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BIOACTIVE POTENTIAL AND ANTIOXIDANT STATUS OF LABORATORY GROWN *Calocybe indica* (MILKY MUSHROOM)

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Abstract

Calocybe indica (Milky mushroom) is one of the easily available and culturable mushrooms at relatively low cost. It comprises economic source of protein enriched with antioxidants and immune enhancing factors required for human health. Medicinal property of C. indica is due to the presence of polysaccharides, proteins, aminoacids, terpenes, terpenoids and phenols. In this study, methanolic extract of C.indica was used for invitro antioxidant, Protein denaturation inhibition, Nitric oxide scavenging and Membrane stabilization assays. Total Antioxidant activity (TAA) and phenolic content of Laboratory grown C. indica by phosphomolybdenum method was equivalent to that of ascorbic acid. Antiinflammatory effect was estimated by protein denaturation inhibition assay exhibits inhibition of 66.80 % at 1000 µg/ml. C.indica also found to possess nitric oxide scavenging activity capable of 69.76 % inhibition at 1000 µg/ml. Membrane stabilization assay revealed that methanolic extract of C.indica provides 55.83 % membrane stabilization.

Key words: *C. indica, Antioxidant, Antiinflammatory, Nitric oxide, Membrane stabilization*

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INTRODUCTION:

Mushrooms are the interesting group of fungi generally consumed as food, since ancient times in India. It forms rich source of protein, unsaturated fatty acids, vitamins and minerals. Apart from essential nutrients, it also comprises of several important bioactive compounds includes polysaccharides, phenolic compounds, terpenes and terpenoids, phenols, peptides, and proteins. Polysaccharides obtained from mushroom were found to possess antioxidant, antidiabetic, antimicrobial, anti-inflammatory, anticancer, and immunomodulatory activity [1,2]. Mushroom β-glucan enhances the phagocytic property of macrophages and activates cytokines release, which in turn proliferates several immune cells like B-lymphocyte and T- lymphocyte [2,3]. Terpenes isolated from mushroom possess anti-inflammatory response. It comprises of volatile mono and sesquiterpenes oils (C10 and C15), (2) less volatile diterpenes (C20), (3) involatile triterpenoids and sterols (C30), and (4) the carotenoid pigments (C40). Terpenes from the mushroom *Ganoderma lucidium* shown potent antioxidative effects [4]. Terpenes with potent anti-inflammatory properties have been also isolated from Inonotus obliquus [5,6]. Mushroom encompassed with phenols like phenolic acids, flavonoids, hydroxybenzoic acids, hydroxycinnamic acids, lignans, tannins, stilbenes, and oxidized polyphenols [7,8]. The phenolic compounds exhibit antioxidant activity in biological systems, acting as free radical inhibitors, peroxide decomposers, metal inactivators, or oxygen scavengers.

C.indica also known as milky mushroom, white in color, with white gills and thick fibrous stalks. Several reports demonstrated the antioxidant property of *C.indica*. Phytochemical and antioxidant potential of *C.indica* var APK2 cultivated in Tamil Nadu was reported [9]. Antioxidant potential of cultivation aspects of C. indica was investigated [10]. I*n vitro* and *in vivo* antiinflammatory activity of the methanolic extract of *Calocybe indica* was evaluated [11]. In this study, laboratory grown mushrooms were evaluated for total antioxidant activity using phosphomolybdenum method, anti-inflammatory effect by protein denaturation inhibition, nitric oxide scavenging activity and RBC membrane stabilization.

MATERIALS AND METHODS:

Mushroom extract preparation

C. indica mushrooms previously grown in laboratory were dried, powdered and cold extracted by immersing it in methanol for 7 days. After extraction, mushroom debris was removed by filtering with whatmann no1 filter paper. The filtrate was collected, and stored at 4º C until further use.

Phosphomolybdenum assay

TAA was estimated by phosphomolybdenum assay [12]. Methanolic extract of *C.indica* in different concentration ranging from 200µg/ml to 1000 µg/ml were added to each test tube individually containing 3 ml of distilled water and 1 ml of Molybdate reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). These tubes were kept incubated at 95°C for 90min. After incubation, these tubes were normalized to room temperature for 20-30 min and the absorbance of the reaction mixture was measured at 695nm. Experiments were done in triplicates. Ascorbic acid was used as the positive reference standard.

Estimation of Total Phenol

The concentration of phenolics in plant extracts was determined using spectrophotometric method [13]. Methanolic solution of the *C.indica* extract in the concentration of 200-1000 µg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% NaHCO3. Blank was concomitantly prepared ,containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO3 . The samples were thereafter incubated in dark for 45 min. The absorbance was measured at 650 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained.

Membrane Stabilization assay (Oyedepo and Femurewa, 1995) [14]

Preparation of Red Blood Cells (RBCs) Suspension

Fresh whole human blood (10 ml) was collected and transferred to the heparinized centrifuged tubes. The tubes were centrifuged at 3000rpm for 10 min and were washed three times with equal volume of normal saline. The volume of the blood was measured and reconstituted as 10% v/v suspension with normal saline.

Heat Induced Hemolysis

The 2ml reaction mixture is consisted of 1ml of *C.indica* test extract at various concentrations and 1ml of 10% RBCs suspension, instead of drug only saline was added to the control test tube. Diclofenac sodium was taken as a standard drug. All the centrifuged tubes containing reaction mixture were incubated in a water bath at 56ºC for 30 min. At the end of the incubation, the tubes were cooled under

running tap water. The reaction mixture was centrifuged at 2500rpm for 5 min and the absorbance of the supernatants was taken at 560nm. The experiment was performed in triplicates. % membrane stabilization activity was calculated by the formula

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Protein denaturation inhibition assay (Tanford, 1968)[16]

The reaction mixture (0.5ml) consisted of 0.45 ml bovine serum albumin (5% aqueous solution) and 0.1 ml of *C.indica* extract at different concentration. The samples were incubated at 37ºC for 30 min. After cooling the samples, 2.5 ml phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660nm for control test 0.05 ml distilled water was used instead of extracts. The percentage inhibition of protein denaturation was

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Nitric oxide scavenging activity

Nitric oxide scavenging activity can be estimated by the use of Griess IlIosvoy reaction [15](Garrat, 1964).The compound sodium nitroprusside decomposes in aqueous solution at physiological pH (7.2) producing NO. Under aerobic conditions, NO - reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations (200-1000 µg/ml) 0.1 ml of methanol extract of *C.indica* were dissolved in methanol and incubated at 30ºC for 2 hours. The same reaction mixture without the extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3P0_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 540nm. Inhibition of nitrite formation by the plant extracts and the standard antioxidant ascorbic acid were calculated relative to the control.

$$\text{Percent inhibition} = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{Control}}} \times 100$$

Statistical Analysis

All experiments were performed in triplcates and the results were expressed as mean ± SD.

RESULT:

Total antioxidant activity of *C. indica* was estimated using Phophomolybdenum method which is based on reduction of Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V), green molybdenum complex. TAA of *C. indica* was slightly higher than the positive reference standard ascorbic acid at varying concentrations (Table 1). These results confirm the presence of TAA in laboratory grown mushroom. Phenolic content of C.indica was also estimated (Table 2).

Table1: Total Antioxidant activity of *C. indica*

Concentration µg/ml	Absorbance at 695nm	
	C. indica methanolic extract	Ascorbic acid
200	2.488 ± 0.08	1.236 ± 0.05
400	2.940 ± 0.06	2.740 ± 0.08
600	3.327 ± 0.05	2.920 ± 0.07
800	3.510 ± 0.04	3.160 ± 0.10
1000	3.620 ± 0.07	3.550 ± 0.09

Table 2: Total phenolic content of *C. indica*

Concentration µg/ml	Absorbance at 695nm	
	C. indica methanolic extract	Ascorbic acid
200	1.683 ± 0.04	2.724 ± 0.03
400	2.138 ± 0.03	2.750 ± 0.02
600	2.424 ± 0.06	2.804 ± 0.02
800	2. 620 ± 0.08	2.892 ± 0.01
1000	2.739 ± 0.02	2.923 ± 0.03

Membrane stabilizing action and protein denaturation inhibition of drugs is a measure of anti-inflammatory effect. *C.indica* has shown moderate membrane stabilization of 53.83% and protein denaturation inhibition of 66.80 % at 1000 µg/ml respectively (Table 3 and 4).

Table 3: Membrane stabilization assay of *C. indica*

Concentration µg/ml	Abs $_{Control}$ @ 560nm	Abs $_{Sample}$ @ 560nm	%Membrane stabilization
200	2.142 ± 0.12	1.982 ± 0.16	7.47 ± 0.72
400		1.821 ± 0.20	14.99 ± 0.80
600		1.530 ± 0.17	28.57 ± 1.12
800		1.268 ± 0.09	40.80 ± 0.91
1000		0.989 ± 0.13	53.83 ± 0.78

Table 4: Protein denaturation inhibition assay of *C. indica*

Concentration µg/ml	Abs $_{Control}$ @ 660nm	Abs $_{Sample}$ @ 660nm	% Inhibition
200	0.988 ± 0.02	0.760 ± 0.01	23.08 ± 0.24
400		0.728 ± 0.04	26.32 ± 0.22
600		0.640 ± 0.03	35.22 ± 0.65
800		0.514 ± 0.09	47.98 ± 0.87
1000		0.328 ± 0.02	66.80 ± 1.10

NO assay based on quenching of NO by drug molecules, thereby preventing the formation of nitrate and nitrite, which could be detected using griess reagent. The chromophore formed during the diazotization of nitrite with sulphanilamide represents the presence of nitrite and nitrate. In other words , higher the chromophore lower the NO percent inhibition. NO assay of *C.indica* has shown 69.79 % inhibition at maximum concentration of 1000 µg/ml (Table 5).

Table 5: NO inhibition assay of *C. indica*

Concentration µg/ml	Abs $_{Control}$ @ 660nm	Abs $_{Sample}$ @ 660nm	% Inhibition
200	1.273 ± 0.06	1.005 ± 0.03	21.05 ± 0.24
400		0.842 ± 0.07	33.86 ± 0.22
600		0.536 ± 0.01	57.89 ± 0.65
800		0.490 ± 0.03	61.51 ± 0.87
1000		0.385 ± 0.08	69.76 ± 1.10

DISCUSSION:

Antioxidants are the important category of biomolecules capable of neutralizing free radicals and reactive oxygen species. Milky mushroom, one of the successfully rearable mushroom species known to harbor several important bioactive compounds like terpenes, terpenoids, proteins, amino acids and polysaccharides. In this study, antioxidant potential, membrane stabilization, protein denaturation inhibition and NO scavenging activity of laboratory grown *C. indica* was determined. *C.indica* was found to possess antioxidant activity equivalent to that of ascorbic acid. It also shown protein denaturation inhibition and membrane stabilization activity. Infact, many inflammatory diseases arises due to denaturation of proteins and also due to disruption of cell membrane. Hence, *C.indica* could be used as possible therapeutic agent for protein denaturation diseases. NO is the important free radical helps in the vasodilation process, while in the picomolar quantity. However, their increases to nanomolar or micromolar possess threat to the living cell, as it may damage DNA and protein and decrease the viability of the cell. Interestingly, *C.indica* compassed appreciable level of NO scavenging activity that helps to combat NO related anomalies.

CONCLUSION:

Bioactive potential of *C.indica* was determined using several invitro assays. Results indicated that this mushroom species could be used as therapeutic agent to eradicate several ROS, inflammatory and protein denaturation related deadly diseases. Future research related to isolation and purification of single molecule with clear therapeutic mechanism and action at genetic level should be determined.

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