

## Original Article

# Mushroom Extracts Induce Human Colon Cancer Cell (COLO-205) Death by Triggering the Mitochondrial Apoptosis Pathway and Go/G1-Phase Cell Cycle Arrest

Shagun Arora PhD<sup>1</sup>, Simran Tandon PhD<sup>2</sup>

## Abstract

**Background:** Functional foods are extensively studied for their cancer preventive effects. In the present study, we compared the anti-cancer activity of aqueous extracts of three species of mushrooms including: *Pleurotus ostreatus* (PAE), *Auricularia polytricha* (AAE) and *Macrolepiota procera* (MAE) on COLO-205 cells.

**Methods:** Various *in vitro* approaches were performed to investigate the most potential mushroom variety that possesses maximum cytotoxic, anti-proliferative and apoptosis inducing properties. MTT assay was used to assess cytotoxicity. IC<sub>50</sub> values were obtained and further used to perform clonogenic survival, wound scratch and apoptosis assays. Gene expression studies of apoptosis and cell cycle related studies were performed by reverse transcriptase PCR, followed by estimation of DNA content by flow cytometric analysis.

**Results:** Our study showed that PAE acts as the most prominent inducer of cancer cell death as compared to other species. Therefore, we performed expression studies for apoptosis and cell cycle to understand the genes which are responsible for their profound activities. Expression studies illustrated increased levels of caspase-9 (1 to 2.1,  $P < 0.01$ ), caspase-3 (1 to 1.7,  $P < 0.01$ ) and Bax (1 to 1.4,  $P < 0.05$ ) genes followed by decreased levels of Bcl-2 (1 to 0.44,  $P < 0.05$ ) gene with PAE treatment and this was attributed to the activation of intrinsic pathway. Along with apoptosis, an arrest at Go/G1 phase was observed through flow cytometric analysis followed by increased expression of inhibitors of cyclin dependent kinases (CKIs), p16 (1 to 1.5,  $P < 0.05$ ) and p21 (1 to 2.4,  $P < 0.01$ ).

**Conclusion:** This study exemplifies the effectiveness of PAE and may serve as a potential therapeutic agent.

**Keywords:** Cell death, colon cancer, COLO-205 cells, mushroom

**Cite this article as:** 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.

## Introduction

Throughout medical history, plants have been a valuable source of anti-cancer agents. Other than plants, fungal species have also been shown to possess therapeutic values that can be addressed as potential anti-cancer agents, especially mushrooms.<sup>1</sup> There are 14,000 varieties of mushroom in the world, 3000 of these are edible and 700 species are known to possess therapeutic potential. Therefore the challenge lies in the identification of such mushroom species which can be both consumable and possess medicinal values. Such mushroom species would be a good source of possible anti-tumor activity.<sup>2</sup>

Colon cancer accounts for third most common cancer in males and second in females worldwide. In India, a total of 64,000 cases were diagnosed, out of which 49,000 people died due to colon cancer.<sup>3</sup> Despite various therapeutic approaches such as chemotherapy, radiotherapy and immunotherapy, the survival rate of patients with malignant colon carcinomas remains poor. This could be either due to the resistance developed by tumor cells or their associated adverse side-effects. Therefore, screening of newer therapeutic strategies is urgently required for successful manage-

ment of this disease. In particular, the search for novel anti-cancer agents that minimize toxicity and reduce adverse side effects are the need of the hour.<sup>4</sup> Substantial evidence has demonstrated, plant extracts or natural products derived from plants possess anti-tumor properties.<sup>5-7</sup> Until now, a number of significant studies have been documented regarding various mushroom species such as, anti-oxidant, cytotoxic, anti-proliferative, anti-diabetic, anti-microbial, anti-inflammatory, apoptotic inductivity and immunomodulatory agents that can be attributed for its medicinal potential.<sup>8-14</sup> The use of mushroom has also been known to counteract the side effects associated with conventional therapies such as nausea, bone marrow suppression, anaemia and lowered resistance.<sup>1</sup> Moreover an inverse correlation between mushroom intake and the risk of developing gastrointestinal cancer have been reported in a clinical trial. In addition, mushroom consumption has also shown to prevent the development of tumor, later in life.<sup>15</sup> Since nature has provided mankind with these valuable anticancer agents, therefore to address their potential we have investigated mushrooms which have been used from ancient times to combat various diseases including cancer.

We selected three varieties of mushrooms i.e. *Auricularia polytricha*, *Macrolepiota procera* and *Pleurotus ostreatus* for our study and investigated their effects on human colon cancer cell line, COLO-205. The criteria for selecting aqueous extract of each species against the experimental cancer model i.e. COLO-205 cells was based on the results of our previous studies. In brief we had seen a greater cytotoxic potential of aqueous extracts of these

**Authors' affiliations:** <sup>1</sup>Jaypee University of Information Technology, Waknaghat, Solan, Himachal Pradesh, India, <sup>2</sup>Amity Directorate of Science and Innovation, Amity University, Noida, India.

**Corresponding author and reprints:** Simran Tandon, PhD, Amity Directorate of Science and Innovation, Amity University, Noida 201313, India.  
Tel: + 914586912-120-, E-mail: standon1@amity.edu, shagun.phd@gmail.com.  
Accepted for publication: 22 April 2015

three species against breast, kidney and colon cancer cells as compared to the ethanolic extracts.<sup>16</sup> In addition, a selective cytotoxic preference towards the cancer cells as compared to normal cells by all three mushroom species was also seen.<sup>17</sup>

The aim of our study was to identify the potential therapeutic properties in mushroom species against COLO-205 cells that have the capacity to inhibit, retard or reverse the multi-step carcinogenesis process either by activating pro-survival and cyclin dependent kinases inhibitor (CKIs) genes or by suppressing anti-survival genes. In this study, the mechanisms underlying growth inhibitory effects induced by the selected medicinal mushrooms are summarized, which provide new insights into the possible therapeutic use of mushrooms against colon cancer.

## Materials and Methods

### Sample Collection

Three species of mushrooms (i.e. *Auricularia polytricha*, *Macrolepiota procera* and *Pleurotus ostreatus*) were procured from the Directorate of Mushroom Research, Solan, Himachal Pradesh, India. The fruiting bodies of these mushrooms were dried and stored in an airtight container.

### Preparation of Extracts

The dried fruiting bodies of mushrooms were ground in a pestle and mortar, followed by grinding in a mixer-grinder until a fine powder was achieved. To prepare 10% (w/v) aqueous extract, 10 grams of the powdered mushroom was dissolved in 100 mL of water using soxhlet apparatus. The extraction procedure was similar for all the three mushroom species. This solution was then lyophilized to obtain the powdered extract. The extracts obtained were termed as PAE (*Pleurotus ostreatus* aqueous extract), MAE (*Macrolepiota procera* aqueous extract) and AAE (*Auricularia polytricha* aqueous extract) and were stored at -20 °C till further use.

For the cell culture based assays the extracts were solubilised in DMSO at a stock concentration of 50 mg/mL and were filtered through 0.22 micron filters prior to adding to cells. The working stocks were prepared in RPMI to give concentrations ranging from 50 – 250 µg/mL.

### Cell Line

COLO-205, a human colon cancer cell line was obtained from NCCS Pune, India. COLO-205 cell line was routinely cultured in the cell culture RPMI 1640 medium (Sigma Aldrich, India) supplemented with 10% fetal bovine serum (FBS) (Gibco, India), and 1% penicillin-streptomycin (Invitrogen, India). Cells were grown under the conditions of 5% CO<sub>2</sub> at 37 °C in a CO<sub>2</sub> incubator.

### Cytotoxicity Assessment by MTT Assay

The cytotoxicity of the extracts was tested upon COLO-205 cell line. Cells were harvested, counted and transferred to 96-well plates and incubated for 24 hours to reach 70% confluence. Cells were then treated with varying concentrations of PAE, AAE and MAE (50 – 250 µg/mL) as well as 5-FU (1 – 4 µg/mL), which was taken as the positive control, and incubated for a further 48-hour. After incubation, 25 µL of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (Sigma, India) (5 mg/mL) was added to each well and incubated for another 4 hours. The formazan product from each well were solubilized with 100 µL DMSO and

the optical density of the wells was determined at 570 nm using a Biorad microplate reader. The concentration which led to a 50% killing (IC<sub>50</sub>) was calculated by plotting a dose response graph of the cytotoxicity values obtained using the formula given below:<sup>18</sup>

$$\% \text{ Cell cytotoxicity} = 100 - \left\{ \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \times 100 \right\}$$

### Growth Kinetics Studies

The cells (1 × 10<sup>5</sup>) were plated onto 12-wells plates. After 24 hours when the density reached 70% confluence, cells were treated with the IC<sub>50</sub> value of PAE, AAE and MAE and 5-FU for 48 hours. For growth kinetic experiments, after the treatment period the culture plates were examined under a phase-contrast microscope (100 X) and photographed.<sup>19</sup>

### *In vitro* Wound Scratch Assay

In order to verify the *in vitro* effect of mushroom extracts on migration of COLO-205 cells, wound healing assay was performed. COLO-205 cells were seeded in 6-well plates at 1 × 10<sup>6</sup> cells/well. Confluent cells were scrapped off using 20 – 200 µL pipette tip along the diameter of the well to create a clear area devoid of cells. Cell debris was removed by washing with PBS. The wells having the wounded monolayer were incubated in the absence (negative control) and presence of PAE, AAE and MAE as well as 5-FU at their IC<sub>50</sub> value for 24 hours. Wound closure by the cells was observed under the microscope at 40X magnification using a Nikon inverted microscope and photographs were captured immediately at 0 hours and after 24 hours treatment. The migrating cells from the scratch in extract-treated wells were compared to the control wells.<sup>20</sup>

### Clonogenic Survival Assay

Single cell suspension of COLO-205 cells were seeded at 5000 cells/ml in a 6-well plate and incubated for 24 hours to allow for attachment. Subsequently, culture medium containing PAE, AAE and MAE extracts and 5-FU at their IC<sub>50</sub> values was added to each well. After a 48-hour treatment, the medium was discarded. Then the cells were washed with PBS and fresh medium was added. On the 10<sup>th</sup> day of incubation, the cells were fixed with 4% (v/v) paraformaldehyde and subsequently stained with 0.1% (w/v) crystal violet solution. Colonies consisting of more than 50 cells were counted by using a colony counter and the results are reported as a percentage of colonies formed using the following equation:<sup>21</sup>

$$\% \text{ Colonies formed} = \frac{\text{Colonies formed in treated sample}}{\text{Colonies formed in untreated sample}} \times 100$$

### Apoptosis Inductivity

#### Hoechst 33258 Staining

The effect of mushroom extracts on nuclear chromatin condensation in COLO-205 cells was assessed by fluorescence microscopy using Hoechst 33258 stain. This dye stains the DNA and distinguishes densely stained and condensed apoptotic nuclei from weakly stained healthy nuclei. The COLO-205 cells were treated with the IC<sub>50</sub> value of either PAE, AAE and MAE and 5-FU for 48 hours. After incubation, the medium was removed and the cells were fixed with 4% paraformaldehyde for 30 minutes. After washing twice with PBS, cells were permeabilized with 0.1% Triton X-100 for 15 minutes. Finally, cells were stained with 5 µg/mL

of Hoechst 33258 dye for 10 minutes at room temperature in the dark. After washing twice with PBS, stained nuclei were observed under a fluorescence microscope (Nikon eclipse Ti) at 100X magnification.<sup>22</sup>

#### Annexin V/ Propidium Iodide (PI) Staining

Annexin V/PI staining was performed to evaluate apoptosis induced by mushrooms. Under normal conditions, Phosphatidylserine (PS) is predominantly located in the innermost layer of the plasma membrane. Upon initiation of apoptosis, PS shifts from the innermost layer to the outermost layer of the plasma membrane, which is one of the indications of apoptosis. Differential analysis of cells was done using a fluorescence microscopy on the basis of uptake of the dye and intensity of fluorescence to give a pattern which was as follows: untreated cells are Annexin -ve/ PI -ve and can be visualised as they show faint impression of green and red coloured cells. Early apoptotic cells are Annexin +ve/ PI -ve and can be visualised by their bright green color. The late apoptotic cells are Annexin +ve/PI +ve with bright green and red color. The experiment was performed according to the manufacturer's protocol (ApoDETECTM ANNEXIN V-FITC KIT) and  $10^5$  cells were seeded into each well of a 12-well plate. Next day, the cells were treated with either PAE, AAE and MAE or 5-FU at their  $IC_{50}$  value and kept for 48 hours. After treatment, both cells in monolayer and suspension were collected and centrifuged at 3000 rpm for 1 minute. The pellet was washed with 1X PBS and centrifuged at 3000 rpm for 1 minute. Then, 190  $\mu$ L of Annexin binding buffer and 10  $\mu$ L of Annexin V dye (20  $\mu$ g/mL) were added to the pellet and incubated for 10 minutes at room temperature. Finally, cells were re-suspended in 190  $\mu$ L of binding buffer containing 10  $\mu$ L of Propidium iodide dye (20  $\mu$ g/mL) and photographed using Nikon eclipse Ti fluorescence microscope at 100X.<sup>23</sup>

#### Qualitative Determination of DNA Fragmentation using Agarose Gel Electrophoresis

Another distinctive feature of apoptosis is DNA fragmentation. This is an early event, which occurs before any shifts in plasma membrane permeability. The DNA fragmentation assay is used to visualize the endonuclease cleavage products of apoptosis on an agarose gel electrophoresis. Cells ( $1 \times 10^6$ ) after treatment with  $IC_{50}$  value of PAE, AAE and MAE and 5-FU for 48 hours were lysed with lysis buffer (10 mM Tris-HCl + 1 mM EDTA + 0.2% Triton -X 100) and centrifuged at 20,000 g for 10 minutes at 4 °C. The supernatant was transferred to a new vial to which an equal volume of isopropanol was added to precipitate the DNA. Then, the sample was centrifuged for 10 minutes at 20,000  $\times$  g. The supernatants were then discarded and the pellets were washed with 70% cold ethanol. DNA samples were electrophoresed on a 1.0% agarose gel. Finally, the gel was examined under UV light following ethidium bromide staining to determine apoptotic DNA fragmentation using a BioRad Gel-Doc system.<sup>24</sup>

#### Quantitative Assessment of Percent Fragmented DNA

The extent of DNA fragmentation was determined spectrophotometrically using diphenylamine reagent.<sup>25</sup> Cells ( $1 \times 10^6$ ) after treatments with  $IC_{50}$  values of PAE, AAE and MAE and 5-FU for 48 hours were lysed with 500  $\mu$ L of lysis buffer (10 mM Tris-HCl + 1 mM EDTA + 0.2% Triton -X 100). The lysate was centrifuged at 13000  $\times$  g for 10 minutes at 4 °C (tube B) to separate the intact and fragmented chromatin. Both the pellet (Tube B) and the su-

pernatant (Tube A) were precipitated overnight at 4 °C with 500  $\mu$ L of 25% trichloroacetic acid (TCA). After that, the precipitates were sedimented at 13 000  $\times$  g, at 4 °C for 10 minutes. On the next day, the DNA precipitates were heated at 83 °C for 20 minutes in 80  $\mu$ L of 5% TCA, followed by mixing with 160  $\mu$ L of diphenylamine solution (1.5% w/v diphenylamine, 1.5% sulphuric acid and 0.01% acetaldehyde in glacial acetic acid) and then left overnight at room temperature. Both optical densities were measured at 620 nm and the percentage of DNA fragmentation was calculated as per the given formula:

$$\% \text{ fragmented DNA} = \frac{\text{O.D tube B}}{\text{O.D tube A} + \text{O.D tube B}} \times 100$$

#### Gene Expression Studies

The extracted RNA from the PAE treated and untreated cells were used as template to examine the expression level of seven different apoptosis specific genes (caspase-9, caspase-3, Bax, Bcl-2, p16, p21 and p27) in the presence of housekeeping gene ( $\beta$ -Actin). Total RNA was isolated using Trizol reagent according to manufacturer's protocol. Thereafter, RNA was quantified (Nanodrop 2000) and reverse transcribed by reverse transcriptase into complementary DNA (cDNA) using reaction mixture of 1x PCR buffer, 0.5 mM deoxy-nucleoside triphosphates (dNTPs), 2.5  $\mu$ M of oligo d(T) primer and 2.5 units of MuLV reverse transcriptase and incubated at 46 °C for 1 hour. Equal amount of untreated and treated cDNAs were used to quantify the amount of change in expression. PCR reactions were carried out in a final volume of 20  $\mu$ L containing 12.5  $\mu$ L of PCR green mix (Fermentas), 1  $\mu$ g cDNA, 1  $\mu$ L of forward and reverse primer (0.1 mM of each primer) and 4.5  $\mu$ L of nuclease free water. The PCR products were run on 1.8% agarose gel using ethidium bromide. The levels of mRNA were expressed as arbitrary units obtained by densitometry using Alpha Innotech Geldoc (Alpha imager EP software). The values for each gene product was normalised to the housekeeping gene,  $\beta$ - actin. The primer sequences of the genes included in our study are shown in Table 1.<sup>26-29</sup>

#### Cell Cycle Analysis

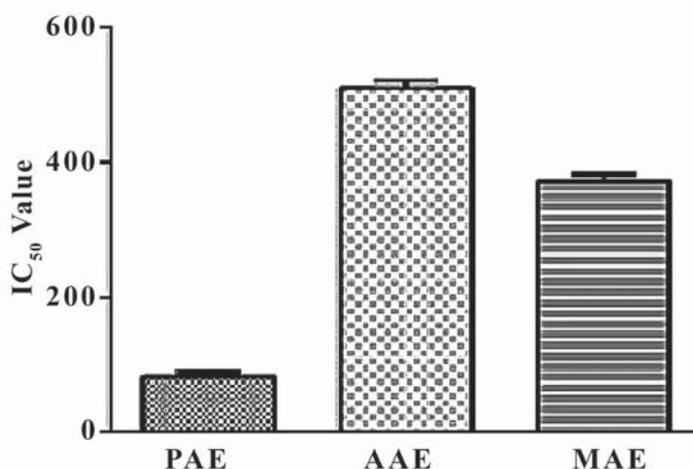
The distribution of cells at different stages in the cell cycle was determined by flow cytometric DNA analysis.<sup>30</sup> Flow cytometric measurements of cellular DNA content was performed with the ethanol (70%) fixed cells using the intercalating DNA fluorochrome Propidium iodide. Briefly,  $10^6$  cells were incubated overnight in 35 mm dishes in a medium containing 10% FBS. After 24 hours, cells were treated with  $IC_{50}$  value of PAE for 48 hours. Cells were harvested at 48 hours, washed twice with cold PBS (pH 7.4) and fixed with 70% ethanol at least 2 hours at -20 °C. The fixed cells were then centrifuged at 300 g for 5 minutes to remove ethanol. The pellet was re-suspended in 1 mL of PBS and stained with 1 mL of PI staining solution (100  $\mu$ g/mL RNAase, 50  $\mu$ g/mL Propidium Iodide and 0.5% Triton-X 100 in PBS) for 30 minutes at room temperature in the dark. A minimum of  $1 \times 10^4$  cells per sample was evaluated, and the percentage of cells in each cell cycle phase was calculated using BD AccuriR C6 Flow Cytometer.

#### Statistical Analysis

The results are presented as means  $\pm$  SD of three independent experiments. One way ANOVA was used to compare treated samples with untreated control samples in case of *in vitro* studies. A

**Table 1.** List of genes used in the study and its cycling parameters

Gene	PCR Programme	Reference
<b><math>\beta</math>-actin</b> F'5AGCCGTGGCCATCTCTTGTCTCGAAG3' R'5 GCCATGTACGTTGCTATCCAGGCTG 3'	94 °C-4 min, 34 cycles of 94 °C-30 sec, 65 °C-30 sec and 72 °C-1min and final extension at 72 °C-10 min; <b>Product Size:</b> 300 bp	26
<b>Caspase-9</b> F'5GTGTCCTACTCTACTTTCCAGGTTTT3' R'5GTGAGCCCACTGCTCAAAGAT 3'	95 °C-5 min, 40 cycles of 95 °C -45 sec, 60 °C-1min and 72 °C-1min and final extension at 72 °C-10 min <b>Product Size:</b> 101 bp	27
<b>Caspase-3</b> F'5 C A G T G G A G G C C G A C T T C T T G 3 ' R'5TGGCACAAAGCGACTGGAT3'	95 °C-5 min, 40 cycles of 95 °C-45 sec, 60 °C-1min and 72 °C-1min and final extension at 72 °C -10 min <b>Product Size:</b> 102 bp	27
<b>Bax</b> F'5TCCACCAAGAAGCTGAGCGA3' R'5 GTCCAGCCCATGATGGTTCT3'	95 °C-5 min, 40 cycles of 95 °C-45 sec, 65 °C-1min and 72 °C-1min and final extension at 72 °C-10 min <b>Product Size:</b> 257 bp	28
<b>Bcl-2</b> F-5'GTGGAGGAGCTCTTCAGGGA3' R-5'AGGCACCCAGGGTGTATGCAA3'	93 °C-2min, 40 cycles of 92 °C-30 sec, 55 °C-40 sec and 72 °C-30 sec and final extension at 72 °C-10 min. <b>Product Size:</b> 304 bp	29
<b>p16</b> F-'ATCTGATCTCCATCGCAGGG' R- 'AAACTGTGCTCCTCCCCTAC'	93 °C-3min, 38 cycles of 93 °C-30 sec, 59 °C-45 sec and 72 °C- 60 sec and final extension at 72 °C-10 min. <b>Product Size:</b> 236 bp	Primer 3'
<b>p21</b> F-'GTTCTACCTCAGGCAGCTCA' R-'AATGAACTGGGGAGGGATGG'	93 °C-3min, 38 cycles of 93 °C-30 sec, 59 °C-45 sec and 72 °C- 60 sec and final extension at 72 °C-10 min. <b>Product Size:</b> 208 bp	Primer 3'
<b>p27</b> F-'GCAAGTACGAGTGGCAAGAG' R-'GTCGCTTCCTTATCCTGCG'	93 °C-3min, 38 cycles of 93 °C-30 sec, 56 °C-45 sec and 72 °C- 60 sec and final extension at 72 °C-10 min. <b>Product Size:</b> 249 bp	Primer 3'



**Figure 1.** Effect of mushroom extracts on cytotoxicity to COLO-205 cells. Cells were treated with varying concentrations of PAE, AAE and MAE for 48 and MTT assay was performed to calculate the concentration leading to 50% cell death (IC<sub>50</sub>). It was noticed that PAE exerted maximal growth inhibitory effect cells with the lowest IC<sub>50</sub>. Data presented as mean ± S.D (n = 3).

minimum *P*-value < 0.05 was considered to indicate a statistically significant difference.

## Results

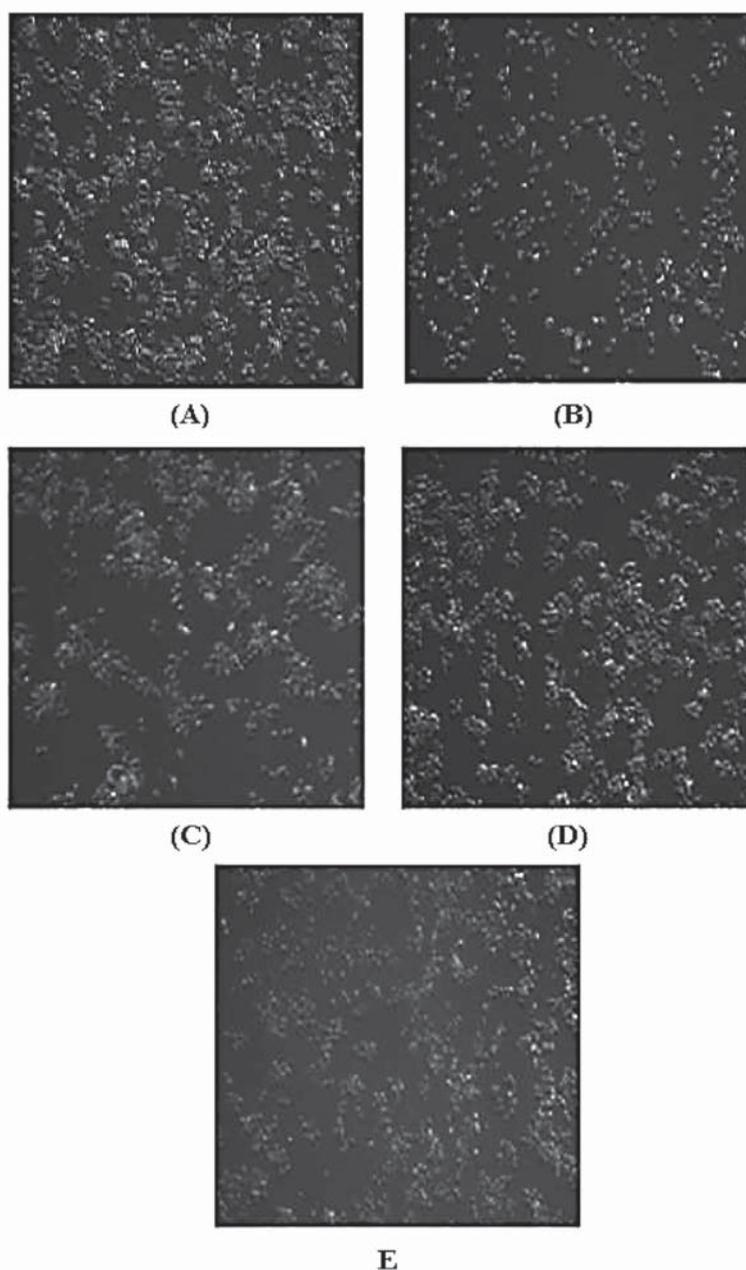
### Cell Cytotoxicity Assay

To examine the effect of PAE, AAE and MAE extracts on COLO-205 colon cancer cell line, the cells were treated with increasing concentration of mushroom extract (50 – 250 μg/mL) and the cell cytotoxicity was determined by MTT assay. As illustrated in Figure 1, the treated cells exhibited a significant decline in viability in comparison with the untreated control cells. The half maximal inhibitory concentration (IC<sub>50</sub>) on tested cell line was calculated

from the concentration- response curve. The results indicated PAE to exhibit maximum cytotoxicity with lowest IC<sub>50</sub> value of 81.2 ± 2.3 μg/mL as compared to AAE (500.9 ± 1.7 μg/mL) and MAE (362.1 ± 1.3 μg/mL). The concentrations were selected for subsequent experiments based on the MTT assay results.

### Growth Kinetics Analysis

Growth kinetics of COLO-205 cells was observed using phase contrast microscopy. COLO-205 cells cultured in the presence or absence of mushroom extracts for 48 hours at their IC<sub>50</sub> values of PAE (81.2 ± 2.3 μg/mL), AAE (500.9 ± 1.7 μg/mL) and MAE (362.1 ± 1.3 μg/mL), showed a reduced cell number as evidenced by the phase contrast photomicrographs. Furthermore the cells in culture seemed to have lost contact with their neighbouring cells



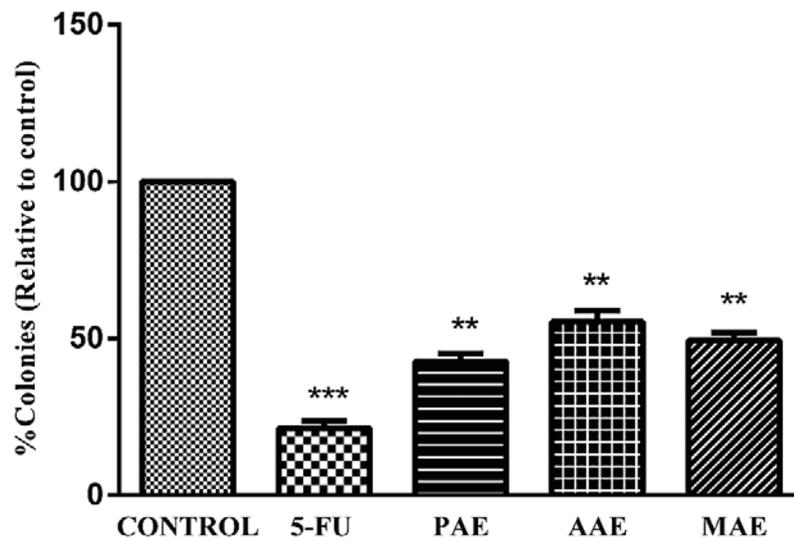
**Figure 2.** Effect on growth of COLO-205 cells. To study the effect of PAE, AAE and MAE on cell growth, COLO-205 cells were treated with extracts and kept for a period of 48 hours; **A**) Control; **B**) 5-FU; **C**) PAE; **D**) AAE and **E**) MAE (Magnification at 100X).

as well as the culture dish (this was reflected by blank areas in the dish which were devoid of cells). Figure 2 shows the representative images of untreated and treated cells at  $IC_{50}$  values of mushroom extracts. The microscopic observation indicated that in comparison to treatment with AAE (Figure 2B) and MAE (Figure 2C), this effect was more evident in PAE (Figure 2C) treated cells. A reduction in cell adhesion capability by PAE as compared to other extracts was seen, while untreated cells continued their normal proliferation pattern (Figure 2A).

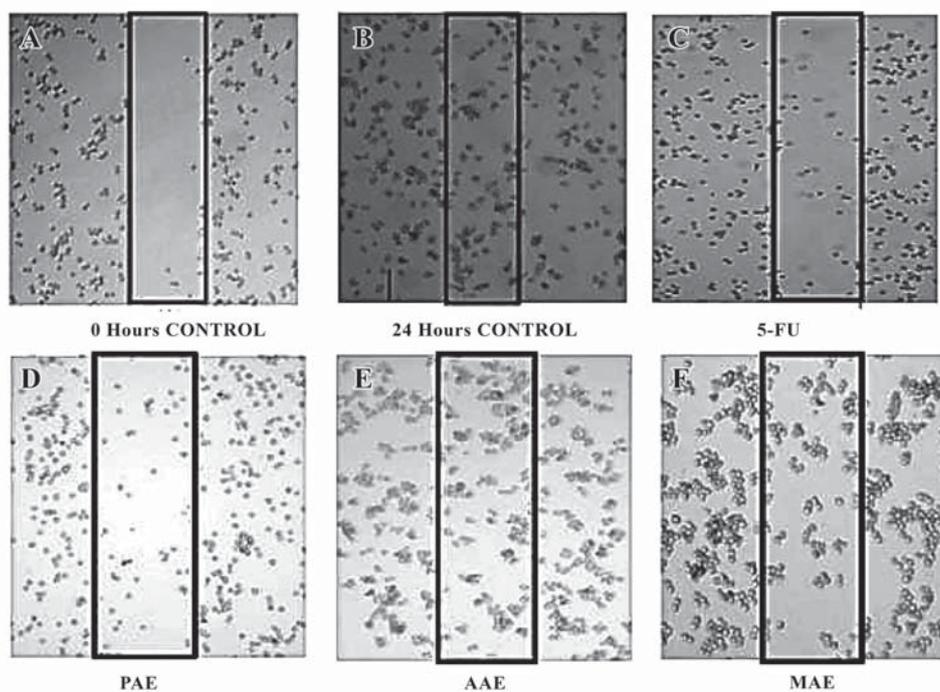
#### Clonogenic survival assay

A cell that retains the property to divide and proliferate by producing a large colony of cells (comprising of at least 50 cells) is referred to as “clonogenic.” Clonogenicity provides an indirect method for evaluating the propensity of cancer cells to undergo

neoplastic transformations. In this study, we determined the effect of mushroom extracts on clone formation in COLO-205 cells. Clonogenicity was determined by plating a fixed number of COLO-205 cells in 6-well plate. Cells were treated with  $IC_{50}$  value of PAE ( $81.2 \pm 2.3 \mu\text{g/mL}$ ), AAE ( $500.9 \pm 1.7 \mu\text{g/mL}$ ) and MAE ( $362.1 \pm 1.3 \mu\text{g/mL}$ ) extracts for 48 hours. After 48 hours, media was removed and cells were replenished with fresh media and maintained in culture for 10 days to allow formation of colonies. As shown in Figure 3, during the 10-day culture period, mushroom treatment reduced the number of growing colonies and visibly decreased the size of growing colonies. PAE exhibited a greater reduction in the number of colonies with  $43.8 \pm 3.5\%$  ( $P < 0.01$ ) as compared with 100% proliferation on untreated cells, whereas AAE and MAE showed  $59.9 \pm 2.6$  ( $P < 0.01$ ) and  $47.7$  ( $P < 0.01$ ) percent colonies.



**Figure 3.** Colony formation inhibition of COLO-205 cells on treatment with mushroom extracts for 14 days. Cells were fixed with 4% paraformaldehyde and stained with crystal violet. Data presented as percent colony formation of mushroom extract treated COLO-205 cells and compared with control (untreated cells) (n = 3). \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



**Figure 4.** Inhibitory effects of mushroom extracts on the migration of COLO-205 cells. The images demonstrate cell migration over time, as the width of the wound (open scratch area) narrowed after 24 hours incubation; **A)** Initial view of scratch at time 0, prior to any treatment; **B)** Control, after 24 hours; **C)** 5-FU treated cells at 24 hours; **D)** PAE treated cells at 24 hours; **E)** AAE treated cells at 24 hours and **F)** MAE treated cells at 24 hours

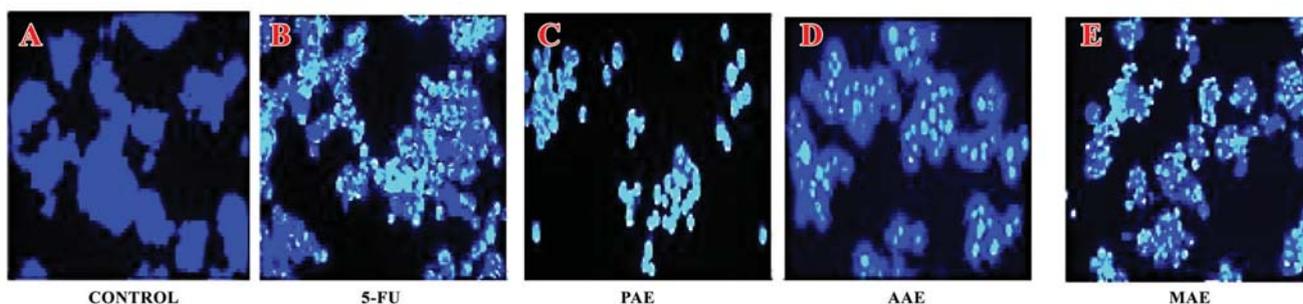
### Cell Migration

This assay means to emphasize the potential of mushroom extracts to suppress the motility of rapidly growing cancer cells and slow down their progression upon treatment as compared to untreated cells. The ability of cells to migrate into the wounded area was assessed over 24 hours. A confluent monolayer of COLO-205 cells was scratched as described in the method section, and then allowed to re-epithelialize in the presence or absence of PAE, AAE and MAE. In the present study, the result describes an effective reduction in the migratory capacity of treated cells as compared to their untreated cells (Figure 4). Treatment of COLO-205 cells with PAE (Figure 4D) resulted in the least migration of cells

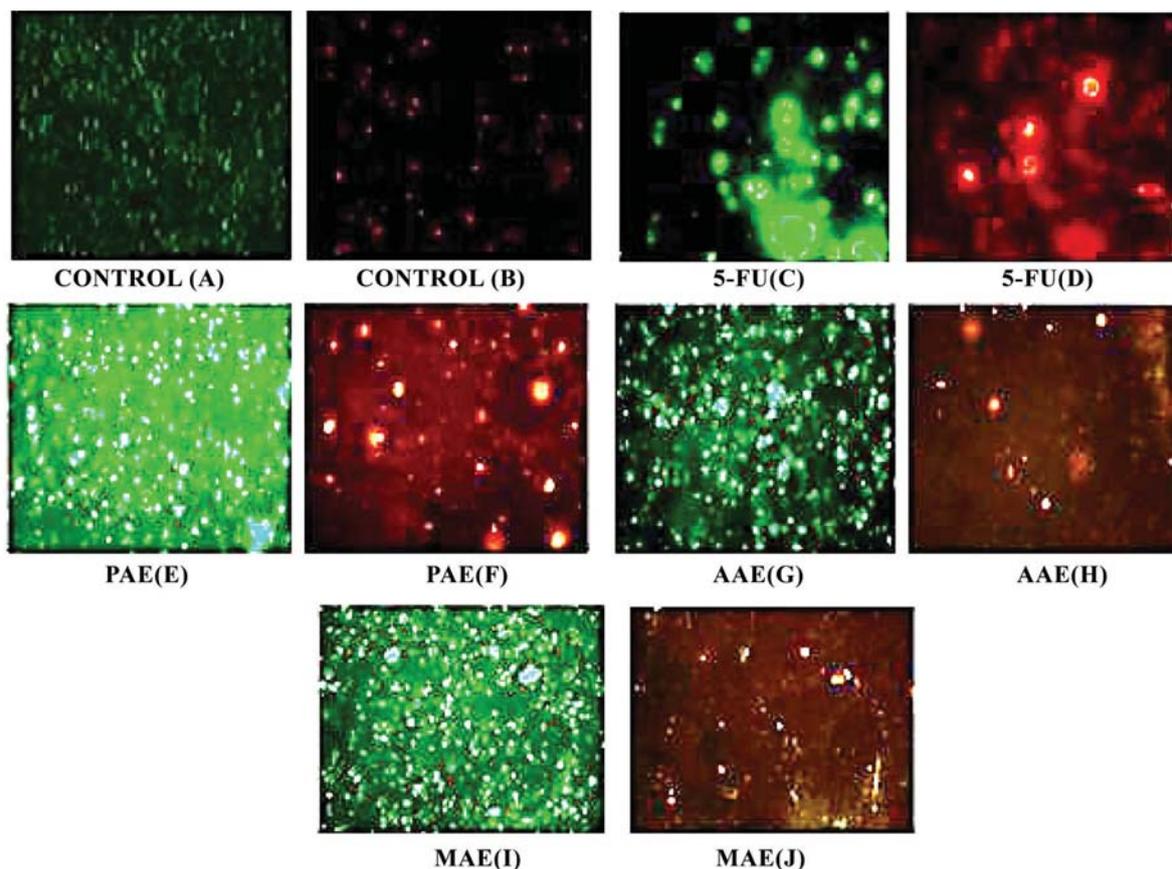
into the wounded area. This indicated a greater inhibitory effect on cell proliferation after 24 hours in comparison to AAE (Figure 4E) and MAE (Figure 4F) treated. On the other hand the distance across the wound narrowed significantly over the 24-hour period in the untreated control COLO-205 cells as compared to 0 hours (Figure 4 A – B).

### Apoptosis Inductivity

As apoptosis mediated cell death is a complex process, more than one approach is required to study the various endpoints and accomplish reproducible observations. Depending on cell type and signalling mechanism involved in this process, not all charac-



**Figure 5.** Evaluation of apoptotic changes in COLO-205 cells by Hoechst 33258 staining. A change in nuclear morphology was observed after 48 hours of treatment with mushroom extracts. Cells were observed under fluorescence microscope at 100 X; **A)** Control; on treatment with **B)** 5-FU; **C)** PAE; **D)** AAE and **E)** MAE.



**Figure 6.** Evaluation of apoptosis changes in COLO-205 cells by Annexin V/PI staining. An exposure of phosphatidyl serine on cell surface was observed after 48 hours treatment with mushroom extracts. Cells were observed under fluorescence microscope at 100 X; **A)** Control; **C)** 5-FU; **E)** PAE; **G)** AAE and **I)** MAE observed for uptake of annexin V dye and **B)** Control; **D)** 5-FU; **F)** PAE; **H)** AAE and **J)** MAE observed for for PI staining.

teristics may be noticeable in one experiment. Morphological observations on the growth inhibition effects of mushroom extracts were studied over a 48-hour period.

#### Hoechst Staining

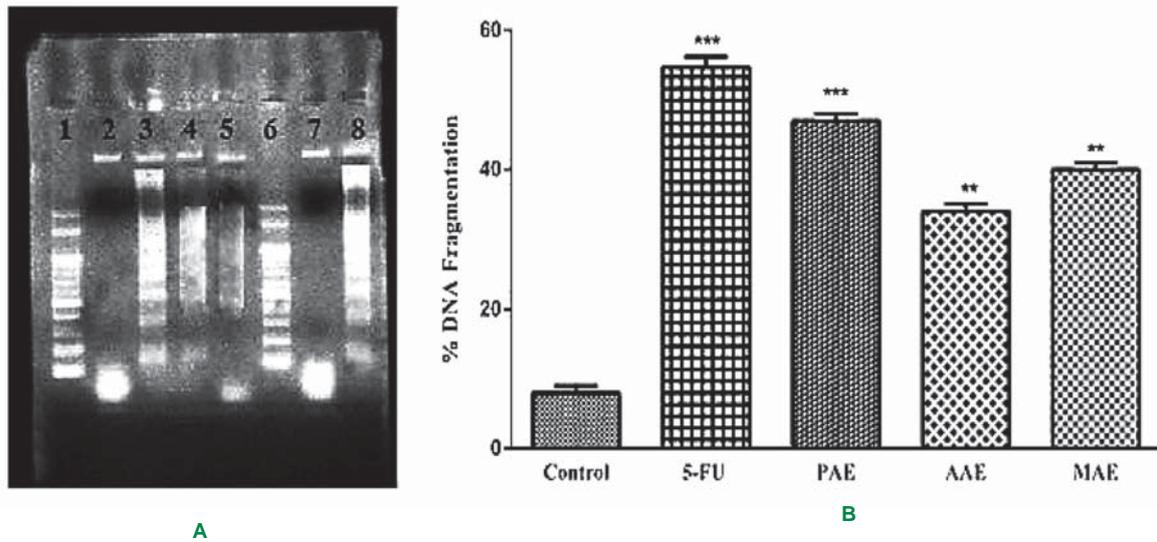
Hoechst 33258 is a blue fluorescent dye that stains the cell nucleus. When cells were treated with Hoechst 33258, live cells appeared to show uniformly light blue nuclei under a fluorescence microscope, while apoptotic cells exhibited bright blue because of chromatin condensation.

Under control conditions, COLO-205 cells appeared normal and the nuclei were round and homogeneous (Figure 5A). After treatment with PAE (Figure 5C), AAE (Figure 5D) and MAE (Figure

5E), the cells exhibited the characteristic features of apoptosis, with nuclear condensation and fragmentation as indicated by hoechst staining.

#### Annexin V/PI Staining

During apoptosis, phosphatidylserine (PS) was translocated from interior leaflet to the exterior leaflet of the plasma membrane. Annexin V is a calcium dependent phospholipid binding protein with high affinity for phosphatidylserine and used to detect early apoptotic cells. PI (Propidium Iodide) is a red fluorescent dye and stains cells that have lost membrane integrity. Therefore, staining cells with annexin V and PI helps in discriminating apoptotic cells from live cells. As shown in Figure 6, treatment with PAE (Figure



**Figure 7.** A Induction of DNA fragmentation in the COLO-205 cells. Fragmentation of genomic DNA was studied in COLO-205 cells exposed to mushroom extracts for 48 hours. Genomic DNA was isolated and electrophoresed as described earlier. Lane 1- Ladder, Lane 2- untreated control cells, Lane 3- PAE treated cells, Lane 4- AAE treated cells, Lane 5- MAE treated cells, Lane 6- Ladder, Lane 7- untreated control cells and Lane 8- 5-FU treated cells; **B)** Quantitative Assessment of DNA fragmentation by DPA method. The percent DNA fragmentation of mushroom extracts treated cells was measured by DPA method and compared control COLO-205 cells. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

6 E-F) led to a more profound apoptotic effect in the COLO-205 cells with greater annexin V and PI positive cells as compared to AAE (Figure 6, G – H) and MAE (Figure 6, I – J).

#### DNA fragmentation by Agarose Gel Electrophoresis

Evaluation of apoptosis was further carried out by performing DNA laddering test, an indicator of the late stage of apoptosis. Treatment of COLO-205 cells with PAE, AAE and MAE revealed the characteristic pattern of DNA laddering. The results of extracted DNA on agarose gel electrophoresis showed typical “DNA ladder”, in COLO-205 treated cells, while a sharp DNA band with no laddering like pattern was seen in untreated control cells (Figure 7A).

#### DNA Fragmentation by DPA method

The impact of treatment with the various mushroom extracts on the integrity of DNA was evaluated by measuring the level of genomic DNA fragmentation using the DPA assay. Compared to control COLO-205 cells, mushroom extract treatment increased the percentage of DNA fragmentation. As seen in the data shown in Figure 7B, PAE treatment contributed to the highest DNA fragmentation as compared to the other extracts. The order of fragmentation was  $47.89 \pm 3.1\%$  ( $P < 0.001$ ), AAE  $34.7 \pm 2.9\%$  ( $P < 0.001$ ) and MAE  $39.9 \pm 2.5\%$  ( $P < 0.001$ ).

Our study demonstrated that colon cancer cell line COLO-205 was highly sensitive to PAE mediated growth inhibition and led to apoptotic cell death. Therefore, the molecular mechanism of PAE action was analysed using gene expression analysis of the apoptosis as well as the cell cycle.

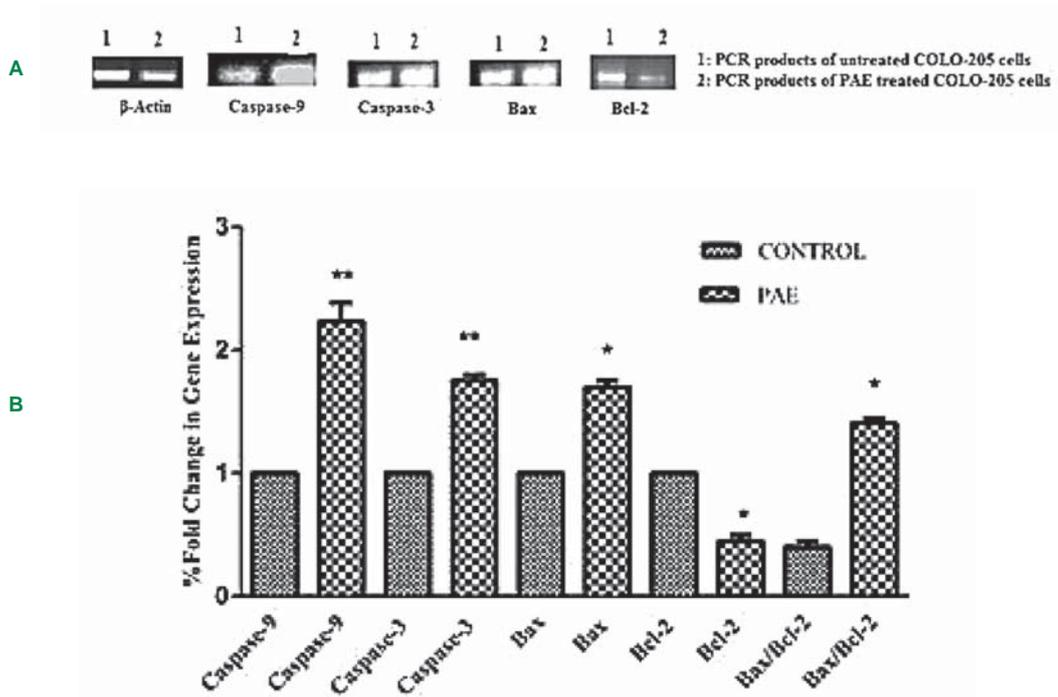
#### Gene Expression Studies

After establishing the mode of apoptosis induced by PAE at its  $IC_{50}$ , next we aimed at delineating the underlying mechanism.

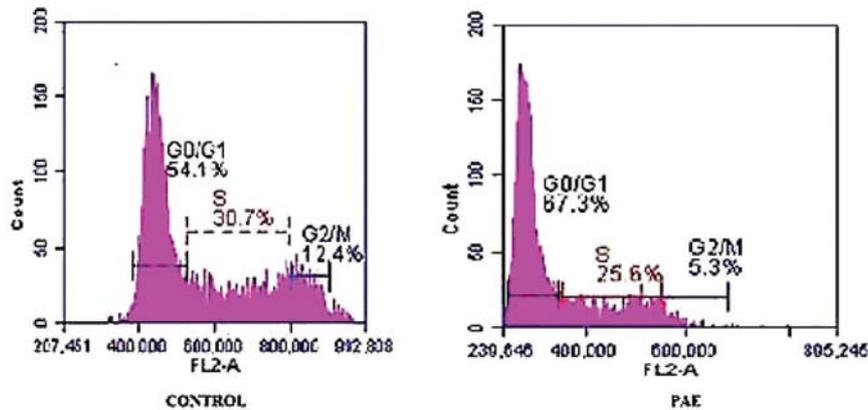
Commencement of the intrinsic pathway of apoptosis is associated with the disruption of the mitochondrial membrane potential and release of mitochondrial contents resulting in activation of caspase-9 and -3, along with decreased levels of Bcl-2 and increased levels of Bax. The PCR products of synthesised cDNAs from the treated and the untreated control cells were subjected to electrophoresis on a 1.8% agarose gel. The gel images are shown in Figure 8A, indicate increased expression in the genes coding for caspase-9, caspase-3, Bax and a reduction in Bcl-2. All samples were normalized with  $\beta$ -actin and quantification of each band was performed by densitometry analysis software to calculate fold changes in gene expression. Figure 8B showed levels of caspase-9, caspase-3 and Bax genes increased to 2.1 ( $P < 0.01$ ), 1.7 ( $P < 0.01$ ) and 1.4 ( $P < 0.05$ ), respectively, in treated sample as compared to untreated cells, whose level was taken as 1 in these experiments. A reduction in the level of Bcl-2 from 1 to 0.44 ( $P < 0.05$ ) was also observed in the PAE treated sample.

#### Cell Cycle Analysis

Since cell cycle regulation plays a critical role in the cancer growth and development, cell cycle arrest is one of the most targeted anti-cancer therapies. The impact of the PAE treatment ( $81.2 \pm 2.3 \mu\text{g/mL}$ ) on different phases of cell cycle in COLO-205 cells was evaluated by flow cytometric analysis. The percentage of the cells at  $G_0/G_1$  phase was remarkably increased after PAE treatment from 54.1% (control) to 67.3% (Figure 9). In order to correlate the arrest at a specific phase of the cell cycle, the expression of key genes involved in the cell cycle was evaluated by RT-PCR. The expression of CKIs was used to confirm the arrest in cell cycle distribution on treatment with PAE. Gel images from Figure 10 showed an evidence of up-regulated expression of p16 and p21 genes after treatment and this increased expression was further quantified by densitometry analysis. Expression of p21



**Figure 8. A)** Activation of intrinsic/mitochondrial apoptotic pathway in COLO-205 cells. Alterations in the expression levels of pro-apoptotic gene and anti-apoptotic gene in PAE treated COLO-205 cells was analysed by agarose gel electrophoresis of PCR products of  $\beta$ - actin; Caspase -9; Caspase-3; Bax and Bcl-2 gene of PCR products of control COLO-205 cells and treated cells. **B)** The fold change in expression of apoptosis-associated gene after PAE treatment. The densitometric analysis was done of the PCR products Caspase -9, Caspase-3, Bax and Bcl-2 gene to measure the fold change in each gene and was normalised to house-keeping gene  $\beta$ -actin. Data presented as percent fold change in gene expression between treated cells and untreated control cells. \* $P < 0.05$ , \*\* $P < 0.01$ .



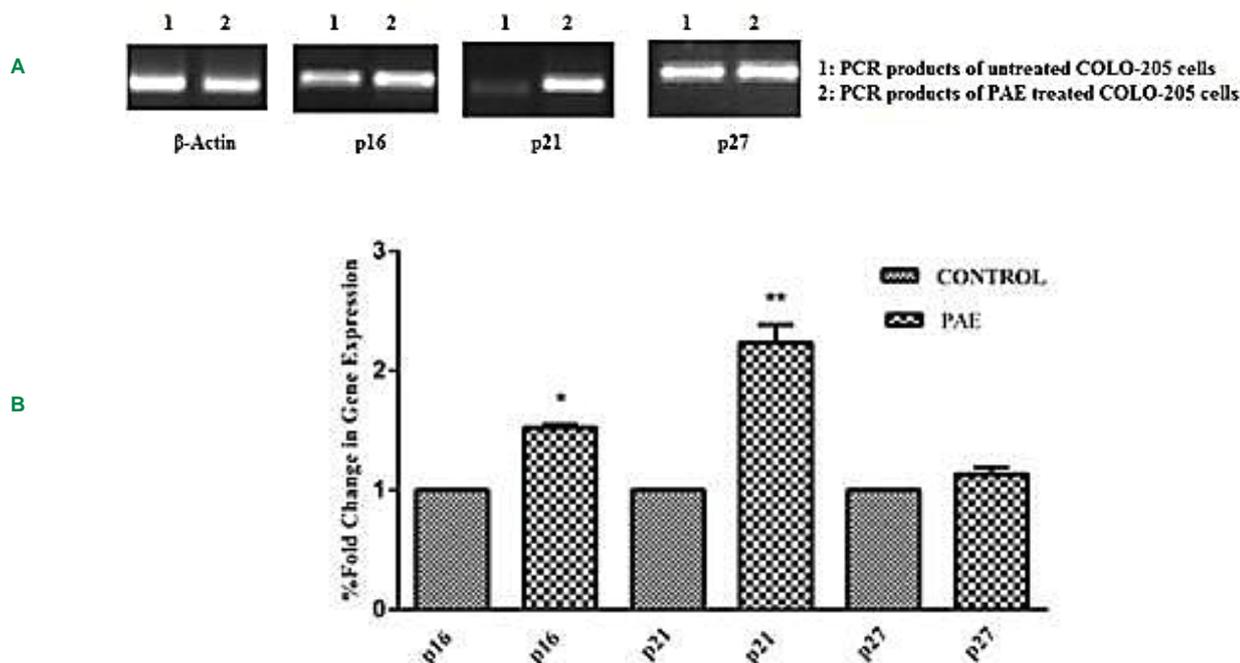
**Figure 9.** Effect on cell cycle by flow cytometric analysis; COLO-205 cells were treated with PAE for 48 hours. Cells were fixed and stained with PI and analysed by flow cytometry. Histogram of cell cycle distribution of control untreated cells and PAE treated cells.

was raised from 1 to 2.4 ( $P < 0.01$ ) after treatment, followed by an increase in p16 expression from 1 to 1.5 ( $P < 0.05$ ). However, no significant difference was observed in the expression of p27 after treatment.

**Discussion**

Despite significant advances in conventional therapies and screening techniques, colon cancer continues to be a major cause

of death worldwide. Agents capable of activating programmed cell death, inhibiting cell survival or modulating cellular signaling cascades are currently used for cancer treatment. It is well known that cancer is a disease in which a number of pathways are abnormal. Therefore, an agent with multiple strategies against cancer cells is considered to be more effective.<sup>31</sup> Recently, major developments have been centred on identification of functional foods as novel complementary and alternative approaches to manage cancer. This would lessen the burden of mortality as well as



**Figure 10. A)** Alterations in the expression levels of CKI's in PAE treated COLO-205 cells was analysed by agarose gel electrophoresis of PCR products of (A)  $\beta$ - actin; p16; p21 and p27 genes of PCR products of control COLO-205 cells and treated cells; **B)** The densitometric analysis was done of the PCR products p16, p21 and p27 gene to measure the fold change in each gene and was normalised to house-keeping gene  $\beta$ -actin. Data presented as percent fold change in gene expression between PAE treated cells and untreated control cells. \* $P < 0.05$ , \*\* $P < 0.01$ .

side-effects conferred by conventional therapies.<sup>32</sup> Among functional foods, mushrooms are one of those species which are both a source of food as well as holding tremendous medicinal value and could be an emerging area for biomedical research.<sup>33</sup> However, the molecular mechanism for their apoptotic effect as an anticancer agent in COLO-205 cells has not yet been clarified. In the present study, we aimed to explore anti-tumor activity of three species of mushrooms against an *in vitro* colon cancer model. Our results demonstrate that all three extracts of mushroom i.e. PAE, AAE and MAE inhibit the growth of human colon cancer cells. According to the results of the cytotoxicity data assessed by the MTT assay, PAE exerted greater efficacy against the cancer cells with the lowest  $IC_{50}$  value as compared to other mushroom extracts. The  $IC_{50}$  concentration of each extract obtained from the MTT assay was used as the treatment concentration for further experiments.

Qualitatively treatment with the various extracts (PAE, AAE and MAE) revealed significant loss of cell viability and an increase in the cells floating in the medium, due to the loss of adherence as seen from the phase contrast images. Anti-proliferative behaviour of cancer cells upon treatment was also analyzed by clonogenic survival and wound scratch assay, which are indirect methods to measure re-epithelialization of cells or to assess dynamics of proliferation of cancer cells after treatment. Clonogenic survival assay was performed to assess antineoplastic potential.<sup>34</sup> The results indicated that PAE, AAE and MAE extracts have cytostatic effects on COLO-205 cells as shown by a gradual decrease in the percentage of colonies after removal of the extracts. The classic method of *in vitro* wound-scratch assay was performed to investigate cellular migration. Cells were exposed to the  $IC_{50}$  concentration of each extract for 24 hours. The representative images at time 0 and 24 hours after wound initiation for both treated and untreated cells, clearly showed reduced cell motility after treatment.

This was in contrast to the control untreated cells, which were able to invade the scratched area and re-colonised the scratch at the end of the 24 hour incubation period. It was interesting to see that PAE exerted the maximum anti-neoplastic and anti-migration activities amongst the aqueous extracts. Most of the available anti-cancer agents act by either inducing cell death or by inhibiting the cell proliferation.<sup>35-37</sup> Our results obtained from treating COLO-205 cells with the mushroom extracts are also in agreement with studies using various extracts on cancer cell lines. The extract of fruiting bodies of *Pleurotus tuber-regium* and *Pleurotus ostreatus* exhibited cytotoxicity and anti-proliferative activity against human acute promyelocytic leukemia cells (HL-60).<sup>38-39</sup> These results, show that extracts from varied sources have the capacity to induce cytotoxicity to cancer cell lines.

One of the major factors regulating the proliferating cancer cells is the inactivation of apoptosis, which is a self-suicidal program. Apoptosis is characterized by morphological and biochemical alterations such as membrane blebbing, cell shrinkage, chromatin condensation, flip-flop translocation and DNA fragmentation.<sup>40</sup> The anti-proliferative and cytotoxic effects are likely to be linked via activation of apoptosis. Treatment with Agaritine, a hydrazine-derivative from hot-water extract of *Agaricus blazei Murill* on human leukemic monocyte lymphoma (U937) cells led to DNA fragmentation, annexin V expression, and cytochrome c release.<sup>41</sup>

In the present study, we investigated whether mushroom extracts could induce apoptosis in COLO-205 cells. Staining with Hoechst 33258 showed condensed bright nuclei in cells treated with mushroom extracts. In addition, cells stained positively for Annexin V and PI, which revealed the extracts induced apoptosis using fluorescence microscopy. Morphological changes were clearly evident during apoptosis. Some of the observations included, chromatin condensation, plasma membrane blebbing and DNA fragmentation. Qualitative DNA analysis in the treated

COLO-205 cells revealed apoptotic cell death with the ladder like pattern. In addition, increased levels of fragmented DNA further confirmed cytotoxicity via apoptotic inductivity. From the above results, it was very clear that among three extracts, PAE exhibited maximum cytotoxicity and apoptotic efficiency. Therefore, we carried out further studies to understand the mechanism of the inhibitory effects exerted by PAE, by examining the expression of apoptosis and cell cycle related genes by RT-PCR.

The apoptosis pathway is an active cellular suicidal mechanism operative within all cells and tightly regulated in terms of both activation and execution. The Bcl-2 protein family is divided into two sub-families i.e. pro-apoptotic proteins which comprise of Bax and Bad, which helps in the activation of the cell death pathway. The other is the anti-apoptotic (Bcl-2), that regulates the mitochondrial membrane integrity, cytochrome c release and caspase activation, finally leading to execution of cell death.<sup>40</sup> Induction of programmed cell death was also observed when HT-29 (colon cancer) cells were treated with aqueous extract of *Pleurotus ostreatus* due to increased levels of the pro-apoptotic molecule Bax.<sup>42</sup> Increased level of caspase-9, caspase-3 and Bax was observed in PAE treated cells, followed by decreased expression of Bcl-2 gene. Furthermore, the relative balance between pro-apoptotic Bax and anti-apoptotic Bcl-2 genes influences the propensity of cells to undergo cell death pathway. An increase in Bax/Bcl-2 ratio in comparison to the untreated cells was seen on treatment, suggesting PAE induced colon cancer cell apoptosis.

Disturbed cell-cycle is a primary characteristic of cancer. Normal cells only multiply in response to signals that indicate a requirement for tissue growth, whereas the proliferation of cancer cells proceeds essentially unchecked. This indicates that cancer cells proliferate because of defects in internal and external proliferation-inhibitory signals. However, there must also be defects in DNA repair systems that should prevent such unregulated proliferation. We now have a detailed molecular picture of normal cell-cycle control, in which a series of kinases promote progression through each phase of the cell cycle. The phosphorylation of a variety of proteins by members of the cyclin-dependent kinase (CDK) family is crucial to this progression. Each CDK is dependent on a particular cyclin; therefore, the activity of each CDK can be controlled by the availability of its cyclin partner, and the expression of a specific CDK inhibitor (CKI).<sup>43</sup> Due to loss of key cell cycle checkpoints, abnormal cell proliferation is an important characteristic of cancer cells. Abnormal cell proliferation facilitates oncogenic transformation, therefore in this study the distribution of cells was measured in different phases of cell cycle by PI staining. Histograms from flow cytometric analysis depicted an arrest at G<sub>0</sub>/G<sub>1</sub> phase after PAE treatment, compared to untreated control cells. The levels of CKIs were also checked to further investigate the genes responsible for this cell cycle arrest. Up-regulated levels of p16, p21 and p27 were observed in cells treated with PAE. A number of CAM sources have exhibited significant inhibitory effects on cancer cells via disruption of cell cycle progression. Amongst various traditional Chinese medicines (TCM), *Ganoderma* is one of the commonly used herbal medicines in Asia. Studies have shown that treatment with the chloroform extract of *Ganoderma* caused G<sub>2</sub>/M cell cycle arrest in Hep3B (human hepatoma) and COLO-205 (human colon cancer) cells as well as the ethanolic extract of *Ganoderma* induced S-phase arrest in H23/0.3 (doxorubicin-resistant human lung adenocarcinoma) cells.<sup>44-46</sup> The oncolytic potential of PAE, could be

attributed to its ability to induce death of cancer cells either by activation of apoptotic pathway or by cell cycle arrest.

In conclusion, a putative mechanism behind the action of the aqueous extract of *Pleurotus ostreatus* on COLO-205 cells can be put forth on the basis of the observed results. This action can be attributed to its regulatory effect on genes responsible for progression through the cell cycle as well as apoptosis. As far as we know, this is the first report to demonstrate that PAE activated caspase dependent pathway by modulating Bcl-2 expression, followed by up-regulating expression of caspase-9, caspase-3 and Bax. Moreover, PAE blocked cell cycle progression at G<sub>0</sub>/G<sub>1</sub> phase by increasing expression of p16 and p21. Therefore, the natural compounds present in PAE could modulate apoptosis and cell cycle pathways that are frequently blocked in human cancers and may provide novel opportunities for cancer drug development.

## Conflict of interest

*We declare that we have no conflict of interest.*

## Acknowledgments

*We are thankful to the Department of Biotechnology and Bioinformatics, Jaypee University of Information and Technology, Wagnaghat Campus for providing facilities for doing this work. We are also thankful to the Directorate of Mushroom Research, Solan, India for providing the mushroom samples.*

## References

1. Patel S, Goyal A. Recent developments in mushrooms as anti-cancer therapeutics: A review. *3Biotech*. 2012; **2**: 1 – 15.
2. Mowsumi FR, Choudhury MR. Oyster mushroom: Biochemical and medicinal prospects. *Bangladesh Journal of Medical Biochemistry*. 2010; **3(1)**: 23 – 28.
3. Colorectal Cancer Incidence and Mortality Worldwide in 2012. *Globocan Cancer Fact Sheet*. *Globocan*. IARC. 2013.
4. Leonard RC, Williams S, Tulpule A, Levine AM, Oliveros S. Improving the therapeutic index of anthracycline chemotherapy: focus on liposomal doxorubicin (Myocet). *Breast*. 2009; **18**: 218 – 224.
5. Sakamoto S, Kudo H, Kuwa K, Suzuki S, Kato T, Kawasaki T, et al. Anticancer effects of a Chinese herbal medicine, *juzen-taiho-to*, in combination with or without 5-fluorouracil derivative on DNA-synthesizing enzymes in 1,2-dimethylhydrazine induced colonic cancer in rats. *Am J Chin Med*. 1991; **19**: 233 – 241.
6. Shukla Y, Singh M. Cancer preventive properties of ginger: A brief review. *Food Chemistry and Toxicology*. 2007; **45**: 683 – 690.
7. Cragg GM, Newman DJ. Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology*. 2005; **100(1-2)**: 72 – 79.
8. Thohinung S, Kanokmedhakul S, Kanokmedhakul K, Kukongviriyapan V, Tusskorn O, Soyong K. Cytotoxic 10-(indol-3-yl)-[13]cytochalasans from the fungus *Chaetomium elatum* ChE01. *Archives of Pharm Research*. 2010; **33**: 1135 – 1141.
9. Jiang J, Sliva D. Novel medicinal mushroom blend suppresses growth and invasiveness of human breast cancer cells. *International Journal of Oncology*. 2010; **37**: 1529 – 1536.
10. Fortes RC, Novaes MRCG. Effects of dietary supplementation with Agaricales mushrooms and other fungi in medicinal therapy against cancer. *Revista Brasileira de Cancerologia*. 2006; **52**: 363 – 371.
11. Barros L, Baptista P, Estevinho LM, Ferreira ICFR. Bioactive properties of the medicinal mushroom *Leucopaxillus giganteus* mycelium obtained in the presence of different nitrogen sources. *Food Chemistry*. 2007; **105**: 179 – 186.
12. Luo KW, Yu ZM, Co NN, Wu SH, Wu P, Fung KP, Kwok TT. Suillin from the mushroom *Suillus placidus* as potent apoptosis inducer in human hepatoma HepG2 cells. *Chemico-Biological Interactions*. 2009; **181**: 168 – 174.
13. Kim SH, Song YS, Kim SK, Kim BC, Lim CJ, Park EH. Anti-in-

- inflammatory and related pharmacological activities of the n – BuOH subfraction of mushroom *Phellinuslinteus*. *Journal of Ethnopharmacology*. 2004; **93**: 141 – 146
14. Naji M, Bassil, Massih RM. *Pleurotus ostreatus* and *Ruscus aculeatus* Extracts Cause Non – Apoptotic Jurkat Cell Death. *Journal of Plant Studies*. 2012; **1**: 14 – 24.
  15. Kim HJ, Chang WK, Kim MK, Lee SS, Choi BY. 2002. Dietary factors and gastric cancer in Korea: A case – control study. *International Journal of Cancer*. 2002; **97**: 531 – 535.
  16. Arora S, Goyal S, Balani J, Tandon S. Enhanced antiproliferative effects of aqueous extracts of some medicinal mushrooms on colon cancer cells. *International Journal of Medicinal Mushrooms*. 2013; **15**: 301 – 314.
  17. Arora S, Tandon C and Tandon S. Evaluation of the cytotoxic effects of cam therapies: An in vitro study in normal kidney cell lines. *The Scientific World Journal*. 2014; 452892.
  18. Li FR, Jiao P, Yao ST, Sang H, Qin SC, Zhang W, et al. *Paris polyphylla* smith extract induces apoptosis and activates cancer suppressor gene connexin26 expression. *Asian Pacific Journal of Cancer Prevention*. 2012; **13**(1): 205 – 209.
  19. Srivastava R, Saluja D, Dwarakanath BS, Chopra M. Inhibition of human cervical cancer cell growth by ethanolic extract of *Boerhaavia diffusa* linn. (punarnava) root. *Evidence-Based Complementary and Alternative Medicine*. 2011; Article ID 427031: 13 pages.
  20. Hsu SC, Kuo CL, Lin JP, Lee JH, Lin CC, Su CC, et al. Crude extracts of *euchresta formosana* radix inhibit invasion and migration of human hepatocellular carcinoma cells. *Anticancer Research*. 2007; **27**: 2377 – 2384.
  21. George VC, Kumar DR, Suresh PK, Kumar S, Kumar RA. Comparative studies to evaluate relative *in vitro* potency of *luteolin* in inducing cell cycle arrest and apoptosis in HaCaT and A375 cells. *Asian Pacific Journal of Cancer Prevention*. 2013; **14**(2): 631 – 637.
  22. Allen J, Sotos M, Sylte J, Czuprynski CJ. Use of hoechst 33342 staining to detect apoptotic changes in bovine mononuclear phagocytes infected with *mycobacterium avium* subsp. paratuberculosis. *Clinical and Diagnostic Laboratory Immunology*. 2001; **8**: 460 – 464.
  23. Silva MS, Filho JM, Anazetti MC, Frungillo L, Haun M, Diniz MF, et al. Xylodiol from *Xylopi langsdorfiana* induces apoptosis in HL60 cells. *Brazilian Journal of Pharmacognosy*. 2011; **2**: 1035 – 1042.
  24. Zinadah OA, Khalil WK, Ashmaoui HM, Abdul F, Alsoud ME. Evaluation of the anti – genotoxicity and growth performance impacts of green algae on *Mugil cephalus*. *Life Science Journal*. 2013; **10**(3): 1543 – 1554.
  25. Azizi E, Abdolmohammadi MH, Fouladdel S, Shafiee A, Amin G, Ghaffari S. valuation of p53 and Bcl – 2 genes and proteins expression in human breast cancer T47D cells treated with extracts of *Astrodaucus persicus* (Boiss.) Drude in comparison to Tamoxifen. *Daru*. 2009; **17**(3): 181 – 186.
  26. Choi SC, Kim KD, Kim JT, Kim JW, Lee HG, Kim JM, et al. Expression of human NDRG2 by myeloid dendritic cells inhibits down–regulation of activated leukocyte cell adhesion molecule (ALCAM) and contributes to maintenance of T cell stimulatory activity. *Journal of Leukocyte Biology*. 2008; **83**(1): 89 – 98.
  27. Tsuan HY, Lu C, Yang JS, Chiang JH, Li TC, Siu Wan IP, et al. Berberine induced apoptosis via promoting the expression of caspase –8, –9 and –3, apoptosis–inducing factor and endonuclease G in SCC–4 human tongue squamous carcinoma cancer cells. *Anticancer Res*. 2009; **29**: 4063 – 4070.
  28. Shang LH, Li CM, Yang ZY, Che DH, Cao JY, Yu Y. *Luffa echinata roxb* induces human colon cancer cell (ht–29) death by triggering the mitochondrial apoptosis pathway. *Molecules*. 2012; **17**: 5780 – 5794.
  29. Ling Q, Xu X, Wei X, Wang W, Zhou B, Wang B, et al. Oxymatrine induces human pancreatic cancer PANC–1 cells apoptosis via regulating expression of Bcl–2 and IAP families, and releasing of cytochrome c. *Journal of Experimental & Clinical Cancer Research*. 2013; **30**: 66.
  30. Pozarowski P, Darzynkiewicz Z. Methods in molecular biology: checkpoint controls and cancer, activation and regulation protocols. *Humana Press Inc*. **281**(2): 303.
  31. Guzman M. Cannabinoids: Potential anticancer agents. *Nature Reviews Cancer*. 2003; **3**: 745 – 755.
  32. Helyer LK, Chin S, Chui BK, Fitzgerald B, Verma S, Rakovitch E, et al. The use of complementary and alternative medicines among patients with locally advanced breast cancer—a descriptive study. *BMC Cancer*. 2006; **6**: 39.
  33. Jedinak A and Sliva D. *Pleurotus ostreatus* inhibits proliferation of human breast and colon cancer cells through p53–dependent as well as p53–independent pathway. *International Journal of Oncology*. 2008; **33**: 1307 – 1313.
  34. Muslim NS, Nassar ZD, Aisha A, Shafaei A, Idris N, Majid AM, et al. Antiangiogenesis and antioxidant activity of ethanol extracts of *Pithecellobiumjiringa*. *BMC Complementary and Alternative Medicine*. 2012; **12**: 210.
  35. Hasan TN, Grace BL, Shafi G, Al–Hazzani AA, Alshatwi AA. Anti-proliferative effects of organic extracts from root bark of *Juglans Regia* L. (RBJR) on MDA-MB-231 human breast cancer cells: role of Bcl-2/Bax, caspases and Tp53. *Asian Pacific Journal of Cancer Prevention*. 2011; **12**: 525 – 530.
  36. Alshatwi AA, Shafi G, Hasan TN, Al-Hazzani AA, Alsaif MA, Al-fawaz MA, et al. Apoptosis–mediated inhibition of human breast cancer cell proliferation by lemon citrus extract. *Asian Pacific Journal of Cancer Prevention*. 2011; **12**: 1555 – 1559.
  37. Patel Y, Naraian R, Singh VK. Medicinal properties of *Pleurotus* species (oyster mushroom): A review. *World Journal of Fungal and Plant Biology*. 2012; **3**(1): 1 – 12.
  38. Wong SM, Wong KK, Chiu LCM. Non–starch polysaccharides from different developmental stages of *Pleurotus Tuber–Reginum* inhibited the growth of human acute promyelocytic leukemia hl–60 cells by cell–cycle arrest and/or apoptotic induction. *Carbohydrate Polymers*. 2007; **68**: 206 – 217.
  39. Venkatakrishnana V, Shenbhagaramanb R, Kaviyarasanb V, Gunasundari D, Radhika K, Dandapani R, et al. Antioxidant and antiproliferative effect of *Pleurotus Ostreatus*. *Journal of Phytology*. 2010; **2**(1): 22 – 28.
  40. Kerr J, Wyllie A, Currie A. Apoptosis: A basic biological phenomenon with ranging implications in tissue kinetics. *British Journal of Cancer*. 1972; **26**: 239 – 257.
  41. Patel S, Goyal A. Recent developments in mushrooms as anti–cancer therapeutics: A review. *3 Biotech*. 2012; **2**(1): 1 – 15.
  42. Lavi I, Friesem D, Geresh S, Hadar Y, Schwartz B. An aqueous polysaccharide extract from the edible mushroom *Pleurotus ostreatus* induces anti-proliferative and pro-apoptotic effects on HT-29 colon cancer cells. *Cancer Letters*. 2006; **244**: 61 – 70.
  43. Cheng T. Cell cycle inhibitors in normal and tumor stem cells. *Oncogene*. 2004; **23**: 7256 – 7266.
  44. Mau J, Lin HC, Chen CC. Antioxidant properties of several medicinal mushrooms. *Journal of Agricultural and Food Chemistry*. 2002; **50**: 6072 – 6077.
  45. Wang G, Zhang J, Mizuno T, Zhuang C, Ito H, Mayuzumi H, et al. antitumor active polysaccharides from the Chinese mushroom *Songshan lingzhi*, the fruiting body of *ganoderma tsugae*. *Bioscience, Biotechnology, and Biochemistry*. 1993; **57**: 894 – 900.
  46. Yu YH, Kuo HP, Hsieh HH. *Ganoderma tsugae* induces S phase arrest and apoptosis in doxorubicin–resistant lung adenocarcinoma H23/0.3 cells via modulation of the PI3K/Akt signaling pathway. *Evidence–Based Complementary and Alternative Medicine*. 2012; **2012**: 371286.