

ANTIPROLIFERATIVE AND APOPTOTIC EFFECT OF ETHANOLIC EXTRACT OF *CALOCYBE INDICA* ON PANC-1 AND MIAPaCa2 CELL LINES OF PANCREATIC CANCER

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Background: Pancreatic cancer is an aggressive malignancy with poor prognosis. New options for its treatment including the use of the substances of natural origin are now on the agenda. **Aim:** An attempt has been taken to examine the effect of ethanolic extract (EE) of edible mushroom *Calocybe indica* on PANC-1 and MIAPaCa2 cell lines of pancreatic cancer *in vitro*. **Materials and Methods:** Study of cell morphology, evaluation of apoptosis by DAPI staining under fluorescence microscope, anti-proliferative study by MTT assay, lactate dehydrogenase assay, pro-apoptotic and anti-apoptotic protein detection by Western blotting were performed to test effects of *Calocybe indica* EE on the cells of these two lines. Effect on *in vitro* migration was studied by scratch method. **Results:** Both cell lines treated with 250 or 500 µg/mL of EE underwent morphological changes — became more round and shrunken, with membrane blebbing and the reduced cell confluence. The nuclei of treated cells became condensed and fragmented. The percentage of apoptotic cells increased as EE concentration increased from 100 µg/mL to 500 µg/mL. Anti-proliferative effect was recorded at all concentrations within 100–750 µg/mL. The effect EE on lactate dehydrogenase leakage was concentration-dependent in both cell lines. Western blotting showed that caspase-3 and -9, and p53 protein levels were increased and Bcl2 protein was decreased after treatment of the cells with EE compared to the control. PANC-1 cell migration was inhibited by $80.12 \pm 4.25\%$ after 24 h treatment of the cells with 500 µg/mL EE compared to the control. **Conclusions:** EE of *Calocybe indica* inhibits the growth and induces apoptosis in pancreatic cancer cell lines *in vitro*.

Key Words: ethanolic extract, anticancer agent, PANC-1, MIAPaCa2, apoptosis, cytotoxicity.

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Pancreatic cancer is one of the leading causes of cancer death worldwide, with 232,000 new cases and 213,000 deaths reported each year. Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States. Although pancreatic cancer is relatively common in Western countries, the epidemiological data demonstrated that the incidence of pancreatic cancer is 0.5–2.4 per 100,000 men and 0.2–1.8 per 100,000 women in most parts of India [1]. As per the data of Cancer Facts and Figures 2019 [2], the incidence rate of pancreatic cancer increases about 1% per year, and the survival rate is very poor (5–6%) in the first 5 years. As pancreatic cancer shows no symptoms, its early detection and diagnosis is not possible [3]. Moreover, due to its very rapid growth, spreading to distant organs, and resistance to most therapies, the management of this cancer is now extremely challenging.

Gemcitabine has been used as standard chemotherapy for pancreatic cancer [4], but most clinical trials have failed to demonstrate markedly improved oncologic outcomes [5]. Folfirinox is an emerging chemotherapeutic agent for metastatic pancreatic cancer treatment [6]. Scientists are now engaged in searching for formulation of new drugs from natural products of plants, bacteria, fungi, animals or other sources. Cancer mycotherapy is an emerging medical field, which deals with anticancer agents originated from

mushrooms. More than 50 mushroom species have already exhibited potential anticancer activity *in vitro* or in animal models and six substances have been tested clinically [7].

Calocybe indica is a mushroom commonly grown in wild and cultivated in West Bengal province of India. Earlier, we demonstrated anticancer effect of *Calocybe indica* extract in breast cancer and Ewing's sarcoma cell lines [8]. Although the antiproliferative activity of the extracts from a few mushrooms has been assayed in human pancreatic cancer cells [9, 10], *Calocybe indica* extract has not been yet studied for this purpose. Therefore, an attempt has been taken to examine the effect of ethanolic extract (EE) of edible mushroom *Calocybe indica* on PANC-1 and MIAPaCa2 cell lines of pancreatic cancer *in vitro*.

MATERIALS AND METHODS

Mushroom collection and extraction. The cultivated basidiocarps of *Calocybe indica* were collected in July 2018 from Rahara market, District-24-Parganas (S), India and carried to laboratory for their identification. The young fruit bodies of the mushroom whose caps were unopened were air dried in oven at 50 °C for 48 h, chopped into pieces and then grinded into powder. 15 g of dried mushroom powder was extracted for 3 days in 150 mL of 90% ethanol at room temperature and filtered through Whatman No 4 and Whatman No 1. The ethanol was removed from the extract using a rotary vacuum evaporator at 40 °C, and the remaining solvent was removed with a freeze-drier. The powder of EE of *C. indica* was kept in airtight condition in refrigerator at 4 °C. Extract

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Abbreviations used: DAPI – 4',6-diamidino-2-phenylindole; EE – ethanolic extract; LDH – lactate dehydrogenase; PBS – phosphate buffered saline.

used for *in vitro* assays was dissolved in Roswell Park Memorial Institute 1640 medium and passed through a 0.22 µm Millipore filter. The prepared EE was further diluted with plain Roswell Park Memorial Institute 1640 medium to certain concentrations just prior to use. The yield of EE of *C. indica* was 3.65 (w/w).

Cell culture. The human pancreatic cancer cell lines (PANC1 and MIAPaCa2) (purchased from NCCS, Pune) were cultured separately in DMEM supplemented with L-glutamine, 10% v/v fetal bovine serum 100 µg/mL streptomycin (Invitrogen, USA) and 250 IU/ml penicillin (Invitrogen, USA), in 75 mm² tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO₂ to the 60–70% confluence. Cells were treated with EE (0–500 µg/mL) and then examined under bright field inverted microscope.

Nuclear morphology and apoptosis evaluation by DAPI staining. 4',6-diamidino-2-phenylindole (DAPI) stain was used to observe nuclear morphology of cells. The cells were washed by cold phosphate-buffered saline (PBS) and fixed with 3.7% (w/v) para-formaldehyde in PBS for 10 min at room temperature. After permeabilization, the cells were stained with a DAPI solution (10 µg/mL) at 37 °C for 30 min. The stained cells were washed with PBS and observed using an inverted fluorescent microscope (Olympus, Japan), photographs were taken using a Magna-Fire digital camera (Optotronics, USA) for analysis. Apoptosis was evaluated by morphological changes of the nuclear structure. The apoptotic cells were observed and counted under an inverted fluorescent microscope; at least five optical fields were counted containing a total of 200 cells. The percentage of apoptotic cells was calculated by the formula:

percentage of apoptotic cells = (number of apoptotic cells/total cells counted) · 100%.

Cell proliferation/cytotoxicity assay. The effects of EE on cell proliferation of PANC-1 and MIAPaCa2 cell lines were evaluated by using dimethyl thiazolyl tetrazolium bromide (MTT) assay (Sigma, USA) following the method of Mosmann [11] with some modification. Briefly, 1 · 10⁴ cells per well of 96-well culture plate were seeded in fresh DMEM medium, containing 10% FBS and antibiotics, overnight to reach 80% confluence. Then the culture was washed with 10% PBS and treated with different concentrations of EE dissolved in DMEM and incubated at 37 °C in 5% of CO₂ and 95% of air for 24 h. Upon incubation, cells were washed with PBS, and 100 µL of 0.5% MTT solution in Roswell Park Memorial Institute 1640 was added to each well and cultures were further incubated for 3 h, then after discarding the media, 100 µL of DMSO was added for dissolving the crystals. The absorbance in the wells was measured in microplate reader (Bio Rad, USA) at 570 nm.

Growth inhibition rate was determined by the formula:

$$\text{Growth inhibition} = \left(1 - \frac{A_{570 \text{ nm treated cells}}}{A_{570 \text{ nm control cells}}} \right) \cdot 100\%$$

IC₅₀ was calculated by plotting a dose response graph of growth inhibition.

Lactate dehydrogenase (LDH) assay. Cells were seeded and grown separately in 96-well plate in medium containing 10% FBS and incubated for 24 h under 5% CO₂ at 37 °C. Then the cells were washed with PBS, 100 µL of the each concentration of EE was added separately to the wells, and 100 µL of medium was added to the control well and incubated for 24 h. The cells were harvested, and the assay was carried out using the LDH kit (Thermo Fisher Sci. Ltd, USA). 100 µL of sample with working reagent was mixed and incubated for 1 min at 37 °C. The absorbance was measured at 340 nm and results of the assay were calculated according to the manufacture's instruction.

Western blot analysis. PANC-1 and MIAPaCa2 (2 · 10⁵) cells were treated separately with 500 µg/mL of EE for 24 h. After treatment, cells were lysed with radioimmunoprecipitation assay buffer (Abcam, USA). The effect of treatment on the detection of certain cell cycle proteins such as p53, and on pro-apoptotic proteins such as caspase-3, caspase-9, and anti-apoptotic Bcl-2 were determined. Proteins were detected by incubation with the corresponding primary antibodies (Santa Cruz Biotechnology, USA: dilution 1:500). The following monoclonal antibodies were used: anti-caspase-9 (1-2): sc-56073 AC; anti-caspase-3 (E-8): sc-7272AC, anti-p53 (DO-1): sc-126, anti-Bcl-2 (C-2): sc-7382 AC, anti-actin(C4): sc-47778 AC), and HRP-conjugated secondary antibody. The blots were then detected using Luminol (Bio-Rad, USA). The intensity of each blots was measured by Image J.

Cell migration assay by scratch method.

The migration assay was performed by the method of Lee *et al.* [12] with some modifications. Briefly, 5 · 10⁴ of PANC-1 cells per well was cultured in 6-well plate. After reaching 90% confluence, the center of the culture dishes was scratched with 100 µL pipette tip. Then the cells were washed frequently by using PBS and incubated with EE at concentration of 500 µg/mL. After 24 h, image of the cells was taken. The relative migration rate was estimated by measuring the distance the cells migrated for 24 h as compared to the cells cultured without EE. The relative migration rate was calculated.

Statistical analysis. GraphPad Prism 5 (GraphPad Software, Inc., USA) was applied for data analysis, and one-way analysis of variance (ANOVA) was done to compare the differences. The results were represented as means ± standard deviation/standard error from triplicate experiments done in a parallel, unless otherwise indicated. All figures were obtained from at least three independent experiments. *P* < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Effects of EE on cell morphology. PANC-1 cells treated with 250 µg/mL EE lose their normal shape, become shrunken, and cell confluency is reduced. More round, shrunken, dead cells and cell debris were observed in cells treated with 500 µg/mL of EE (Fig. 1, a, b, c). Similar effects were obtained in MIAPaCa2 (Fig. 1, d, e, f).

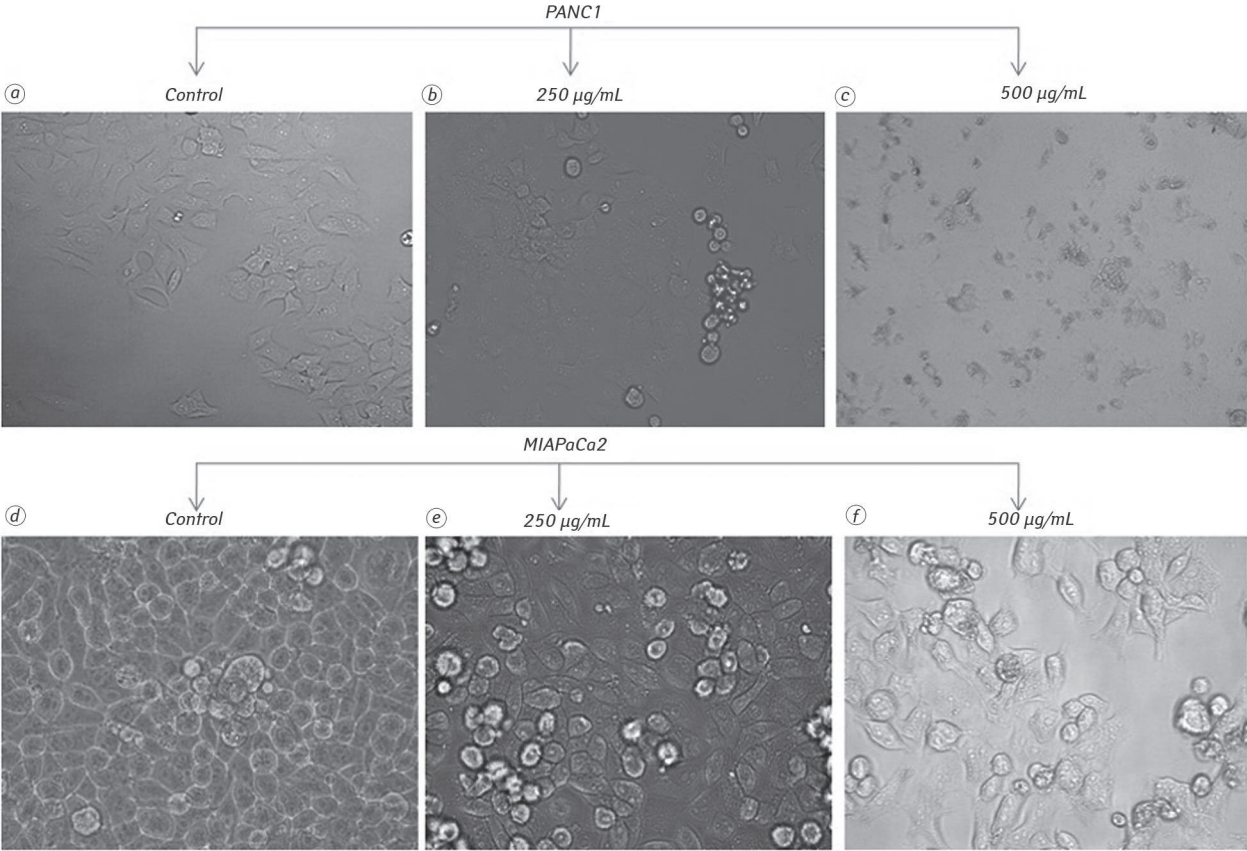


Fig. 1. Morphological changes of PANC-1 (a–c) and MIAPaCa2 (d–f) cells treated with EE for 24 h: a, d — control (negative); b, e — 250 µg/mL; c, f — 500 µg/mL. x 200

Evaluation of apoptosis by DAPI staining. When DAPI-stained cells were examined under fluorescent microscope, nuclei of the cells of negative control appeared normal taking light blue color, and were round and homogeneous, while nuclei of cells treated with EE (250 or 500 µg/mL) were condensed and in few cases irregular and fragmented (Fig. 2, a–d). The percentage of apoptotic cells of PANC-1 and MIAPaCa2 cells

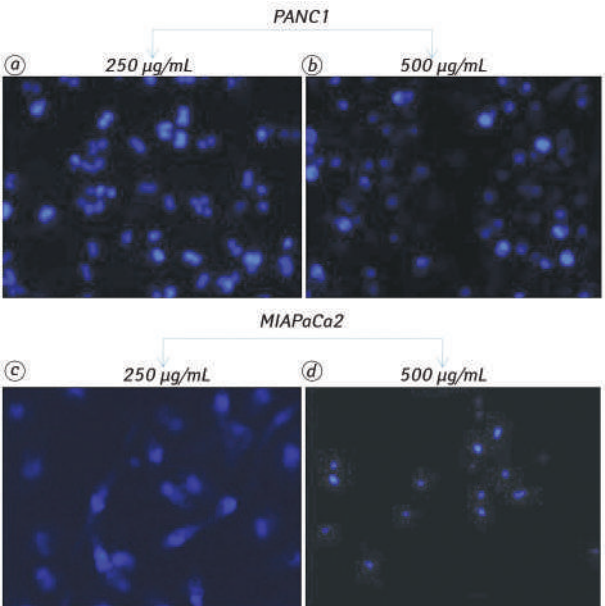


Fig. 2. Morphological changes of nuclei of DAPI-stained PANC-1 (a, b) and MIAPaCa2 (c, d) cells treated with EE for 24 h: a, c — 250 µg/mL; b, d — 500 µg/mL

treated with different concentrations of EE is given in Table 1. All the values are significantly higher than in control cells ($p < 0.05$) and dose dependence may be followed.

Antiproliferative effect of EE against PANC-1 and MIAPaCa2 cells lines. The data presented in Table 2 show that the proliferation of two cell lines was reduced gradually as the concentration of EE increased from 100 µg/mL to 750 µg/mL. Based on these data, IC₅₀ values of EE against PANC-1 and MIAPaCa2 were calculated as 245 µg/mL and 332 µg/mL, respectively.

LDH assay. Fig. 3 demonstrates that LDH leakages from these two cell lines occur in dose-dependent manner. PANC-1 cells were more sensitive to EE (500 µg/mL) than MIAPaCa2 cells.

Table 1. Percentage of apoptotic PANC1 and MiaPaca2 cells upon treatment with EE for 24 h

EE concentration (µg/mL)	% of apoptotic cells ± SE	
	PANC1	MiaPaca2
Control	0.45 ± 0.01	0.35 ± 0.02
100	45.54 ± 3.11	40.12 ± 2.10
250	57.00 ± 3.34	51.56 ± 3.06
500	77.23 ± 4.23	75.32 ± 5.01
Gemcitabine 50 µg/mL	90.12 ± 5.34	88.56 ± 6.00

Table 2. Percentage of growth inhibition of PANC-1 and MIAPaCa2 cell lines by EE upon treatment for 24 h

EE concentration (µg/mL)	% of growth inhibition ± SD	
	PANC-1	MIAPaCa2
100	11.00 ± 1.21	8.22 ± 1.01
250	54.23 ± 6.65	44.68 ± 5.22
500	78.12 ± 7.11	62.78 ± 6.22
750	80.34 ± 8.99	71.23 ± 7.55
Gemcitabine 50 µg/mL	96.12 ± 11.54	90.39 ± 10.30

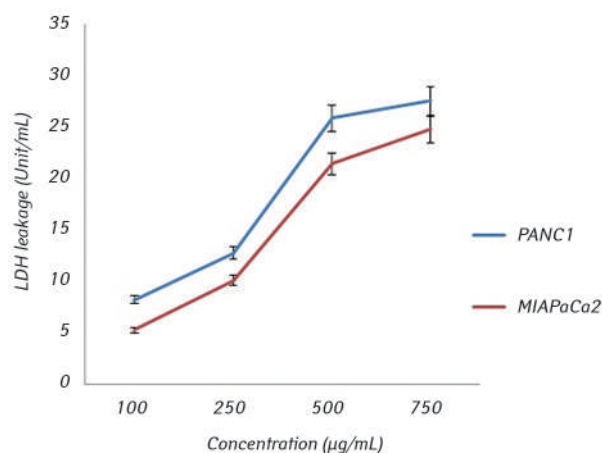


Fig. 3. LDH leakage (unit/ml) from PANC-1 and MIA PaCa2 cells after treatment with EE for 24 h

Expression of apoptosis-related proteins.

Western blot analysis demonstrated the decreased expression of Bcl-2 protein in PANC-1 and MIA PaCa2 cells exposed to EE, while the expression of caspase-3 and caspase-9 as well as p53 increased in both cell lines (Fig. 4, a, b).

Migration inhibition of PANC-1 cells by wound healing/scratch assay. Cell migration is an important property of cancer cells. The effect of EE on cell migration was examined by a wound healing or scratch assay *in vitro*. Untreated PANC-1 cells filled the scratched space (Fig. 5, a) after the overnight culture (Fig. 5, c), while treatment with EE (500 µg/mL) reduced cell migration of PANC-1 cells evidenced by the wider open space left between two sides of the cells (Fig. 5, b). The relative cell migration rate inhibition calculated in comparison with control amounted to $80.12 \pm 4.25\%$ ($p < 0.05$).

DISCUSSION

In our experiments, EE of *C. indica* caused morphological changes of cells, induced apoptosis and inhibited proliferation of PANC-1, MIA PaCa-2 pancreatic cancer cell lines. Although this is the first work on the effect of *C. indica* extract in pancreatic cancer cells, mushroom extract from *Cyathus striatus* was recorded to inhibit the viability of other human pancreatic cell lines such as HPAF-II and PL 45 in low concentration and with short exposure [9]. Cheng *et al.* [13] demonstrated

that mixture of triterpenes extracted from *Poria cocos* and three pure triterpenes such as pachymic acid, dehydropachymic acid and polyporenic acid C acted as anti-proliferative agents against four pancreatic cell lines (PANC-1, MIA PaCa-2, AsPc-1 and BxPc-3). Antiproliferative effect including ballooning (morphologic changes) by MMH01, a compound isolated from *Antrodia cinnamomea* against BXPc3 cell line was also shown [10], while Yu *et al.* [14] showed that antroquinonol, a ubiquinone derivative isolated from the same mushroom, inhibited cell proliferation of PANC-1 and AsPc-1 cells in a dose dependent manner. In this experiment in both cell lines morphological changes like rounding, shrinking, and membrane blebbing (bulbing) and the reduction of cell confluence were noted in 250 and 500 µg/mL of EE. Similar observation were recorded when HeLa cells were treated with 500 µg/mL/750 µg/mL of methanolic extract of *Agaricus bisporus* for 24 h at same conditions [15].

EE also caused leakage of LDH from treated cancer cells as evidenced from our LDH assay experiment. LDH levels measured by Sharvit *et al.* [9] suggested the lack of a necrotic effect of the extract of *Cyathus striatus* in cell line. In our previous work [8], ethanolic, methanolic and aqueous extracts of *Calocybe indica* were recorded as potent anti-cancerous substances against MCF-7 (breast cancer), MHH-ES1 (Ewing's sarcoma) cells. On some other human cancer cell lines, Maiti *et al.* [16] reported antiproliferative action of *C. indica*. Similarly, the extract of this mushroom exhibited potent inhibitory effect against induced inflammation in the experimental mice model [17]. From the work of Rosendahl *et al.* [18], it was also found that polysaccharide K (PSK) from *Coriolus*

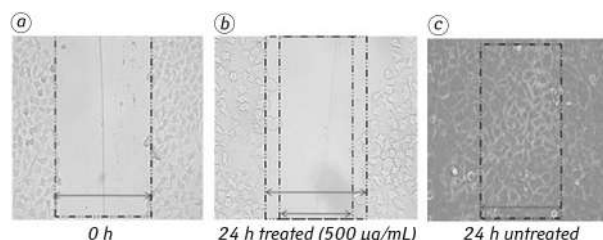


Fig. 5. Inhibition PANC-1 cell migration after treatment with 500 µg/mL of EE for 24 h; a — 0 h no migration; b — migration inhibition 24 h after treatment; c — 24 h, untreated cells or negative control, scratch portion fully packed by cells

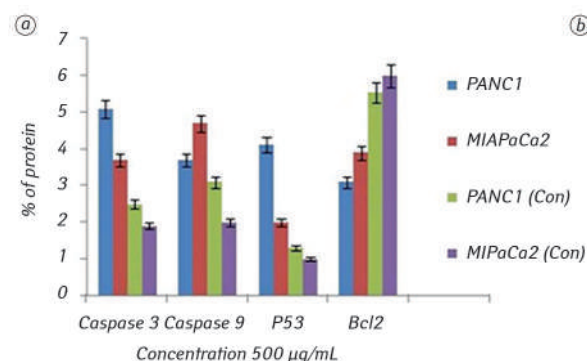
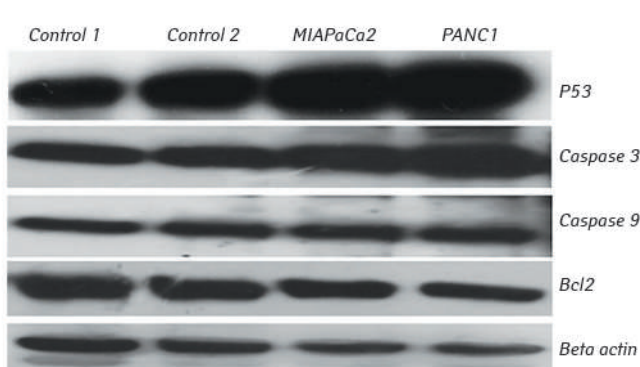


Fig. 4. Relative levels of caspase-3, caspase-9, p53 and Bcl-2 in PANC-1 and MIA PaCa2 cells treated with EE (500 µg/mL) for 24 h ($\pm$ SE bar at 5% level has been inserted) (a). Western blots of proteins (caspase-3, caspase-9, p53 and Bcl2) in PANC-1 and MIA PaCa2 cells treated with EE (500 µg/mL) for 24 h (b); control 1 & control 2 mean untreated PANC-1 & MIA PaCa2 cells, respectively



versicolor induced apoptosis in pancreatic cancer cells by increasing p21 WAF/Cip1 and antigrowth effect of PSK on PANC-1, BxPC-3, AsPC-1 and MIAPaCa-2 was recorded by up-regulating cell cycle regulatory p21 WAF/Cip1 and pro-apoptotic protein Bax levels, as a result cell cycle arrest and induction of apoptosis occurred.

To identify possible molecular targets of EE from *C. indica*, we treated PANC-1 and MIAPaCa-2 cells with EE, performed Western blotting analysis and observed that EE increased the protein products of pro-apoptotic genes such as caspase-3 and -9 while reduced the protein product of anti-apoptotic gene Bcl2. Another thing was that EE increased the level of p53 protein, which guards the cell from oncogenic action. In our migration experiment by scratch method, we observed that EE inhibited migration of PANC-1 cells by about 80%. Similarly, PSK inhibited invasion or migration of NOR-P1 pancreatic cell line by down-regulation of invasion-promoting factors such as TGF- β 1 and MMPs (matrix metalloproteinase) as observed by Zhang *et al.* [19] in Matrigel-coated filters-invasion experiment.

Mushrooms are rich in natural bioactive compounds such as polysaccharides, terpenoids, proteins, peptides, polysaccharopeptide etc. The commercialized polysaccharide from *Phellinus linteus* showed the effectiveness in postoperative adjuvant chemotherapy with surgical resection in pancreatic cancer patients [20]. The biologically active substances of mushrooms responsible for immunomodulation and direct cytotoxicity toward cancer cell lines and the mechanisms of their antitumor action are worth of studying [21]. The present study provides new evidence that EE from mushroom *C. indica* inhibits growth and migration of pancreatic cancer cells.

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CONTRIBUTION OF AUTHORS

SKG designed the experimental research work, investigated the whole work and wrote the manuscript. TS have done the experimental works, data collection, statistical analysis, and figure preparation.

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