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Research Article

POTENTIAL ANTIOXIDANT EFFICACY OF THE SECONDARY METABOLITES ISOLATED FROM *CALOCYBE INDICA* (VAR.APK2): AN EDIBLE MACROFUNGI

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ABSTRACT

Objective: Mushrooms have been valued as traditional sources of natural bioactive compounds for many centuries and targeted as promising therapeutic agents. The bioactive mycomolecules of mushrooms are reported to have antioxidant, antitumor, antidiabetic, anti-inflammatory, and antimicrobial activity, which are the important medicinal targets in terms of drug discovery today. Hence, an attempt was made in the present study, to evaluate the immunomodulatory and oxidative process of secondary metabolites from the milky mushroom *Calocybe indica* (P&C) var.APK2 using radical scavenging assays.

Methods: The fruiting bodies of milky mushrooms were found to produce an array of mycomolecules such as phenols, flavonoids, alkaloids, tannins, terpenoids, steroids, and saponins in their methanolic extract which was confirmed using Fourier-transform infrared spectrophotometer (FT-IR) analysis and standard phytochemical studies; hence, chromatography fractions of these mushroom seem greatly promising biological activities including antioxidants.

Results: The functional analysis of the secondary metabolites of these macrofungi was evaluated by the separation of potential fractions using preparative thin-layer chromatography (TLC) that revealed seven distinct bands with Rf values of 0.14, 0.26, 0.31, 0.42, 0.52, 0.70, and 0.82; the antioxidant activity was determined through TLC *in situ* bio autography. The quenching property of metabolite compound which was ranging from 19% to 77.9% and the half effective concentration values of 2,2-diphenyl-1-picrylhydrazyl and hydroxyl radical scavenging activity was recorded as 64.26 µg/ml and 54.5 µg/ml sample concentration, respectively. The active mycomolecules of *C. indica* from the TLC was, further, confirmed using Gas Chromatography–Mass Spectrometry studies.

Conclusion: The present investigation of the study revealed that the antioxidant efficacy of edible milky mushroom may be further proceeded for *in vivo* studies for novel drug discovery.

Keywords: Thin-layer chromatography, 2,2-diphenyl-1-picrylhydrazyl, Fourier-transform infrared spectrophotometer analysis, Reactive oxygen species, *Calocybe indica,* Secondary metabolites.

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INTRODUCTION

Mushrooms have been regarded as gourmet cuisine across the world since ancient era for their unique taste and subtle flavor. However, their legendary effects in promotion of good health and vitality are being supported by contemporary studies only. Mushroom species which are considered as miniature pharmaceutical factories have a number of biologically active compounds with therapeutic effects Lee *et al.* [1]. Besides, high molecular weight compounds such as polysaccharides and their low molecular weight metabolites, namely, the secondary metabolites are reported to have various pharmacological functions including immunomodulation and radical scavenging activity Liu *et al.* [2]. Around 1800 species of mushroom have known medicinal properties. Up to 85% of all the therapeutic mushroom products are derived from the fruiting bodies which have been either commercially farmed or collected from the wild Mizuno [3]. Several pharmacologically active compounds have been identified and isolated from Phylum Basidiomycota with a wide spectrum of biological activities and health promoting immunomodulatory, antioxidant, antiviral, cholesterol lowering, anticancer, and anti-inflammatory activities Ferreira *et al.* [4].

The mycomolecules from the edible mushrooms also have been suggested to contribute to the enhancement of immunity and tumor-retarding effects. The antioxidants including vitamin E (α-tocopherols), vitamin A (carotenoids), polyphenol, triterpenes, ascorbic acid (Vitamin C), and glutathione from macrofungi also play a key role in inhibiting the oxidation reaction of free radicals by exchanging one of their own electrons with the free radical molecules to stabilize them and prevent the diseases Halliwell [5]. Hence, the antioxidant compounds can be incorporated into food regime as functional additives representing an alternative source of food to prevent damage promoted by Reactive Oxygen Species (ROS) Augusto and Muntz Vaz, Sánchez [6,7].

Calocybe indica (P&C) var.APK2 (Summer white mushroom or milky mushroom) is one of the most economically important edible mushrooms that grow predominantly in hot humid climates in Tamil Nadu, India, Krishnamoorthy [8]. It is considered as a valuable health food and as a nutraceutical. Moreover, the bioactive compounds from the mushrooms have been emerged as a wonderful source of antioxidant activity Wang *et al*. and Selvi *et al*. [9,10]. The milky mushroom contains low moisture content, rich in carbohydrate, protein, fiber, and Vitamin (B) and it is a good source of minerals (Ca, K, Mg, Na, and P) and trace elements (Cu, Fe, Mn and Zn). The cultivation technology of milky mushroom is very simple involving less cost. So far, there are no scientifically proven reports on the milky mushroom for functional analysis of secondary metabolites against. Hence, the present study is aimed to look for the mycomolecules of *C. indica* to act against the reactive oxygen species by neutralizing the free radicals in *in vitro* antioxidant studies.

METHODS

Collection of mushrooms

Freshly cultivated fruiting bodies of milky mushroom *C. indica* (P&C) var.APK2 were obtained from RPJ Mushroom farm, Usilanakottai, Ramanathapuram, Tamil Nadu, India and authenticated by Dr. A. S. Krishnamoorthy, Professor and Head, Department of Plant pathology, Tamil Nadu Agricultural University, Coimbatore. A voucher specimen has been deposited at the Mushroom Unit, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.

The sample was washed thoroughly, dried by blotting, chopped in to small pieces, shade dried and pulverized into fine powder in sterile condition, stored in a desiccator, and protected from sun light until further analysis.

Extraction
Various solvents such as hexane, chloroform, ethyl acetate, and methanol were used for extraction. 100 g dried mushroom powder was transferred to a Soxhlet apparatus and extracted with the above solvents individually (60–86°C) for 4–6 h. The extracts were concentrated under rotary vaccum evaporator at 25°C and stored under 4°C. The solvent which showed maximum extractive value was chosen to carry out further investigations.

$$Extractive\ value\ (\%) = \frac{Weight\ of\ the\ dried\ extract}{Weight\ of\ the\ sample} \times 100$$

Evaluation of secondary metabolites from *C. indica*
The qualitative phytochemical analysis of secondary metabolites, namely, alkaloids, phenols, flavonoids, tannins, terpenoids, saponins, glycosides, and polysaccharides of the mushroom extract was evaluated using standard method Harborne [11].

Fourier-transform infrared spectrophotometer analysis
Fourier-transform infrared spectrophotometer (FT-IR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined.

Ten milligram of the dried crude extract powder was encapsulated in 100 mg of KBr pellet, to prepare translucent sample disks. The powdered sample of each fraction specimen was loaded in FT-IR spectroscope with a scan range from 400 to 4000/cm with a resolution of 4/cm Kishore Kumar *et al*. [12].

Optimization of solvent system using thin-layer chromatography
In this method, pre-coated silica gel plates (60 F_{254} Merck) were employed. The dried plates were spotted with mushroom extracts with the help of capillary tube at a point of about 1 cm from the bottom of the plates. Spots were dried in front of air blower. After the saturation of chamber with mobile phase, the spotted thin-layer chromatography (TLC) plates were kept in the chamber and allowed for the solvents to run on the plate. When they reached out ¾ of the TLC plates, they were removed from the chamber and examined visually under UV transilluminator at 254 and 366 nm. Spotted TLC plates were developed under various solvent systems to get best resolution Mensor [13].

The solvent systems optimized for TLC were as follows:
- Solvent System 1: Hexane (5): Chloroform (2): Methanol (1)
- Solvent system 2: Chloroform (8): Ethanol (2)
- Solvent system 3: Hexane (5): Ethyl acetate (5).

Bioautography of antioxidant activity on thin-layer chromatography
It is a rapid method for screening of antioxidant compounds using 2,2-diphenyl-1-picrylhydrazyl and hydroxyl (DPPH) spray detection method. For this examination, 0.2% of DPPH solution in methanol was sprayed over the surface of developed TLC plates and incubated for 10 min at room temperature. The active antioxidant constituents were detected as pale yellow spots produced against purple background by bleaching of DPPH on the TLC plates. After visual comparison of the intensity of bleached color of the TLC band, the antioxidant strengths of the mushroom constituents were tentatively categorized as strong and weak activities. All detected antioxidant constituents were noted according to their Rf values Nair [14].

The bands that tested positive for antioxidant compounds in the preparative TLC were scratched off, mixed with 5 ml of absolute ethanol, allow to stand for 10 min, and then filtered with Whatmann No.1 filter paper and collected in glass vials for antioxidant assays Mittal [15].

Following this technique, the positive fractions were analyzed by gas chromatography–mass spectrometry (GC-MS) techniques to identify the active secondary metabolites.

***In vitro* antioxidant studies using potential mushroom fraction**
Preparation of sample for antioxidant assays
The recovered antioxidant compounds from the TLC plates were diluted with sample concentration of 20, 40, 60, 80, and 100 µg/ml sample concentration.

DPPH radical scavenging assay
Briefly, the potential mushroom metabolite fraction at various concentrations (20–100 µg/ml) was mixed with 0.1 mM solution of DPPH in methanol. After incubation at room temperature for 30 min in dark condition, the absorbance of the mixture was read at 517 nm using UV-Visible spectrophotometer. The control was prepared as above without sample; ascorbic acid was used as an antioxidant standard. The percentage of free radical scavenging activity of the sample was calculated using the following formula Brand-Williams [16].

DPPH scavenging effect (%) = $[(A_0 - A_1)/A_0] \times 100$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the standard sample. The EC_{50} (half Effective Concentration) value represented the concentration of the compound that causes 50% inhibition of the DPPH radical formation.

Hydroxyl radical scavenging assay
The mixture containing 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 solution was mixed with different concentration of metabolite fraction (20–100 µg/ml) shaken vigorously and incubated at 37°C for 30 min. Then, the absorbance of the reaction mixture was determined at 510 nm. Ascorbic acid was used as the positive control. The hydroxyl radical scavenging activity was calculated by the following formula Zhong [17].

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 solution). The EC_{50} value represented the concentration of the compounds that caused 50% inhibition of the hydroxyl radical formation.

Identification of functional compounds against reactive oxygen species
Gas chromatography–mass spectrometry analysis
The positive fractions from preparative TLC of the fruiting bodies of the *C. indica* was investigated using GC-MS analysis, using Clarus 500 Perkin-Elmer (Auto system XL) gas chromatography equipped and coupled to a mass detector Turbo mass gold-Perkin Elmer 5.2 spectrometer with Elite-5 MS (5% diphenyl/95% dimethyl poly siloxane), 30 m × 0.25 µm DF of capillary column. The instrument was set to an initial temperature of 110°C and maintained at this temperature for 2 min. At the end of this period, the oven temperature was rose up to 280°C at the rate of an increase of 5°C/min and maintained for 9 min. Injection port temperature was ensured as 200°C and Helium flow rate as 1 ml/min. The ionization voltage used was 70 eV. The sample (potential antioxidant fraction) was injected in split mode as 10:1 Mass spectral scan range was set at 45–450 m/Z Kalimuthu [18].

Interpretation of the GC-MS was conducted using the data base of National Institute of Standard and Technology (NIST) 2011, having more than 62,000 patterns. The spectrum of the chosen sample was compared with the spectrum of the known compounds stored in the NIST library. The name, structure of the compounds, and molecular weight of the tested material were ascertained.

Statistical analysis
All the *in vitro* experiments were conducted using triplicates values and parameters were given as mean ± standard deviation (SD) values. All

the experimental results were given as mean ± SD along with standard error using the statistical package of Microsoft Excel Version 2010 and graphs were plated using origin ver. 6 software.

RESULTS AND DISCUSSION

Nature has proven and continues to be proving itself as a source for the discovery of bioactive compounds which are important for the development of new pharmaceuticals. Exploration of novel bioactive compounds from the natural sources has been an emerging field of medicine all over the world. Chang *et al*. [19] reported the nutraceutical, nutriceutical, and pharmaceutical nature of edible mushrooms that would enhance the immune response of the body, thereby increasing resistance to diseases and also can cause regression.

Mushroom, as functional food, is a compendium of current research on its chemistry and biology. Their biological activities and chemical background of novel secondary metabolites are being investigated in recent years. As a result, mushrooms are considered as medicinals with anti-cancerous, anti-inflammatory, immunomodulatory, antibacterial, antifungal, antiviral, hepatoprotective, and hypoglycemic activities Lin; Chang and Olusegun [20-22]. In recent decades, interesting compounds from fruit bodies of either wild or commercially grown Basidiomycota were found to exhibit antitumor, antibacterial, antifungal, antiviral, and other pharmacological activities Francia; Rapior; Bao and De silva [23-26]. Milky mushroom *C. indica* (P&C) var. APK2 is a tropical edible mushroom and researchers claim this milky mushroom as a nutritive food, dietary supplement, and an immune enhancer for its biological activities Krishnamoorthy[8].

Extraction of fruit bodies

Extraction of dried fruiting bodies of *C. indica* with the organic solvents, namely, hexane, chloroform, ethyl acetate, and methanol recorded pale yellow to dark brown color, with gummy texture (Table 1).

Fruiting bodies of milky mushroom *C. indica* were subjected for their mycomolecules based on the relative solubility in suitable solvents. Among the different solvents used, methanol was found to show high extractive value of 9.3% ± 0.23% (Table 1). The mycomolecules of milky mushroom recorded in this study might be useful in therapeutic application.

Evaluation of secondary metabolites from milky mushroom

Qualitative phytochemical analysis

In the phytochemical screening, the fruiting bodies of *C. indica* revealed the presence of multiple phytochemical constituents (Table 2) and these fruit bodies were found to be the potential sources of phenols, flavonoids, alkaloids, tannins, terpenoids, steroids, and saponins.

Studies revealed that these milky mushroom have significant pharmacological functions due to their bioactive molecules, namely, phenols, triterpenoids, alkaloids, saponins, polysaccharides, and proteins Prabu and Kumuthakalavalli; Sumathy *et al*. and Parveen Nisha and Kumuthakalavalli [27-29]. Similarly, the mycomolecules of milky mushroom recorded in this study also might possess wide range of pharmacological applications.

Deepak Rahi and Malik [30] reported that the fruiting bodies of basidiomycetes possess useful therapeutic metabolites including alkaloids, flavonoids, saponins, tannins anthroquinones, and steroids. These therapeutic metabolites make these mushrooms as a popular and functional food resource. Hence, their presences in the mushroom fruit bodies make them valuable in drug development. Phenolic compounds exhibits its antioxidant activity by free radical inhibitors, peroxide decomposers, metal inactivators, or oxygen scavengers in *in vivo* system Yagi; Dziezak; Xu and Beelman [31-33]. Alkaloids, observed in the chosen milky mushrooms, are another type of mycomolecules with complex and heterogeneous group of compounds. Trease and Evans [34] stated the remarkable pharmacological properties of mushroom alkaloids against malignant diseases, infections, and malaria.

Table 1: Extractive value of mushroom fruit bodies

Extracting solvents	Yield of extractive materials (%)
Hexane	1.5±0.08
Chloroform	1.7±0.15
Ethyl acetate	2.8±0.04
Methanol	9.3±0.23

Fourier-transform infrared spectrophotometer analysis of crude extract of *C. indica*

The FT-IR spectroscopy has proven to be a valuable tool for characterization and identification of compounds and functional groups present in the unknown mixture of samples. The FT-IR spectroscopy analysis of the crude mushroom extract shown that the unique chemical bonds (Table 3), namely, phenols, amines, alcohols, carboxylic acids, aldehydes, esters, and others functional groups which are responsible for the active secondary metabolites from the range between 550 and 3640 cm^{-1} (Fig. 1).

Scientist suggested that the wavelength of 1200–800 is responsible for polysaccharides, C-H stretching, and 3200 stretching of primary and secondary amides with N-H stretch of triterphenes, 1150 C-O stretch for proteins Sangeetha [35].

This FT-IR spectrum of *C. indica* reveals that the active secondary metabolites for antioxidant compounds such as phenols and triterpenes and diatary supplements such as proteins and polysaccharides were recorded. Similarly, the *G. lucidum* and its active metabolites were recorded using FT-IR analysis.

Similarly, Israilides *et al*. [36] reported that the phenolic compounds such as flavonoids and tannins were determined in the fruiting bodies of *Lentinulaedodes* using FT-IR analysis.

Identification of functional compounds against reactive oxygen species

Optimization of the solvent system using thin-layer chromatography

In the optimization study among the three solvent systems (Solvent system 1, 2, and 3) tested, the chloroform: ethanol solvent system with 8:2 ratio was found to be potential enough to separate the phytocompounds from the crude mushroom extract in TLC and chosen for further studies (Table 4).

Bioautography of antioxidant activity on thin-layer chromatography

TLC screening is a separation technique for the rapid identification of bioactive components in crude mushroom extracts. In the present study, chromatography separation of the milky mushroom was the first step in the separation of individual component from the mixture of compounds, the crude methanolic extract of *C. indica* was separated using TLC technique (Chloroform – ethanol 8:2 ratio) and seven compounds could be identified with different Rf value such as 0.14, 0.26, 0.31, 0.42, 0.52, 0.70, and 0.82. The antioxidant compound from the separated fractions was identified with the Rf value of 0.14, 0.26, and 0.31 using *in situ* DPPH spray detection method. In this method, the development of pale yellow color against purple by the conversion of which indicates the scavenging potential of the antioxidants in the fraction. This might to due to the active metabolites of *C. indica* can prevent or inhibit the oxidation mechanism by several actions including hydrogen ion or single electron transfer mechanism. This was, further, evaluated by administering *in vitro* antioxidant studies.

Xu *et al*. [37] stated that the isolated and purified compounds from therapeutic mushrooms are a crucial task for prevention of dreadful diseases including cancer. Shirmila Jose and Radhamany [38] also adopted similar method with bioluminescent mushroom *Omphalotusnidiformis* where, in the mushroom, extract was loaded on TLC plates and the antioxidant potential was confirmed by spraying with DPPH on TLC plates and the active antioxidant constituents were detected as yellow spots by the reduction of DPPH with the Rf value of 0.39, 0.49, 0.67, and 0.77. Agatonovic-Kustrin *et al*. [39] stated that TLC hyphenated with an appropriate bioassay could identify the antioxidant

Table 2: Qualitative phytochemical analysis of milky mushroom

Compound tested	Name of the test	Hexane	Ethyl acetate	Chloroform	Methanol
Phenols	Lead acetate test	++	+	+++	+++
Flavonoids	Ferric chloride test	++	+	++	+++
Alkaloids	Dragendroff's test	+	+	+	++
Tannins	Ferric chloride test	++	+	+++	+++
Terpenoids	Liebermann-Burchard test	-	-	++	+++
Steroid	Liebermann Burchard test	-	-	+	++
Quinones	Ammonia test	-	-	-	-
Glycosides	Keller-Killani test	-	-	+	-
Saponins	Froth test	-	+	+	++
Polysaccharide	Anthrone test	-	-	-	-

+++: Instant end point, ++: Slightly delayed end point, +: Delayed end point, -: No end point

Table 3: Fourier-transform infrared spectrophotometer absorption for crude extract

Wave length (/cm)	Functional groups	Bond
803–550	Alkyl halides	C-Cl stretch
1160–980	Carboxylic acid	C-O stretch
1250–1020	Aliphatic amines	C-N stretch
1320–1000	Alcohols, carboxylic acids	C-O stretch
1370–1350	Esters, ethers	C-H rock
1650–1550	Primary amines	N-H bend
1740–1720	Aldehydes and saturates	C=O stretch
2850–3000	Alkanes	C–H stretch
3400–3250	Primary and secondary amines and amides	N–H stretch
3640–3610	Alcohols and phenols	O–H stretch, free hydroxyl

Table 4: Optimization of the solvent system for chromatography studies

Solvent systems and Rf values		
Chloroform: ethanol (8:2)	**Hexane: Chloroform: Ethanol (5:2:1)**	**Hexane: ethyl acetate (5:5)**
0.14	0.14	0.14
0.26	0.26	-
0.31	-	-
0.42	0.42	-
0.52	-	0.52
0.70	-	-
0.82	0.82	-

compounds with Rf values ranging from 0.1 to 0.3 in fermented foods.

***In vitro* antioxidant studies using potential mushroom fraction**

Antioxidants are defined as the potential molecules that are capable of removing free radicals and can prevent or repair the process of oxidation formed in the human body during energy production in the mitochondrial electron transport chain, phagocytosis, arachidonic acid metabolism, ovulation, fertilization, and in xenobiotic metabolism Blois [40].

2,2-diphenyl-1-picrylhydrazyl and hydroxyl radical scavenging assay

Halliwell and Gutteridge [41] stated that DPPH is a stable radical in solution and appears purple color absorbing at 515 nm in methanol and the assay is based on the principle that DPPH on accepting a hydrogen (H) atom from the scavenger molecule (antioxidant) resulting into reduction of DPPH to DPPH2, to produce yellow color from purple color and the color change is monitored by spectrophotometric method. In the present study, mushroom metabolite fraction and the standard, namely, ascorbic acid at various concentrations ranging from 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml were subjected for DPPH radical scavenging assay. As a result, DPPH radical scavenging activity of mushroom fraction and standard ascorbic acid was increased in increased concentrations of the extracts

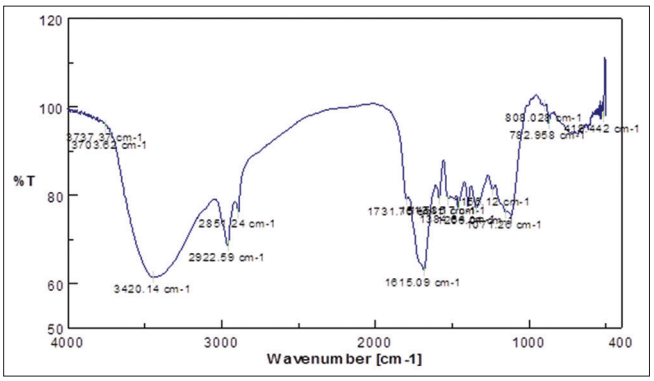

Fig. 1: FT-IR frequency and functional group of crude extract of *Calocybe indica* crude

(20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml) exhibited their scavenging activity as 21.42% ± 0.51%, 29.26% ± 0.43%, 42.43% ± 0.27%, 64.26% ± 0.31%, and 77.9% ± 0.03%, respectively. Similar trend of increase was noted in the standard also. The results revealed that antioxidant positive fraction has maximum radical scavenging activity of 77.9% ± 0.03% at 100 µg/ml concentration and the half maximum effective concentration (EC_{50}) was found to be 64.26 µg/ml sample concentration (Fig. 2).

Liu *et al*. [42] reported that the macrofungi *Ramaria flava* (Broom fungus) was extracted using ethanol and sequentially partitioned with ethyl acetate and petroleum and the potential fraction recorded the EC_{50} for DPPH and hydroxyl radical scavenging activity as 5.86 g/ml and 18.08 g/ml, respectively.

Hydroxyl radical scavenging assay

Hydroxyl radical the most reactive radical can attack and damage almost every biomacromolecules in living cells and, hence, used to evaluate the antioxidant potential of selected mushroom. In the present study, 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml, and 100 µg/ml of the mushroom metabolite fraction of *C. indica* were tested for their antioxidant activity by scavenging hydroxyl radical. The scavenging activity of both the mushroom fraction and ascorbic acid (Control) was increased with the increase in their concentration. The inhibition of hydroxyl radical by mushroom fraction showed steady increase from 19.16% ± 0.45%, 26.0% ± 90.62%, 40.46% ± 0.58%, 54.5% ± 0.52%, and 67.46% ± 0.6% in 20 µg/ml to 100 µg/ml concentration, respectively. Maximum hydroxyl radical scavenging activity of 67.46% ± 0.6% was observed in antioxidant positive fraction of mushroom at 100 µg/ml concentration and the EC_{50} value was observed as 54.5µg/ml sample concentration (Fig. 3).

Hydroxyl radicals (•OH) in the human body such as hydrogen peroxide (H_2O_2), superoxide anion (O_2•−), hydroxyl radical, and ROS have the ability to oxidize easily with a cellular components, especially nick DNA to damage essential enzymes and structural proteins to provoke uncontrolled chain reactions such as lipid peroxidation or auto oxidation reactions Bijalani [43]. Among ROS, the OH exhibits the strongest

oxidative activity. Zahid *et al*. [44] reported that milky mushrooms possess vitamin C, an inhibitor of lipid peroxidation with free radical scavenging activity and vitamin E an antioxidant that protects membrane. The GSH (reduced glutathione) of milky mushroom play an essential role in the protection against free radical Selvi *et al*. [10]. Maiti *et al*. [45] isolated a protein fraction, namely, Cibacron Blue

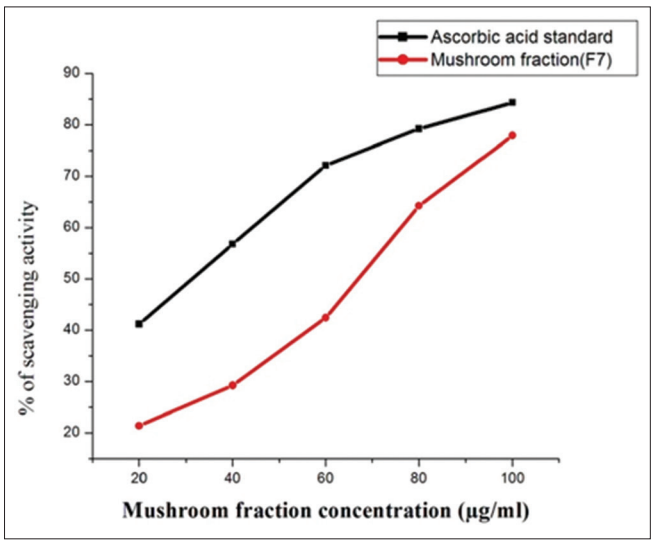

Fig. 2: 2,2-diphenyl-1-picrylhydrazyl and hydroxyl radical scavenging assay of potential fraction

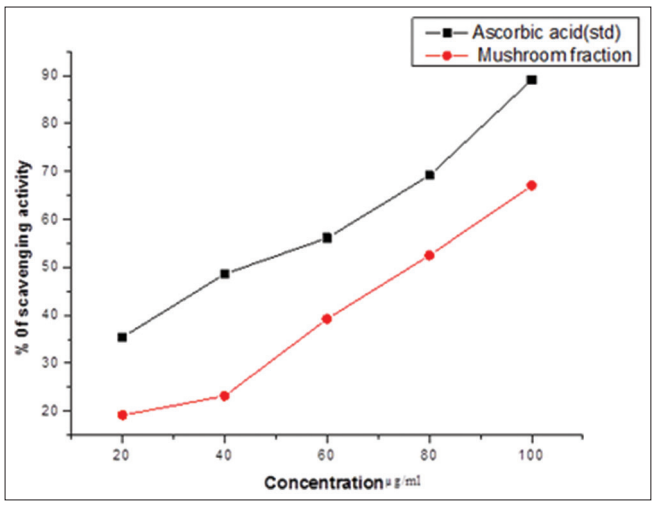

Fig. 3: Hydroxyl radical scavenging assay potential fraction

Affinity Eluted Protein from *C. indica* that exhibited antiproliferative and immunomodulatory activities. In this present study, the evaluation of mycomolecules and its active metabolite against reactive oxygen species was determined using *in vitro* radical scavenging assays.

These results clearly indicated that the active metabolites from the milky mushroom can scavenge the hydroxyl radicals by virtual sharing the ionic bonds.

Identification of bioactive mycomolecules from the potential mushroom fraction

Gas chromatography–mass spectrometry analysis

The mushroom metabolite fraction of *C. indica* was further investigated for the evaluation and identification of its bioactive compounds using GC-MS. Using computer searcher (NIST) version 2011MS data library and comparing the spectrum obtained through GC-MS, two major compounds were recorded (Fig. 4) and they were identified as steroid and triterpene compounds such as 12-Oleanen-3-yl acetate (β Amyrin acetate Fig. 5) and 9,19-Cyclolanost-24-en-3-ol (Fig. 6) and their chemical structure have been derived (Table 5).

Today, the modern chromatographic techniques are more effective to interpret the volatile compositional characteristics. GC–MS is useful techniques to detect the compounds from the mixture of sample. GC–MS proved as suitable tools for the interpretation of the volatile compositional characteristics of the mushrooms Zhang *et al*. [46].

In this study, GC-MS analysis of the pale yellow colored fraction of milky mushroom clearly indicated the presence of triterpene derivatives such as 12-oleanen-3-yl acetate (33.55%) and 9, 19-cyclolanost-24-en-3-ol (cycloartenol) (25.3%). The results of GC-MS analysis proved that terpenes are the broad group of widespread secondary metabolites present in milky mushroom. Triterpenes are considered as potentially useful in cancer pharmacotherapy. Triterpenes isolated from Ganoderma, namely, ganoderic acid, lucidimols, and ganoderiol F have been reported as antioxidants which were confirmed by TLC and GCMS analysis Sliva [47].

9,19-cyclolanost-24-en-3-ol (Cycloartenol) and 12-Oleanen-3yl acetate (β Amyrin acetate) are the important classes of triterpenoid class, found in plants. The biosynthesis of cycloartenol starts from squalene that possesses strong antioxidant propertyand12-Oleanen-3yl acetate commonly known as beta-amyrin acetate which acts as scavenger of DPPH radical and has cytotoxic potential Oluwatoyin [48].

The compound cycloartenol was isolated from the fruit *Ficus racemosa* and its scavenging activity was determined using column chromatography Jain *et al*. [49]. The results of GC-MS analysis stated that the seeds of *Pimpinella anisum* L. and endemic species of *Kopsia singapurensis* Ridl, which possesses strong antioxidant compound, namely, β Amyrin acetate Farzaneh *et al*. and Shan *et al*. [50,51]. The polar solvent extraction from Bridelia ferruginea leaves shown that, the

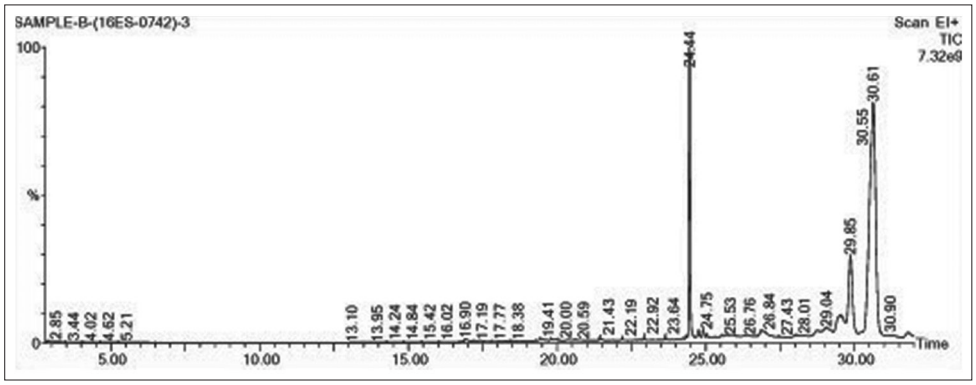

Fig. 4: GCMS study on *C. indica* fraction

Table 5: Bioactive constituents of potential fraction of *Cannabis indica* by gas chromatography–mass spectrometry analysis

Name of the constituents peak	RT	Area (%)	Molecular formula	Molecular weight (g/mol)	Class
12-Oleanen-3-yl acetate	30.55	33.55	$C_{32}H_{52}O_2$	468.766	Steroid
9,19-Cyclolanost-24-en-3-ol	24.44	25.03	$C_{30}H_{50}O$	426.717	Triterpene

RT: Retention time

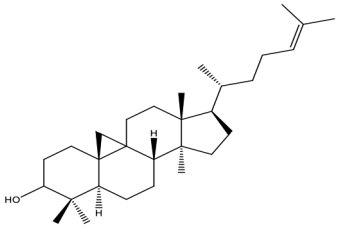

Fig. 5: 9,19-CYCLOLANOST-24-EN-3-OL

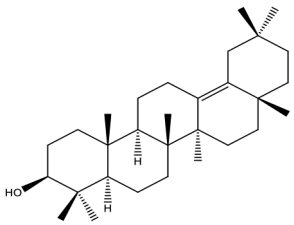

Fig. 6: 12-OlEANEN-3-YL ACETATE

strong antioxidant compound namely, beta-amyrin acetate which acts as a scavenger of DPPH radical Fabiyi O [52].

Edible mushrooms like *Agrocybe aegerita* were found to have sterols with new cyclooxygenase (COX) inhibitory and antioxidant activity Zhang *et al.* [53]. Wu *et al.* [54] reported two novel secoergosterols, namely, tylopiol A and tylopiol B that were isolated from the fresh fruit bodies of *Tylopilus plumbeoviolaceus* McMorris [55]. Several researchers suggested that the steroids of mushrooms along with the polyketides and triterpenes have the activity on physiopathology of several oxidative stress-related diseases like cancer. Hence, they have been used as antimicrobial, antioxidant, and antitumor agents Aleksandra and Karaman [56].

Terpenoids are one among the bioactive molecules of macro fungus. In this present study, triterpenes were recorded in milky mushrooms. These metabolites include volatile mono and sesquiterpenes oils, less volatile diterpenes, triterpenes, sterols, and carotenoid pigment that show structural diversity due to their stereo chemical arrangements Paliya [57]. In this present study, triterpenoid derivatives such as 9,19-cyclolanost-24-en-3-ol and 12-oleane-3yl acetate were recorded from milky mushrooms.

Triterpenes isolated from *Ganoderma* species such as *G. lucidum* have been identified as potent anticancer and immunomodulatory agents Nonaka *et al.*; Cheng *et al.* and Watanabe *et al.* [58-60]. Some triterpenes, namely, ganoderic acid C and derivatives from *G. lucidum* are able to inhibit biosynthesis of cholesterol Wu *et al.* [56]; contribute to atherosclerosis protection Komoda *et al.* [61]; and also show antiviral, antibacterial activity Morigiwa and el-Mekkawy [62,63]. Different sterols of fruit bodies of *Inonotus obliquus* were recorded with anti-inflammatory properties Niedermeyer *et al.* and Park *et al.* [64,65]. Anticancer activities of several triterpenes from the sclerotia of *I. obliquus* have also been reported Van *et al.*, Ma *et al.* [66,67]. Hence, the triterpenoids recorded in milky mushrooms may also possess antioxidants and thereby used as anticancer and immunomodulatory agents.

CONCLUSION

In the present study, the milky mushroom *C. indica* var.APK2 represents a vast source of mycomolecules with immunomodulating and anticancer activity and can represent a growing segment of pharmaceutical industry. From the qualitative phytochemical investigation of crude extract, the active antioxidant metabolite compound was separated using preparative TLC and identified with the Rf value of 0.14, 0.26 and 0.31 using *in situ* DPPH spray detection method. Due to the above findings, the mushroom metabolite fraction was subjected for *in vitro* scavenging activity using DPPH and hydroxyl scavenging assays. In DPPH assay, the mushroom fraction recorded maximum radical scavenging activity of 77.9% ± 0.03% at 100 µg/ml concentration and the half maximum effective concentration (EC_{50}) of 64.26µg/ml sample.

Similarly, the above fraction recorded the maximum hydroxyl radical scavenging activity of 67.46% ± 0.6% at 100 µg/ml concentration with the EC_{50} value of 54.5 µg/ml sample concentration. The outcome of the study may be used for the effective utilization of these milky mushrooms *C. indica* as adjuvants to treatment and also for development of new nutraceutical bioactive products.

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