Dragendroff's reagent were added. A reddish brown color and turbidity with the reagent indicated the presence of alkaloids.

Test for Tannins

Ferric chloride test:To 1 ml of the extract, few ml of 5 % ferric chloride was added. The development of dark bluish black color indicated the presence of tannins.

Test for Terpenoids

Liebermann - Burchard's test : Extract was treated with a few drops of acetic anhydride, boil and cool. Concentrated sulfuric acid was added from the sides of the test tube which showed a brown ring at the junction of two layers, and the formation of deep red color indicated the presence of terpenoids.

Test for saponins

Frothing test: 1 ml of filtrate was diluted with 4 ml of distilled water and the mixture was shaken vigorously and observed for persistent foam which lasted for atleast 15 min which indicated the presence of saponins.

Test for glycosides

Keller - Killani test: 2 ml of extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution. The mixture was then poured into a test tube containing 1 ml of conc. H_2SO_4. A brown ring at the inter-phase indicated the presence of a deoxy sugar, characteristic of cardenolides.

Test for Polysaccharides

2ml of mushroom extract was mixed with 0.1ml of anthrone and 1 drop of concentrated sulphuric acid. The mixture was warmed gently. Formation of green colour indicated the presence polysaccharides.

Reducing power assay

The reducing power was determined according to the method of Oyaizu[18] with some modifications. Reaction was carried out in a mixture containing 1 mL of sample (20-100 µg/ml), 2.5 ml of 0.1 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1%, w/v potassium ferro cyanate [$K_3Fe(CN)_6$] by incubating at 50°C for 20 min. After addition of 2.5 ml trichloroacetic acid (10%, w/v), the mixture was centrifuged at 5000g for 10 min. The upper

layer (5 ml) was mixed with 0.5 ml of fresh $FeCl_3$ (0.1%, w/v), and the absorbance at 700 nm was measured against a blank. Gallic Acid was used as the positive control.

DPPH radical scavenging assay

The free radical scavenging activity of the aqueous extract of *C. indica* was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using the method described by Shimada[19]. Briefly 0.1 mM solution of DPPH in ethanol was prepared; 1ml of the solution was added to 1ml of extract in water at different concentrations (20-100 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 (%) = $[(A_0-A_1)/A_0] \times 100$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the standard sample or extract. The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of DPPH radical formation.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was determined according to the method of Zhong[20] with some modifications. The mixture containing 1 ml of sample (0.1–0.5 mg/ml), 1 ml of 9 mM $FeSO_4$ and 1 ml of 0.3% H_2O_2 in 0.5 ml of 9 mM salicylic acid–ethanol solutions was shaken vigorously and incubated at 37°C for 30 min. Then, the absorbance of the reaction mixture was determined at 510 nm. Gallic Acid was used as the positive control. The hydroxyl radical scavenging activity was calculated by the following formula:

Scavenging activity (%) = $[1- (A_1 - A_2)/ A_0] \times 100$

where A_0 is the absorbance of the control (ethanol instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (salicylic acid–ethanol solution instead of $FeSO_4$ and H_2O_2 solutions). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of hydroxyl radical formation.

Superoxide radical scavenging assay

The superoxide radical scavenging activity was performed by the method of Jing and Zhao[21] with some modifications. Reaction was carried out in a mixture containing 4.5 ml of 50 mM Tris–HCl buffer (pH 8.2), 0.4 ml of 25 mM pyrogallol solution and 1 ml of sample (0.5–5 mg/mL) by incubating at 25°C for 5 min. Finally, 1 ml of 8 mM HCl solution was dripped into the mixture promptly to terminate the reaction. The absorbance of the mixture was measured at 420 nm. Gallic Acid was used as the positive control. The superoxide radical scavenging activity was calculated by the following formula:

Scavenging activity (%) = $[1- (A_1- A_2)/A_0] \times 100$

where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (Tris–HCl buffer instead of pyrogallol solution). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of superoxide radical formation.

H_2O_2 scavenging assay

The H_2O_2 scavenging activity was determined according to the method of Ruch[22] with some modifications. The mixture containing 1 ml of sample (0.1– 2 mg/mL), 2.4 ml of phosphate buffer (0.1 M, pH 7.4) and 0.6 ml of H_2O_2 solution (40 mM) was shaken vigorously and incubated at room temperature for 10 min. Then, the absorbance of the reaction mixture was determined at 230 nm. Gallic Acid was used as the positive control. The H_2O_2 scavenging activity was calculated as follows:

Scavenging activity (%) = $[1- (A_1 - A_2)/ A_0] \times 100$

Where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample only (phosphate buffer instead of H_2O_2 solution). The IC_{50} value represented the

concentration of the compounds that caused 50% inhibition of H_2O_2.

β-carotene linoleate bleaching assay

The antioxidant activity of *C.indica* extracts was assayed based on the β-carotene bleaching method described by Cheung[23]. In the β-carotene bleaching assay, linoleic acid produces hydroperoxides (ROS), and this oxidizes the β- carotene at 50°C. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be inactivated by antioxidants from extracts. Gallic acid was used as the standard. β- Carotene (0.2 mg in 1 ml of chloroform), linoleic acid (0.02 ml), and Tween 80 (0.2 ml) were transferred into a round-bottomed flask. Chloroform was removed at room temperature using a rotary evaporator. Following evaporation, 50 ml of distilled water was added to the mixture and shaken vigorously to form an emulsion. Two milliliters of aliquots of the emulsion was pippeted into test tubes containing of mushroom extracts (different concentration of 20–100 µg/ml) and immediately placed in a water bath at 50°C. The absorbance was read at 20 min intervals for 2 h at 470 nm using UV-vis spectrophotometer. The antioxidant activity (I) was expressed as a percent of inhibition relative to the control, using the following formula:

I (%) = (Ac – As)/Ac X 100

Where Ac and As represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively.

Lipid peroxidation Assay

The inhibition of lipid peroxidation was determined according to the method of Abdullah[24]. The reaction mixture contained 1 mL of fowl egg yolk emulsified with phosphate buffer (pH 7.4) to obtain a final concentration of 25 g/L, sample (different concentration of 20–100 µg/ml), and 100 μL of 1000 μM $FeCl_2$. The mixture was incubated at 37°C for 1 h before being treated with 0.5 ml of freshly prepared 15% trichloroacetic acid (TCA) and 1.0 ml of 1% thiobarbituric acid (TBA). The reaction tubes were further incubated in boiling water bath for 10 min. Once cooled to room temperature, the tubes were centrifuged at 3500 g for 10 min to remove precipitated protein. The absorbance at 532 nm was determined spectrophotometrically. Gallic acid was used as positive control. The percentage inhibition was calculated from the following equation: Inhibition (%) = $[(Ab - As)/Ab] \times 100$, Where Ab is the absorbance of the blank without the extract or ascorbic acid and As is the absorbance in the presence of the extract or gallic acid.

Statistical Analysis

All experiments were conducted in triplicates and the data are shown as the mean ± standard deviation (SD), using the statistical package within Microsoft® Excel Version 2007.Ink and the graphs were plotted using software Origin 8.0.

RESULTS AND DISCUSSION

Mushrooms have continued to generate a lot of interest particularly in their consumption as food and in the cure of disease that stems from the bioactive molecules present in them. Results on the present investigation on the presence of phytochemicals in the HWE and CWE of mushroom (*C.indica*) were given in Table:1. As shown in table, among the two extracts analysed hot water extract showed higher presence of phytochemicals compared to that of the cold water extract. Totally eight compounds were analysed in which HWE showed the presence of six compounds whereas the CWE showed the presence of only three compounds. The major compounds in the HWE were polysaccharides, phenols and Flavonoids. Other important compounds were terpenoids, saponins and alkaloids. Similar results were reported for *C.indica*[13]. These phytochemicals present in many other mushrooms were reported to be active as potential antioxidants[25] Keeping the presence of phytochemicals as the base, the HWE of mushroom was subjected to free radical

scavenging activity analysis by various *in vitro* assays. Since, natural foods contain an array of classes and types of antioxidants, various antioxidant assays can be used to determine the total antioxidant capacity and usage of multiple assays provide evidence of the antioxidant capacity within the food material that are able to scavenge various biologically significant free radicals. Antioxidant activities of compounds have been attributed to various mechanisms in the body. Among them the reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In the present study, the reducing power of aqueous extract and gallic acid increased with the increase of concentrations (Figure: 1). A similar trend was observed for the reducing power capacity for *C.indica*[15,13] and for *Agaricus bisporus*[2]. Model of scavenging DPPH radical is a widely used method to evaluate the free radical scavenging activities of antioxidants. The DPPH scavenging activities are ascribed to their hydrogen donating abilities. As given in Figure:2, the scavenging activities on DPPH radical of the aqueous extract and gallic acid increased with the increase in concentration. At the concentration 0f 100µg/ml the DPPH scavenging activity for the extract and GA was 78.72±0.40 and 81.24±0.02% respectively. The half maximal inhibitory concentration (IC_{50}) of the mushroom was found to be 62.45 µg/ml. The results obtained herein was in conformity with the earlier reports[15,13] . Hydroxyl radical, the most reactive radical known can attack and damage almost every biomacromolecule in living cells[26]. In this study, the scavenging activities of aqueous extract and gallic acid increased with the increase in concentration (Figure:3). At the concentration of 100µg/ml the hydroxyl scavenging activity for the mushroom extract and GA was 66.94±0.06 % and 79.23±0.02% respectively. The half maximal inhibitory concentration (IC_{50}) of the mushroom was found to be 66.92 µg/ml. Earlier researchers reported that there was no hydroxyl radical scavenging activity was observed for extracts of *Clitocybe maxina*, *Pleurotus ferulae* and *Pleurotus ostreatus*[27]. The aqueous extracts of *C.indica* showed a moderate hydroxyl radical scavenging activity than the methanolic extract[15].Thus the mushroom *C.indica* can be considered as good scavenger of hydroxyl radicals. Superoxide radical, arising either through metabolic process or from oxygen activation by physical irradiation is considered as the primary ROS, as a result of formation they induce oxidative damage in lipids, proteins and DNA[26]. The superoxide radical scavenging activities of aqueous extract and GA were shown in Figure: 4. The scavenging activities of all samples were correlated well with the increase of concentrations and the scavenging activity of gallic acid was higher than that of aqueous extract. At the concentration of 100µg/ml the scavenging activity for the extract and GA was 58.94±0.03 % and 79.23±0.02 % respectively. The half maximal inhibitory concentration (IC_{50}) of the mushroom was found to be 72.32µg/ml. Reports obtained were concomitant with the earlier findings of Mirunalini[15]. H_2O_2 plays an important role as the radical forming intermediate in the production of ROS molecules. Although H_2O_2, is not very reactive, its high penetrability in cellular membrane leads to hydroxyl radical formation, which is one of the main inducer of cellular aging and could attack many cellular energy producing systems[28]. As depicted in Figure:5, aqueous extract of *C.indica* and GA both exerted concentration dependent H_2O_2 scavenging activities. At the concentration of 100µg/ml the scavenging activity for the extract and GA was 62.42±0.30 % and 79.23±0.02 % respectively. The half maximal inhibitory concentration (IC_{50}) of the mushroom extract was found to be 78.45 µg/ml. Till now only very few investigators focused on the H_2O_2 radical scavenging activity of *C.indica*. The present study indicates that the aqueous extract of *C.indica* have moderate H_2O_2 scavenging activity, similar to *A.bisporus*[2]. The β-carotene linoleic acid bleaching assay is one of the rapid methods to screen the antioxidant, which is mainly based on the principle that linoleic acid which is an unsaturated fatty acids gets oxidized by ROS produced by oxygenated water. The products

formed will initiate the β-carotene oxidation which leads to discoloration reaction[29]. This fact is shown in the antioxidant activity of aqueous extract of *C.indica*, in comparison with the well known antioxidant gallic acid (Figure: 6). At the concentration of 100µg/ml the scavenging activity for the extract and GA was 66.41±0.02 % and 71.19±0.0 % respectively. The half maximal inhibitory concentration (IC_{50}) of the mushroom was found to be 74.22 µg/ml. Results obtained were in accordance with the earlier reports on many mushrooms like *A.bisporus*[2]; *Pleurotus sajor-caju*[30] and *Boletus edulis*[31]. The study on lipid peroxidation (LPO) processes has become a rapidly growing field in medicine and biology. The first phase of this process is represented by peroxidation of poly unsaturated fatty acid compounds which can bring about biological change at molecular level[24]. The ability of the *C.indica* in egg yolk is presented in Figure:7, the peroxidation of aqueous extract and gallic acid increased with the increase in concentration. At the concentration of 100µg/ml, the lipid peroxidation activity for the extract and GA was 46.91±0.02 % and 57.23±0.02 % respectively. The half maximal inhibitory concentration (IC_{50}) of the mushroom was found to be 107.9 µg/ml. The results demonstrated that the aqueous extract of *C.indica* has capacity to inhibit lipid peroxidation significantly than the species namely *Leucopaxillus giganteus* and *Sarcodon imbricatus*. These data were in concurrence with the previous studies[14,32]. As previously described[33,34], the use of different methods is necessary in antioxidant activity assessment. The present study showed that no single testing method is sufficient to estimate the antioxidant activity of a studied sample. The combination of seven methods applied in this study was a good choice to evaluate the antioxidant activity of aqueous extract of *C.indica*. The antioxidant power depends on the chosen method; on the concentration; on the nature and physicochemical properties of studied antioxidants. From the present it was evident that the same antioxidant samples exhibit different antioxidative values depending on the concentration and the measured parameters. And also results indicate that the *C.indica* could be in use as potential resource of natural antioxidants. This indicates the potential of the mushroom as panacea for many diseases and also reveals a novel potential fight against ROS mediated diseases.

Table 1
Qualitative Phytochemical Analysis of Calocybe indica

Parameters	HWE	CWE
Phenol	+++	+
Flavonoid	++	+
Alkaloid	+	-
Tannin	-	-
Terpenoid	+	-
Saponin	+	-
Polysaccharide	+++	+
Glycosides	-	-

HWE –Hot Water Extract; CWE-Cold Water Extract
Note: "+++" Occurrence very high concentration; "++" Occurrence high concentration; "+" Occurrence low concentration; "-" Denotes absent.

Figure 1
Reducing Power of Calocybe indica extracts

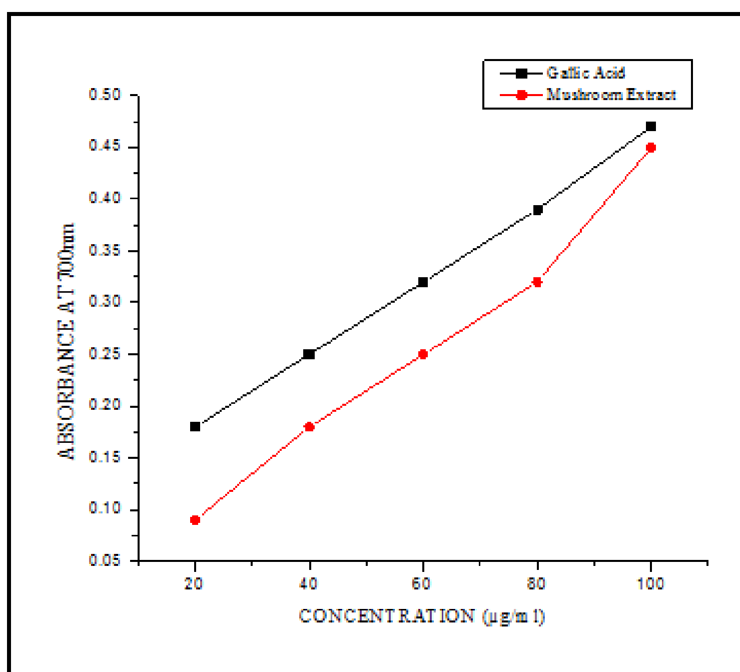


Figure 2
DPPH radical scavenging activity of Calocybe indica extracts

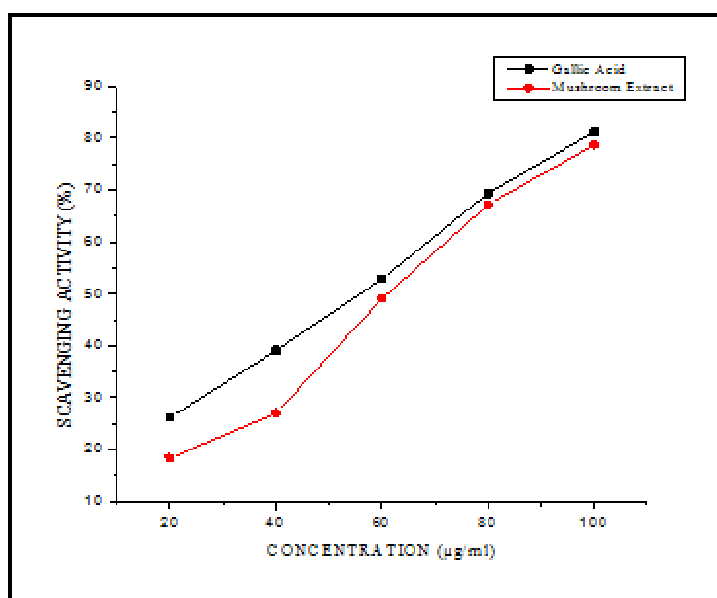


Figure 3
Hydroxyl radical scavenging activity of Calocybe indica extracts

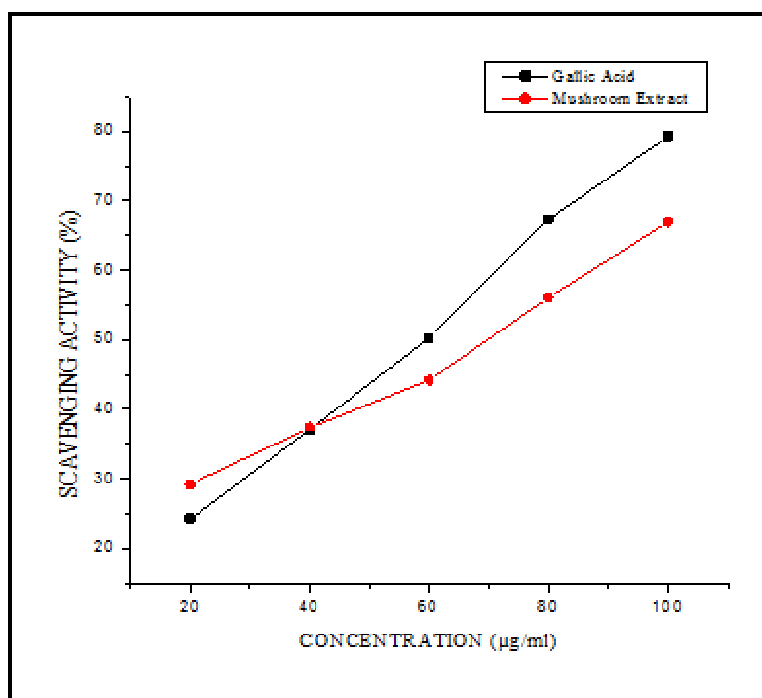


Figure 4
Superoxide radical scavenging activity of Calocybe indica extract

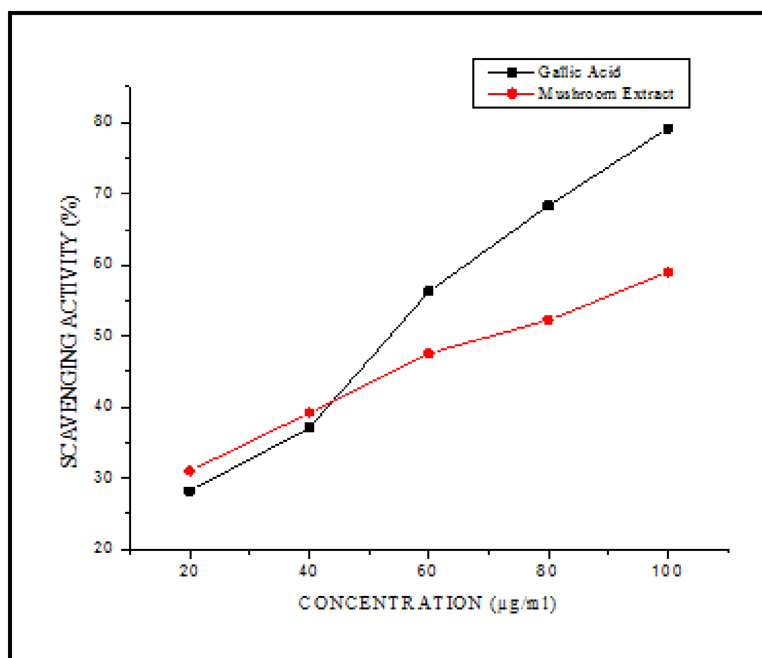


Figure 5
H_2O_2 radical scavenging activity of Calocybe indica extract

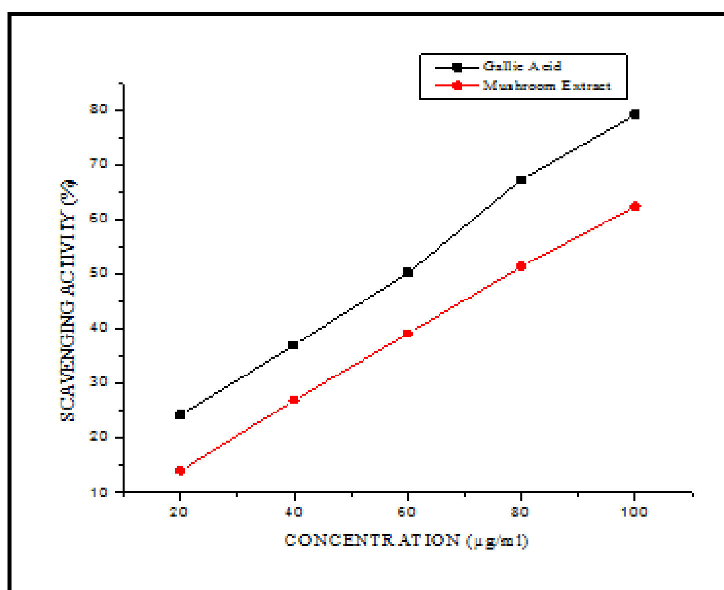


Figure 6
Inhibition of β-carotene of Calocybe indica extract

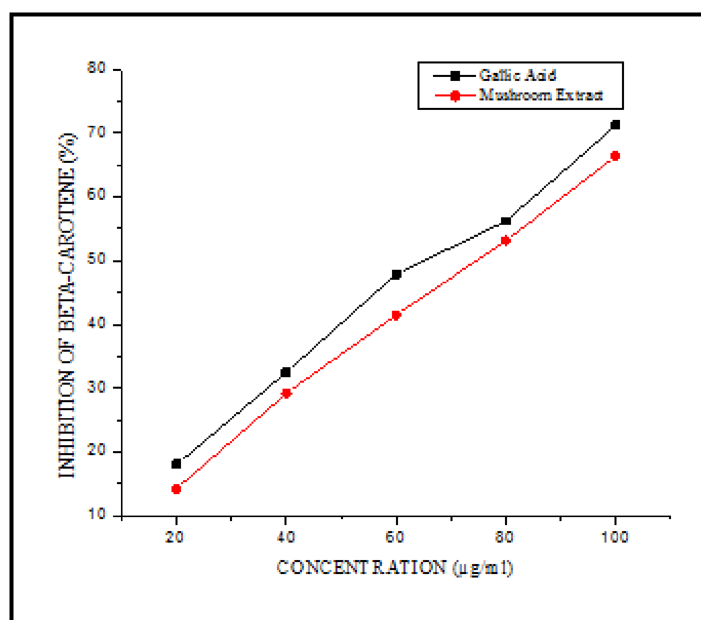
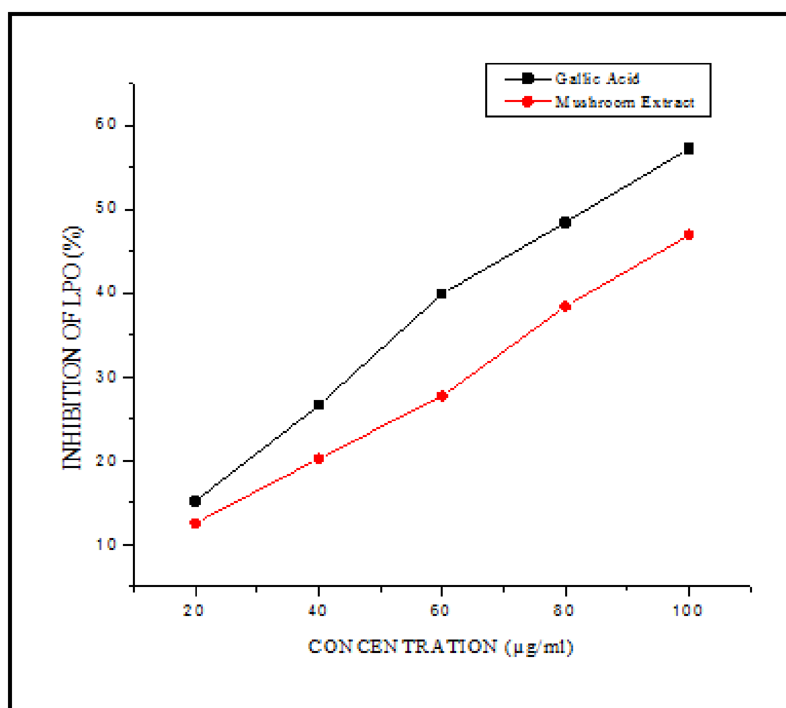


Figure 7
Inhibition of lipid peroxidation of Calocybe indica extract



REFERENCES

1. Deng J., Wang X., Qian F., Vogel S., Xiao L., Ranjan R., Park H., Karpurapu M., Ye RD., Park GY., Christman JW. Protective role of reactive oxygen species in endotoxin-induced lung inflammation through modulation of IL-10 expression. J. Immunol., 188(11):5734-40., (2012).
2. Liu J., Jia L., Kan J., Jin C., In vitro and in vivo antioxidant activity of ethanolic extract of white button mushroom (*Agaricus bisporus*). Food and Chemical Toxicology, 51: 310–316, (2013).
3. Javed Ahmad Wagay and Deepak Vyas., Phenolic quantification and anti-oxidant activity of *Morchella esculenta.* Int. J.phar & Biosci, 2(1): 188-197, (2011).
4. Ferreira ICFR., Barros L., Abreu RMV., Antioxidants in wild mushrooms. Curr. Med. Chem, 16: 1543–1560, (2009).
5. Pan Y., Wang K., Huang S., Wang H., Mu X., He C., Ji X., Zhang J., Huang F., Antioxidant activity of microwave-assisted extract of longan (*Dimocarpus longan* Lour.) peel. Food Chem, 106:1264-1270, (2008).
6. Pokorny J., Natural antioxidant for food use. Trends in Food Science Technology, 9: 223–227, (1991).
7. Kulisic T., Radonic A., Katalinic V., Milos M., Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem, 85: 633–640, (2004).
8. Grice HC., Safety evaluation of butylated hydroxyanisole from the perspective of effects on for stomach and oesophageal squamous epithelium. Food Chem. Toxicol, 26: 717–723, (1988).
9. Palacios I., Lozano M., Moro C., D'Arrigo M., Rostagno MA., Martínez JA., García-Lafuente A., Guillamón E., Villares A.,

Antioxidant properties of phenolic compounds occurring in edible mushrooms. Food Chem, 128:674– 678, (2011).
10. Sarikurkcu C., Tepe B., Semiz DK., Solak MH., Evaluation of metal concentration and antioxidant activity of three edible mushrooms from Mugla, Turkey. Food Chem. Toxicol, 48: 1230–1233, (2010).
11. Murugkar AD., Subbulakshmi G. Nutritional value of edible wild mushrooms collected from the Khasi hills of Meghalaya, Food Chem, 89:599-603, (2005).
12. Pushpa H., Purushothoma KB., Nutritional analysis of wild and cultivated edible medicinal mushrooms. World J. Dairy Food Sci. 5(2): 140-144, (2010).
13. Prabu M., Kumuthakalavalli R. Nutritional and phytochemical studies on *Pleurotus florida* (mont.) Singer and C*alocybe indica* P&C. W.J.Phramaceutical research, 3(3): 4907-4913, (2014).
14. Selvi S., Umadevi P., Suja S., Sridhar K., Chinnaswamy P., Inhibition of *in vitro* lipid peroxidation (LPO) evoked by *Calocybe indica* (milky mushroom). Ancient Science of Life Vol : XXVI (1&2): 41-45, (2006).
15. Mirunalini S., Dhamodharan G., Deepalakshmi K. Antioxidat potential and current cultivation aspects of an edible milky mushroom – *Calocybe indica*. Int.J.PharmPharm Sci, 4 (1): 137-143, (2010).
16. Krishnakumari S., Rajeshwari P., Kathiravan S., Ameliorative effect of C*alocybe indica,* a tropical Indian edible mushroom on hyperglycemia induced oxidative stress. International Scholarly and Scientific Research & Innovation, 7(7): 279 – 282, (2013).
17. Harborne JB, Phytochemical Methods: A guide to modern techniques of plant analysis (1st Edn). Published by Chapman and Hall, an imprint of Thomson Science, 2-6 Boundary Row, London, UK. (1973).
18. Oyaizu M.,Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. Jpn. J. Nutr, 44:307–315, (1986).
19. Shimada K., Fujikawa K., Yahara K., Nakamura T., Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. J. Agric. Food Chem, 40: 945–948, (1992).
20. Zhong XK., Jin X., Lai FY., Lin QS., Jiang JG., Chemical analysis and antioxidant activities in vitro of polysaccharide extracted from *Opuntia ficus* indica Mill cultivated in China. Carbohydr. Polym, 82: 722–727 (2010).
21. Jing TY., Zhao XY., The improved pyrogallol method by using terminating agent for superoxide dismutase measurement. Prog. Biochem. Biophys, 22: 84–86, (1995).
22. Ruch RJ., Cheng SJ., Klauning JE., Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. J.Carcinogenesis, 10: 1003–1008, (1989).
23. Cheung LM., Cheung PCK., Ooi VEC., Antioxidant activity and total phenolics of edible mushroom extracts. Food Chem, 81: 249–255, (2003).
24. Abdullah SM., Ismail N., Aminudin AS., Shuib B., Lau B., Evaluation of selected culinary-medicinal mushrooms for antioxidant and CE inhibitory activities. *Evidence-Based Complementary and Alternative Medicine*, Article ID 464238, 12 pages, (2012).
25. Mujic Z., Zekovic Z., Lepojevic S., Vidovic., Antioxidant properties of selected edible mushroom species. J Central European Agriculture, 11: 387-392, (2010).
26. Valko M., Leibfritz D., Moncol J., Cronin MTD., Mazur M., Telser J., Free radicals and antioxidants in normal physiological

functions and human disease. Int. J. Biochem. Cell Biol, 39: 44–84, (2007).

27. Tsai SY., Huang SJ., Lo SH., Wu TP., Lian PY., Mau JL., Flavour components and antioxidant properties of several cultivated mushrooms. Food Chem, 113: 578–584, (2009).
28. Liu YT., Sun J., Luo ZY., Rao SQ., Su YJ., Xu RR., Yang YJ., Chemical composition of five wild edible mushrooms collected from Southwest China and their antihyperglycemic and antioxidant activity. Food Chem. Toxicol, 50: 1238 1244, (2012).
29. Alam N., Jahan Bristi N., Rafiquzzaman R., Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal, 21:143–152, (2013).
30. Kanagasababapathy G., Malek SNA., Kuppusamy UR., Vikineswary S., Chemical composition and antioxidant properties of extracts of fresh fruiting bodies of *Pleurotus sajor-caju* (Fr.) Singer. J. Agric. Food Chem, 59: 2618–2626, (2011).
31. Vamanu E., Nita S., 2013. Antioxidant Capacity and the Correlation with Major Phenolic Compounds, Anthocyanin, and Tocopherol content in various extracts from the wild edible *Boletus edulis* mushroom. BioMed Research International. Article ID 313905, 11 pages (2013).
32. Selvi S., Umadevi P., Devipriya D., Chinnaswamy P., *In vitro* Antioxidant and Antilipidperoxidative potential of *Calocybe indica.* Journal of Natural Remedies, 10(1): 27 – 31, (2010)
33. Frankel EN., Finley JW., How to standardize the multiplicity of methods to evaluate natural antioxidants. J Agric Food Chem, 56:4901–4908, (2008).
34. Koleva II., van Beek, TA., Linssen JPH., de Groot A., & Evstatieva LN., Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochemical Analysis, 13: 8–17, (2002).