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ANTICANCER ACTIVITY OF MILKY MUSHROOM (*CALOCYBE INDICA* VAR.APK2) AGAINST A549 HUMAN LUNG CANCER CELL LINE STUDIES

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ABSTRACT

Methanolic extract of fruiting bodies of *Calocybe indica* Var.APK2 was evaluated for their anticancer activity against lung carcinoma (A549) cell line. The preliminary phytochemical characterization of the extract using FT-IR recorded the presence of different bioactive compounds, namely alcohols, phenols, aromatics, alkanes, unsaturated esters, carboxylic acid and alkyl halides in their major peaks. The anti-oxidant potential of *Calocybe indica* in terms of DPPH radical scavenging assay was evolved as 60% and hydroxyl radical scavenging activity as 66 % using ascorbic acid as standard. The IC_{50} value of the sample to A549 lung cancer cell line was expressed as 4.9 µg/ml. Apoptosis profile of *C.indica* was indicated as 80.13% and maximum rate of inhibition (54%) was observed in S phase. These results indicate that *C. indica* possesses bioactive metabolites and phytochemicals capable of scavenging free radicals and inhibit the growth of A549 lung cancer cell line and suggest their potential for medicinal purpose.

KEY WORDS: **FT-IR, Free radicals, MTT assay, Flow cytometer**

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INTRODUCTION

Cancer remains to be one of the leading causes of death around the world. Many factors may increase the risk of cancer such as inadequate diet, excessive light intensities and toxins[1]. The major characteristic is the lack of control of the cell proliferation, differentiation, and death including invading organs and tissues. There are many difficulties in the treatment of cancer such as drug resistance, toxicity, and low specificity. Most of the cancer treatments rely heavily on chemotherapy, however chemotherapy has limitations. Chemotherapeutic drugs lack of selectivity and they can also kill normal cells. Hence, there is an urgent need for new drugs that are highly effective and possess low toxicity. In this regard, several studies have reported the cytotoxic activity of organic extracts of spores, vegetative bodies[2]and fruiting bodies[3] from several species of macro fungi against cancer cells. Lung cancer, also known as carcinoma of the lung or pulmonary carcinoma, is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung. If left untreated, this growth can spread beyond the lung by process of metastasis into nearby tissue or other parts of the body. Lung cancer accounts for about 27% of all cancer deaths and is by far the leading cause of cancer death among both men and women. New concepts have appeared in this trend, such as nutraceuticals nutritional therapy, phytonutrients and phytotherapy. Functional foods and phytomedicines play positive role in maintaining well-being, enhancing health and modulating immune function to prevent specific disease. Macro fungi have long been used as valuable food source and as traditional medicines around the world, especially in the Orient[4]. Mushrooms are highly nutritive, low calorie food with good quality protein, vitamin, and minerals[5]. Macro fungi are known to produce large and diverse variety of secondary metabolites[6]. Several pharmacologically active compounds have been identified and isolated from Basidiomycota with a wide spectrum of biological activities and health promoting properties including immuno modulatory, antioxidant, antiviral, cholesterol lowering, anticancer and anti-inflammation activities[7].*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 amino acids. 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[8]. The present study has been aimed to determine *invitro* anticancer activity of *Calocybe indica* Var APK2 against A549 lung cancer cell line.

MATERIALS AND METHODS

(i) Collection of Macro fungus

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

(ii) Preparation of Methanolic extract of C.indica

Powdered fruiting body (100g) of *C. indica* was extracted with 500 ml of methanol by using soxhlet apparatus for 6h at 50-60^{0}C and the extract was concentrated under vaccum by using rotary evaporator and stored under 4^{0}C[9].The crude compound was dissolved in 4% of DMSO (Dimethyl sulfoxide) and stored at -20^o C for further studies.

(iii) Preliminary phytochemical screening

The powdered fruiting bodies were mixed with potassium bromide (pellets) and subjected at spectra frequency region from 4000-400cm^{-1} through FT-IR studies[10].

(iv) Evaluation of antioxidant activity

Antioxidants, namely the free radical scavengers are highly reactive chemicals that have the potential to harm cells. Free radicals can be hazardous to the body and damage all major components of cells, including DNA, proteins and cell membranes[11].The Antioxidant potential of *C.indica* was evaluated by DPPH assay and hydroxyl radical scavenging assay.

(v) DPPH radical scavenging assay

Free radical scavenging activity of methanolic extracted *C. indica* was performed using DPPH assay. 0.1mM solution of DPPH was prepared in 100% methanol, and 1 ml of this solution was added to 4 ml of sample in 40% methanol at various concentrations (10-160 µg/ml). This mixture was shaken vigorously and incubated for 30 min at 37°C in the dark. The reduction of the DPPH radical was measured by continuous monitoring of the decrease of absorption at 517 nm using ELISA reader. The L-ascorbic acid was used as standards[12].

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.

(*vi) Hydroxyl radical scavenging assay*

This assay was carried out according to the method Halliwell[13]. To the reaction mixture containing deoxyribose (3 mM, 0.2 ml), ferric chloride (0.1 mM, 0.2 ml), EDTA (0.1 mM, 0.2 ml), ascorbic acid (0.1 mM, 0.2 ml) and hydrogen peroxide (2 mM, 0.2 ml) in phosphate buffer (pH, 7.4, 20 mM), 0.2 ml of various concentrations of extracts or standards in DMSO were added to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37°C. After incubation, ice- cold tri-chloroacetic acid (0.2 ml, 15 % w/v) and thiobarbituric acid (0.2 ml, 1 % w/v), in 0.25 N Hcl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm.

(vii) Cell culture

Cytotoxic activity of C.indica on lung cancer cell line

The A549 human lung adenocarcinoma epithelial cell lines were purchased from National Centre for Cell Science, Pune, India. These A549 cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat-inactivated Fetal Bovine Serum 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 ºC in a humidified atmosphere of 95% air and 5% CO_2

(viii) Morphological observation

Morphological changes of cells in both of the treated group and control group were analyzed at 8 h; 24 h under the OLYMPUS 1×71 Inverted Fluorescence microscope with 10× magnification[14].

(ix) Cytotoxic activity of C. indica on lung cancer cell line

MTT Assay

The cytotoxic effect of methanolic extraction of *C. indica* against A549 lung cancer cell line was assayed by MTT assay.A549 cells were grown in 96 well micro titer plate ($5x10^3$ cells well) for 24h after seeding. The plates were incubated with methanolic extract with different concentration for 24h and 48 h (check to put h) respectively. The medium was refreshed and 20µl of MTT (5mg/ml) was added. The plates were incubated for three h in dark. The formazan crystals developed were solubilized with 100µl of DMSO and the plates were kept in dark for another 5-10 min. The colour developed was measured in an ELISA reader at 570 nm. The IC50 concentration was determined as the drug concentration that is required to reduce the absorbance to half that of the control[15].

(x) Cell cycle analysis

A549 lung cancer cells were seeded in 6 or 12 well culture plates. After cell adherence, cells were treated with mushroom extract (500µg/ml) for 24 h and, the cells were harvested using trypsin (0.05%)-EDTA (0.54 mM) solution. Later, the cells were washed using media thoroughly and centrifuged at 1200 rpm for 5 min, the pellets were collected and 300 µl of PBS was added. These cells were added with 700 µl ice cold ethanol drop by drop and the cells were fixed with ethanol and stored overnight at 4°C. For flow cytometry analysis, the cells were washed with PBS. Triton X 556 µl containing RNase 20 µl were added with the cells and incubated for an hour. After incubation, propidium iodide 24 µl was added and incubated in dark for 45 min and the sample was analyzed using flow cytometer[16].

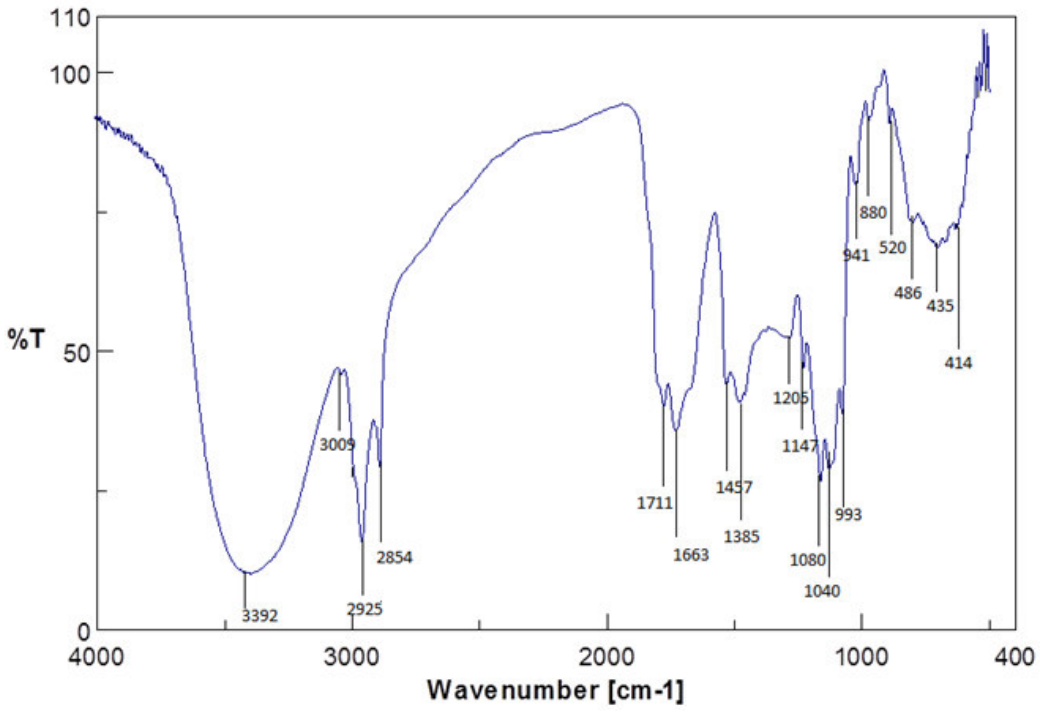
RESULT AND DISCUSSION

Mushrooms are highly nutritive, low calorie food with good quality of protein and vitamins. Macro fungi are known to produce a large and diverse variety of secondary metabolites[17]. These secondary metabolites have health promoting properties such as antioxidant, antimicrobial, anticancer, cholesterol lowering and immunostimulatory effects[18]. Radiation therapy is commonly used in the treatment for cancer, in order to damage the DNA of the tumour cell to inhibit proliferation and induced apoptosis. The main side effect of using radiation therapy is DNA damage to the surrounding healthy tissues. Hence protection of non – cancerous tissue against radiation is vital for reducing the side effect. The present study was undertaken to elucidate the antioxidant effect and *in vitro* cytotoxic potential of edible mushroom *Calocybe indica* varAPK2.Therefore organic extractions, such as methanol, ethyl acetate, chloroform and aqueous extraction were taken. In FT-IR profile of all the solvent extracts, the methanolic extraction eluted the potential phytocompounds such as alcohols, phenols, alkanes, aromatics, unsaturated esters, carbonyl groups, esters, ethers, primary amines and alkyl halides (Table 1). The methanolic extraction of fruiting bodies of *Ganoderma lucidum* produced two fold increased cytotoxic activity in mouse melanoma cell line[19]. Similarly, phytochemical screening of methanolic extraction of the fruiting bodies of *Cantharellus cibarius* showed phenols, flavonoids, saponins and anthroquinones[20]. The phenols and flavonoids have reported to provide the protection against oxidative stress induced diseases and responsible for the antioxidant activity of the mushroom[21]. Alkaloids are suggested to stimulate the anesthetic effect in ophthalmology[22]. Flavonoids and phenols have been reported to provide protection against oxidative stress induced diseases and majorly responsible for the antioxidant activity of mushrooms[23].The synergistic effect of the phytochemicals and nutraceuticals present in mushroom has been implicated their antimicrobial and antioxidant activity[24].

Table 1
FT-IR frequency and functional group of Calocybe indica

S. No	Peak Value	Functional Groups
1.	3500-3200	Alcohol and phenol
2.	3100-3000	Aromatics
3.	3000-2850	Alkanes
4.	3000-2850	Alkanes
5.	1730-1715	αandβ unsaturated esters
6.	1760-1665	Carbonyl(general)
7.	1500-1400	Aromatics
8.	1400- 1300	Aromatics
9.	1320-1000	Alcohols, carboxylic acid esters and ethers
10.	1320-1000	Alcohols, carboxylic acid, esters and ethers
11.	1320-1000	Alcohols, carboxylic acid, esters and ethers
12.	1320-1000	Alcohols, carboxylic acid, esters and ethers
13.	950-910	Carboxylic acid
14.	900-665	Primary and Secondary amines
15.	690-515	Alkyl halides

Figure 1
FT-IR analysis of Calocybe indica



(1) Antioxidant Assays

Oxidative stress is an important parameter that increases cancer risk. Free radicals are known to induce oxidative damage in biomolecules and play an important role in aging, cardiovascular disease and unbalanced immune system[25]. Antioxidant activity is manifested in wide variety of actions such as inhibition of oxidation enzymes, chelating of transition metals and enzyme detoxification of ROS [26]. The scavenging activity of the methanolic extraction of *C.indica* was compared with L-Ascorbic acid as standard. The DPPH radical scavenging activity was increased at sample concentration 50 to 250µg/ml. The dose dependent of DPPH radical scavenging activity of methanolic extract of *C.indica* recorded the higher scavenging activity (Figure 2). The EC_{50} value of methanolic extract of the *C.indica* was expressed as 89µg/ml. The hydroxyl radical is one of the representative reactive oxygen species generated in the body by the common biological reaction of Iron (II) based Fenton reaction. The hydroxyl radicals are considered to be major harmful Reactive Oxygen Species (ROS) that may cause the oxidative damage of the biomolecules[27]. The EC50 value of *C.indica* was expressed as 68µg/ml (Figure 3).The radical scavenging capacity may be attributed to phenolic compounds in methanolic extract with the ability to accept electrons, which can combine with free radical competitively to decrease hydroxyl radical. Methanolic extraction of *Phillusrimosus* showed significant hydroxyl radical scavenging activity and the EC_{50} value ranging from 25.3 µg/ml[28].

Figure 2
DPPH scavenging activity

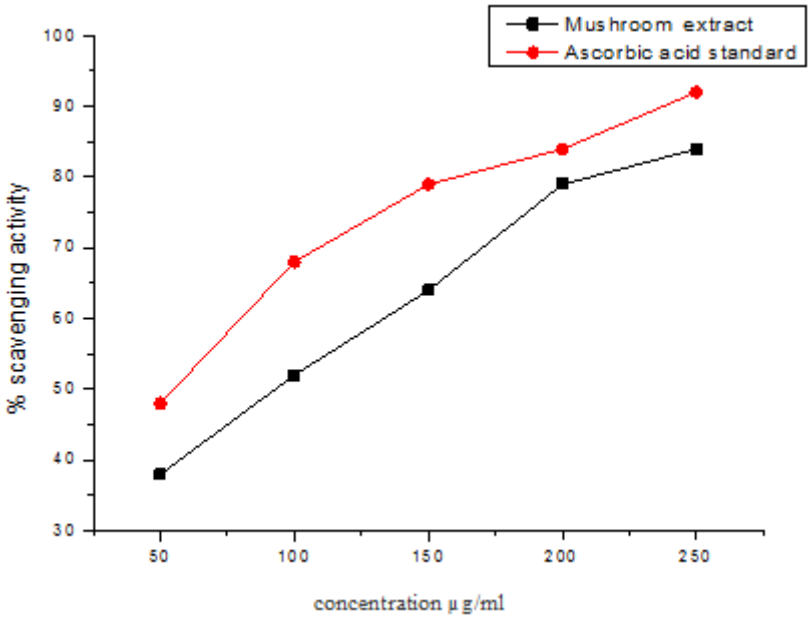
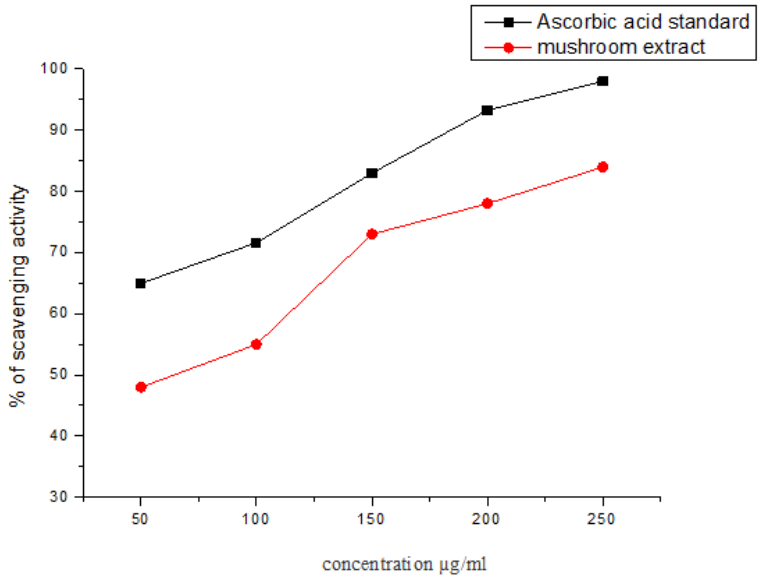


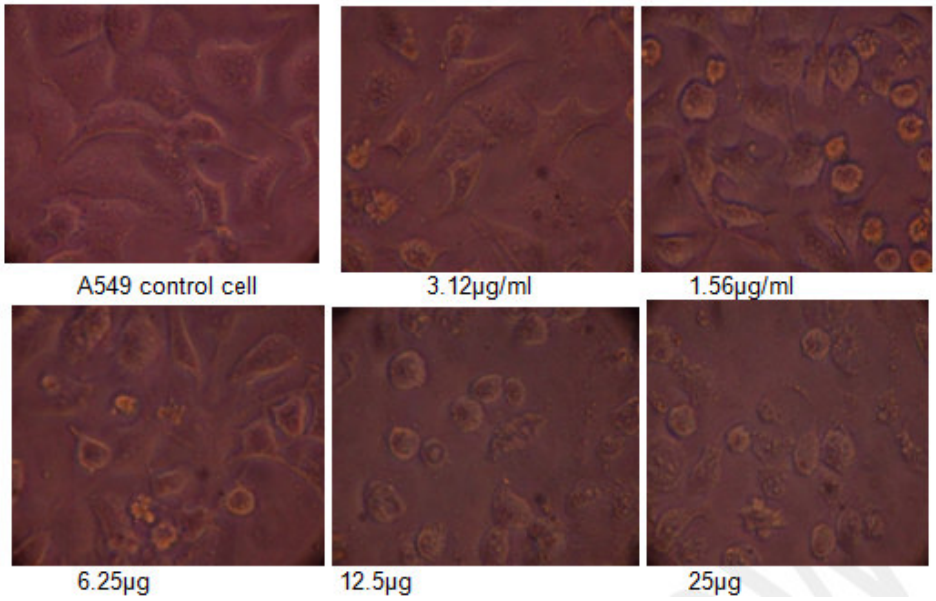
Table: 3
Hydroxyl radical scavenging assay



(2) Cytotoxic activity

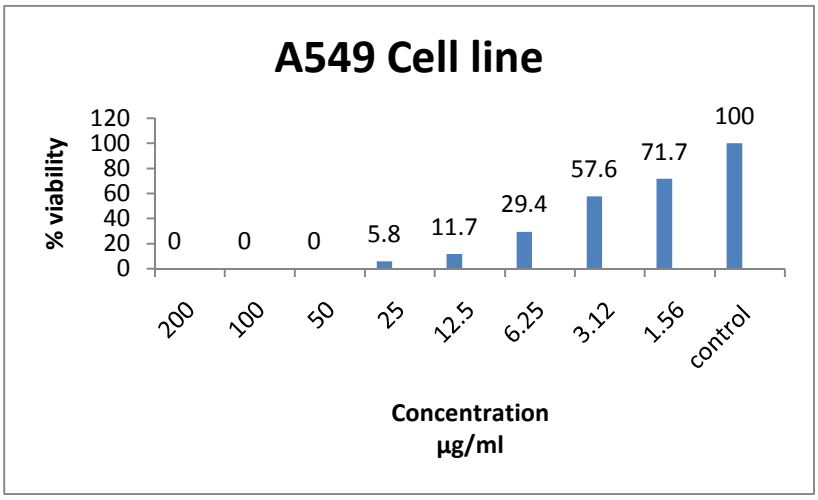
Antiproliferative activity of A549 lung cancer cells against *Calocybe indica* was assessed using MTT assay as it depends mainly on the mitochondrion dehydrogenase which is the important parameter to assess the viability of the cells[29]. The mushroom extract expressed the potent cytotoxic activity against human lung cancer cell line (Table 2).Morphological observation of A549cell was determined by the changes induced by the *C.indica*. Changes such as shrinking of the cells, membrane, ballooning, were observed in predicting the apoptotic mechanism for cell death (Figure 4).

Figure 4
Morphological changes of A549 control and *C.indica* treated cells



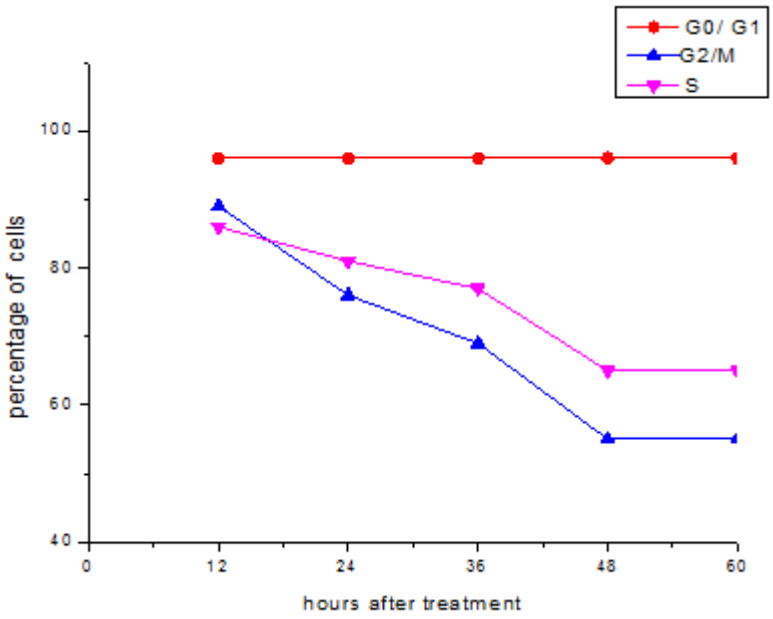
The fruiting bodies of C. indica expressed maximum cytotoxic activity of 71.7% at 1.56µg/ml concentration and IC50 value of 4.9µg/ml. The methanolic extraction of fruiting bodies of Pleurotus ostratus expressed maximum cytotoxic activity of 87% and the IC50 value was 39µg/ml[30]. Moreover the cytotoxic activity of Cordyceps taii (Caterpillar mushroom) against A549 lung and human gastric cancer cells in dose dependent manner from 1.9 to 250µg/ml[31].

Table 2
Cytotoxic activity of C.indica (MTT assay)



Cancer cells have a deregulated cell cycle leading to uncontrolled cell proliferation. In mammalian cells, the cell cycle generally occur in regular fashion from G1 to S and M phase in the presence of Cyclin dependent protein kinase (Cdks) and accelerated by the dependent kinase inhibitors P16, P21 and P27, retenoblasoma, tumor suppressor protein, D type cyclins such as D1,D2 and D3 are involved in regulation of transition from suppressor protein. Anticancer agents can arrest the cell cycle at G0/G1, S or G2/M phase reduce the rate of cell proliferation[32]. To investigate the basis antiproliferative properties of methanol extract of *Calocybe indica*, recorded accumulation of cells in G0-G1 phase. In G2/M phase (56%), the number of cells decreased gradually from 12-48h. Similar trend has been noticed in S phase also (68%) (Figure 5). Antiproliferative properties have been reported in alcoholic extract of *Ganoderma lucidum* which induces cell cycle arrest at G1 phase in MCF7 cells[33].

Figure 5
Cell cycle arrest (Flow cytometer assay of C.indica)



CONCLUSION

The milky mushroom *Calocybe indica* could be used as the promising functional food and phyto-medicine as it recorded to have the cytotoxic effect against A549 lung cancer cell line through *invitro* studies. However further studies should be extended to identify the molecular targets, optimum dosage, efficacy, safety etc for the invention of pharmaceutical drugs.

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CONFLICTS OF INTEREST

Disclosure the authors report no conflicts of interest in this work.

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