

Antiproliferative, Apoptotic, and Antimigration Property of Ethyl Acetate Extract of *Calocybe indica* against HeLa and CaSki Cell Lines of Cervical Cancer, and its Antioxidant and Mycochemistry Analysis

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Abstract

Background: The incidence rate of cervical cancer is increasing and its existing drugs are becoming more and more resistant. Therefore, we extracted the fruiting body of *Calocybe indica* edible mushroom in 90% ethyl acetate extract (EAE) and evaluated it as an anticancer property against HeLa and CaSki.

Method: We performed cytotoxicity assay by MTT, cell morphological study by phase contrast microscope, and apoptosis study by nuclear morphology via DAPI staining under inverted microscopy; the expressions of proapoptotic and antiapoptotic genes and p53 were examined by Western blotting, cell cycle analysis, and cologenic and cell migration assay. Antioxidant content and activity assays were performed and for mycochemistry analysis of EAE, thin layer chromatography (TLC) was done.

Results: EAE-treated HeLa and CaSki cells became round and showed condensed and fragmented nuclei. They inhibited the cell proliferation of both cancer cell lines in a dose-dependent manner. At maximum dose (1250 µg/mL) after 24 h, the cell inhibition percentages of HeLa and CaSki cells were 97.12±10.01 and 98.52±10.08 ($P<0.05$), respectively. They upregulated the expression of p53, caspase 3, and caspase 9 while down-regulating Bcl2 gene. Cell cycle became arrested at G2/M checkpoint of both cancer cell lines by EAE. EAE inhibited colony formation and cell migration. The antioxidant assay showed that EAE contained good amounts of phenolic compounds, flavonoids, and ascorbic acids and had good antioxidant activity. TLC supported the presence of bioactive components.

Conclusion: The EAE of *C. indica* exerts very potent anticervical cancer effects. It is urgent that future studies analyze its bioactive compounds in detail and examine them in animal models.

Keywords: Mushroom, Cervical cancer, Cytotoxicity, Apoptosis, Metastasis, Bioactive compounds

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Introduction

The modern research on molecular mechanisms associated with the progression and metastasis of cancer has prompted oncologists to search for new natural compounds. Certain important well-adapted experiments such as inhibition of proliferation, induction of apoptosis, and antimetastasis in cancer cells have been conducted to prove the anticancerous activities of these natural compounds.¹ Mushrooms were primarily used as foods for the unique taste. Later, humans realized that some also have medicinal properties. Following the research of Byerrum,² continuous investigations have been focused on different mushrooms in search of anticancer natural products. Currently, it is established that some edible mushrooms are good sources of anticancer agents against many cancers.^{3,4,5,6} Three major anticancer drugs, Krestin from cultured mycelium of *Trametes versicolor*, Lentinan from the fruiting bodies of *Lentinus edodus*, and Schizophyllan from *Schizophyllum commune*, were developed.⁷⁻⁹ There exist more than 14,000 mushrooms out of 5.1 million estimated fungi, among which around 700 exhibit medicinal properties.¹⁰ Since many of the compounds have been shown to act synergistically,¹¹ it is worth testing the antiproliferative effects of the whole mushroom extracts rather than its individual components. Liu et al.¹² and other colleagues¹³⁻¹⁵ used the ethanolic extract of mushroom against various human cancer cell lines; however, ethyl acetate extract (EAE) was

used less for this purpose. Statistics show that one third of the newly diagnosed cancers among women in India are of cervical type.¹⁶ In India, every year, out of the 122,844 women diagnosed with cervical cancer, 67,477 die from the disease.^{17,18} Therefore, we conducted some experiments to evaluate the anticancer properties associated with the EAE of *C. indica* regarding important parameters such as antiproliferation, induction of apoptosis, anticolonization and anti migration on HeLa and CaSki cells of cervical cancer. *Calocybe indica* is an edible mushroom, widely found and cultivated in West Bengal, but its anticancerous characteristic on HeLa and CaSki cells is reported for the first time in our study, which is the novelty of our work.

Materials and Methods

Mushroom collection and identification

The wild basidiocarps were collected in July 2018 from Khardah market, District-24-Parganas (N), India, and carried to Laboratory in biodegradable polythene bag. All morphological characteristics such as color, shape, and size were recorded, spore print and anatomical observations were noted, and this mushroom was identified by following published identification keys.¹⁹⁻²¹

Extraction

As per Liu et al.¹² we dried the fruiting bodies after chopping into pieces and ground them into powder using a mixer grinder (Beckmen mixture).

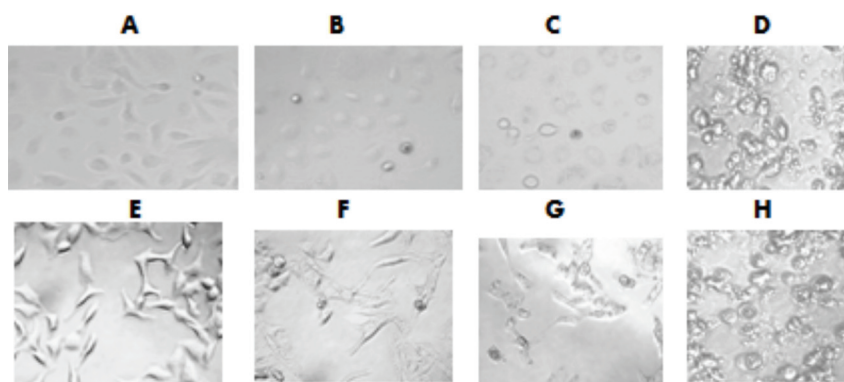


Figure 1. Morphology of CaSki and HeLa cells under phase contrast microscope; A, E. Negative control shows the normal cells of CaSki and HeLa, respectively; B, F. 500 µg/mL of EAE treatment shows the round and floating cells of CaSki and HeLa. C, G. Cell confluency decreased when cells treated with 750 µg/mL of EAE, cells are round, plasma membrane blebbing of CaSki and HeLa, respectively. D, H. In 1000 µg/mL dead cells with debris of CaSki.

Table 1. Evaluation of the percentage of apoptotic HeLa and CaSki cells at different concentrations of EAE after 24h

Concentration(mg/ml)	% of apoptotic cells \pm SD	
	HeLa	CaSki
250	27.20 \pm 2.78	20.06 \pm 2.32
500	35.50 \pm 3.90	30.45 \pm 3.34
750	45.15 \pm 5.98	44.78 \pm 5.56
Control(negative)	0.24 \pm 0.01	0.07 \pm 0.34
Control(positive-adriamycin -25 μ M)	58.67 \pm 7.34	57.12 \pm 7.56

Note: The data are mean \pm SD of three replicated plates. Statistical significance at $P < 0.05$.

In short, 15 grams of dried mushroom powder was dipped in 150 mL of 90% ethanol under shaking conditions at room temperature. Following 48 h, it was filtered through Whatman No.4 followed by Whatman No.1 filter papers. A rotary vacuum evaporator removed the ethyl acetate from the extract at 40°C, and the remaining solvent was removed with a freeze-drier. We kept the powder of EAE of *C. indica* in airtight conditions in refrigerator at 40 C.

Cell culture

The Human Cervical Cell Lines HeLa and CaSki, with respective passage numbers 170 and 90 (purchased from NCCS, Pune), were cultured in DMEM (Dulbecco's Modified Eagle media) and supplemented with L-Glutamine (Himedia), 10% v/v fetal bovine serum (Himedia), 100 mg/mL streptomycin (Invitrogen), and 250 IU/mL penicillin(Invitrogen); we kept them in 75 square mm tissue culture flasks at 37°C in a humidified atmosphere of 5% CO₂ until 80-90% confluency.

For maintenance, cultures were passaged weekly, and the culture medium was changed twice a week.²²

Cell morphological study under Phase-contrast microscope

Cancer cells were grown in 60-mm tissue culture plates and treated with EAE (0-1000 μ g/mL). We examined cells under a Phase-contrast microscope and, for analysis, took photographs by a Magna-Fire digital camera (Optotronics, Goleta, CA, USA).

Nuclear morphology and evaluation of apoptosis by DAPI staining under inverted fluorescent microscope

4', 6-Diamidino-2-phenylindole (DAPI) staining was done to examine the nuclear morphology of the treated cells. The examined HeLa and CaSki cells were washed with cold PBS and fixed with 3.7% (w/v) paraformaldehyde in PBS for 10 min at room temperature. Following

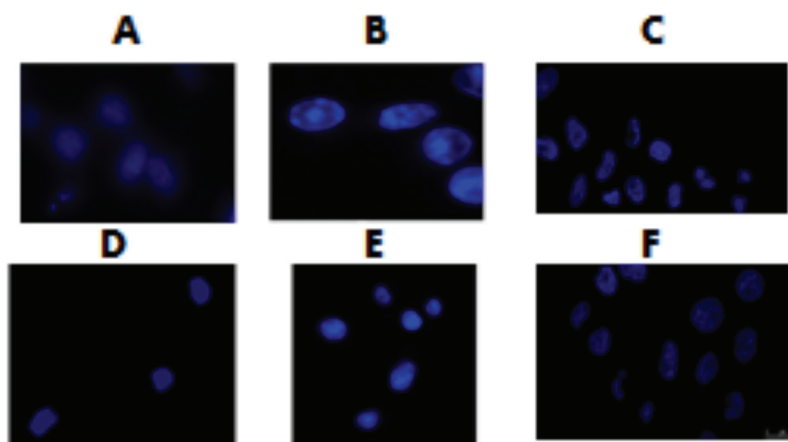


Figure 2. Nuclear morphology of HeLa and CaSki cells after DAPI staining under inverted fluorescence microscopy; A,D. Negative control shows the normal nuclei of HeLa and CaSki; B, C. 500 and 750 μ g/mL of EAE treatment shows apoptotic nuclei (chromatin condensed, fragmented nuclei) of HeLa; E, F. 500 and 750 μ g/mL of EAE treatment shows apoptotic nuclei of CaSki (chromatin condensed, fragmented nuclei).

Table 2. Cytotoxicity (% of cell viability) or antiproliferative (% of cell inhibition) effect of EAE on HeLa and CaSki cells and their IC50 value at 24 h

Treatment (mg /mL)	HeLa		CaSki		IC 50 value ($\mu\text{g}/\text{mL}$) \pm SD	
	Cell viability(%) \pm SD.	Cell inhibition(%) \pm SD.	Cell viability(%) \pm SD.	Cell inhibition(%) \pm SD	HeLa	CaSki
250	55.86 \pm 4.12a	44.14 \pm 3.57c	67.00 \pm 5.23a	33.00 \pm 3.00d	506 \pm 15.20	520 \pm 18.32
500	50.56 \pm 3.98b	49.44 \pm 4.00c	55.56 \pm 4.78b	4 4.44 \pm 3.43c		
750	30.45 \pm 3.00c	69. 55 \pm 5.80b	36.00 \pm 3.99c	64.00 \pm 5.18 b		
1000	14.45 \pm 2.11d	85.55 \pm 9.84a	11.00 \pm 1.98d	89.00 \pm 9.93a		
1250	2.88 \pm 0.98e	97.12 \pm 10.01a	1.48 \pm 0.56e	98.52 \pm 10.08a		
Positive control (Adriamycin)	1.95 \pm 0.50e	98.05 \pm 10.01 a	2.48 \pm 0.66e	97.03 \pm 10.09a		
Negative control		0.000		0.000		

Note: The same letter in the same row indicates no statistical difference, but a different letter indicates statistical difference as per Duncan analysis ($P=0.05$ level)

permeabilization, we stained the cells with a DAPI (10 $\mu\text{g}/\text{mL}$) solution at 37°C for 30 min. They were then washed with PBS and examined by an inverted fluorescent microscope (Olympus, Tokyo, Japan); photographs were taken by use of a Magna-Fire digital camera (Optotronics, Goleta, CA, USA) for analysis.

Apoptosis was assessed based on the morphological changes in the nuclear structure stained with DAPI. In each experiment, we observed and counted the apoptotic cells under an inverted fluorescent microscope; we counted a minimum of five optical fields, containing a total of 200 cells, in each experiment.

The percentage of apoptotic cells was calculated by the formula: percentage of apoptotic cells = [number of apoptotic cells / total cells counted (200 usually)] \times 100%.

Cytotoxicity/cell proliferation /viability assay

We evaluated the effect of EAE on the cell proliferation of HeLa cell and CaSki line using

Dimethyl thiazolyl tetrazolium bromide (MTT) assay (Sigma, USA) and following the method of Akiyama et al.²³ with certain modifications. In short, 10×10^3 cells per well of 96-well culture plate were seeded overnight with fresh DMEM medium containing 10% FBS and antibiotics to reach 80% confluency. The culture was then washed with 10% PBS and treated with EAE dissolved in DMEM at various concentrations (0, 250, 500, 750, 1000 and 1250 $\mu\text{g}/\text{mL}$) and incubated at 37°C in 5% of CO_2 and 95% of moisture for 24 h. Afterwards, the cells were washed with PBS, 100 μL of 0.5% MTT solution (dissolved in RPMI 1640) was added to each well, and cultures were further incubated for 3 h; after discarding the media, 100 μL of DMSO was added to dissolve the crystals. The plate was read by a micro plate reader (BioRad) at 570 nm absorbance. Normal human peripheral mononuclear leukocytes were collected from whole peripheral blood. We obtained the blood sample by venipuncture from a healthy volunteer blood donor. To isolate the lymphocytes, the gradient density

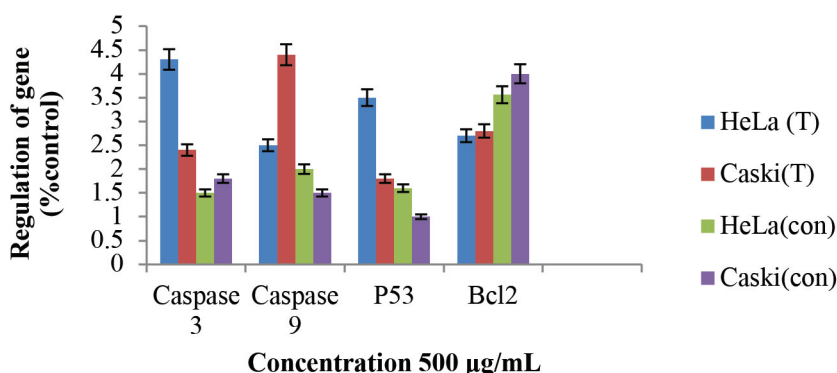


Figure 4. p53 expression in immunostaining under fluorescence microscope, A. control, B. 250 $\mu\text{g}/\text{mL}$, C. 500 $\mu\text{g}/\text{mL}$, D. 750 $\mu\text{g}/\text{mL}$ and E. Bar diagram (Concentration versus color intensity) at 12 h ($***P<0.001$).

Table 3. Effect of EAE on cell cycle distribution of HeLa and Caski

EAE($\mu\text{g/ml}$)	HeLa			CaSki		
	G0/G1	S	G2/M	G0/G1	S	G2/M
0.0(Negative control).	73.00 \pm 4.99	21.10 \pm 1.50	05.90 \pm 0.55	56.66 \pm 2.99	33.00 \pm 1.99	10.34 \pm 0.99
250	77.25 \pm 5.94	20.21 \pm 1.81	07.54 \pm 0.87	67.32 \pm 3.90	25.46 \pm 2.00	15.32 \pm 1.23
500	72.24 \pm 3.90	18.76 \pm 1.05	17.00 \pm 0.75	52.00 \pm 2.11	20.25 \pm 1.67	27.75 \pm 1.89

Note: Cell cycle distribution in % (G0/G1, S, G2/M). The data are mean \pm SD of three experiments. Statistical significance at $P < 0.05$

method was applied through the use of the Hystopaque 1077 solution (from Sigma Aldrich).²⁴

Growth inhibition rate was determined by the formula:

$$\text{Growth inhibition} = [1 - A_{490 \text{ nm of treated cells}} / A_{490 \text{ nm of control cells}}] \times 100\%$$

The concentration which led to 50% killing (IC_{50}) was calculated through plotting the dose-response graph of the cytotoxicity values obtained using the below formula:

$$\% \text{ Cell cytotoxicity} = 100 - [(A_{\text{control}} - A_{\text{test}}) / A_{\text{control}}] \times 100]$$

Data points represent the mean \pm SD in one experiment at least thrice repeated

Western blot analysis

CaSki and HeLa (2×10^5) cells were treated with 500 $\mu\text{g/mL}$ of EAE for 24 h. After treatment, cells were lysed with RIPA buffer (Abcam, UK). We also determined the effect of treatment on the expression of certain cell cycle proteins such as p53, and on apoptotic proteins such as Bcl-2, procaspase-3, procaspase-9, and PRAP (Santacruz Biotechnology, USA). Protein expression levels were specified using the mean fluorescence intensity values of the samples via FV10 ASW 1.7 software (Olympus Corporation, Tokyo, Japan.). The protein concentration was 750 $\mu\text{g/mL}$. Proteins were separated by SDS-PAGE and detected through incubation with the corresponding primary antibodies and those followed by blotting with the HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were then detected using Luminol (Bio-Rad).

Immunofluorescence and microscopic analysis

CaSki cells (1×10^5) were seeded on circular cover slips and treated with 500 $\mu\text{g/mL}$ EAE for 12 h. After washing with Hank's solution (Thermo

Fisher Scientific, Inc. USA), the cells were fixed with 4% paraformaldehyde solution for 30 min at room temperature and permeabilized with 0.1% Triton X-100 (dissolved in PBS) for 10 min. Next, the cells were washed with PBS three times and blocked with 5% BSA dilution in PBS for 1 h at RT. After blocking, they were incubated with 50 μl anti-p53 antibody (1:100) at 4°C overnight. Afterwards, they were washed three times with PBS and incubated with Alexa 555-conjugated secondary antibodies (1:200; Cat. No 21428; Invitrogen; Thermo Fisher Scientific, Inc. USA) for 1 h at room temperature in the dark. Immunofluorescent images were observed and analyzed using the Olympus inverted fluorescent microscope (Olympus Corporation, Tokyo, Japan).

Cell cycle analysis

Cells (7.5×10^5) were seeded in 100 mm dishes and cultured in DMEM media with 10% FBS for 24 h, followed by incubation with EAE (0, 250 and 500 $\mu\text{g/mL}$) at 37°C for 24 h. Following incubation, the cells were harvested, washed with PBS containing 1% FBS, and resuspended in propidium iodide (PI) (50 $\mu\text{g/mL}$).

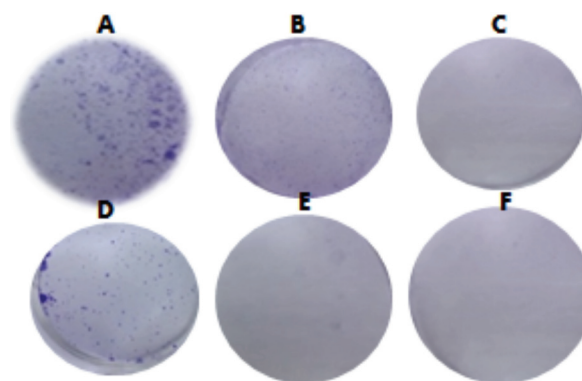


Figure 5. Anticolonization effect of EAE on both CaSki and HeLa cell line. A-C. CaSki, A Control (negative), B 500 $\mu\text{g/mL}$, C 750 $\mu\text{g/mL}$; D-F HeLa, D Control (negative). E 500 $\mu\text{g/mL}$, F 750 $\mu\text{g/mL}$ ($P < 0.05$).

***:Highly significant

Table 4. Anticolonization of EAE on CaSki and HeLa

EAE	Colony formation in agar plate			
	CaSki	CFE (%)	HeLa	CFE (%)
	Colony±SD Sq inch		Colony±SD Sq inch	
Control	120±9.0	100	96±8.0	100
500	57±5.0	47	30±3.5	31.25
750	1.5±0.5	4.1	2.6±1.0	2.70

Note: Colony numbers of mean ±standard deviation of five replicated plates, CFE (Colony forming efficacy). Statistical significance at $P<0.05$

Samples were analyzed on a FAC Star PLUS flow cytometer (Becton-Dickinson, San Jose, CA, USA). We presented the fractions of cells in different cell cycle phases (G0/G1, S, and G2/M) as a percentage of the total cells analyzed.

Cologenic assay

CaSki and HeLa (500) cells per well were seeded separately in a six-well cell culture plate containing agar medium and incubated at 37 °C. Immediately after attachment, the cells were treated with (0, 500 and 750 µg/mL) EAE for four days of interval. After that, they were treated with the same extract concentration, and the colonies were then fixed with 3.7% formaldehyde and stained with (0.5% w/v) crystal violet. Viable colonies were counted using Olympus inverted fluorescence microscope (CKX 53). Through the use of a colony counter, we counted the colonies comprised of more than 50 cells; the results were reported as a percentage of colonies formed using the following equation: % Colonies formed = Colonies formed in treated sample / Colonies formed in untreated sample × 100.

Cell migration assay by scratch method

We performed the migration assay according to the method of Lee et al.²⁵ with certain modifications. Briefly, 50×10^3 per well of HeLa cell was cultured in a 24-well plate. After reaching 90% confluency, the center of the culture dishes was scratched with 100 µL pipette tip. The cells were then washed frequently via PBS and incubated with EAE at a concentration of 500 µg/mL. After 24 h, the image of all cells was taken under ×100 magnification. We then compared the degree of cell spreading and directional migration. The relative migration rate was estimated by measuring the distance of the cells migrated for 24 h, control was also set for comparing with treatment. The relative migration rate was calculated as compared with the control group.

Determination of antioxidant chemical components Total phenolic content

The total phenolic content of EAE of *C. indica* was measured using Folin-Ciocalteu reagent method.^{26,27} We added 50 µL of extract to 250 µL of pre diluted Folin-Ciocalteu reagent with distilled water (1:9). After 1 min, 750 µL of 20%

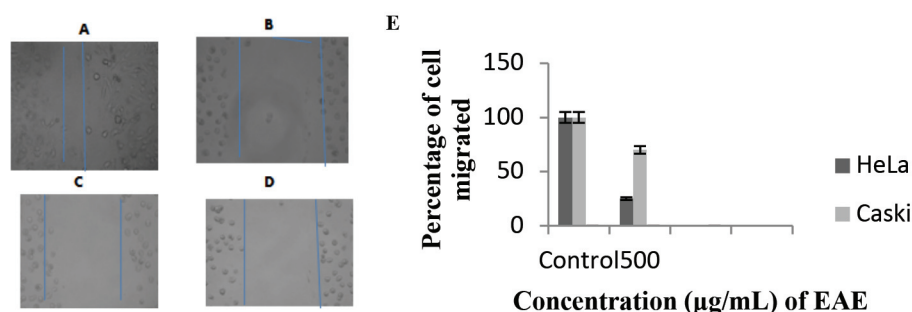


Figure 6. Antimigration against cell line; A and B, treated CaSki at 24 h and 0 h; C and D, treated HeLa cells at 24 h and at 0 h; E, Bar diagram showing percentage of cell migration against control and treated. The treatment dose was 500 µg/mL of EAE; ± SE at 5% level.

Table 5. Determination of antioxidant component and content (mg/g dry weight) of EAE of *C.indica*

Antioxidant component	Antioxidant content (mg/g dry weight±SD) of EAE of <i>C.indica</i>
Phenolic	15.45± 3.45 GAEs mg/g
Flavonoid	10.24 ± 3.15quercetin mg/g
Ascorbic acid	4.09±0.98 mg/g

(w/v) aqueous Na₂CO₃ was added and the volume was made up to 5.0 mL by adding 3.95 mL of ethanol. The control contained all the reaction reagents except the extract and then all the reaction mixtures were incubated for 2 h at 25°C; the absorbance was then measured at 760 nm and compared to a Gallic acid calibration curve. Finally, the result was expressed as mg of Gallic acid equivalent (GAEs) present per g of mushroom extract.

Total ascorbic acid content

Total ascorbic acid content of EAE was measured using Folin-Ciocalteu reagent.²⁸ We mixed 500 µg of mushroom with 0.2 mL of Folin-Ciocalteu reagent; the volume was adjusted to 2 mL by use of distilled water. The mixture was then incubated in dark for 10 min at room temperature; we ultimately read the absorbance at 760 nm by a spectrophotometer. Different concentrations of ascorbic acid (0-10 µg/mL) were used to make a standard curve. Finally, the results were expressed as mg of ascorbic acid equivalent (AAEs) present per g of mushroom extract.

Total flavonoid content

Total flavonoid content of EAE was measured employing aluminium chloride colorimetric method.²⁹ We mixed 500 µg of mushroom extract with 40 µL of 10% aluminium chloride and 1 M potassium acetate; the volume was adjusted to 2 mL with the help of distilled water. Afterwards, the mixture was incubated for 20 min, and the absorbance was finally read at 415 nm by a spectrophotometer. Different concentrations of quercetin (0-10 µg/mL) were used as a standard. Ultimately, the result was expressed as mg of quercetin equivalent (QEs) present per g of mushroom dry weight.

Assay of antioxidant potential of EAE DPPH (1,1-diphenyl-2-picrylhydrazyl) assay

The scavenging activity of different EAE on DPPH radicals was measured by the method proposed by Chiou et al.³⁰ Different concentrations (50, 75, 100, 125 and 150 mg/mL) of EAE powder were prepared with ethanol. Here, 0.9 mL of DPPH solution (0.1 mM) was added to a test tube with 100 µL of each concentration of EAE powder separately. Control was prepared with ethanol instead of EAE. The reaction mixture was incubated for 2h at RT, and the absorbance was measured at 515 nm with a spectrophotometer. The scavenge potentiality of EAE was ultimately measured.

The percentage of DPPH reduction was calculated according to the following equation: % DPPH reduction = $[(Ac-As)/Ac] \times 100$ (where As is the absorbance of sample and Ac is the absorbance of control).

Analytical thin-layer chromatography

We performed thin layer chromatography (TLC) as per the method of Lihua et al.³¹ with minor modifications. A 10×1.5 cm size of TLC plate was heated up to 100°C for 10 min for activation and cooled down to RT. Pencil lines were drawn 1.5 cm from one edge of the plates. EAE and standard samples were spotted using thin capillary pipettes onto the pencil line. Next, we placed the plates in a development chamber with solvent systems. The solvent systems with their used proportions were I. Chloroform-ethanol-acetic acid (90:10:1) II. Chloroform-ethyl acetate-acetic acid (50:50:1), and III. Butanol-acetic acid-water (20:10:5).³² The solvent front was allowed to move until about 1 cm from the top end. The TLC plates were taken off and the solvent front was marked using a soft pencil. They were air dried and spots were detected through exposing the developed plates to UV

Table 6. Antioxidant activity of EAE by scavenging activity (% of inhibition) of DPPH radical in comparison with standards

Solvent extract/Standard	% of inhibition (\pm SD) of DPPH radical at different concentration (μ g/mL)					DPPH EC50 (μ g/mL) \pm SD
	50	75	100	125	150	
EAE	30.12 \pm 3.99	52.45 \pm 6.78	65.95 \pm 7.19	70.56 \pm 7.95	86.12 \pm 9.67	70.12 \pm 10.56
Ascorbic acid	41.67 \pm 4.78	61.45 \pm 7.15	67.90 \pm 7.19	75.45 \pm 8.01	88.56 \pm 10.00	58.36 \pm 7.45
BHA	55.24 \pm 6.96	68.05 \pm 7.81	70.86 \pm 7.99	83.47 \pm 9.11	90.15 \pm 11.41	43.78 \pm 6.34

Note: Statistical significance at $P < 0.05$

light at 254 and 366 nm. Afterwards, we marked the chromatograms /spots, noted their color in different exposures, and calculated and recorded the retention factors (R_f). The resultant chromatograms were captured on camera.

The R_f values of the different bands were then calculated using the equation:

$$R_f \text{ value} = \frac{\text{Distance of spot from origin}}{\text{Distance of solvent front from origin}}$$

Results

Yield of EAE

Using 95% ethyl acetate solvent, we obtained 5% (W/W) yield of EAE /product of *C. indica*.

Studying the morphological changes of HeLa and CaSki cells by EAE under phase contrast microscopy

Untreated CaSki and HeLa cells (negative control) exhibited normal elongated (Figure 1 A) and spindle shapes (Figure 1E), reaching 90% confluency following 24 h of culture. CaSki cells treated with 100 μ g/mL and 250 μ g/mL of EAE were more or less similar to the control cells with elongated shapes except for a few cell numbers. However, CaSki cells treated with 500 μ g/mL /750 μ g/ mL of EAE became round and shrunk. We also observed membrane blebbing, reduced cell confluency, and certain floated cells (Figure 1B, C); however, when cells were treated with 1000 μ g/ml of EAE of mushroom, many cell debris were seen and dead cells were floating (Figure 1D). Similar trends associated with the morphological changes of treated HeLa cells were further noted (Figure 1F, G, H). Cells treated with Adriamycin (positive control) became round and died (Not shown in Figure). In this screening trial, the EAE of this mushroom's fruiting body

showed no cytotoxicity on normal lymphocyte cells. Moreover, this mushroom is eaten by the locals of the collected zone.

Nuclear morphology study and apoptosis evaluation by DAPI staining

When cells were treated with DAPI stain, live cells became light blue but apoptotic cells exhibited a bright blue color because of chromatin condensation under fluorescence microscope. The nuclei of the untreated HeLa (Figure 2A) and CaSki (Figure 2D) appeared normal (having a light blue color), and were round and homogeneous; however, the nuclei of both HeLa (Figures 2B and C) and CaSki (Figures 2E and F), separately treated with EAE (500 and 750 μ g/mL), were irregular and fragmented in few cases and condensed in each case.

The percentages of the apoptotic cells of HeLa by different concentrations of EAE (250, 500 and 750 μ g/mL) were 27.20 \pm 2.78, 35.50 \pm 3.90, and 45.15 \pm 5.98, respectively and in negative control and positive control (Adriamycin), the percentages of the apoptotic cells were 0.24 \pm 0.01 and 58.67 \pm 7.34, respectively. In case of CaSki cells, the percentages of the apoptotic cells by different concentrations of EAE (250, 500 and 750 μ g/mL) were 20.06 \pm 2.32, 30.45 \pm 3.34, and 44.78 \pm 5.56, respectively (Table 1). In negative and positive controls, the apoptotic nuclei percentages of CaSki were 0.07 \pm 0.34 and 57.12 \pm 7.56. This indicates that the exposure of HeLa and CaSki cells to different doses of EAE for 24 h resulted in increased apoptosis rates.

Cytotoxicity/antiproliferative of EAE against HeLa and CaSki cell line by MTT assay

We determined whether or not EAE affected HeLa and CaSki cell survivability. The HeLa

Table 7. Analytical TLC of EAE, its different spots or compounds, and their R_f values in different solvent systems

Solvent System	Run Time	Sample	Visualization					
			UV 366 nm			UV 254 nm		
			Spot no	Spot col	R_f value	Spot no	Spot col	R_f value
Chloroform:	30min	Galic acid	0	0	0	1.	Blk	0.21
Methanol: 33s		Cinnamic acid	1	0	0.65	1.	Blk	0.65
Acetic Acid:		Quercetin	1	Bl	0.62	2.	Blk	0.62,
:90:10:1.		Ascorbic acid	0	0	0	1.	Blk	0.04
		Calocybe EAE	4	Bl Gr	0.89,0.85,0.80,0.65	7.	Blk	0.89,0.85,0.80,0.65,0.32,0.15,0.07
Chloroform:	18min	Galic acid	0	0	0	1	Blk	0.17
Ethyl acetate:	34s	Cinnamic acid	0	0	0	1	Blk	0.47
Acetic Acid:		Quercetin	1	Bl	0.4	2	Blk	0.4
: 50:50:1		Ascorbic acid	0	0	0	1	Blk	0
		Calocybe EAE	4	Bl Gr	0.77,0.70,0.58,0.25	8	Blk	0.83,0.77,0.70,0.58, 0.42, 0.25, 0.17,0.11
Butanol:	1hr	Galic acid	0	0	0	1.	Blk	0.70
Acetic acid:	5 min	Cinnamic acid	0	0	0	1	Blk	0.77
Water:: 20:10:5	10 s	Quercetin	0	0	0	2	Blk	0.77,0.49
		Ascorbic acid	1	Bl	0.57	1	Blk	0.57
		Calocybe EAE	1	Bl	0.78	6	Blk	0.80,0.68,0.64,0.57,0.50,0.41

Note: Spot colour Br= Brown, Blk=Black, Bl=Blue, Gr=Green

cells treated with EAE showed that in 250, 500 and 750, 1000 and 1250 $\mu\text{g/mL}$, the proliferation of cells were $55.86 \pm 4.12a$, $50.56 \pm 3.98b$, $30.45 \pm 3.00c$, $14.45 \pm 2.11d$, and $2.88 \pm 0.98e$, respectively ($P < 0.05$) [as per Duncan analysis ($P = 0.05$ level)] after 24 h (Table 2). The CaSki cells treated with EAE showed that in 250, 500, 750, 1000, 1250 $\mu\text{g/mL}$ concentration, proliferation percentages were $67.00 \pm 5.23a$, $55.56 \pm 4.78b$, $36.00 \pm 3.99c$, $11.00 \pm 1.98d$ and $1.48 \pm 0.56e$, respectively ($P < 0.05$) [as per Duncan analysis ($P = 0.05$ level)] in 24 h (Table 2). The percentage of inhibition was further calculated. The comparative analysis of the effect of maximum concentration 1250 $\mu\text{g/mL}$ of EAE showed $97.12 \pm 10.01a$ and $98.52 \pm 10.08a$ percentages of growth inhibition associated with HeLa and CaSki cells, respectively, after 24 h; the IC_{50} values of this extract against HeLa and CaSki were 506 ± 15.20 $\mu\text{g/mL}$ and 520 ± 18.32 $\mu\text{g/mL}$, respectively (Table 2). Moreover, at maximum concentration (1250 $\mu\text{g/mL}$), EAE showed no cytotoxicity on normal lymphocytes.

Studying the induction of apoptosis by gene expression in Western blotting assay

We found that treatment with EAE (500 $\mu\text{g/mL}$) reduced the expression of gene Bcl-2 (Figure 3) while increasing the expression of pro-apoptotic genes caspase 3 and caspase 9 (Figure

3) in both HeLa and CaSki cells in vitro. Moreover, p53 gene was upregulated by EAE. Using western blotting, it was observed that the levels of p53 protein and its downstream target proteins were upregulated; whereas, the levels of another antiapoptotic protein, Bcl-2, decreased (Figure 3).

P53 expression by immunostaining in CaSki cell line

Figure 4 shows that increasing EAE concentration from 250 to 750 $\mu\text{g/mL}$ increased the fluorescence intensity (Figures 4B, C and D) of p53 protein expression. The color intensity was 50 in the control (negative) and 80, 100, and 200 in treatment with EAE 250, 500, and 750 $\mu\text{g/mL}$, respectively (Figure 4E). This implies that EAE product increased about four times the expression of p53 gene/protein ($***P < 0.001$).

Cell cycle arrest

Table 3 shows that under 500 $\mu\text{g/mL}$ concentration of EAE, HeLa and CaSki cells were arrested (17.00 ± 0.75 and 27.75 ± 1.89 ($P < 0.05$), respectively) at G2/M stage; whereas in control, it was minimal (05.90 ± 0.55 and 10.34 ± 0.99 ($P < 0.05$), respectively).

Anticolonization by EAE in HeLa cells

We examined the capacity of EAE to inhibit

the colony formation of HeLa and CaSki cells on six-well plates. The colony numbers of CaSki on agar plate in control, 500, and 750 $\mu\text{g/mL}$ EAE were 120 ± 9.0 , 57 ± 5.0 , and 1.5 ± 0.5 colony/Sq. inch, respectively. The percentages of colony efficiency (PCE) were 100, 47, and 4.1 %, respectively (Figures 5 A, B, C and Table 4). However, in the case of HeLa, colony numbers were 96 ± 8.0 , 30 ± 3.5 , and 2.6 ± 1.0 colony/Sq. inch at control, 500 and 750 $\mu\text{g/mL}$, while the percentages of colony efficiency (PCE) were 100, 31.25, and 2.7%, respectively (Figures 5D, E, F and Table 4).

Antimigration by EAE against HeLa and CaSki cells by wound healing /scratch assay

Cell migration is an important property of cancer cells. The effect of EAE on cell migration was examined by a wound healing or scratch assay in vitro. The migration of cells was photographed and presented in figure 6. Cells in the control (untreated/complete medium) filled the scratched space following an overnight culture, while treatment with EAE reduced the cell migration of both CaSki and HeLa cells as evidenced by the wider open space left between the two sides of the cells (Figures 6 A, B, C, and D). The inhibitory effect of EAE on cell migration was dose-dependent. The cell migration rate in the treated group was calculated comparing with the control (untreated) group. The data presented in the bar diagrams (Figure 6E) showed that HeLa cells migrated $25\pm 1.24\%$, while CaSki cells migrated $70\pm 2.78\%$ ($P < 0.05$) at 500 $\mu\text{g/mL}$ of EAE. This indicates that EAE was more effective in inhibiting the migration of HeLa compared with CaSki cells.

Antioxidant content

Ascorbic acid, flavonoid, and total phenolic contents in EAE

The EAE showed that it contained high ascorbic acid content, i.e. 4.09 ± 0.98 mg/g dry weight of extract. As major naturally occurring anticancer compounds, flavonoids comprise a large group of phenolic compounds with antioxidant activity. This EAE showed that it

contained high flavonoid, i.e. 10.24 mg/g dry weight of extract. Phenolic contents were the major naturally occurring antioxidant components found in the mushrooms. The total phenolic content was expressed as mg of GAEs mg/g of dry weight of extract. The phenolic content of EAE was 15.45 GAEs mg/g of dry weight of extract (Table 5), indicative of its good radical scavenging ability.

Antioxidant activity assay

DPPH assay

The DPPH percentage related to the inhibition capacity of EAE was compared to standard pure compound ascorbic acid and BHA and also measured against 0.1 mM DPPH free radical. The results presented in table 6 show that at all concentrations (50-150 $\mu\text{g/mL}$), EAE was able to scavenge the radical, with the inhibition percentages being 20.56 ± 3.99 , 47.45 ± 6.78 , 61.95 ± 7.19 , 70.42 ± 7.95 , and 86.23 ± 9.67 , respectively. In highest concentration (150 $\mu\text{g/mL}$), percentages of the inhibition of standard ascorbic acid and BHA were 88.56 ± 10.00 and 90.15 ± 11.41 , respectively. The calculated EC50 values of EAE, ascorbic acid, and BHA were 70.12 ± 10.56 , 58.36 ± 7.45 , and 43.78 ± 6.34 , respectively (Table 6).

Analytical TLC of EAE

The analytical TLC showed (Table 7) that in the EAE of *C. indica*, when run on chloroform: Methanol: Acetic Acid: 90:10:1 solvent system, four spots were visualized under UV 366 nm with R_f values of 0.89, 0.85, 0.80 and 0.65, along with seven spots under UV 254 nm with R_f values of 0.89, 0.85, 0.80, 0.65, 0.32, 0.15, and 0.07. The common spots with their R_f values were 0.89, 0.85, 0.80, and 0.65. Therefore, seven spots or compounds were present in the EAE of this mushroom. Four standard compounds (gallic acid, cinnamic acid, quercetin, and ascorbic acid) were used along with the sample in this solvent system. Among the compounds, cinnamic acid gave one spot with an R_f value of 0.65 under both UV light, matching perfectly with one spot of one compound of EAE (R_f 0.65). Therefore, we may

note that of the seven compounds, one may be cinnamic acid. When EAE and standard compounds were run in chloroform: ethyl acetate: acetic acid:: 50:50:1 system, it gave four spots under UV 366 nm with R_f values of 0.77, 0.70, 0.58, and 0.25 and eight spots under UV 254 nm with R_f values of 0.77, 0.70, 0.58, 0.25, 0.83, 0.42, 0.17, and 0.11. The common spots with their R_f values were 0.77, 0.70, 0.58, and 0.25. Therefore, eight spots or compounds were present in the EAE of this mushroom. We ran four standard compounds (gallic acid, cinnamic acid, quercetin and ascorbic acid) along the sample in this solvent system. Of these compounds, gallic acid gave one spot with an R_f value of 0.17 under UV 254 nm light, matching perfectly with one spot of one compound of EAE (R_f 0.17). The R_f value of quercetin was 0.40, while one spot of one compound of EAE (R_f) (0.42 under 254 nm UV) was close to it. Therefore, EAE also may contain quercetin and gallic acid. The data presented in table 7 also show that seven spots (one in UV 366 nm and six in 254 nm light) with R_f values of 0.78, 0.80, 0.68, 0.64, 0.57, 0.50, and 0.41 were present in EAE in butanol: acetic acid: water:: 20:10:5 solvent system. Here, the R_f value of ascorbic acid was 0.57, matching exactly with one spot of EAE under 254 nm UV light. Under these three solvent systems, EAE of *C. indica* showed eight compounds(spots) and four compounds, which might be cinnamic acid, gallic acid, quercetin, and ascorbic acid.

Discussion

In the present study, the cytotoxicity assay revealed that maximum 1250 $\mu\text{g/ml}$ of EAE showed 97.12 ± 10.01 and 98.52 ± 10.08 growth inhibition percentage in HeLa and CaSki cells, respectively, after 24 h; also, the IC_{50} values of this extract against HeLa and CaSki were 506 ± 15.20 $\mu\text{g/mL}$ and 520 ± 18.32 $\mu\text{g/mL}$, respectively. Apoptosis evaluation showed that exposure of HeLa and CaSki cells to different doses of EAE for 24 h resulted in increased apoptosis rates. This was supported by cell cycle arrest at G2/M point and validated by our gene expression data which exhibited down regulated

antiapoptotic genes Bcl-2 and upregulated proapoptotic genes, caspase 9, and tumor suppressor p53 gene. The yield of ethyl acetate (EAE) of *C. indica* was 1%, but in line with the results presented by Wang et al.,³³ which yielded 6%; however, their sample was *Pleurotus ferulae*. Few studies have been done on in vitro anticancer effect of EAE of *C. indica*. The morphology changes of cancer cells on exposure to any chemical are the primary indication of its anticancer properties. In this experiment, we observed cell morphological changes from normal spindle or elongated shape to round or irregular, and reduced confluency levels as seen from phase contrast microscopy analysis in both HeLa and CaSki.

Similarly, Jedinak and Sliva reported *P. ostreatus* changed the morphology of MCF-7 and HT-29 cells, but the mushroom extract was the methanolic extract of the fruiting body.³⁴ In our previous study, we found that ethanolic extract and methanolic extract of *A. bisporus* changed the cell morphology of CaSki cell line from normal elongated to round.³⁵ So far, literature survey has shown that ethanol extract (EE) of *C. indica* induces apoptosis in T24 cell line,³⁶ and its methanolic extract triggers the apoptosis of lung carcinoma (A549) cell line.³⁷ Ghosh²⁴ reported that the extracts (WE and ME) of *C. indica* were anti-proliferative against sarcoma and breast cancer cell lines, and other spp of edible mushrooms such as *P. ostreatus* suppressed the proliferation of breast cancer (MCF-7, MDA-MB-231) and colon cancer (HT-29, HCT-116) cells by its methanolic extract.³⁴ Nisha and Kumuthakalavalli recorded that the IC_{50} value of the EAE of *C. indica* to A549 lung cancer cell line was 4.9 $\mu\text{g/mL}$.³⁷ Apoptosis profile of *C. indica* was shown to be 80.13%, and maximum rate of inhibition (54%) was observed in S phase. The nucleus condensation, fragmentation, and DNA cleavage are indicative of apoptosis.³⁸ In our experiments, DAPI staining showed nuclear condensation and fragmentation in both CaSki and HeLa under treatment by EAE. Induction of apoptosis is suggested to be one of the major action mechanisms of chemotherapeutic anticancer

drugs on malignant cells.³⁹ Wang et al.³³ also observed that EE of *P. ferulae* induced apoptosis by caspase 3 activity, by reduction of mitochondrial membrane potential (MMP), cell cycle progression, migration, and gene expression by EE of *P. ferulae*. Xu reported the effects of EAE of WBM (white button mushroom) against four human cancer cell lines such as MCF 7 (breast cancer), H1299 (lung cancer), LNCaP (prostate cancer).⁴⁰ There are two types of death pathways in apoptosis: the extrinsic pathway and the intrinsic mitochondria-mediated pathway. The latter triggers the release of proapoptotic mitochondrial proteins into the cytosol, thereby activating caspase-dependent and caspase independent pathways and inducing cell apoptosis and necrosis. In this study, we observed that the increased concentration of EAE augmented apoptotic rate in vitro. Liu and Zhong reported that ganoderic acids isolated from *Ganoderma lucidum* triggered the apoptosis of HeLa cells.⁴¹ Our findings showed that the anticancer bioactivity of EAE of *C. indica* was mediated by induced apoptosis. This may be through ROS formation and MMP reduction as previous authors reported that mushroom products generated ROS and reduced mitochondrial membrane potential triggering apoptosis.⁴⁰ The mechanisms behind the anticancer activity were found to be the downregulation of antiapoptotic genes Bcl-2 and upregulation of proapoptotic genes caspase 3, caspase 9, and tumor suppressor p53 gene, which is one of the important molecules involved in apoptosis induction. Our microscopic immunostaining experiment of p53 showed that increase in the dose of EAE against CaSki cell line increased the intensity or expression of p53 compared with control (negative). Many studies have raised the possibility that cells lacking p53 activity due to mutation might be more resistant to cancer chemotherapy.^{42,43} It was very interesting to note that in our cell cycle experiment, EAE arrested cell cycle at G2/M checkpoint of both HeLa cells and CaSki. Similarly, other scientists have observed that the methanolic extract of mycocomplex,⁴⁴ *Ganoderma lucidum* in prostate,⁴⁵ hepatoma,⁴⁶ and bladder cancer cells⁴⁷

induce significant cell cycle arrest at G2/M phase. Moreover, cell cycle arrest at G2/M was induced by *A. blazei* in gastric epithelial cells,⁴⁸ or by cordycepin isolated from *Cordyceps militaris* in colon cancer cells;⁴⁹ whereas, ethanolic extract of *P. ferulae* arrested the cell cycle of melanoma cancer B16F10 cells at G0/G1.^{33,34} On the other hand, Dudhgaonkar et al.⁵⁰ isolated triterpenes from *G. lucidum* and observed that they induced cell cycle arrest at G0/G1 and G2/M phase in macrophages; therefore, it is suggested that cell cycle arrest depends on the specific biologically active compounds as well as particular cells.

Colony formation and migration of cancer cells are two important hallmarks of metastasis. Cologenic assay and antimigration assay against both cell lines revealed that the EAE of *C. indica* had antimetastasis property as this extract inhibited the colony formation and migration of both cell lines. Ganoderic acids from *G. lucidum* demonstrated inhibition on the colony formation of invasive breast cancer cells.⁵¹ *Pleurotus ostreatus* (aqueous extract) exhibited a reduction in the number of colonies of COLO-205 (oral cancer) cells with 43.8±3.5% in contrast to 100% proliferation of untreated cells.⁵²

Many authors have suggested that mushrooms rich in antioxidant activity play an important role in cancer prevention.^{53,54} Xu proposed phenolic compounds to be the major effective anticancer compounds.⁴⁰ Our antioxidant content determination experiment showed that EAE contains total phenolic compounds such as 10.05±2.45 mg GAE/g extract, which is lower than the earlier report of Xu; however,⁴⁰ other antioxidant flavonoids and ascorbic acids were also present in high amounts. Our TLC analysis also validated the presence of phenolic compounds, flavonoids, and ascorbic acids. Keleş et al.⁵⁵ recorded the total phenolic content of *A. bisporous* ethanolic extract to be 4.020 mg/g. Flavonoids have been shown to be highly effective scavengers of most types of oxidizing molecules, including singlet oxygen and various free radicals.⁵⁶ Flavonoids are able to alter peroxidation kinetics and reduce the fluidity of membranes.⁵⁷ Similarly Keleş, et al.⁵⁵ reported 67.86% of the

scavenging capacity of this mushroom (concentration 25 mg/mL of ethanolic extract) along with other mushrooms with more or less capacity. Using ascorbic acid as standard, the antioxidant potential of *Calocybe indica* in terms of DPPH radical scavenging assay was evolved as 60% and hydroxyl radical scavenging activity as 66%.³⁷ In our experiment, scavenging capacity of EAE of this mushroom (concentration 150 µg/mL) was 86.23%, which is far better than earlier reports. The phenolic compounds are also responsible for cell cycle arrest during anticancer effects as studied by other researchers.⁵⁸⁻⁶¹ Our TLC analysis of the EAE of *C. indica* exhibited some active compounds, including quercetin, gallic acid, and ascorbic acids. We propose that these phenolic compounds may play a role in the inhibition of cervical cancer cell growth by inducing apoptosis and arresting the cell cycles of both HeLa and CaSki at G2/M stage.

In conclusion, EAE of edible mushroom *C. indica* inhibits proliferation, induces apoptosis, arrests cell cycle at G2/M point of HeLa and CaSki cells, and has antimetastatic properties. The good amount of antioxidant content (phenolic compounds, flavonoids and ascorbic acid) and activity of EAE further supported its anticervical activity. The mycochemistry analysis of EAE by TLC indicated the presence of eight compounds, including phenolic compounds such as gallic acid, cinnamic acid, quercetin, and acetic acid. Our work suggests that *C. indica* mushroom may be a novel promising agent for the treatment of cervical cancer. Our work is probably the first in medical literature to focus on the anticervical cancer potential of EAE of *Calocybe indica*. In the future, more analytical chemistry studies of EAE will probably identify a novel class of mushroom constituent with broad-spectrum antitumor activity for cervical cancer management.

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Conflict of Interest

None declared.

Reference

1. Li GX, Hu H, Jiang C, Schuster T, Lü J. Differential involvement of reactive oxygen species in apoptosis induced by two classes of selenium compounds in human prostate cancer cells. *Int J Cancer*. 2007; 120(9):2034-43. doi: 10.1002/ijc.22480.
2. Lucas EH, Ringler RL, Stevens JA, Clarke DA, Stock CC. Tumor inhibitors in *Boletus edulis* and other *Holobasidiomycetes*. *Antibiot (Northfield)*. 1957; 7(1):1-4.
3. Ghosh SK. Cancer and its remedy by mushrooms. In: Chanda I; Rakshit A, editors. *Biotechnology in human Welfare* Memari, Burdwan, Kolkata: Avenel Press; 2018. p. 29-38.
4. Ghosh SK, Chakrabarty AM. Bioactive compounds of mushrooms as immune enhancer in human against cancer: an emerging avenue for immunotherapy. In: De D, Roy S, Bera GC, editors. *Biotechnology and nature*. Kolkata: Kabitika; 2018. p. 260.
5. Akgul H, Sevindik M, Coban C, Alli H, Selamoglu Z. New approaches in traditional and complementary alternative medicine practices: *Auricularia auricula* and *Trametes versicolor*. *J Tradit Med Clin Natur*. 2017; 6(239):2. doi: 10.4172/2573-4555.1000239.
6. Aly AH, Debbab A, Proksch P. Fifty years of drug discovery from fungi. *Fungal Divers*. 2011; 50(1):3. doi: 10.1007/s13225-011-0116-y.
7. Wasser SP. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl Microbiol Biot*. 2002; 60(3):258-74. doi:10.1007/s00253-002-1076-7.
8. Mizuno T. The extraction and development of antitumor-active polysaccharides from medicinal mushrooms in Japan. *Int J Med Mushrooms*. 1999; 1(1). doi: 10.1615/IntJMedMushrooms.v1.i1.20.
9. Mo S, Wang S, Zhou G, Yang Y, Li Y, Chen X, et al. Phelligradins 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(5):823-8. doi:10.1021/np030505d.
10. Wasser SP. Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. *Appl Microbiol Biot*. 2011; 89(5):1323-32. doi: 10.1007/s00253-010-3067-4.
11. Yamasaki KI, Sone S, Yamashita T, Ogura T. Synergistic induction of lymphokine (IL-2)-activated killer activity by IL-2 and the polysaccharide lentinan, and therapy of spontaneous pulmonary metastases. *Cancer Immunol Immunother*. 1989; 29(2):87-92. doi:10.1007/BF00199282.
12. Liu K, Wang JL, Gong WZ, Xiao X, Wang Q. Antioxidant activities in vitro of ethanol extract and

- fractions from mushroom, *Lenzites betulina*. *J Food Biochem*. 2013; 37(6):687-93.
13. Ivanova TS, Krupodorova TA, Barshteyn VY, Artamonova AB, Shlyakhovenko VA. Anticancer substances of mushroom origin. *Exp Oncol*. 2014; 36(2):58-66.
 14. Wu HT, Lu FH, Su YC, Ou HY, Hung HC, Wu JS, et al. In vivo and in vitro anti-tumor effects of fungal extracts. *Molecules*. 2014;19(2):2546-56. doi: 10.3390/molecules19022546.
 15. Fan MJ, Lin YC, Shih HD, Yang JS, Liu KC, Yang ST, et al. Crude extracts of *Agaricus brasiliensis* induce apoptosis in human oral cancer CAL 27 cells through a mitochondria-dependent pathway. *In Vivo*. 2011; 25(3):355-66.
 16. Ajith TA, Janardhanan KK. Indian medicinal mushrooms as a source of antioxidant and antitumor agents. *J Clin Biochem Nutr*. 2007; 40(3):157-62. doi: 10.3164/jcbn.40.157.
 17. Bruni L, Barrionuevo-Rosas L, Serrano B, Brostons M, Albergo G, Osano R, et al. Human papillomavirus and related diseases in India. ICO Information Centre on HPV and Cancer (HPV information center), Summary Report. 2014.
 18. Sreedevi A, Javed R, Dinesh A. Epidemiology of cervical cancer with special focus on India. *Int J Women's Health*. 2015; 7:405. doi: 10.2147/IJWH.S50001.
 19. Purkayastha R P, Chandra A. Manual of Indian edible mushrooms today & tomorrow'. New Delhi, India: Printer and Publishers; 1985.p.267.
 20. Watling R. Identification of the larger fungi. Edinburgh: Hulton Educational Publications Ltd.; 1973. p. 60-65.
 21. Pacioni G, Lincoff GH. Simon & Schuster's guide to mushrooms; 1981.p. 69-71.
 22. Freshney RI. Culture of animal cells. A manual for basic technique and specialized application. 7th ed. New York: Wiley-Blackwell; 2017.p. 728.
 23. Akiyama H, Endo M, Matsui T, Katsuda I, Emi N, Kawamoto Y, et al. Agaritine from *Agaricus blazei* Murrill induces apoptosis in the leukemic cell line U937. *Biochim Biophys Acta*. 2011; 1810(5):519-25. doi: 10.1016/j.bbagen.2011.02.010.
 24. Ghosh SK. Study of anticancer effect of *Calocybe indica* mushroom on breast cancer cell line and human Ewings sarcoma cancer cell lines. *NY Sci J*. 2015; 8(5):10-5.
 25. Lee SJ, Kim SK, Choi WS, Kim WJ, Moon SK. Cordycepin causes p21WAF1-mediated G2/M cell-cycle arrest by regulating c-Jun N-terminal kinase activation in human bladder cancer cells. *Arch Biochem Biophys*. 2009; 490(2):103-9. doi: 10.1016/j.abb.2009.09.001.
 26. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Amer J Enol Viticult*. 1965;16(3):144-58.
 27. Olajire AA, Azeez L. Total antioxidant activity, phenolic, flavonoid and ascorbic acid contents of Nigerian vegetables. *Afr J Food Sci Technol*. 2011;2(2):22-9.
 28. Klein BP, Perry AK. Ascorbic acid and vitamin A activity in selected vegetables from different geographical areas of the United States. *J Food Sci*. 1982; 47(3):941-5. doi.org/10.1111/j.1365-2621.1982.tb12750.x.
 29. Jagadish LK, Krishnan VV, Shenbhagaraman R, Kaviyaran V. Comparative study on the antioxidant, anticancer and antimicrobial property of *Agaricus bisporus* (JE Lange) Imbach before and after boiling. *Afr J Biotechnol*. 2009; 8(4). doi:10.5897/AJB2009.000-9111.
 30. Chiou A, Karathanos VT, Mylona A, Salta FN, Preventi F, Andrikopoulos NK. Currants (*Vitis vinifera* L.) content of simple phenolics and antioxidant activity. *Food Chem*. 2007; 102(2):516-22. doi.org/10.1016/j.foodchem.2006.06.009.
 31. Lihua G, Tao W, Zhengtao W. TLC bioautography-guided isolation of antioxidants from fruit of *Perillafrutescens* var. *acuta*. *LWT-Food Sci Technol*. 2009;42:131-6. doi.org/10.1016/j.lwt.2008.04.006.
 32. Amarowicz R, Kozłowska H, Shimoyamada M, Okubo K. Chromatographic analysis of rapeseed glucoside fractions. *Pol J Food Nutr Sci*. 1992;1(01).
 33. Wang W, Chen K, Liu Q, Johnston N, Ma Z, Zhang F, et al. Suppression of tumor growth by *Pleurotus ferulae* ethanol extract through induction of cell apoptosis, and inhibition of cell proliferation and migration. *PLoS One*. 2014;9(7). doi.org/10.1371/journal.pone.0102673
 34. Jedinak A, Sliva D. *Pleurotus ostreatus* inhibits proliferation of human breast and colon cancer cells through p53-dependent as well as p53-independent pathway. *Int J Oncol*. 2008; 33(6):1307-13.
 35. Ghosh SK, Sanyal T, Chakrabarty A. Antiproliferative and apoptotic effect of ethanolic and methanolic extract of edible mushroom *Agaricus bisporus* against CaSki cell line of cervical cancer. *Plant Cell Biot Mol Biol*. 2018; 19(7&8):372-82.
 36. Selvi S, Umadevi P, Murugan S, Senapathy JG. Anticancer potential evoked by *Pleurotus florida* and *Calocybe indica* using T 24 urinary bladder cancer cell line. *Afr J Biotechnol*. 2011; 10(37):7279-85. doi: 10.5897/AJB10.1750.
 37. Nisha AP, Kumuthakalavalli R. Anticancer activity of milky mushroom (*Calocybe indica* Var. *Apk2*) against A549 human lung cancer cell line studies. *Int J Pharm Bio Sci*. 2016;7(3):73-80.
 38. Kerr JF, Wyllie AH, Currie AR. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 1972;26:239-57. doi:10.1038/bjc.1972.33.
 39. Evan GI, Vousden KH. Proliferation, cell cycle and

- apoptosis in cancer. *Nature*. 2001; 411(6835):342. doi:10.1038/35077213.
40. Xu T. Anti-cancer effects of phenolic-rich extracts of button mushrooms (*Agaricus bisporus*). [dissertation] Pennsylvania State University, USA; 2013.147 p.
 41. Liu RM, Zhong JJ. Ganoderic acid Mf and S induce mitochondria mediated apoptosis in human cervical carcinoma HeLa cells. *Phytomedicine*. 2011; 18(5):349-55. doi: 10.1016/j.phymed.2010.08.019.
 42. López-Lázaro M. Dual role of hydrogen peroxide in cancer: possible relevance to cancer chemoprevention and therapy. *Cancer Lett*. 2007; 252(1):1-8. doi:10.1016/j.canlet.2006.10.029.
 43. Fisher DE. Apoptosis in cancer therapy: Crossing the threshold. *Cell*. 1994; 78:539-42. doi:10.1016/0092-8674(94)90518-5.
 44. Jiang J, Sliva D. Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells. *Int J Oncol*. 2010;37(6):1529-36. doi: 10.3892/ijo_00000806.
 45. Jiang J, Slivova V, Valachovicova T, Harvey K, Sliva D. *Ganoderma lucidum* inhibits proliferation and induces apoptosis in human prostate cancer cells PC-3. *Int J Oncol*. 2004; 24(5):1093-9.
 46. Lin SB, Li CH, Lee SS, Kan LS. Triterpene-enriched extracts from *Ganoderma lucidum* inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. *Life Sci*. 2003;72(21):2381-90. doi:10.1016/s0024-3205(03)00124-3.
 47. Lu QY, Jin YS, Zhang Q, Zhang Z, Heber D, Go VL, et al. *Ganoderma lucidum* extracts inhibit growth and induce actin polymerization in bladder cancer cells in vitro. *Cancer Lett*. 2004;216(1):9-20. doi:10.1016/j.canlet.2004.06.022.
 48. Jin CY, Choi YH, Moon DO, Park C, Park YM, Jeong SC, et al. Induction of G2/M arrest and apoptosis in human gastric epithelial AGS cells by aqueous extract of *Agaricus blazei*. *Oncol Rep*. 2006;16(6):1349-55.
 49. Lee SJ, Moon GS, Jung KH, Kim WJ, Moon SK. c-Jun N-terminal kinase 1 is required for cordycepin-mediated induction of G2/M cell-cycle arrest via p21WAF1 expression in human colon cancer cells. *Food Chem Toxicol*. 2010; 48:277-83. doi: 10.1016/j.fct.2009.09.042.
 50. Dudhgaonkar S, Thyagarajan A, Sliva D. Suppression of the inflammatory response by triterpenes isolated from the mushroom *Ganoderma lucidum*. *Int Immunopharmacol*. 2009;9(11):1272-80. doi: 10.1016/j.intimp.2009.07.011.
 51. Jiang J, Grieb B, Thyagarajan A, Sliva D. Ganoderic acids suppress growth and invasive behavior of breast cancer cells by modulating AP-1 and NF- κ B signaling. *Int J Mol Med*. 2008; 21(5):577-84.
 52. Arora S, Tandon S. Mushroom extracts induce human colon cancer cell (COLO-205) death by triggering the mitochondrial apoptosis pathway and Go/G1-phase cell cycle arrest. *Arch Iran Med*. 2015;18(5): 284-295. doi: 0151805/AIM.006.
 53. Wasser SP, Weis AL. Therapeutic effects of substances occurring in higher Basidiomycetes mushrooms: a modern perspective. *Crit Rev Immunol*. 1999;19(1).
 54. Kidd PM. The use of mushroom glucans and proteoglycans in cancer treatment. *Altern Med Rev*. 2000;5(1):4-27.
 55. Keleş A, Koca I, Genççelep H. Antioxidant properties of wild edible mushrooms. *J Food Process Technol*. 2011;2(6):2-6. doi: 10.4172/2157-7110.1000130.
 56. Ferreira IC, Barros L, Abreu R. Antioxidants in wild mushrooms. *Curr Med Chem*. 2009; 16(12):1543-60. doi:10.2174/092986709787909587.
 57. Arora A, Byrem TM, Nair MG, Strasburg GM. Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. *Arch Biochem Biophys*. 2000; 373(1):102-9. doi:10.1006/abbi.1999.1525.
 58. Zi X, Grasso AW, Kung HJ, Agarwal R. A flavonoid antioxidant, silymarin, inhibits activation of erbB1 signaling and induces cyclin-dependent kinase inhibitors, G1 arrest, and anticarcinogenic effects in human prostate carcinoma DU145 cells. *Cancer Res*. 1998;58(9):1920-9.
 59. Sahu RP, Batra S, Srivastava SK. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *Br J Cancer*. 2009;100:1425-33. doi: 10.1038/sj.bjc.6605039.
 60. Casagrande F, Darbon JM. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: regulation of cyclin-dependent kinases CDK2 and CDK1. *Biochem Pharmacol*. 2001; 61(10):1205-15. doi:10.1016/s0006-2952(01)00583-4.
 61. Ren L. Anticancer ability of mushroom polysaccharides. [dissertation] The University of Auckland, Symonds Street, Auckland 1142, New Zealand; 2014.283 p.