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Der Pharmacia Sinica, 2015, 6(2):6-11

Der Pharmacia Sinica
ISSN: 0976-8688
CODEN (USA): PSHIBD

Effect of phytochemicals and antioxidant compounds enriched extract from *Calocybe Indica* var. APK2 on proliferation of human MCF-7 breast carcinoma cells

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ABSTRACT

The present study was conducted to evaluate the antiproliferative activity of aqueous extract of Calocybe indica Var. APK2. Mushroom extract was screened for the presence of phytochemicals and antioxidant compounds. The extract was found to contain significant amount of phenolics (7.22 mg GAE/g), flavanoid (1.54 mg quercetin Equivalent/g), saponin (0.076/g), terpenoid(0.099mg/g) and alkaloid (2.15 mg/g). Mushroom extract analysed for enzymatic antioxidants was found to contain superoxide dismutase (625.27µg/g); catalase (352.8 µg/g); glutathione S transferase (825.26 µg/g); glutathione peroxidase (756.41µg/g) and glucose 6 phosphatase (0.19 µg/g) and. The non enzymatic antioxidant compounds like β-carotene (112.73 µg/100g) and lycopene (22.42 µg/100g) respectively. The above listed compounds enriched extract of the mushroom C.indica was subjected to assess the antiproliferative activity against MCF-7 breast cancer cell line using MTT assay and Tryphan blue dye exclusion method. Results on MTT assay showed that as the concentration increased from 0.125 to 1.0 mg/ml; cell viability decreased from 81.13±0.22% to 29.72±0.02% and 64.31±0.44% to 18.22 ±0.85% at 24h and 48h of incubation respctively. Results on Tryphan blue dye exclusion study revealed that the total cell count of MCF-7 cells decreased with increase in concentration of the mushroom extract indicating the inhibitory activity. All these findings point out for the first time that the mushroom, Calocybe indica is an excellent source of natural chemopreventive agents in the treatment of breast cancer.

Keywords: Breast cancer, β-carotene, lycopene, MTT, Tryphan blue dye exclusion.

INTRODUCTION

Human body is constantly generating free radicals and has the power to neutralize them. An imbalance between the free radicals and the ability of the body to neutarlise this imbalance leads to oxidative stress. Oxidative stress may cause various problems and diseases such as diabetes, Alzheimer's disease, Parkinson's disease, aging and cancer. Oxidative stress can be quenched by using antioxidants. An Antioxidant is a any substance that delays, prevents or removes oxidative damage to a target molecule; can scavenge free radicals; provide protection against the diseases [1].Antioxidants can be classified into a number of different groups as enzymatic and non-enzymatic strategies. The enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase,

while non-enzymatic antioxidants include the tocopherol, β-carotene, flavanoids, lycopene and phenolic compounds [2].

Cancer is a heterogeneous disease of varied etiology. Breast cancer is a group of neoplasm originating from the epithelial cells lining the milk ducts and are most common malignancies which causes cancer deaths in women worldwide, consisting of approximately 18% of all female cancers [3]. In India about four out of five breast cancer cases are treated at very advanced stages. Breast cancer is the 2nd most common cancer among women in India and accounts for 7% of global burden of breast cancer and one-fifth of all cancers among women in India [4]. Chemotherapy, surgery and radiotherapy have remained the mainstay of human cancer treatment that lead to increase survival time for many cancer patients [5,6,7]. Yet, these treatments are harmful to normal cells. The major problem involved in current chemotherapy is the development of drug resistance. Some of the cancer types are intrinsically resistant to various anticancer drugs while others develop multidrug resistance upon treatment. Treatment for cancer is frequently an assault to the immune system that leads to weakening of the body's immune system resulting in immunosuppression that can significantly increase a patient's risk for infection. Thus the development of chemoresistance remains a major obstacle for cancer treatment and as the plateau of cancer death continues, the need for new approaches with less or no side effects to prevent this hazardous disease become imperative.

Exploration of natural sources in search for biologically active compounds that could be applied in anticancer therapy or chemoprevention has been practiced with success for a long time [8]. Fungi are well known for their nutritional and medicinal value due to their content of variety of bioactive substances with pharmacological properties [9]. Mushrooms, the macro fungi used for many years in natural medicine, have especially been screened for phytochemicals with beneficial properties on human health [10]. Bioactive compounds present in the mushrooms have been reported to possess anti-tumor, anti-cancer, anti-viral, anti-coagulant, anti-inflammatory, anti-diabetic and immuno-modulatory activities [11].

The mushroom *Calocybe indica* Var.APK2 is an edible and commonly grown and consumed mushroom in Tamilnadu, India. They are reported to possess many medicinal properties like anti-microbial, anti-diabetic, anti-inflammatory and antioxidant properties [12,13,14]. The purpose of the recent research was to evaluate the effects of a hot water extract of *C.indica* on the proliferation of MCF-7 human breast cancer cells. The study also included quantifying the phytochemicals, enzymatic and non-enzymatic antioxidants from the chosen mushroom.

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 studied samples were authenticated by Dr.A.S.Krishnamoorthy, Professor, Department of Plant Pathology, Tamilnadu Agricultural University, Coimbatore, Tamilnadu. 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 extract of *C.indica*

Aqueous (Hot water extract) extraction was prepared using 5g of *C.indica* powder stirred with 50ml of sterile distilled water at 60°C for 1h. The 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 supernatants were pooled together and concentrated in rotary evaporator at 60°C. The dried extract thus obtained was used for the determination of phytochemicals, antioxidant compounds and *invitro* cytotoxicity.

Determination of Phytochemicals

The sample was analyzed for their phytochemical components using the standard procedures – phenol [15]; flavanoid [16]; alkaloid [17], saponin and terpenoid [18].

Determination of β-carotene and lycopene [18]

For β-carotene and lycopene determination, the dried aqueous extract (100 mg) was vigorously shaken with an acetone hexane mixture (4 : 6, 10 mL) for 1 min and filtered through Whatman no. 1 filter paper. The absorbance of

the filtrate was measured at 453, 505, and 663 nm. β Carotene and lycopene content were calculated according to the following equations:

lycopene (mg/100 mL) = (−0.0458 × *AA*663) + (0.372 × *AA*505) – (0.0806 × *AA*453)
β-carotene (mg/100 mL) = (0.216 × *AA*663) – (0.304 × *AA*505) + (0.452 × *AA*453)
The results were expressed as µg of carotenoid/100 g of sample.

Enzymatic Antioxidants
Catalase activity was assayed following the method described by Sinha [19]; glutathione peroxidase activity was assayed according to the method of Rotruck [20]; Superoxide dismutase activity was determined by using the method described by Das [21] and the activity of glutathione-S-transferase and glutathione - 6 - phosphatase were assayed based on the method given by Habig [22] and Suganya and Suryavathana [23].

Cell Culture
MCF-7 cells (isolated in 1970 from a breast adenocarcinoma of a 69 year old Caucasian female) exhibit the features of the mammary gland epithelium; so they make a good model of breast cancer *in vitro*. The MCF-7 cell line used in this study, was purchased from National Centre for Cell Sciences, Pune, India and cultured according to the provider's recommendations, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1mM Sodium pyruvate (SP), 2mM L-glutamine, 2-fold Minimal Essential medium (MEM), vitamins and 10% heat-inactivated FBS (56°C for 30 min). The monolayer cultures were maintained in 75-cm^2 tissue culture flasks at 37°C in a humidified 5% CO_2 incubator.

Anti-proliferation Assay
Cell proliferation was determined by MTT assay [24]. Approximately 12,000 cells per well were seeded on a 96-well plate and incubated at 37 °C overnight in a humidified environment of 5% CO2 and 95% air. Fresh medium was then replaced and the cells were exposed to 20 to 100 µg/ml of aqueous extract of *C.indica* for 48 hours. Subsequently, 20 µl of sterilized MTT (5 mg/ml) in phosphate buffered saline (PBS) buffer (pH 7.4) was spiked into each well and incubated at 37 °C for 4 hours. The supernatant was then carefully removed, and 200 µl of dimethyl sulfoxide (DMSO) was added into each well to dissolve the MTT formazan (blue colour) at the bottom of the wells. After 15 min, the absorbance at 540 nm with 690 nm as background absorbance was measured with an ELISA microplate reader. The complete growth medium was the blank, and cells incubated only in culture medium without mushroom extracts were denoted as positive control.

Tryphan Blue Dye Exclusion – for total cell concentration
Total cell concentration of MCF-7 cells after treatment with different concentrations of aqueous extract of *C.indica* was assessed by Tryphan blue dye exclusion method described by Sheeja [25]. Briefly after treatment with extracts for 24h, the cells were stained with 0.4% tryphan blue and 100 cells were counted at various fields in the hemocytometer for each concentration. The viable cells were transparent and non-viable cells were dark blue in color.

Statistical Analysis
All experiments were conducted in triplicates and the parameters were given as mean ± standard error (SE) values. Both mean and standard deviation performed were appropriate, using the statistical package within Microsoft® Excel Version 2007.Ink, and the graphs were plotted using software Origin 8.0.

RESULTS AND DISCUSSION

Edible mushrooms are commonly thought to have ample range of secondary metabolites with unlimited medicinal value [26,27]. In this context, phytochmeicals present in the aqueous extract of *Calocybe indica* were analysed quantitatively and the results of the analaysis revealed the presence of phenolics, flavanoids, alkaloids, saponins and terpenoids (Table:1). Amount of phenolics (7.26 ± 0.43 mg GAE/g) was found to be high followed by flavanoid (1.54±0.62 mgQE/g); alkaloid (2.15±0.73 mg/g); terpenoid (0.099±0.84 mg/g) and saponin (0.076±0.61 mg/g). Results obtained were in agreement with the earlier reports of Mirunalini et al. [13] who stated that the *C.indica* mushroom contain wide array of phytochemicals. Results were in conformity with the earlier reports [28,29].

The level of enzymatic antioxidants such as SOD,CAT, GPx, GST and G6P values and non-enzymatic antioxidants such as β-carotene and lycopene were shown in Table:2 and Table 3 respectively.SOD and CAT in the aqueous extract of *C.indica* was found to be 625.27±0.41 U mg^{-1} and 352.89±0.12 U mg^{-1} respectively. The activity of GPx, GST and G6P was found to be 825.26±0.33 U mg^{-1}; 756.41±0.24 U mg^{-1} and 0.19±0.17 U mg^{-1} respectively. Activity of the tested enzymes in the mushroom extract was in the order GPx>GST>SOD>CAT>G6P.Mushroom were found to contain non enzymatic antioxidant compounds like β-carotene (112.73±0.15µg/100g) and lycopene (22.42±0.09µg/100g) respectively. More or less similar reports on antioxidant compounds of various mushrooms like *Calocybe indica* and *Agaricus bisporus* have been reported [23]; *Boletus edulis*, *Ganoderma tsuage* and *Micoporous xanthopus* [30].Differences in concentration between the studies may be due to particle size, type of solvents used, polarity of solvent, time and temperature of extraction and solvent to solid ratio [31,32,33]. Findings were also in conformity with the earlier reports [34,35,36] which stated that the enzymes can be a good choice of natural antioxidants

From our earlier studies and the present investigation on the aqueous extract of *Calocybe indica*, divulges the presence of broad array of antioxidant compounds which may well quench the free radicals, that have an important role in pathogenesis of a wide range of diseases including cancer. Thus our further study has been carried out with the objective to study the anti-proliferative activity of the aqueous extract of *Calocybe indica* on MCF-7 (breast adeno carcinoma) human cancer cell line. The aqueous extract enriched with antioxidant compounds showed significant anti-proliferative activity using a colorimetric MTT-based assy (Figure:1) and the cell count was analysed by tryphan blue dye exclusion method (Table:4). When MCF-7 cells were treated with the mushroom extract, there was a concentration dependent cytotoxic effect. As the concentration increased from 0.125mg/ml to 1.0mg/ml, percentage of inhibition increased from 18-81%. At the concentration of 1.0mg/ml there was a significant decrease in cell viability, ie. 29% and 18% for cells incubated at 24h and 48h respectively.

Similarly the MCF-7 cells were treated with mushroom extracts at various concentrations 0.125mg/ml to 1.0mg/ml for 24h and 48h and the live and dead cells were counted using hemocytometer. The total cell count of extract treated cells was decreasing with the increase in concentration of mushroom extract, indicating an inhibitory effect. There was a decrease in the cell count from $40x10^4$ cells/ml (control) to $11x10^4$ cells/ml (1.0mg/ml) after treatment with extract for 24h, whereas the cells treated with extract (1.0mg/ml) for 48h showed very low cell count of $3x10^4$ cells/ml. thus by the above two *in vitro* analysis it can be concluded that the aqueous extract of *Calocybe indica*, the milky mushroom has momentous anti-proliferative activity towards the human cancer cells. Even though the role of *Calocybe indica* in many diseased conditions is documented, its role as an anti-cancer agent towards breast cancer has not been scientifically studied and reported as far as the literature reviewed by our research team. However there are many reports on edible and medicinal mushrooms that cure breast cancer like *Ganoderma lucidum* [37,38] ; *Agaricus bisporus* [39]; *Lentinus polychrous* [40]; *Tricholoma giganteum massee* [41]. It is also reported from the earlier works of Aesun Shin [42], that diet with *Pleurotus ostreatus*, *Lentinus edodes*, *Agaricus bisporus* and *Flammulina velutipes* mushroom intake was associated with lower risk of breast cancers among premenopausal women. Research shows that, 30 to 35% of all cancers can be prevented by eating well, being active and maintaining a healthy body weight (Canadian Cancer Society). As fresh mushrooms are low in calories and fat as well as being versatile and great tasting, they are a good addition to a healthy eating pattern.

TABLE:1 PHYTOHEMICAL COMPOSITION OF *CALOCYBE INDICA VAR APK2*

Phytochemicals	Concentration mg/g
Phenolics(GAE)	7.26 ± 0.43
Flavanoid (QE)	1.54±0.62
Saponin	0.076±0.61
Terpenoid	0.099±0.84
Alkaloid	2.15±0.73

TABLE:2 ENZYMATIC ANTIOXIDANTS OF *CALOCYBE INDICA VAR APK2*

Enzymatic antioxidant compounds	Concentration U mg^{-1}
Superoxide Dismutase (SOD)	625.27±0.41
Catalase (CAT)	352.89±0.12
Glutathione Peroxidase (GPx)	825.26±0.33
Glutathione-S-Transferase(GST)	756.41±0.24
Glucose – 6 – Phosphatase (G6P)	0.19±0.17

Unit of enzyme activity is expressed as mg-1 glucose released mg-1 enzyme protein.

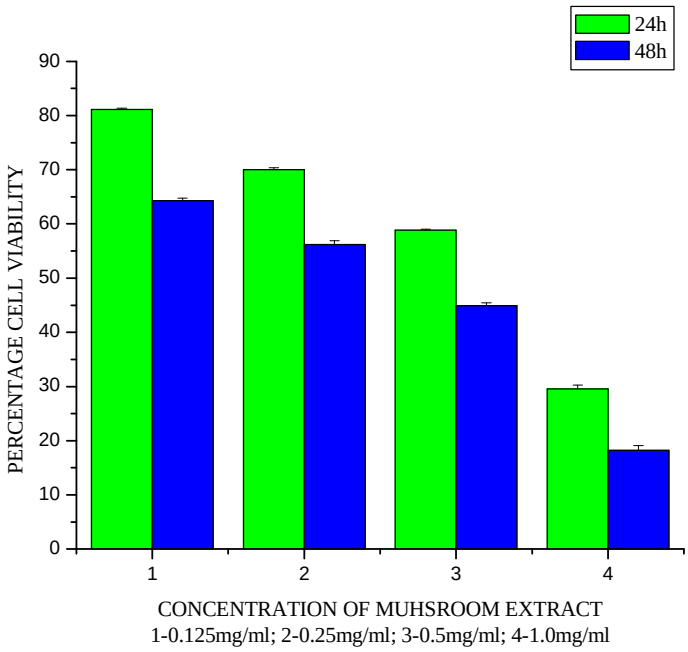
TABLE:3 NON-ENZYMATIC ANTIOXIDANTS OF *CALOCYBE INDICA VAR APK2*

Non - Enzymatic antioxidant compounds	Concentration µg/100g
Beta carotene	112.73±0.15
Lycopene	22.42±0.09

TABLE:4 EFFECT OF MUSHROOM EXTRACT ON TOTAL CELL CONCENTRATION OF MCF-7 CELLS

Concentration of Mushroom Extract (mg/ml)	Control	24h	48h
0.125	40 x 10^4 cells	36x10^4 cells	28 x 10^4 cells
0.25	40 x 10^4 cells	28 x 10^4 cells	17 x 10^4 cells
0.50	40 x 10^4 cells	19 x 10^4 cells	11 x 10^4 cells
1.0	40 x 10^4 cells	11 x 10^4 cells	4 x 10^4 cells

FIGURE:1 ANTI-PROLIFERATIVE ACTIVITY OF *CALOCYBE INDICA VAR APK2* ON HUMAN BREAST CANCER CELLS



CONCLUSION

In summary, this is the first report that our data support the hypothesis; the phytochemical and antioxidant compounds of the aqueous extract of *C.indica* are capable of inhibiting proliferation of MCF-7, human breast cancer cells. Future work can be designed to focus on animal studies; results proved on animal, can be taken for phase I/II/III clinical trials for convention of *Calocybe indica* as a potent nutraceutical that could reduce the proliferation of cancerous cells and in addition could fight reducing the risk of getting breast cancer.

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