

Study of anticancer effect of *Calocybe indica* mushroom on breast cancer cell line and human Ewings sarcoma cancer cell lines

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Abstract: *Calocybe indica* is one kind of basidiomycetous edible mushroom. Its inhibitory and cytotoxic effects have been tested on human Ewing's sarcoma MHH - ES - 1 and breast cancer MCF-7 cell lines, using vincristine and tamoxifen as reference positive controls. Inhibition of MHH - ES - 1 and MCF-7 cells by *C. indica* was determined individually by the MTT colorimetric assay method and was employed to evaluate cell viability in this cytotoxic assay. Micromolar concentrations of both WE and ME of *C.indica* (10 mg to 100mg) inhibited the growth of both cell lines in a dose-dependent manner. The IC50 values of WE and ME in MHH - ES - 1 cell line were 55.25 ± 1.201 mg/ml and 46.56 ± 0.134 mg/ml respectively while these values of MCF7 cell line were 52.12 ± 0.15 mg/ml and 47.94 ± 0.09 mg/ml respectively. It indicates that both WE and ME of this mushroom fruit body are effective for inhibition of both cancer cell lines. This action suggests that the extract of *C indica* might be a new promising agent in the treatment of both human sarcoma and breast cancer.

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1. Introduction

Mushroom is 'a macrofungus with a distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand' (Chang & Miles. 1992). More than 30 species of medicinal mushrooms are currently identified as sources for biologically active metabolites with potential anti-cancer properties (Dilani et al 2012). More than 50 mushroom species have yielded potential immunocuticals that exhibit anticancer activity *in vitro* or in animal models and of these, six have been investigated in human cancers (Parris. 2000). Six mushroom preparations have shown clinically significant efficacy against human cancers: lentinan (*Lentinula edodes*), Schizophyllan (*Schizophyllum commune*), Active Hexose Correlated Compound (AHCC) (*Lentinula edodes*), Maitake D-Fraction (*Grifola frondosa*), Polysaccharide-K (*Coriolus versicolor* (formerly *Trametes versicolor*, *Polyporus versicolor*), and Polysaccharide-P (*Coriolus versicolor* (formerly *Trametes versicolor*, *Polyporus versicolor*). Since lentinan and schizophyllan have limited oral bioavailability, and therefore fail to meet the definition of immunocutical, they will only be given a cursory review. AHCC and Maitake D-Fraction are still in the early stages of investigation. The remaining two have been subjected to in-depth application against cancers in humans (Parris. 2000).

The number of mushroom species on the earth estimated to be 140 000, suggesting that only 10% are known. Assuming that the proportion of useful

mushrooms among the undiscovered and unexamined mushrooms will be only 5%, which implies 7000 yet undiscovered species will be of possible benefit to mankind (Hawksworth 2001). Ghosh et al (2012) reported 47 mushroom species from West Bengal, Cancer is a leading cause of death worldwide. The current anti-cancer drugs available in market are not target specific and pose several side-effects and complications in clinical management of various forms of cancer, which highlights the urgent need for novel effective and less-toxic therapeutic approaches. In this context, some prized mushrooms with validated anti-cancer properties and their active compounds are of immense interest. Ghosh (2014) isolated polysaccharides and terpenoids from basidiomycota mushrooms and found their antimicrobial property, There are, however, still countries and regions that have not been studied for their diversity of mushrooms. This is particularly true in tropical regions of the world (Hawksworth 2001; Hyde 2001; Aly et al. 2010; Boonyanuphap and Hansawasdi 2010). There is a need to explore tropical countries for the presence of mushrooms and to assay their bioactive metabolites that can be used as possible remedies for cancer treatments.

Cytotoxicity has been defined as the cell killing property of a chemical compound independent from the mechanism of death (Graham-Evans *et al.*, 2003). Cytotoxicity assay is an appropriate method for screening new substances within a short time in order to determine cytotoxicity on cancer cells (Alley *et al.*,

1988). Usually in oncology research and clinical practices, *in vitro* testing is preferred prior to *in vivo* testing. *In vitro* cultures can be cultivated under a controlled environment (pH, temperature, humidity, oxygen / CO₂ balance etc.) resulting in a homogenous batches of cells and thus minimizing experimental errors. MTT assay has been described as rapid, simple and reproducible method, widely used in the screening of anticancer drugs and to measure the cytotoxic properties. Hence, in the current study, the cytotoxic properties of *C. indica* was evaluated using this assay.

Therefore, the objective of this work is to bioassay of *Calocybe indica* mushroom as anticancer agent against two cancer cell lines *in vitro*.

2. Materials and Methods

Mushroom collection and identification

The wild fruit bodies /basidiocarps were collected in August 2014 from Badu regions of Barasat, District, 24-Parganas (N), India and carried to Laboratory in biodegradable polythene bags. Their color, size, shape and attachment to the substrates were recorded. Some biochemical tests were conducted and morphological and anatomical observation were noted as per published keys of mushrooms. The mushrooms were phenotypical identified consulting with published keys.

Extraction

The collected fruit bodies were dried in oven at 60⁰C for two days. The dried fruit bodies were cut in to pieces, and were then ground to powder using mortar and pestle. The powders were extracted with two types of solvents (distilled water or methanol). In both cases, 100 grams of the dried mushroom powdered were filled separately in the thimble and extracted with methanol/water (250 ml) using a Soxhlet apparatus followed by distillation and evaporation. The source of heating was a mantle heater with highly regulated thermostat. The powder is placed in a cellulose thimble in an extraction chamber, which is placed on top of a collecting flask beneath a reflux condenser. Methanol is added to the flask, and the setup is heated under reflux. When a certain level of condensed solvent has accumulated in the thimble, it is siphoned into the flask beneath. The obtained extract solution was then evaporated at 50⁰C using a rotary evaporator, and the resultant crude extract was stored at -20⁰C until use to prepare the required concentrations.

Preparation and sterilization of serial concentration of mushroom extracts

The five concentrations (10.0, 20.0, 40.0, 100.0 and 200 mg/ml) of each mushroom extract were prepared and the solvent was DMEM medium. In the

case of methanol extract (ME), it was first dissolved in drops of DMSO, and then the volume was made-up with DMEM medium.

All concentrations of both extract (WE and ME) solutions were passed through sterilized Millipore filter (0.22µm).

Chemicals

3-(4,5- dimethyl thiazol-2-yl)-5-diphenyl tetrazolium bromide (MTT), Fetal Bovine Serum (FBS), Phosphate Buffered Saline (PBS), Eagle s minimum essential medium (DMEM) and Trypsin were obtained from sigma Aldrich Co, St. Louis, USA. EDTA, glucose, Trichloroacetic acid (TCA), Acetic acid, Tris base and antibiotics from Himedia Laboratories Ltd., Mumbai, Dimethyl Sulfoxide (DMSO) and Propanol from E. Merck Ltd., Mumbai, India, Vincristine and tamoxifen.

Cell lines, culture medium and subculture of cell line

Human Ewing's sarcoma MHH - ES - 1 having passage number 170 and breast cancer MCF-7 cell lines having passage number 185 were grown and maintained in DMEM (Dulbecco's Modified Eagle media) supplemented with L Glutamine, 10% v/v fetal bovine serum, sodium carbonate in 75 square mm tissue culture flasks to the 60-70% confluence. The media also supplemented with 100 µg/ml streptomycin (Invitrogen) and 250 IU/ml penicillin (Invitrogen) at 37⁰c in a humidified atmosphere of 5% CO₂. Maintenance cultures were passage weekly, and the culture medium was changed twice a week. (Freshney, 2000).

Normal human peripheral mononuclear leukocytes were obtained from whole peripheral blood. The sample was obtained by venipuncture from a healthy volunteer blood donor. Lymphocytes were isolated with the gradient density method, using the Hystopaque 1077 solution (from Sigma Aldrich).

Sterility test

Sterility test was done initially to check the extracts for the contamination. 35 mm culture dish was plated with MHH - ES - 1 and MCF-7 cell suspension in 2ml of DMEM media and allow the cells to adhere. The crude plant extracts were added into culture dishes and incubated at CO₂ incubator (5%) for 24 hours.

Cell Treatment Procedure

The monolayer cells were detached with trypsin ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of 1×10^6

cells / ml. One hundred micro litres per well of cell suspension were seeded in to 96-well plates at plating density of 10,000 cells / well and incubated to allow for cell attachment at 37 °C, 5% CO₂, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentration of the test samples.. Aliquots of 100µl of these different sample dilutions were added to the appropriate wells already containing 100µl of medium, resulted the required final sample concentrations (5, 10, 20, 50 and 100mg/ml). Following treatment with methanolic extract of *C. indica*, the plates were incubated for an additional 48 h at 37° C, 5% CO₂, 95% air and 100% relative humidity. The medium without samples were served as negative control and medium treated with vincristine/ tamoxifen as positive control; triplicate was maintained for all concentrations.

MTT assay

Inhibition of MHH - ES - 1 and MCF7 cell cells to *C. indica* was determined individually by the MTT colorimetric assay (Mosmann 1983). Cells were seeded in a flat-bottomed 96-well plate and incubated for 24 h at 37°C and in 5% CO₂. Both cell lines were treated with different concentration of extract of *C. indica*. The solvent DMSO treated cells served as control. Cells were then treated with 15µ l of MTT (5g / ml) in PBS (phosphate buffered saline) for 4 h at 37°C and then DMSO (200 µl) was added to each well to dissolve the formazan crystals. The assay is based on the cleavage of the tetrazolium salt MTT to form purple color coloured formazan dye by viable cells. Thus DMSO soluble formazan is produced. The amount of soluble formazan product generated from MTT is proportional to the number of living cells in the sample and the optical density (OD) was recorded at 492 nm in a microplate reader. (a BioTek Synergy 2 multiplate reader.). Percentage of residual cell viability was determined as $[1 - (\text{OD of treated cells} / \text{OD of control cells})] \times 100$. The Inhibitory concentration required for 50% cytotoxicity (IC₅₀) value was analysed using sigmaplot software. Thus MTT assay provides a quantitative measurement of viable cells by determining the amount of formazan produced by metabolically active cells.

For the normal human lymphocytes, which are in suspension, the cytotoxicity was evaluated using the water-soluble MTS (Voraue, 1997) dye.

Cell morphological studies

To determine the morphological changes of cells DAPI staining was performed. The cells were seeded on glass slide and treated with each concentration of

both WE and ME for 24 hours. Untreated and treated cells were rinsed with phosphate buffered saline, fixed with ice- cold 10% tri chloroacetic acid and further washed with cold 70%, 80% and 90 % of ethanol. The cells were stained with 1 µg /ml 4- 6 – diamidino – 2 – phenylindole (DAPI) for 3 minutes cover slipped with 90% glycerol and observed under florescence microscope.

Statistical analysis

The IC₅₀ (50% cytotoxicity) is the concentration of the toxic compound that reduces the biological activity by 50%. The IC₅₀ value was obtained from MTT assay and calculated using Microsoft excel software. The values were expressed in geometric mean. Differences were considered to be a statically significant when $P < 0.05$ and $P < 0.01$.

3.Result and Discussion

On the basis of phenotypical (morphological, biochemical and anatomical) characters of the collected fruit body of mushrooms and comparing with the published keys of mushrooms (www.britmycolsoc.org.uk. www.mycokokey.com). it was identified as *Calocybe indica*.

The inhibitory/cytotoxicity properties of water and methanolic extracts are compared with standard tamoxifen for MCF-7 cell line and vincristine on MHH - ES - 1 cell line (Tables 1, 2,3 and 4) respectively. The maximum concentration (mg/ml) used in the study was 100 mg/ml. The results presented in the table1 showed that 100mg/ml concentration of WE of *C. indica* caused 71.2 % of MHH-ES cell death while 5mg/ml has no effect. 100mg/ml Vincristine showed 98.02 % of cell death. ME (100mg/ml) gave 81.20 % of cell death (table2) and it was better inhibition than WE (100mg/ml). In case of MCF7 cell line, the effect of both WE and ME of this mushroom have the same trends. WE (100mg/ml) gave 88.20 % of cell death (table 3) while ME (100mg/ml) gave 89.20% (table 4). The IC₅₀ values of WE and ME in MHH - ES - 1 cell line were 55.25±1.201mg/ml and 46.56±0.134mg/ml respectively while these values of MCF7 cell line were 52.12±0.15mg/ml and 47.94±0.09mg/ml respectively.

The percentages of cancer cell death are found to be concentration dependent (table 1,2,3, and 4). A linear relationship between the formazan generated and the number of viable cells was demonstrated, together with time-dependent growth characteristics for MCF-7 cells by Ferrari *et al.*, (1990).

Table 1. Effect of water extract(WE) of *C. indica* on MHH - ES - 1 cell line

Treatment(mg/ml)	% of Dead cell±SE	% of viable cells	IC50
05	0.00	100	55.25±1.201
10	0.52 ±0.001	94.80±1.20	
20	23.00±0.20	73.00±1.003	
50	48.00±0.20	52.00±0.70	
100	71.2.0±0.23	28.80±0..914	
100 Vincristine	98.02±1.0	01..08±.0001	
Negative control	00.00	100.00	

Table 2. Effect of methanolic extract(ME) of *C. indica* on MHH - ES - 1 cell line.

Treatment(mg/ml)	% of Dead cell±SE	% of viable cells	IC50(mg/ml)
05	0.00	100	46.56±0.134
10	5.52 ±0.001	94.48±1.20	
20	33.00±0.20	67.00±1.003	
50	58.00±0.20	42.00±0.70	
100	81.20±0.23	18.80±.914	
100 (positive control)Vincristine	98.02±1.0	01..08±.0001	
Negative control	00.00	100.00	

Table 3. Effect of water extract(WE) of *C. indica* on MCF7 cell line.

Treatment(mg/ml)	% of dead cell±SE	% of viable cells	IC50(mg/ml)
05	0.00±0	100±0.0	52.12±0.15
10	4.52 ±0.001	95.48±2.10	
20	30.00±0.20	70.00±4.003	
50	49.00±0.20	41.00±0.50	
100	88.20±0.23	11.80±.5.02	
100(tamoxifen)(Positive control)	97.13±1.0	02..87±.0001	
Negative control	00.00	100.00	

Table 4. Effect of methanolic extract(ME) of *C. indica* on MCF7 cell line

Treatment(mg/ml)	% of Dead cell±SE	% of viable cells	IC50(mg/ml)
05	0.00	100	47.94±0.09
10	15.52 ±0.00	84.48±2.10	
20	43.00±0.11	57.00±2.01	
50	68.00±0.13	32.00±0.80	
100	89.20±0.11	10.80±.8.14	
100(tamoxifen)(positive cotrol)	97.13±1.0	02..87±.0001	
Negative Control	00.00	100.00	

Lucas et al (1957) first demonstrated the anti-cancer activity of mushrooms. They used extracts of fruiting bodies of *Boletus edulis* and other mushrooms in tests against implanted Sarcoma 180 line in mice. The anti-cancer calvacin, a conjugated protein containing one or more carbohydrate residues was extracted from the giant puffball *Calvatia gigantea* and tested against many experimental tumors, such as Sarcoma 180, mammary adenocarcinoma 755,

leukemia L-1210, and HeLa cell lines presenting significant growth inhibition on those types of cancer (Lucas et al 1957). Ikekawa et al. (1969) published one of the first scientific reports on antitumor activity of extracts of mushrooms against implanted Sarcoma 180 in animals. Soon after, three major anticancer drugs, Krestin from cultured mycelium of *Trametes* (*Coriolus versicolor*), Lentinan from fruiting bodies of *Lentinus edodus* and Schizophyllan from

Schizophyllum commune, were developed (Wasser 2002; Mizuno, 1999, Mo et al 2004).

Inflammatory breast cancer (IBC) is the most lethal and least understood form of advanced breast cancer. Michelle et al (2011) reported the effects of *G. lucidum* (Reishi) on viability, apoptosis, invasion, and its mechanism of action in IBC cells (SUM-149). Results show that Reishi selectively inhibits cancer cell viability although it does not affect the viability of noncancerous mammary epithelial cells. Maiti et al (2008) reported cytotoxicity effect of *C. indica* on some cancerous cell lines. The methanolic extract of *Calocybe indica* showed significant inhibitory effect against induced inflammation in the experimental mice model (Prabu and Kumuthakalavalli, 2014). This screening of *C. indica* as anticancer agent also supported them.

Based on MTT assay results, DAPI staining was conducted to investigate *C. indica* induced changes in cell structures. Cells were incubated with both extracts (WE & ME) of *C. indica* separately, and morphological alterations were confirmed via fluorescence microscope. After 24 h of incubation with various concentrations of extract of *C. indica*, many of the cells showed cytoplasmic shrinkage and loss of normal nuclear architecture, became detached and found floating in the medium. As a result, the number of cytotoxic cells increased with WE and ME-concentration, with the highest having the most pronounced inhibitory effect on cell proliferation than the control. In this screening trial, WE and ME of fruit body of this mushroom were found no cytotoxicity on normal lymphocyte cells. Moreover, this mushroom is eaten by locals of collected zone.

4. Conclusion

A nutraceuticals can be defined as a substance that may be considered a food or part of a food that provides medical or health benefits like the prevention and treatment of disease. These results suggest a possible use of extracts from this mushroom to identify compounds of possible interest in the treatment of breast cancer and sarcoma, so that the anticancer agent may be clinically successful. The finding of its active compound against cancers and their isolation and purification will be future course of works.

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References

1. Alley MC, Scudiro DA, Monks A, Hursey ML, Czerwinski MJ, Fine, DL. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res.* 1988;48: 589-601.
2. Blackwell M The fungi: 1, 2, 3 ... 5.1 million species? *Am J Bot* 2011; 98 (3):426-438.
3. Boonyanuphap J, Hansawasdi C. Spatial distribution of β -glucan containing wild mushroom communities in subtropical dry forest. Thailand. *Fungal Divers* 2010; 46:29-42.
4. Chang ST, Miles PG. Mushrooms biology—a new discipline. *Mycologist.* 1992;6:64-5.
5. Dilani D D S, Sylvie R, Françoise, Ali H., Kevin D H. Medicinal mushrooms in supportive cancer therapies: an approach to anti-cancer effects and putative mechanisms of action. *Fungal Diversity*, 2012;55:1-35.
6. Ferrari M, Fornasiero MC, Isetta AM. MTT colorimetric assay for 111 testing macrophage cytotoxic activity in vitro. *J. Immunological Methods.* 1990; 131:165-70.
7. Freshney RI. Culture of Animal Cells. A Manual for Basic Technique, 2000, 4th ed. Wiley-liss, A John Wiley and Sons. Inc. Publication, New York.
8. Ghosh SK. Isolation of Polysaccharides and terpenoids from some Basidiomycota and their antibacterial activities. *British Microbial Research Journal* 2014; 4(2): 151-162,
9. Ghosh SK, Chakravarty A, Talukdar J, Sandhya, S S, Bera M. Sarkar D, Barmon Roy. Study of Diversity of Basidiomycota Mushrooms in West Bengal of India. *Transient –A J of Natural Science and Allied Subjects* 2012;2:73-96.
10. Graham-Evans B, Tchounwou PB, Cohly, HH Cytotoxicity and proliferation studies with arsenic in established human cell lines: keratinocytes, melanocytes, dendritic cells, dermal fibroblasts, microvascular endothelial cells, monocytes and T cells. *Int. J. Mol. Sci.* 2003;4: 13-21.
11. Hawksworth DL. Mushrooms: the extent of the unexplored potential. *Int J Med Mushrooms.* 2001;3:333-7.
12. Ikekawa T., Uehara N., Maeda, Y., Nakanishi M., Fukuoka F. Antitumor activity of aqueous extracts of edible mushrooms. *Cancer Res.*, 1969;29, 734-735.
13. Lucas EH, Ringler RL, Byarrum RU, Stevens JA, Clarke DA, Stock CC. Tumor inhibitors in *Boletus*. DA, Stock CC. Tumor inhibitors in

- Boletus edulis* and other Holobasidiomycetes, *Antibiotics Chemother* 1957;7:1-14.
14. M, Bhutia SK, Mallick SK, Kumar A, Khadgi N, Maiti TK. 2008 Antiproliferative and immunostimulatory protein fraction from edible mushrooms *Environ Toxicol Pharmacol*. 2008; 26(2):187-91.
 15. M, Raysa Rosario Acevedo, Elisa Otero-Franqui, Luis. A. Cubano, and Suranganie F. Dharmawardhane Ganoderma lucidum (Reishi) Inhibits Cancer Cell Growth and Expression of Key Molecules in Inflammatory Breast Cancer *Nutr Cancer*. 2011; 63(7): 1085–1094.
 16. Mizuno, T. The extraction and development of antitumoractive polysaccharides from medicinal mushrooms in Japan (review). *Int. J. Med. Mushr* 1999; 1: 9-30.
 17. Mo S, Wang S, Zhou G, Yang Y, Li Y, Chen X, Shi J. Phelligidins C-F: cytotoxic pyrano[4,3-c][2]benzopyran-1,6-dione and furo[3,2-c]pyran-4-one derivatives from the fungus *Phellinus igniarius*. *J Nat Prod* 2004;67:823–828.
 18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65: 55–63.
 19. Parris K. The Use of Mushroom Glucans and Proteoglycans in Cancer Treatment, *Alternative Medicine Review*, 2000; 5(1) :4-27.
 20. Prabu M and Kumuthakalavalli R, *in vitro* and *in vivo* antiinflammatory activity of the methanolic extract of *calocybe indica* p.&c. *World j of phar and pharma sciences* 2014;3(5):776-783.
 21. Virag P, Brie I, Postescu ID, Perde-Schrepler M, Fischer-Fodor E, et al. Comparative study of two evaluation methods for the genotoxic effects of environmental heavy metals on normal cells. *Toxicol Ind Health* 2009;25: 253–258.
 22. Vorauer K, Steindl F, Jungbauer A, Hahn R, Katinger H. Cytokine activity assay by means of proliferation measured in plane convex microtiter wells. *J Biochem Biophys Methods* 1996;32: 85– 96.
 23. Wasser SP. Medicinal mushrooms as source of antitumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.*, 2002; 60: 258-274.

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