

# Mycochemistry, antioxidant content, and antioxidant potentiality of the ethanolic extract of *Pleurotus florida* and its anti-cancerous effect on HeLa cancer cell line, and antitumor effect on HeLa-implanted mice

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## ABSTRACT

**Objectives:** Cervical cancer is increasing worldwide and is becoming resistant to the existing drugs in clinical practice. Here, ethanolic extract of fruit body of *Pleurotus florida* was evaluated as antioxidant, anticancer agent against HeLa cell lines and anti-tumor against cervical cancer in mice model.

**Methods:** Fruit bodies of *P. florida* in 90% ethanol, and the *P. florida* ethanolic extract (PFEE) was subsequently investigated for its antioxidant content and activity, anticancer properties against the cervical cancer cell line, HeLa, and antitumor activity against HeLa implanted mice.

**Results:** The antioxidant activity bioassay showed that the  $IC_{50}$  of PFEE was  $41.17 \pm 1.42a$   $\mu\text{g/ml}$ . The cytotoxicity assay revealed that PFEE caused inhibition of cell proliferation. At the highest dose (1,250  $\mu\text{g/ml}$ ) after 24 h, 48 h, or 72 h of treatment, the percentages of cell growth inhibition were 75.22%, 77.77%, and 84.65%, respectively. It revealed that PFEE-treated cells became rounded and the nuclei became fragmented. PFEE induced intracellular generation of reactive oxygen species and reduced the mitochondrial membrane potential. PFEE also led to an up regulation of the apoptotic genes for caspases-3, -9, and Bax, whereas Bcl-2 gene was down regulated, and it also promoted the expression of p53. Cell cycle analysis revealed that cell cycle was arrested at the  $G_0/G_1$  checkpoint. PFEE suppressed metastasis and colonization. At a dosage of PFEE of 50 mg/kg of body weight, a 66.72% reduction in the size of tumors and an 87.44% reduction in the tumor weight were observed in mice.

**Conclusions:** It has demonstrated that PFEE is a highly potent anti-cervical cancer agent *in vitro* and *in vivo*.

**Keywords:** Apoptosis, colonization, Cytotoxicity, metastasis, migration

## Introduction

More than two decades have passed since the concept of “functional foods” was first introduced in food science.<sup>[1]</sup> At present, consumers are interested in bioactive food products that are beneficial to health and reduce the risk of disease. Some epidemiological studies revealed that ideal diet containing vegetables, whole grains, fruits, seeds, and herbs, may decrease the risk of the progression of many ailments such as diabetes, cancer, and cardiovascular disease.<sup>[2]</sup> Alhumaydi<sup>[3]</sup> reported that honey has profound protective effect against toxicity caused by application of chemotherapeutant in BALB/C mice. Mushrooms have been recognized as one of the important functional foods for humans. They have been collected and cultivated for

hundreds of years in Asian countries, such as China and Japan<sup>[4]</sup> and they have a long history of use in view of their health promotion benefits.<sup>[5]</sup> Numerous reports have been published in recent years on mushroom biochemistry, and on their nutritional and functional properties.<sup>[6,7]</sup> The number of different mushroom species on earth is estimated at 140,000, of which perhaps only ~10% are known to science. Furthermore, of those ~14,000 species currently recorded, ~50% are considered to possess varying degrees of edibility.<sup>[8]</sup> At present, mushrooms, or the extracts derived thereof, are used globally in the form of dietary supplements for cancer management.<sup>[9,10]</sup> Oyster mushrooms (of the *Pleurotus* genus) are edible and are widespread throughout the hardwood forests of the world.<sup>[11]</sup> Now cultivation and popularity of the *Pleurotus* species has increased markable

throughout the world as it is easy to cultivate and it has high nutritional and medicinal values. It is the 3<sup>rd</sup> largest cultivated mushroom in the world.<sup>[12]</sup> *P. florida* belongs to the family *Pleurotaceae* and order *Agaricales* and is one of the widely cultivated mushroom species, although the number of studies that have been conducted on its anticancer activity in the cervical cancer cell line, HeLa, and other cancer cell lines, are very few.<sup>[11]</sup> Other species of *Pleurotus*, however, have drawn the attention of scientists in terms of investigating their anticancer properties. *Pleurotus ostreatus* is an edible mushroom that has been widely investigated for a variety of properties, including its antitumor effects.<sup>[13]</sup> Ren<sup>[14]</sup> reported that water-soluble polysaccharide extracts of *Pleurotus eryngii*, *Pleurotus austral* and 14 other species of mushroom exhibited direct antitumor activity against the C32 and WM-266-4 cell lines. Since a large number of the compounds have been shown to act synergistically, it is worth testing the anti-proliferative effects of the whole mushroom extract, rather than simply its individual components. This principle (synergy) is compatible with similar natural biological products, such as the essential oils, which are more effective when used as whole products, as potential unwanted side-effects are quenched, or nullified, by the presence of individual components. Previously, numerous researchers have worked with various solvent extracts of mushrooms (ethanolic, methanolic, water, hexane, etc.) on different human cancer cell lines, although the consensus opinion is that ethanolic extracts derived from mushrooms are most effective for this purpose.<sup>[15,16]</sup> The use of the ethanol for extraction has some advantages over other polar solvents as it is able to dissolve both polar and non-polar substances and rather non-toxic in comparison to methanol while the use of methanol is limited due to its toxic effects. Water use for extracting requires care for evaporating.<sup>[17,18]</sup> Rather ethanolic solvent is more effective for extracting maximum antioxidant content.<sup>[19]</sup> On the other hand, among women in India, approximately one-third of the total number of newly diagnosed cases of cancer are of cervical cancer, resulting from infection with certain types of human papilloma virus. This disease is also called as “hidden or silent” cancer as the patient is generally asymptomatic. More than 50% of international cases occur within the Asia-Pacific region, and India has the top predicted figure of cases of this cancer.<sup>[20]</sup> Every year in India, 122,844 women are diagnosed with cervical cancer, and 67,477 die from the disease.<sup>[21,22]</sup> Cancers of the cervix and the uterus are a major problem for women in West Bengal.<sup>[23]</sup> Therefore, in the present study, *P. florida* (fruit body) ethanolic extract (PFEE) was evaluated for its antioxidant content, activity, anticancer properties, including anti-proliferative effects and other properties, such as induction of apoptosis, mitochondrial membrane potential (MMP) reduction, generation of reactive oxygen species (ROS), and anti-colonization and anti-migration effects. To this end, the antitumor effects of PFEE were investigated on the HeLa cervical cancer cell line, and on HeLa cell-implanted Swiss albino mice.

## Materials and Methods

### Mushroom collection and identification

The cultivated basidiocarps were collected from Baruipur market, District-24-Parganas(S), Kolkata, India and carried to the laboratory in a biodegradable polythene bag. All morphological characteristics, such as color, shape, and so on, were recorded, a spore print was taken, and anatomical characters were noted, identifying them by consulting the published keys.<sup>[24-26]</sup> The genomic DNA was isolated<sup>[27]</sup> and purified from fresh mushroom samples, and ITS1-5.8S-ITS2 marker zone of rRNA was amplified by PCR protocol, modified from Gardes and Bruns<sup>[28]</sup> with primers ITS-1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS4 (5'TCCTCCGCTTATTGATATGC3') The PCR products were sent to the SCI GENOME laboratory in Kerala for sequencing. The obtained sequence was subjected to BLAST and submitted to GenBank, NCBI, Baltimore, USA.

### Solvent extraction

The collected fruit bodies of the mushroom were thoroughly washed with tap-water, and subsequently air-dried in an oven at 50°C for 48 h. The dried fruit bodies were chopped into pieces, and then grinded into powder using a mixer grinder.

#### Dip method

700 g of dry mushroom powder was weighed and then it was dipped in ethanolic solvent in proper ratio (1:10), stored at incubator 45°C for 7 days. After 7 days, the mixture was filtered through Whatman No. 4, followed by Whatman No.1 filter papers (GE Healthcare Life Sciences, Little Chalfont, UK). The ethanol was removed from the extract using a rotary vacuum evaporator at 40°C, and the remaining solvent was removed using a freeze-drier. The resultant powder is recognized as PFEE (*Pleurotus florida* Ethanolic Extract) was kept in an airtight condition in a refrigerator at 4°C. Extract to be used in the *in vitro* assays was dissolved in plain Dulbecco's Modified Eagle medium (DMEM) (Himedia Laboratory), and sterilized using a 0.22 µm Millipore filter. The prepared extract was further diluted with DMEM to the desired concentrations prior to use.

#### Soxhlet extraction method

Dried mushroom powder (600 g) was packed into a Soxhlet apparatus and decoction with 500 mL of ethanol at 45°C for 6 h. The elicitation was strained through Whatman filter paper No. 1, and the filtrate was kept and the ethanol was removed from the extract using a rotary vacuum evaporator at 40°C, and the remaining solvent was removed using a freeze-drier. The further steps were as earlier method.

### Mycoscreening of PFEE

Mycoscreening was performed to identify the myco-compound/secondary metabolites in the PFEE sample in this present observation by way of color checks.

## Alkaloid test

A quantity of 0.2 g PFEE was weighed and warmed in 2% sulfuric acid in test tube for 2 min, and a few drops of Dragendorff's reagent were added and the presence of orange red precipitation indicated the presence of alkaloid.<sup>[29]</sup>

### Test for steroids

A few drops of acetic acid anhydride were added to 0.2 g of PFEE in a test tube. Concentrated H<sub>2</sub>SO<sub>4</sub> was carefully delivered drop by drop to the examination tube via the inner wall. The presence of a brown ring on the surrounding floor, which indicated the presence of steroids.<sup>[30]</sup>

### Test for terpenoids

Approximately 0.2 g of PFEE was confiscated and solvated in 2 ml of chloroform in test tube; after filtration, filtrate was added by 3 ml of concentrated sulfuric acid in a test tube and in the end perceived reddish brown shade which indicated for the presence of terpenoid.<sup>[31]</sup>

### Test for flavonoid

Around 0.2 g of PFEE was confiscated and solvated with 4 ml of distilled water in a test tube then appended with diluted sodium hydroxide and diluted hydrochloride solution delivered and described a yellow coloration which implied for the presence of flavonoid. It became colorless while on addition of a few drops of dilute acid which changed into yellow color indicating the presence of flavonoid.<sup>[32]</sup>

### Test for saponins

About 2 ml of the PFEE were mixed with 10 mL of distilled water in a test tube and that was shaken vigorously for about 5 min, and then it turned into stored for 30 min and found for honey comb forth, which turned into indicating the presence of saponin.<sup>[29]</sup>

### Test for tannin

A quantity of 0.2 g of PFEE turned into mingled with 4 ml of distilled water in a test tube and heated on water bath. The aggregate was blended and ferric chloride turned into appended to the filtrate and described for dark inexperienced answer that indicated the presence of tannin.<sup>[33]</sup>

### Test for phenol

Approximately 5 ml of sample PFEE mixed with 5% ferric chloride in a test tube and kept for the genesis of deep blue color which stipulated the presence of phenol.<sup>[34]</sup>

### Test for phlobatannins

Almost 2 ml of PFEE solution have been mixed with dilute HCl in a test tube and located for pink precipitation that indicated the presence of phlobatannins.<sup>[29]</sup>

## Divergent biochemical assay

### Total phenol content assessment

Folin-reagent Ciocalteu's method was used to determine the total phenol content of various solvent extracts.<sup>[35]</sup> The mixture of 0.1 ml Folin-reagent Ciocalteu's (0.5 N) reagent and 0.5 ml PFEE solution was incubated for 15 min at room temperature. Then 2.5 ml of saturated sodium carbonate solution was added, and the mixture was incubated at room temperature for 30 min. At 760 nm, the absorbance was measured and compared to a Gallic acid calibration curve. In the end, the result was expressed as mg of Gallic acid equivalent (GAEs) per gram of dry mushroom weight and it was presented as the mean  $\pm$  SD of triplicate.

### Total flavonoid content assessment

The TFC of the PFEE was determined using a standard method proposed by Meda *et al.*<sup>[36]</sup> Briefly in a test tube, equal amount of 2% AlCl<sub>3</sub> (made in absolute methanol) and extract (1 mg/ml) were incubated for 10 min at room temperature. The mixture was then incubated for 10 min and the absorbance was measured at 415 nm. Different concentrations of quercetin (0–10  $\mu$ g/ml) were used as a standard. The result was expressed as mg of Quercetin equivalent (QEs) per g of mushroom dry weight, and it was presented as the mean presented as the mean  $\pm$  SD of triplicate.

### Total ascorbic acid content assessment

Total ascorbic acid content was determined following the Folin–Ciocalteu reagent method by Jagota and Dani<sup>[37]</sup> with slight modifications. The PFEE (0.5 ml) were combined with 0.8 ml of 10% trichloroacetic acid and rapidly shaken, then refrigerated for 5 min before centrifugation at 3000 rpm for 5 min. The extract (0.2 ml) was then dilute to 2 ml with distilled water. Folin-Ciocalteu 2.0 M was diluted 10 times in distilled water. The combination was then given 0.2 ml of the diluted reagent. The mixture was then incubated for 10 min at room temperature. The absorbance was measured at 760 nm. Different concentrations of ascorbic acid (0–10  $\mu$ g/ml) were used to make a standard curve. Finally, the results were reported as mg of ascorbic acid equivalent (AAEs) present per g of mushroom extract, and presented as the mean  $\pm$  SD of triplicate.

## Antioxidant activity assay

### 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay

The radical scavenging activity (RSA) of PFEE was assessed using the DPPH method. DPPH, a stable free radical with an at 515 nm, was used to study the radical scavenging effect of decoction. As antioxidants deliver protons to these radicals, their absorption reduces. The drop in absorbance is regarded as a measure of the magnitude of radical-scavenging. The free-radical scavenging capacities of the tracts were calculated using the DPPH test. The RSA of DPPH was evaluated using

a modified Bondet *et al.* technique.<sup>[38]</sup> Different concentrations (50–150 µg/ml) of PFEE were made, and here, 0.9 ml of DPPH solution (0.1 mM) was added to a test tube with 100 µl of each concentration of PFEE powder separately. Control was prepared with ethanol. The reaction mixture was incubated for 2 h at RT, and the absorbance was measured at 515 nm with a spectrophotometer. The scavenge potentiality of PFEE was ultimately measured. The synthetic antioxidant BHA (butylated hydroxyanisole) was used as positive control. The percent reduction in DPPH was estimated using the following formula:

$$\% \text{ DPPH} = [(A_c - A_s) / A_c] \times 100$$

As is the absorbance of sample and  $A_c$  is the absorbance of control. Then the  $IC_{50}$  value (50% inhibition) was calculated.

## Anti-cancerous activity

### Cell culture

The human cervical cell line, HeLa, at passage number 30 (purchased from The National Centre for Cell Science, Savitribai Phule Pune University, Pune, India) was cultured in DMEM supplemented with L-glutamine, 10% (v/v) fetal bovine serum, 100 µg/ml streptomycin, and 250 IU/ml penicillin (all purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India) in 75-mm tissue culture flasks at 37°C in a humidified atmosphere of 5%  $CO_2$  until the cells reached 70–80% confluence.<sup>[39]</sup> For maintenance, cultures were passaged weekly, and the culture medium was changed twice a week.

### Cytotoxicity/cell proliferation assay

The effect of PFEE on cell proliferation of the HeLa cell line was assessed using a methyl thiazolyl tetrazolium (MTT) assay (reagents provided by HiMedia Laboratories Pvt Ltd).<sup>[40]</sup> Briefly,  $10 \times 10^4$  cells/well of a 96-well culture plates were seeded with fresh DMEM medium containing 10% FBS and antibiotics overnight until the cells reached 80% confluence. Subsequently, the culture was washed with 10% phosphate-buffered saline (PBS) and treated with PFEE dissolved in DMEM at various concentrations (250, 500, 1000, and 1250 µg/ml), and the cells were then incubated at 37°C in an atmosphere of 5%  $CO_2$  and 95% air, where DMEM used as vehicle control. After 24 h, 48 h, and 72 h of treatment, cells were washed with phosphate-buffered saline, and 100 µl of 500 µg/ml MTT solution (dissolved in phenol red-free DMEM) was added to each well. The cells were subsequently further incubated for 3 h, and after having discarded the media, 100 µl dimethyl sulfoxide (DMSO) was added to dissolve the crystals. The plate was read using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at an absorbance of 570 nm. The HEK 293 cell line was taken and subjected to MTT assay at the highest tested concentration of PFEE (1250 µg/ml) to check the cytotoxicity of this extract toward a normal human cell line. The  $IC_{50}$  value (i.e., the concentration that led to a 50% killing of the cells) was calculated by plotting

a dose-response graph of the cytotoxicity values obtained using the following formula:

$\% \text{ Cell cytotoxicity} = 100 - [A_{\text{Control}} - A_{\text{test}} / A_{\text{Control}}] \times 100 \times 100$   
The data points represent the mean  $\pm$  standard deviation of each experiment, and the experiments were repeated at least 3 times.

### HeLa cell morphology examined under a phase contrast microscope

To investigate the cell morphology,  $2 \times 10^5$  HeLa cells/well were seeded into 6-well plates, and at 80–90% confluence the cells were treated with various concentrations of PFEE (500, 750, and 1000 µg/ml) where DMEM used as vehicle control. After 24 h, images of the cells were captured using an Olympus phase contrast microscope (at a magnification of  $\times 20$ ; Olympus Corporation, Tokyo, Japan).<sup>[41]</sup>

### HeLa nuclear morphology examined by Hoechst-33342 staining under a fluorescence microscope

To determine the morphological changes (chromatin condensation) of the cell nuclei by PFEE, Hoechst-33342 (Thermo Fisher Scientific, Inc.) staining was performed, according to the method described by Allen *et al.*<sup>[42]</sup> In brief,  $2 \times 10^5$  HeLa cells/well were seeded onto a 6-well plate, and after having reached 95% confluence, the cells were treated with two concentrations of PFEE (500 and 1000 µg/ml) supplemented with DMEM, where DMEM used as a vehicle control. After 48 h incubation, the cells were washed with PBS, followed by fixing of the cells with 3.7% formaldehyde for 25 min. After washing twice with PBS, cells were permeabilized with 0.1% Triton X-100 for 15 min. Subsequently, the cells were stained with 5 µg/ml Hoechst 33342 dyes for 10 min at room temperature in the dark. After washing twice with PBS, stained nuclei were observed under an inverted Olympus fluorescence microscope (Olympus Corporation) at a magnification of  $\times 20$ .

### Cell cycle analysis

It was based on flow cytometric DNA analysis of cells that distributed at different stages in the cell cycle as described by Pozarowski and Darzynkiewicz.<sup>[43]</sup> In brief, HeLa cells ( $7.5 \times 10^5$ ) were seeded in 100 mm dishes, and cultured in DMEM containing 10% FBS for 24 h, followed by incubation with PFEE (500, 750, and 1000 µg/ml) at 37°C for 24 h, where DMEM used as vehicle control. After the period of incubation, the cells were harvested, washed with Dulbecco's PBS containing 1% FBS, and re-suspended in 50 µg/ml propidium iodide. Samples were analyzed on a fluorescence-activated cell sorting (FACS Calibur BD Bioscience, USA). The fractions of cells in the various phases of the cell cycle ( $G_0/G_1$ , S, and  $G_2/M$ ) are shown as a percentage of the total cells analyzed.

### Cell migration assay using the scratch method

The migration assay was performed following the method described by Lee *et al.*<sup>[44]</sup> but with certain modifications.

Briefly,  $10 \times 10^3$  HeLa cells/well were cultured in a 24-well plate. After having reached 90% confluence, the center of the culture dishes was scratched with a 200  $\mu$ l pipette tip. The cells were subsequently washed 2 times with PBS, prior to incubation with PFEE at concentrations of 500 and 1000  $\mu$ g/ml. After 24 h incubation, images of the cells were captured at a magnification of  $\times 10$ . The relative migration rate was estimated by measuring the distance the cells had migrated for 24 h, with the data being normalized against cells cultured in DMEM alone as the control. The relative migration rate was calculated as compared with the control group.

#### Clonogenic assay

This assay was performed following the method described by Crowley *et al.*<sup>[45]</sup> In brief, harvested HeLa cells were seeded in 6-well plates at a concentration of 500 cells/well. Subsequently, culture medium containing PFEE at various concentrations (50, 75, 100, and 125  $\mu$ g/ml) was added to each well soon after the cells had attached themselves to the wells. One negative control (DMEM vehicle without PFEE) set of experiments was also performed. Colony formation was assessed after incubation for 14 day(s) in culture medium, which was replaced every 4 days. Colonies were fixed and stained with 3.7% formaldehyde and 0.5% crystal violet; subsequently, the colonies were counted manually under a microscope, and images of the cells were captured. Colonies consisting of  $>50$  cells were counted using a colony counter, and the results are reported as a percentage of colonies formed using the following equation:

$$\% \text{ Colonies formed} = \left( \frac{\text{colonies formed in treated sample}}{\text{colonies formed in untreated sample}} \right) \times 100.$$

#### Microscopic determination of ROS

ROS was determined by staining using 2,7-dichlorodihydrofluorescein di-acetate ( $H_2DCFDA$ ) and 4',6-diamidino-2-phenylindole (DAPI; both purchased from Thermo Fisher Scientific, Inc.). In brief,  $2 \times 10^5$  cells/well were seeded onto a 6-well plate, and after having reached 90% confluence, cells were treated with PFEE at various concentrations (500, 750, and 1250  $\mu$ g/ml) before incubation at  $37^\circ C$  for 2h, where DMEM used as vehicle control. After the 2 h incubation, the existing medium was discarded. The cells were subsequently re-suspended in serum-free DMEM media, 1 $\mu$ l  $H_2DCFDA$  and DAPI were added, and the cells were further incubated for 30 min. Finally, the cells were washed 3 times with PBS, serum-free media were added, and the cells were observed under an inverted fluorescence microscope. Ascorbic acid and hydrogen peroxide ( $H_2O_2$ ) were used as positive and negative controls, respectively.<sup>[46]</sup>

#### Qualitative determination of DNA fragmentation using agarose gel electrophoresis or DNA laddering assay

DNA fragmentation a distinctive feature used to determine the extent of apoptosis. 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. For this assay,  $4 \times 10^5$  cells were seeded on to a 35 mm plate at  $37^\circ C$ . After 24 h, cells were treated with different concentrations of PFEE (500, 825, and 1000  $\mu$ g/ml), and subsequently incubated for 24 h, 48 h, or 72 h. After each respective time of incubation, cells were washed with PBS buffer, trypsinized and finally the total genomic DNA of the cells was extracted using radio-immunoprecipitation (RIPA) assay buffer (Abcam, Cambridge, UK). The extracted DNA was resolved on a 1.5% agarose gel containing ethidium bromide (HiMedia Laboratories Pvt. Ltd.) in 40 mM Tris-acetate buffer (pH 7.5), electrophoresis at 50V for 4 h. Images of the DNA fragments were captured using a gel documentation system (Bio-Rad Laboratories, Inc.).<sup>[47]</sup>

#### MMP analysis

Cells ( $2 \times 10^5$ /well) were seeded on to a 24-well plate in DMEM. After a 24 h incubation at  $37^\circ C$  in an atmosphere of 5%  $CO_2$ , cells were treated with (500, 750, and 1000  $\mu$ g/ml) PFEE, followed by incubation for 12 h, where DMEM used as vehicle control. At the end of the incubation period, cells were washed with  $1 \times$  PBS, and subsequently incubated with 10  $\mu$ l 200  $\mu$ M JC-1 at  $37^\circ C$  in 5%  $CO_2$  and 95% air for 30 min. Finally, the cells were examined under an Olympus fluorescence microscope with a red and green filter (Olympus Corporation).<sup>[48]</sup>

#### Protein isolation and western immunoblotting

Cells ( $4 \times 10^5$  cells/well) were seeded on to a 6-well plate, followed by an overnight incubation ( $37^\circ C$ ). After discarding the media, cells were washed with  $1 \times$  PBS and treated with 500, 750, and 1000  $\mu$ g/ml PFEE for 24 h, where DMEM used as vehicle control. After 24 h incubation, each well was washed with  $1 \times$  PBS (1 ml), and subsequently the cells were trypsinized (1X) and incubated at  $37^\circ C$  in a cell culture incubator for 4 min. Following trypsinization, 2 ml fresh DMEM media were added to each well, and the cells were collected in a 1.5 ml centrifuge tube. Each centrifuge tube was centrifuged at 5000 rpm,  $4^\circ C$  for 5 min, and after discarding the supernatant, the cell pellets were dissolved in 1 ml  $1 \times$  PBS, followed by a further centrifugation at 5000 rpm for 5 min. Total protein isolation was performed in 1X RIPA buffer (Abcam) as per the manufacturer's protocol, and the protein concentration was measured using the Puregene<sup>®</sup> bicinchoninic acid (BCA) method. Protein samples (100  $\mu$ g) were subjected to SDS-PAGE: Protein separation was accomplished on a 10% TGX stain-Free<sup>™</sup> Fast Cast Acrylamide gel (Bio-Rad Laboratories, Inc.) at 200V for 45 min. Following protein separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.) using a Trans-Blot<sup>®</sup> Turbo<sup>™</sup> transfer system (Bio-Rad Laboratories, Inc.). Subsequently, the membranes were washed with TBS buffer for 5 min, and blocked with 5% bovine serum albumin (BSA) for 1 h. The effect of PFEE treatment on the expression of certain genes as proteins, like p53, and on anti-apoptotic and apoptotic proteins, such as Bcl-2, Bax, pro-caspase-3, pro-caspase-9, cleaved-caspase-3,

and cleaved-caspase-9 (Santacruz Biotechnology, USA), were determined. Each membrane was then incubated with the respective primary antibody at a 1:500 dilutions (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in 1× antibody dilution buffer (5% BSA and 0.01% thiomershal) at 4°C with agitation. After an overnight incubation, membranes were washed 3 times (10 min each wash) in 1X TBS/Tween 20 (TBST) and incubated for 1h with the respective secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Again, membranes were washed 3 times (10 min each wash) with 1X TBST, and finally the protein expression of each membrane was imaged on photographic film (Carestream) using enhanced chemi-luminescence buffer (Bio-Rad Laboratories, Inc.).<sup>[49,50]</sup>

**Antitumor effects of PFEE in HeLa cell-implanted mice**  
Swiss albino mice (8–10 weeks old) weighing ~22–26 g were purchased. Animal experiments were conducted on experimental mice. All mice were housed under hygienic/pathogen-free air conditions in a room maintained at 24°C with 50% relative humidity and a 12 h/12 h light-dark cycle. All animal experiments were approved and performed according to the regulations of the Institutional Animal Ethics Committee, Government of India (approval no: 12/P/S/IAEC/2018). The mice were divided into six groups: i.e., control mice supplemented with pellet (control + vehicle;  $n = 6$ ); ii) controls supplemented with PFEE (500 mg.kg<sup>-1</sup>) (control + PFEE;  $n = 6$ ); iii) HeLa cell-implanted mice supplemented with pellet (HeLa + vehicle;  $n = 6$ ); iv) HeLa cell-implanted mice supplemented with PFEE (10 mg.kg<sup>-1</sup> body weight) (HeLa + PFEE 10;  $n = 6$ ); v) HeLa cell-implanted mice supplemented with PFEE (25 mg/kg) (HeLa + PFEE 25,  $n = 6$ ); and vi) HeLa cell-implanted mice supplemented with PFEE (50 mg/kg) (HeLa + PFEE 50,  $n = 6$ ).

For tumor generation, a suspension of  $2 \times 10^6$  HeLa cells in 0.2 mL DMEM was subcutaneously injected on the dorsal surface of the right-hand legs of the Swiss albino mice, whereas the control (DMEM vehicle) groups were injected with DMEM. The tumors were measured with Vernier calipers every 3–4 days using the formula  $a^2 \times b \times 0.52$  (where  $b$  is the shortest diameter and  $b$  is the longest diameter).<sup>[51]</sup> When the tumor volume was measured to be 8.0–10.0 mm<sup>3</sup>, the mice were randomized. Subsequently, the mice were supplemented daily with vehicle or PFEE at the doses of 10, 25, and 50 mg/kg of body weight for 28 days. After 28 days, all the mice in groups 3–6 were sacrificed, and the tumors were excised from their legs and then weighed. Visceral organs (i.e., the kidney, liver, spleen, and lungs) were morphologically observed for possible side effects of PFEE in the mice.

## Statistical analysis

All data were analyzed using Graph Pad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), and one-way

analysis of variance was performed to compare differences between the groups. All results are presented as the mean  $\pm$  standard error from three independent experiments performed in a parallel manner, unless otherwise indicated. Significant level of all data in the presented figures was obtained after Tukey's test statistical analysis ( $P < 0.05$ ) from three independent experiments. Statistically significant differences ( $P < 0.05$ ) among means of experiment results were analyzed by ANOVA and means compared by Duncan's Multiple Range Tests.

## Results

### Identification of mushroom sample

The Phenotypically mushroom sample was identified as *P. florida* by consulting the published keys.<sup>[24-26]</sup> ITS1-5.8S-ITS2 based identification showed that this mushroom was *Pleurotus sp.* and published in GenBank, NCBI, Baltimore, USA with Accession no MF045429.1.

### Yield of ethanolic extracts

The solvents were evaporated using a rotary evaporator once the extraction was completed, yielding semi-solid bulk crude extracts. The extraction yields of ethanol solvent for *P. florida* were obtained and presented in the Table 1, it showed that the yields in dip method and Soxhlet method were  $0.55 \pm 0.01$  and  $0.54 \pm 0.01\%$ , respectively, and they are same statistically ( $P = 0.05$ ).

### Mycoscreening study (qualitative)

The result presented in Table 2 exhibited that the chemical compounds present in the ethanolic extract were alkaloid, steroid, terpenoid, flavonoid, saponin, tannin, and phenolics but this extract did not contain any phlobatannins.

### Total Phenol, flavonoid, and Ascorbic acid content assay

Total phenolic content of ethanolic extract was  $7.98 \pm 0.78$  mg GAEs/g, total flavonoid content of the ethanolic extract was  $2.031 \pm 0.024$  mg quercetin equivalent/g. Ascorbic acid content in the ethanolic extract was  $0.167 \pm 0.03$  mg AAAs/g [Table 3].

### Antioxidant activity by DPPH method

The data presented in Table 4 showed percentage (%) of inhibition ( $\pm$ SD) of DPPH radical at different concentration ( $\mu$ g/ml) of PFEE, ascorbic acid, and BHA. Furthermore, the IC<sub>50</sub> (50 percent inhibition) of natural antioxidant of PFEE was  $41.17 \pm 1.42a$   $\mu$ g/ml, while the IC<sub>50</sub> of synthetic antioxidant BHA and ascorbic acid (positive control) were  $31.28 \pm 1.03b$  and  $39.00 \pm 1.22a$   $\mu$ g/ml, respectively. It clearly indicated that PFEE and ascorbic acids are statistically ( $P = 0.005$ ) same as per IC<sub>50</sub> activity but BHA is different from other two.

**Table 1:** Percentage of yield of PFEE (ethanolic extract) powder by dipping and Soxhlet extraction method

Method of extraction	Ratio of solute and solvent	Initial weight of Mushroom powder (gm)	Final crude weight (gm) (±SD)	Percentage of yield (%)
Dipping method	1:10	700	3.86±0.15	0.55±0.01a
Soxhlet extraction		600	3.26±0.3	0.54±0.01a

Same letter in the same column indicates they are statistically same but different letter indicate they are statistically different ( $P=0.05$ ) as per Duncan's multiple test

**Table 2:** Qualitative mycochemical tests of PFEE

Mycocompound/class	Test	Ethanol extract
Alkaloid	Dragendroff Test	+
Steroid	Lieberman acid test	+
Terpenoid	Salkowski's Test	+
Flavonoid	Ammonium Test	+
Saponin	Foam test	+
Tanin	Feric Chloride Test	+
Phenolics	Feric Chloride Test	+
Phlobatannins	HCl test	-

+ indicates presence, - indicates absence

**Table 3:** Total phenol, flavonoid, and ascorbic acid content of the ethanolic extract of *Pleurotus florida*

Antioxidant type	Amount
Flavonoid	2.03±0.024 mg QEs/g
Ascorbic acid	0.167±0.03 mg AAEs/g
Total Phenol	7.98 ± 0.78 mg GAEs/g

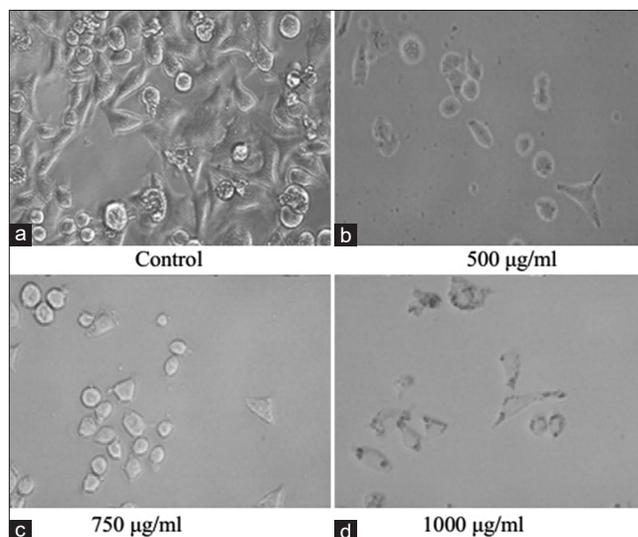
## Anticancer activity

### Effect of PFEE on HeLa cell morphology as measured using phase contrast microscopy

HeLa cell line, treated with various concentrations of PFEE and after 24 h incubation, was examined under a phase contrast microscope to monitor their morphology. The untreated cells (DMEM vehicle control) exhibited the normal spindle shape and reached 90% confluence after 24 h culture [Figure 1a]. Cells treated with 500 and 750 µg/ml PFEE were revealed to be smaller in size; they also lost their spindle shape, and the cell confluence was reduced [Figure 1b and c]. More rounded, shrunken cells were observed for the experiment group treated with 1000 µg/ml PFEE; furthermore, a lot of cell debris was observed, and the cell confluence was markedly lower [Figure 1d].

### Effect of PFEE on the nuclei of HeLa cells as observed under an inverted fluorescence microscope

Hoechst-33342 is a fluorescence stain that yields a blue color under a fluorescence microscope. When cells were stained with Hoechst-33342, live cells took up a light blue coloration uniformly, whereas apoptotic cells were stained a deeper shade of blue due to chromatin condensation. Figure 2a revealed that the nuclei of the untreated HeLa cells appeared normal, assuming a light-blue color, and were round and homogeneous, whereas the nuclei of cells treated with PFEE were observed to take up more of the deep-blue stain, and were condensed [Figure 2b], also being irregular in shape and fragmented



**Figure 1:** Effect of PFEE on HeLa cell morphology under phase contrast microscope (20×). (a) Control cells showed normal spindle shaped morphology but (b-d) treated cell showed mostly round shaped morphology after 24 h treatment of PFEE

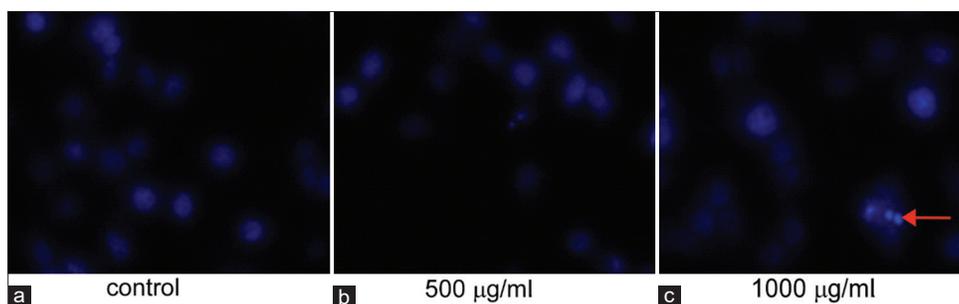
[Figure 2c]. These results demonstrated that PFEE was effective in inducing apoptosis of the HeLa cells.

### Cytotoxicity/anti-proliferation effects of PFEE against the HeLa cell line as determined by the MTT assay

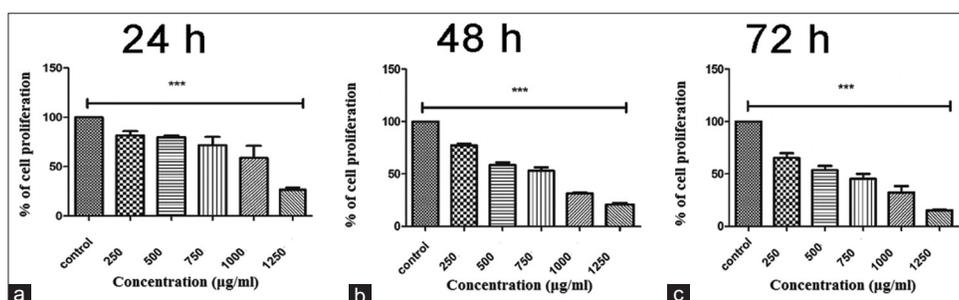
Whether PFEE affected HeLa cell proliferation or not was subsequently investigated. The cell proliferation bar diagram [Figure 3] revealed that the negative control (DMEM vehicle) cells (untreated) proliferated during the course of the observed time periods (24 h, 48 h and 72 h). By contrast, cells treated with PFEE at all tested concentrations exhibited a reduced rate of proliferation in a concentration- and time-dependent manner. Cells treated with the highest concentration (1,250 µg/ml) of PFEE tested proliferated the least. The reduction in cell proliferation was observed starting from 24 h after treatment and became more pronounced as the duration of the treatment was extended. Control cells without PFEE treatment exhibited no indication of any decrease in terms of their growth and proliferation. Cells treated with 1250 µg/ml revealed 75.22%, 77.77%, and 84.65% growth inhibition at the 24 h, 48 h, and 72 h time points, respectively [Figure 3a-c]. The highest dose tested of PFEE (1,250 µg/ml) did not reveal any indications of cytotoxicity in the normal HEK 239 cell line at 24 h, 48 h, or 72 h (data not shown).

### Cell cycle arrest by PFEE

The cell cycle distribution of HeLa cells was subsequently investigated using flow cytometry with the PI staining method



**Figure 2:** Effect of PFEE on HeLa nuclear morphology under Inverted Fluorescence microscopy. (a) Control showed Hoechst-33342 stained round nucleus of HeLa cell. (b and c) showed Hoechst-33342 stained nucleus of PFEE (500 and 1000 µg/ml) treated HeLa cell and they were condensed and fragmented. “Arrow” indicates nuclear fragmentations



**Figure 3:** Cytotoxic effect of PFEE on HeLa cell at (a) 24 h, (b) 48 h, (c) 72 h. *in vitro* cytotoxic effect of PFEE (0–1250 µg/ml) was measured by MTT assay on HeLa cell line. Bar graphs shows % of viable cells (Y axis) against PFEE concentration (X axis). (a-c) demonstrates decreasing percentage of viable cells with increasing concentration of PFEE as compared to untreated cell. Data are representative of three independent experiment and bar graph showed mean ±SEM (\*\* $P < 0.05$ , \*\*\* $P < 0.001$ , ns not significant)

**Table 4:** Percentage of inhibition (±SD) of DPPH radical at different concentrations (µg/ml) of PFEE, ascorbic acid, and BHA along their IC<sub>50</sub> values

Solvent extract/Standard	% of inhibition (±SD) of DPPH radical at different concentration (µg/ml)					IC <sub>50</sub> (µg/ml) ±SD
	50	75	100	125	150	
PFEE	54.78±3.45	60.56±5.67	71.07±6.90	77.09±6.75	79.00±6.95	41.17±1.42a
Ascorbic acid	58.35±4.78	64.07±5.89	75.00±7.01	81.07±7.90	87.12±7.45	39.00±1.22a
BHA	61.05±5.09	72.56±6.73	81.90±7.92	87.00±8.12	90.89±8.23	31.28±1.03b

Same letter in the same column indicates they are statistically same but different letters indicate they are statistically different ( $P=0.05$ ) as per Duncan’s multiple range test

[Figure 4]. The data presented in Table 5 revealed that, for the negative control (DMEM vehicle), the percentage of cells distributed in the G<sub>0</sub>/G<sub>1</sub> phase of the cycle was 63.1 ± 5.23%, although on treatment with PFEE at concentrations of 500, 750, and 1000 µg/ml, the percentages of cells distributed in the G<sub>0</sub>/G<sub>1</sub> phase increased gradually, to 70.6 ± 6.67%, 72.7 ± 6.79%, and 77.9 ± 7.01%, respectively. Hence, the arrest of cell cycle was happened at G<sub>0</sub>/G<sub>1</sub> by this PFEE.

**Effect of PFEE on induction of apoptosis as measured by protein expression with a western blotting assay**

To understand how PFEE induced apoptosis of cancer cells and inhibited cell proliferation, the gene expressions of pro- and anti- apoptosis genes such as, P53, Pro-caspase 3, Pro-caspase 9, Cleaved caspase 3, Cleaved caspase 9, Bax and Bcl-2, were detected by bands in western blotting. The density of bands between the target protein and actin (control) was calculated. In control, band densities of P53,

Bcl-2, Pro-caspase 3, Pro-caspase 9, Cleaved caspase 3, Cleaved caspase 9, and Bax, were 1.86 ± 0.45, 0.95 ± 0.22, 1.79 ± 0.49, 1.00 ± 0.49, 0.74 ± 0.05, 0.64 ± 0.02, and 0.55 ± 0.04%, respectively. In highest dose, band densities of P53, Bcl-2, Pro-caspase 3, Pro-caspase 9, Cleaved caspase 3, Cleaved caspase 9, and Bax were 4.15 ± 0.65, 0.39 ± 0.04, 0.42 ± 0.01, 0.35 ± 0.01, 1.95 ± 0.89, 1.69 ± 0.67, and 1.44 ± 0.45%, respectively. It indicated that gradual increase of dose of PFEE increased the band density of proteins (%) except Bcl2, Pro-caspase 3, and Pro-caspase 9. In these three cases, reverse trend was noticed. We found that treatment with PFEE decreased the expression of gene Bcl-2, while increased the expression of pro-apoptotic genes Caspase 3 and Caspase 9 but decreased the pro-caspase 3 and 9 [Table 6] in HeLa cells *in vitro*. Moreover p53 gene, one of important cancer guard genes showed high expression, that is, it was upregulated by PFEE. Using western blotting, it was observed that the levels of p53 protein and its downstream target proteins were up

regulated. The expression of Bax was increased gradually as concentrations increased [Table 6].

#### Effect of PFEE on intracellular ROS generation

To investigate the intracellular levels of ROS, the cell-permeable probe H<sub>2</sub>DCFDA was utilized. Non-fluorescent H<sub>2</sub>DCFDA, which is hydrolyzed to DCFH inside the cells, yields highly fluorescent DCFDA in the presence of intracellular H<sub>2</sub>O<sub>2</sub> and associated peroxides. Whether or not PFEE increased or decreased the generation of ROS was subsequently examined. As shown in Figure 5, PFEE treatment

increased the generation of ROS in HeLa cells, as determined by monitoring DCF fluorescence, when the dose of PFEE was gradually increased.

#### Effect of PFEE on the MMP

Changes in the MMP are key events in measuring levels of cell apoptosis. Therefore, alterations in the MMP were investigated in the present study using the JC1 dye, after 12 h of PFEE treatment in the HeLa cell line. Figure 6 revealed that non-treated cells exhibited a high level of JC1 aggregates (indicated by the red fluorescence), whereas cells that were treated with PFEE exhibited a red-to-green (monomeric) shift in the fluorescence. This experiment also revealed that the MMP change was concentration-dependent.

#### DNA fragmentation assay

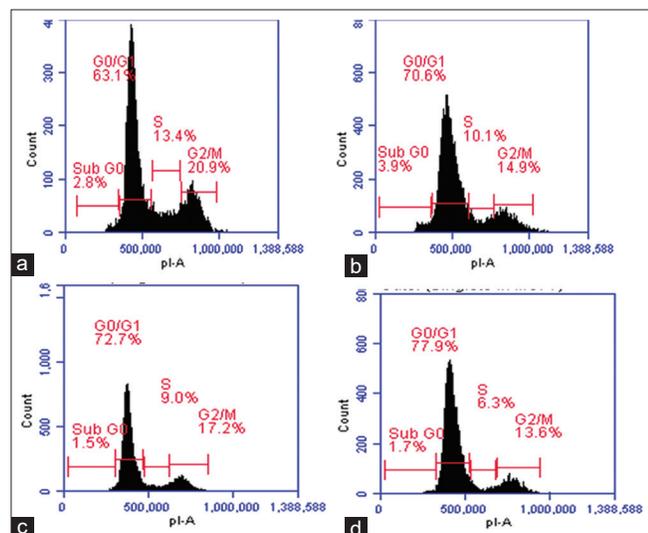
DNA fragmentation of HeLa cells treated with PFEE was easily detected as DNA laddering, prominent in the agarose gel electrophoresis experiments [Figure 7]. Increases in the concentration of PFEE led to increase in the extent of the laddering.

#### Anti-migration effects mediated by PFEE against HeLa cells as determined from the wound healing/scratch assay experiment

Cell migration is an important property of cancer cells. The effect of PFEE on cell migration was examined using a wound healing/scratch assay *in vitro*. Images were captured to show the migration of cells, and these were presented in Figure 8a and b. Cells in the control experiment (untreated/DMEM vehicle) filled the scratched space after the 24 h period of incubation, whereas treatment with PFEE reduced cell migration, as evidenced by the wider open space left between the two sides of the cells [Figure 8a]. In the case of treatment with 500 µg/ml PFEE, 52% of the cells migrated to the scratch, whereas on 1000 µg/ml PFEE treatment, only ~25% cells migrated into the scratched area [Figure 8b]. These results demonstrated that the inhibitory effect of PFEE on cell migration was dose-dependent.

#### Effect of PFEE treatment on anti-colonization in HeLa cells

Subsequently, the ability of PFEE to inhibit colony formation of the HeLa cells on 6-well plates was examined. Figure 9 revealed that PFEE gradually decreased the capability



**Figure 4:** Flow cytometric detection of the effect of PFEE on HeLa cell cycle phase distribution. (a) Control. (b) 1000 µg/ml. (c) 1250 µg/ml (d) 1500 µg/ml shows interfere with cell cycle population by inducing G<sub>0</sub>/G<sub>1</sub> arrest of HeLa cell *in vitro*, in 24 h

**Table 5:** Effect of PFEE on cell (percentage) distribution in cell cycle

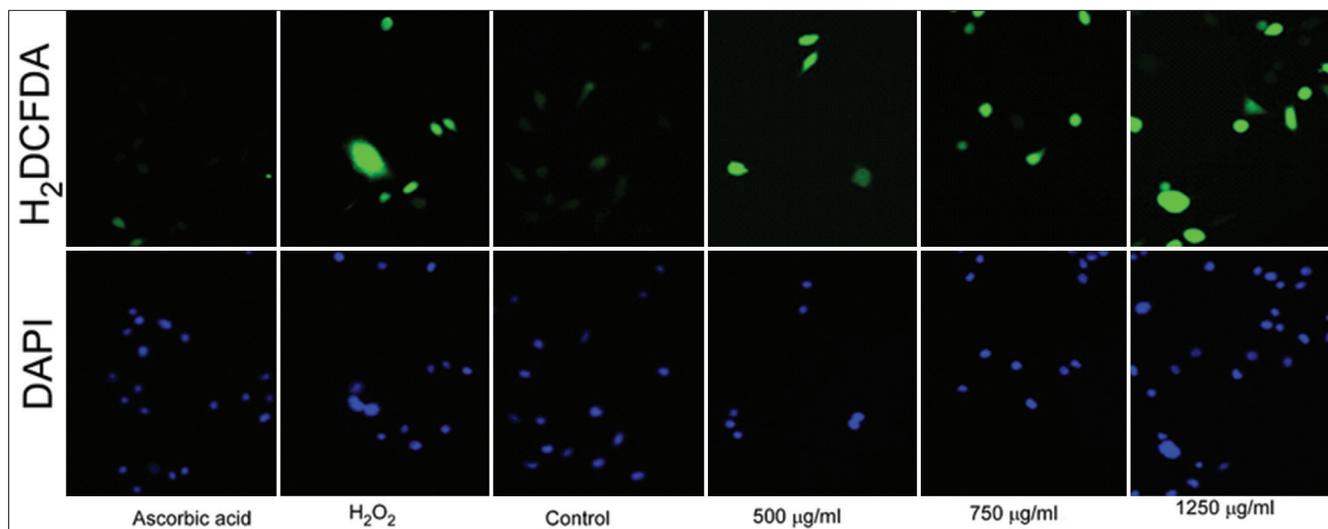
PFEE (µg/ml)	Percentage (%) of cells		
	G <sub>0</sub> /G <sub>1</sub>	S	G2/M
0.0(Negative control)	63.1±5.23	13.4±0.92	20.9±1.90
500	70.6± 6.67	10.1±0.89	14.9±1.07
750	72.7±6.79	9.0±0.76	17.2±1.08
1000	77.9±7.01	6.3±0.45	13.6±0.98

Cell distribution in percentage (%) in G<sub>0</sub>/G<sub>1</sub>, S, and G2/M. The data are mean ±SD of three experiments. Statistical significance P<0.05

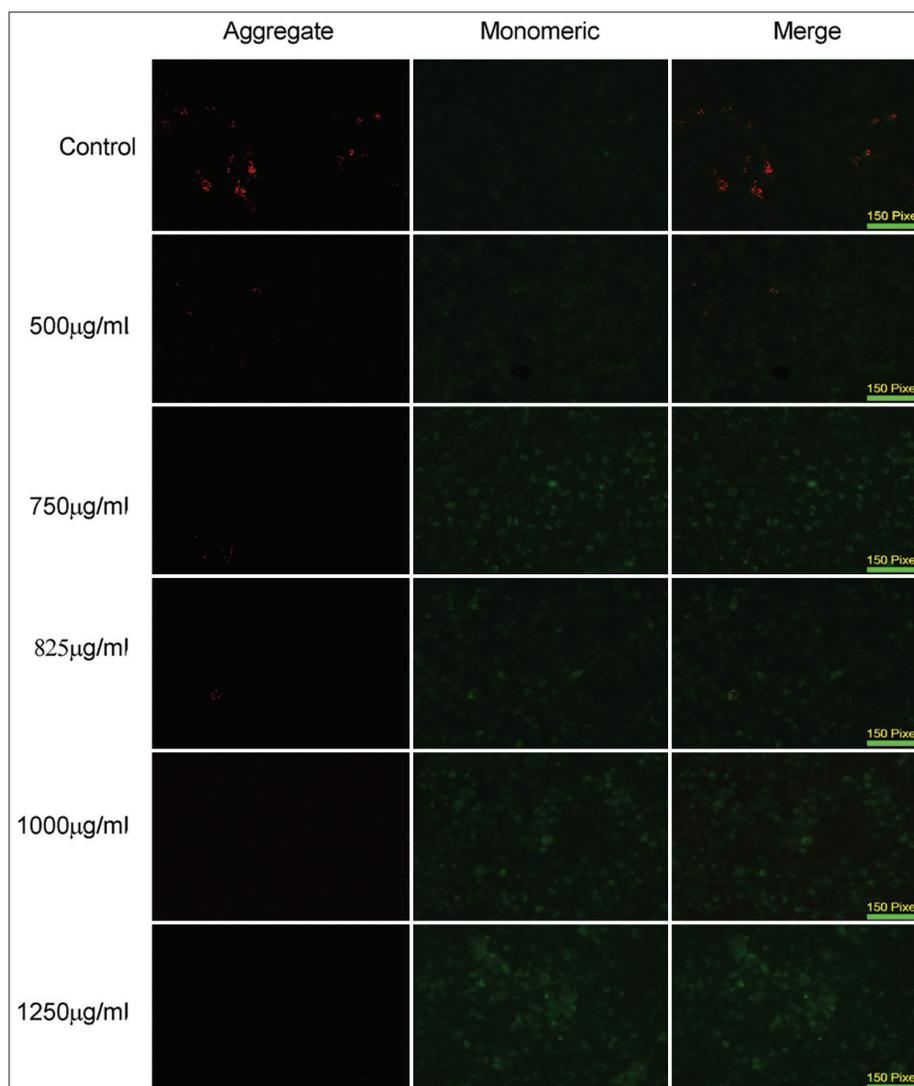
**Table 6:** Quantification of apoptosis related protein expression level (%) in HeLa cells treated with different concentrations of PFEE

Treatment/control	P53	Bcl-2	Pro-caspase 3	Pro-caspase 9	Cleaved caspase 3	Cleaved caspase 9	Bax
Control(beta- actin)	1.86 ±0.45	0.95±0.22	1.79±0.49	1.00±0.49	0.74±0.05	0.64±0.02	0.55±0.04
250 µg/ml	1.88±0.53	0.90±0.23	1.50±0.55	1.11±0.67	0.85±0.08	0.72±0.03	0.65±0.05
500 µg/ml	2.75±0.57	0.75±0.34	0.86±0.09	0.56±0.08	1.22±0.78	0.87±0.07	0.95±0.05
750 µg/ml	3.00±0.62	0.70±0.07	0.54±0.02	0.44±0.06	1.29±0.77	1.22±0.45	1.22±0.33
1000 µg/ml	4.15±0.65	0.39±0.04	0.42±0.01	0.35±0.01	1.95±0.89	1.69±0.67	1.44±0.45

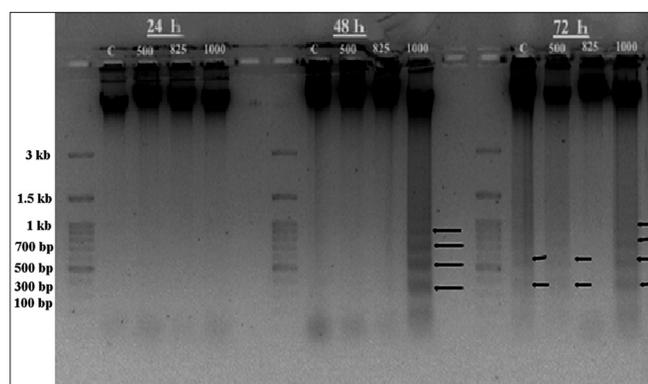
The results are shown as the mean ± SD. Ratio of protein levels was observed by Image J program. The density of bands (%) between the target protein and β-actin was calculated. Cells treated only with 0.1% DMSO was used as control. β-actin was used as a housekeeping gene. \*P<0.05 was regarded statistically meaningful (vs. control)



**Figure 5:** Demonstrates intracellular ROS generation by PFEE in HeLa cell. Treated cells showed high-intensity green fluorescent as compared to control cell, here ascorbic acid and H<sub>2</sub>O<sub>2</sub> used as a negative and positive control



**Figure 6:** Fluorescence microscopic (10×) images of HeLa cells after JC1 staining. Control cell showed no MMP change as they are mostly red (JC1 aggregate), but treated cells are mostly green (JC1 monomeric)

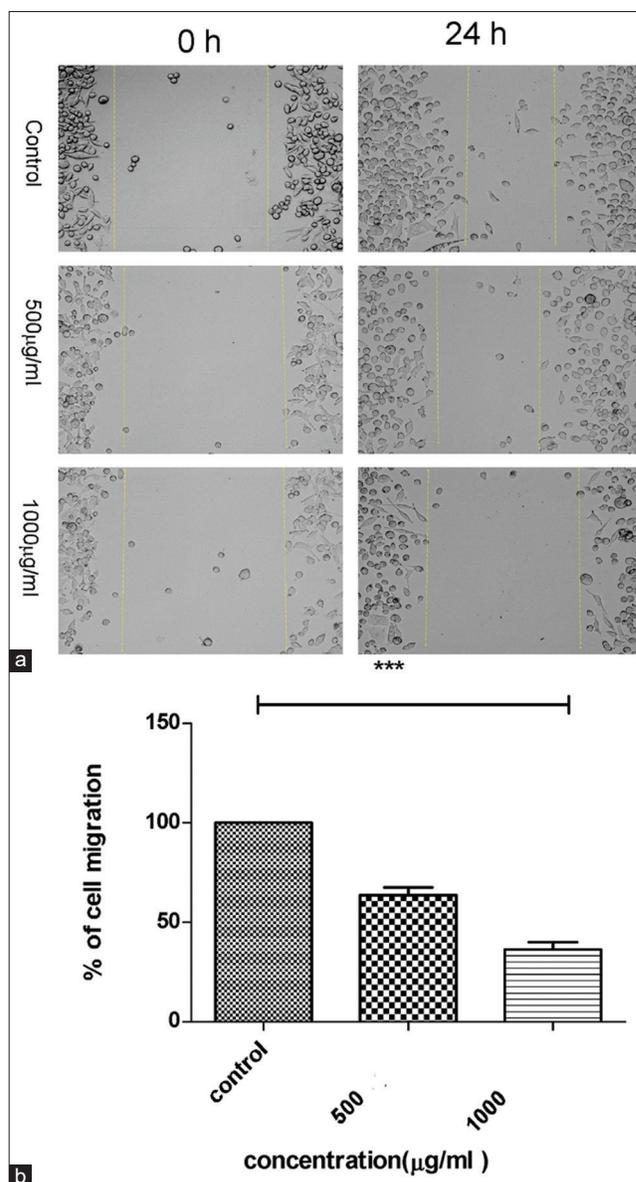


**Figure 7:** Agarose gel (1.5%) electrophoresis of isolated HeLa cell DNA for 24,48 and 72 h. Control (c) lane showed no fragmented DNA but 1000 µg/ml showed heavily fragmented DNA in the form of ladder after 72 h.

of these cancer cells to form colonies. When the dose of PFEE was gradually increased up to a maximum dose of 125 µg/ml, a minimum number of cells were identified upon 14 day(s)' treatment [Figure 9a]. The percentages of colony formation with respect to untreated (control) were calculated for each concentration of PFEE. At the lower concentration (50 µg/ml), the percentage of colony formation was 53.25, and this decreased concomitantly with an increase in the concentration of PFEE. At the highest concentration (1,250 µg/ml), colony formation was reduced to only 12.45% [Figure 9b]. Anchorage-independent growth of cancer cells *in vitro* is one of the most important properties that cancer cells have, and colony formation is also associated with the *in vivo* oncogenic potential of cancer cells.

#### Antitumor effects of PFEE treatment in HeLa-implanted mice

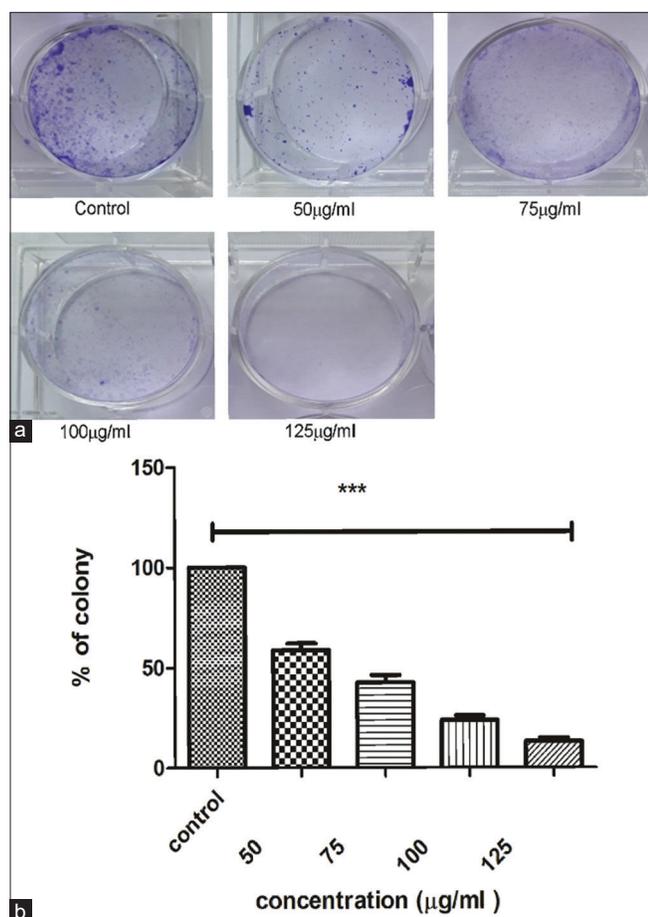
Growth of the tumors in HeLa cell-implanted mice is shown in Figure 10, and data of this experiment have been plotted in line plot [Figure 11]. Tumors in the HeLa + vehicle group increased in size after 7 days ( $12.62 \pm 2.65 \text{ mm}^3$ ), reaching a maximum of  $25.52 \pm 3.23 \text{ mm}^3$  after 28 days. The tumor sizes for the 50 mg/kg PFEE treatment groups on days 11, 15, 21, and 28 were  $10.03 \pm 1.5$ ,  $8.89 \pm 1.12$ ,  $6.26 \pm 1.02$ , and  $4.20 \pm 0.25 \text{ mm}^3$ , respectively [Table 7]. When the weight of the tumors in this group was measured on day 28, following the sacrifice of the mice, the tumors were of the minimum weight ( $280 \pm 14.32 \text{ mg}$ ), whereas, in the HeLa + vehicle control group (negative), the tumors attained the maximum size ( $2230 \pm 30 \text{ mg}$ ). Treatment with 50 mg/kg PFEE led to a marked reduction in the size (66.72%) and weight (87.44%) of the tumors. However, the reduction of tumor growth in the HeLa + PFEE10 treatment group ( $22.87 \pm 2.92 \text{ mm}^3$ ) did not reach a significant level compared with the HeLa+ vehicle group ( $25.52 \pm 3.23 \text{ mm}^3$ ). The visceral organs (kidney, liver, spleen, and lungs) were morphologically observed, and they were revealed to be normal and intact: No disorganization, no lesions and no abnormal growth were identified.



**Figure 8:** Effect of PFEE on migration of HeLa Cells in scratch method. (a) At 0 h no cell migration in control and treated (500 and 1000 µg/ml) and after 24 h control showed scratch/distance is almost filled by migrated cells but treated showed some cells are migrated in the scratch. (b) Showed bar graph of percentage of cell migrated (Y axis) against concentration of PFEE (X axis). Data are representative of three independent experiments and bar graph shows mean  $\pm$  SEM (\*\* $P < 0.05$ , \*\*\* $P < 0.001$ , not significant).

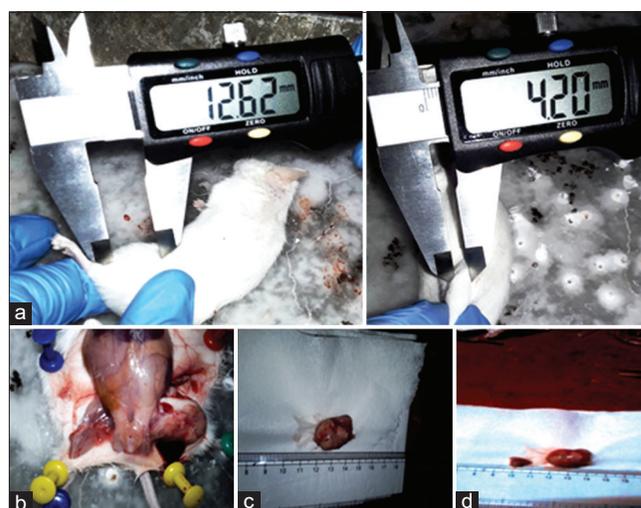
## Discussion

The antioxidant chemical content and antioxidant property of any food are now prime choice of customers and many mushrooms have antioxidant chemicals and antioxidant property. The nutritional and functional values of mushroom depend on the antioxidant content.<sup>[52]</sup> Here two extracting procedures such as dip and Soxhlet method were applied but the yield of PFEE by two methods were more or less similar. The several qualitative tests for mycochemistry of PFEE exhibited that it was positive for terpenoids, alkaloids, flavonoids, tannins,

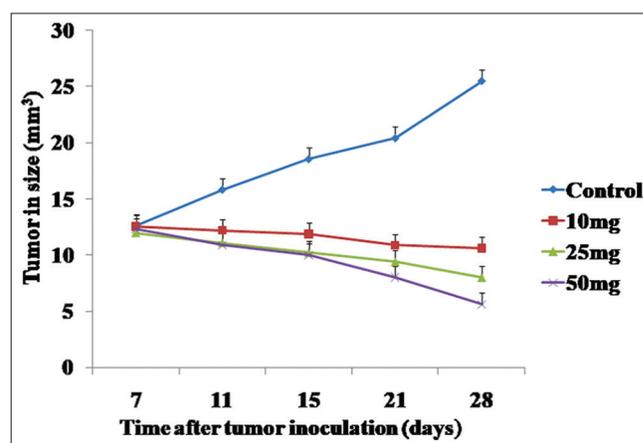


**Figure 9:** Effect of PFEE on colony formation of HeLa cells *in vitro*. (a) Showed dose dependent inhibition of clonogenicity in HeLa cell. (b) Showed bar graph of percentage of colonies (Y axis) against concentration of PFEE (X axis). Data are representative of three independent experiments and bar graph shows mean  $\pm$  SEM (\*\* $P < 0.05$ , \*\*\* $P < 0.001$ , ns not significant)

and phenolic compounds but negative for Phlobatannins. TPC of fruiting body and mycelial extracts of *Pleurotus* sp was of  $4.62 \pm 0.08$  and  $2.02 \pm 0.02$  mg GAE/g extract, respectively,<sup>[11]</sup> while Tsai *et al.*<sup>[53]</sup> recorded higher content of fruit body of this mushroom ( $7.11 \pm 0.24$  mg GAE/g ethanolic extracts). In our strain of *P. florida* TPC was more or similar to other ( $7.98 \pm 0.78$  mg GAEs/g). Differences in the phenolic content may be related to the extraction method, species, strain, etc., of mushroom.<sup>[54]</sup> In the literature; total flavonoid content of methanolic extract of fruit body of *P. ostreatus*, *Pleurotus sajor caju*, and *Pleurotus sapidus* were 1.82, 1.88, and 1.39 mg QE/g, respectively, and it varied due to species of mushroom<sup>[55]</sup> but total flavonoid content of *P. ostreatus* was below 1mg.g<sup>-1</sup><sup>[56]</sup> In our experiment, total flavonoids were greater than earlier workers ( $2.031.85 \pm 0.024$  mg QEs/g) but our species were *P. florida* and solvent was ethanol. The detailed chemistry and biological effects of flavonoids and phenolic acids have been studied by some workers.<sup>[57,58]</sup> The scavenging activity of flavonoids against several free radicals and maximum kinds of oxidizing molecules, including singlet



**Figure 10:** Effects of PFEE on tumor growth. PFEE suppresses the growth of cervical cancer tumors in Swiss albino mice. Mice were inoculated subcutaneously in the right flank with  $2 \times 10^6$  HeLa cells. Tumor volume was measured twice/week using a caliper and calculated as (width)  $2 \times$  length/2. Representative images were captured at the end of therapy, (a) shows measurement of tumor treated (50mg PFEE/Kg) and control (HeLa+ vehicle), (b) sacrificed mouse (dissected), (c) tumor from PFEE (10 mg/kg) treated mouse, (d) tumor from PFEE (50 mg/kg) treated mouse



**Figure 11:** Graphical representation (line plot) of the effects of PFEE on tumor growth *in vivo* mg/kg indicates mg PFEE per kg body weight of mice. The data represented as mean  $\pm$ SD for the three different experiments performed in triplicate. Error bars of  $\pm$  SD are inserted in figure

oxygen have been observed.<sup>[59]</sup> Furthermore, flavonoids have ability to alter peroxidation kinetics and reduce the fluidity of membranes.<sup>[60,18]</sup> Research revealed that flavonoids were prospective compounds against cancer as they inhibited cancer cell proliferation, triggered apoptosis, checked angiogenesis, and metastasis for anti-cancer therapy. The presence of ascorbic acid in different species of *Pleurotus* was reported to be variable, if we go through the literature survey. Tsai *et al.*<sup>[53]</sup> recorded that its content was relatively higher in *P. citrinopileatus* (6.76 mg/g) and *P. ostreatus* (6.55 mg/g), *P. eryngii* (5.88 mg/g) but in other species such as *P. sajor-*

**Table 7:** Tumor progression and reduction in size (diameter and volume) and percentage of reduction at various doses (mg)/Kg body weight of mice) of PFEE

Treatment No. of (day)	Tumor size in diameter (average of a and b in mm ± SD and volume in cm <sup>3</sup> ±SD							
	HeLa + vehicle Group		10 mg PFEE/Kg Group		25 mg PFEE/Kg Group Dia		50 mg PFEE/Kg Group Dia	
	Dia (mm)	Vol (cm <sup>3</sup> )	Dia (mm)	Vol (cm <sup>3</sup> )	(mm)	Vol (cm <sup>3</sup> )	(mm)	Vol (cm <sup>3</sup> )
1 <sup>st</sup> (7th day)	12.62±2.65	0.99±0.07	12.55±2.55	0.89±0.06	11.99±2.22 (0.0)	0.85±0.07	12.62±2.65 (0.0)	0.99±0.08
2 <sup>nd</sup> (11th day)	15.84±2.74	1.63±0.08	16.11±2.98	2.01±0.09	11.12±2.01 (7.25±1.13)	0.71±0.05	10.03±1.5 (20.52±3.22)	0.52±0.03
3 <sup>rd</sup> (15th Day)	18.56±2.97	2.93±0.08	17.94± 2.90	2.84±0.09	10.23±1.66 (14.67±2.72)	0.54±0.06	8.89±1.12 (29.55±4.01)	0.32±0.02
4 <sup>th</sup> (21st Day)	20.42±3.11	4.33±1.01	19.01±2.99	3.33±1.0	9.42±1.56 (21.43±2.90)	0.42±0.05	6.26±1.02 (50.39±6.55)	0.12±0.02
5 <sup>th</sup> (28th Day)	25.52±3.23	7.4±1.62	22.87±2.92	5.97±1.22	7.45±0.87 (37.86±5.34)	0.20±0.03	4.20±0.25 (66.72±6.76)	0.04±0.01

Data within parentheses indicate percentage of tumor reduction or regression in diameter or volume. The percentage of reduction of tumor size has been calculated in case of for the 25 mg PFEE/kg and 50mg PFEE/kg groups on respect of tumor size (7 days) after HeLa implantation; "a" means shortest length of tumor while "b" means longest length of tumor; Dia means diameter and Vol means volume

*caju*, *Pleurotus flabellatus*, and *P. florida*, its presence was lower (4.20, 4.15, 3.89, and 3.76 mg/g, respectively) and even not presence in some extracts such as ethanolic and hot water extracts of *P. ferulae* and *P. ostreatus* fruit bodies.<sup>[53]</sup> In our PFEE of *P. florida*, its presence was very low in amount (0.167 ± 0.03 mg AAES/g). From this study, it was found that *P. florida* contains good amount of antioxidant chemicals such as phenolic compound, flavonoids, and ascorbic acids. Furthermore, PFEE showed good activity of free-radical scavenging (antioxidant) potentiality. The mushroom products among the various natural compounds have been recognized as the useful candidates for the searching of effective antioxidant with radical scavenging potentiality<sup>[61-63]</sup> and Vieira *et al.*<sup>[64]</sup> also recorded antioxidant content and activity of effects of the oyster mushroom *P. ostreatus*. Antioxidant properties of different edible mushroom species including *P. eryngii* have been recorded by some workers.<sup>[12]</sup> The antioxidant capacity of the methanolic extracts of *P. florida* and *Calocybe indica* was determined by the DPPH method and different concentrations of *P. florida* and *C. indica* (200–1000 µg/ml) showed maximum DPPH RSA of 37.04 ± 0.15 and 28.04 ± 0.41%, respectively.<sup>[65]</sup> The IC<sub>50</sub> values of fruiting body extracts of wild *Pleurotus* (0.45 ± 0.04 µg/ml) which was more higher than those of mycelial extracts.<sup>[66]</sup> Similarly, Reis *et al.*<sup>[67]</sup> recorded that fruiting body extracts of *P. ostreatus* had much higher DPPH scavenging potentiality than its mycelial extracts. Here, our PFEE of cultivated *P. florida* (fruit body) exhibited its IC<sub>50</sub> activity as 41.17 ± 1.42 µg/ml which was far lower than other reports. Many authors have suggested that mushrooms rich in antioxidant activity play an important role in cancer prevention.<sup>[68-70]</sup> Xu<sup>[71]</sup> proposed phenolic compounds to be the major effective anticancer compounds. Phenolic compounds have been found to inhibit TNF-α gene expression and their production.<sup>[72,73]</sup> Differences in the antioxidant activity may be related to the extraction method, species, strain, etc., of mushroom.<sup>[54]</sup> Furthermore, it should be noted that temperature influences antioxidant property of substance. In general, the activity of antioxidants has been found due to heating; but, changes in temperature may affect the mode of mechanism of some antioxidants depending on the environment in which they are present.<sup>[74]</sup> Preserving the mushroom or plant products at different low temperatures (20°C, 4°C, -10°C, -20°C, and -40°C) before estimation of antioxidant, has an effect on

activity of antioxidant.<sup>[75-77]</sup> As from reports of earlier workers, the reducing the storage temperature of frozen food caused the antioxidant activity to fall down. According to Sharpe *et al.*,<sup>[54]</sup> the stability for antioxidant activity of antioxidant-rich mushroom extracts on the shelf of market may lose much of their antioxidant activity.

The *in vitro* anticancer effects of the ethanolic extracts of other species of *Pleurotus*, such as *P. sapindus*, on HeLa cells and several other human cancer cell lines, including MGC-803 (gastric cancer) and A549 and SPC-a-1 (lung cancer) cells,<sup>[78]</sup> and of *P. ferulae* on B16F10 (melanoma) and BGC-823 (gastric cancer) cells,<sup>[49]</sup> have been reported. The methanolic extract of *P. ostreatus* was shown to suppress the proliferation of breast cancer (MCF-7, MDA-MB-231) and colon cancer (HT-29, HCT-116) cells.<sup>[13]</sup> The cytotoxicity assay in the present study revealed a maximum inhibition of HeLa cell growth of 84.65% upon PFEE treatment, and cell morphological changes were observed, from the normal spindle shape to either rounded or irregular shapes; furthermore, decreases in the confluence level were also observed based on the phase contrast microscopy analysis. Similarly, Jedinak *et al.*<sup>[13]</sup> reported that *P. ostreatus* changed the morphology of MCF-7 and HT-29 cells, although their study was based on a methanolic extract of the fruit body. Bhat *et al.*<sup>[79]</sup> obtained a water-soluble extract from *P. florida*, prepared gold nanoparticles (AuNPs) and applied them to HeLa and other cell lines for 24 h. Their results revealed that water extract (10–30 µg/ml) had no effect on HeLa and other cell lines, although their AuNPs (30 µg/ml) exhibited good cytotoxicity on all cell lines. In the present study, the cytotoxicity of the ethanolic extract from the same mushroom species was investigated at a dose of 1250 µg/ml for 24 h. This revealed an inhibition rate of 75.22% against HeLa cells. Therefore, in comparison with the study by Bhat *et al.*,<sup>[79]</sup> and without AuNP preparation of PFEE, our PFEE yielded an improved percentage of inhibition after 24 h treatment at highest dose. Inconsistent PFEE concentrations for different experiments have been used, that is, one kind of limitations of our study.

Chromatin condensation and DNA cleavage are major indicators of apoptosis.<sup>[80]</sup> Induction of apoptosis is suggested to be one of the major modes of action of chemotherapeutic

anti-cancer drugs on malignant cells.<sup>[81-83]</sup> Ethanolic extracts of *P. ferulae* were reported to induce apoptosis through caspase 3 activity and by reduction of the MMP.<sup>[49]</sup> Only one previously published report has demonstrated that PFEE induced apoptosis in the T24 cell line.<sup>[84,85]</sup> which also suggested that water and methanolic extracts of *C. indica* could provide a promising agent in the treatment of human sarcoma and breast cancer. In the present study, treatment of the HeLa cells with PFEE led to an induction of apoptosis, as determined by nuclear condensation and the irregular shape of the cells (based on Hoechst staining). Loss of the mitochondrial trans-membrane potential ( $\Delta\psi_m$ ), leading to damage of the mitochondrial membrane, is the most important step for the mitochondria-dependent apoptotic pathway. In the present study, PFEE reduced the MMP and led to mitochondrial membrane damage, suggesting that the mitochondrial pathway was involved in PFEE-induced apoptosis. In this experiment, we have not work on isolated single mitochondria for MMP study. Wang *et al.*<sup>[49]</sup> also reported a loss of MMP in the B16F10, BGC 823, and GES-1 cell lines when treated with the ethanolic extract of *P. ferue*. A carboxy methylated P-glucan from the sclerotia of *P. tuberregium* exhibited anti-proliferative, apoptotic activity against human breast carcinoma MCF-7 cells *in vitro*.<sup>[86,87]</sup> Several groups have reported DNA fragmentation of different cancer cells by different mushroom extracts, as evidenced by treatment with ethyl acetate extract (10 g/ml) of *Cyathus striatus* for 24 h on HPAF-II and PL45 cells,<sup>[88]</sup> the extract of *Agaricus blazei* against human leukemia NB-4 and K-562 cells<sup>[89]</sup>, extract from button mushrooms on LNCaP cells,<sup>[90]</sup> and three other mushroom extracts on COLO-205 cells.<sup>[91]</sup> The results from our DNA laddering assay have, therefore, corroborated the results from previously published studies.

There are two types of death pathways in apoptosis: The extrinsic pathway and the intrinsic mitochondria-mediated pathway. The intrinsic pathway triggers the release of pro-apoptotic mitochondrial proteins into the cytosol. Consequently, this leads to activation of caspase-dependent and -independent pathways, there by inducing cell apoptosis. In the present study, it was observed that PFEE increased the apoptosis of cells *in vitro*. Comparison of the effects of polysaccharides and ethanol extracts of *Tremella mesenterica* revealed that only the ethanol extract induced apoptosis in A549 cells. This was shown to be mediated through activation of a mitochondrial pathway: Disruption of the MMP, the production of ROS, and the activation of caspase-3 protein in A549 cells.<sup>[92]</sup>

Gu and Belury<sup>[16]</sup> investigated selective induction of apoptosis in murine skin carcinoma CH72 cells by an ethanol extract of *Lentinula edodes*. Cordycepin from *Cordyceps militaris* induced apoptosis of human leukemia cells through a signaling cascade involving a ROS-mediated caspase pathway.<sup>[93]</sup> According to Xu,<sup>[71]</sup> intracellular ROS were produced in cancer cells by phenolic compounds. The results of experiments

performed by Ahmad *et al.*<sup>[94]</sup> in androgen-dependent ALVA-41, and androgen-independent PC-3, prostate cancer cells demonstrated that intracellular ROS increased caspase-3 activity led to a release of cytochrome-c from mitochondria, and subsequently induced apoptosis. Selenium-containing compounds were shown to cause apoptosis and block cell cycle in LNCaP prostate cancer cells through alteration of the cellular redox status by elevating intracellular ROS.<sup>[95,96]</sup> Already marketed anti-cancer drugs, such as cisplatin and paclitaxel, kill cancer cells through an elevated generation of intracellular ROS.<sup>[97-99]</sup> Xu<sup>[71]</sup> observed an increase in the levels of ROS upon treatment with brown button mushroom ethyl acetate extract (BBEA) from *Agaricus bisporus* in LNCaP cells. In the present study, the treated cells exhibited higher levels of intracellular ROS formation compared with untreated cells, and therefore ROS formation is one of the important factors contributing to an upregulation of caspases-3 and -9, and to apoptosis. The results regarding ROS formation corroborated those of previously published studies performed with different cell lines, and involving different mushroom extracts. To the best of our knowledge, intracellular ROS production by PFEE in the HeLa cell line has not been previously investigated. Our findings revealed that the anti-cancer bioactivity of PFEE was mediated through an induction of apoptosis, through ROS formation, and through MMP reduction. These conclusions were also supported by our protein expression data, which revealed a downregulation of the anti-apoptotic gene, Bcl-2, and an upregulation of the pro-apoptotic genes, Bax, caspase 3 and caspase 9, and an upregulation of the tumor suppressor p53 gene. p53 is one of the pivotal molecules involved in apoptosis induction. Numerous studies have raised the possibility that cells lacking p53 activity due to mutation may be more resistant to cancer chemotherapy.<sup>[100-102]</sup> The quantification and statistical analysis of the blotting assay has not been done presently. In the cell cycle experiments performed in the present study, PFEE arrested cell cycle at the  $G_0/G_1$  checkpoint of HeLa cells. Similarly, Jiang and Sliva<sup>[103]</sup> observed that the methanolic extract of myco-complex induced significant cell cycle arrest at the  $G_2/M$  phase. Furthermore, cell cycle arrest at  $G_2/M$  was induced by *A. blazei* in gastric epithelial cells,<sup>[104]</sup> and by cordycepin isolated from *C. sinensis* in bladder cancer cells,<sup>[44]</sup> whereas methanolic extract of *P. ostreatus* induced cell cycle arrest at the  $G_0/G_1$  stage of breast cancer cells (MDA-MB-231 and MCF-7), and of colon cancer cells (HCT-116 and HT-29).<sup>[16]</sup> Similarly, Wang *et al.*<sup>[49]</sup> demonstrated that ethanolic extract of *P. ferulae* arrested the cell cycle of melanoma cancer B16F10 cells at the  $G_0/G_1$  stage. It is noteworthy that different extracts from *G. lucidum* demonstrated specific effects on cell cycle progression. Thus, extracts from *G. lucidum* were shown to induce cell cycle arrest at the  $G_0/G_1$  phase in breast cancer cells,<sup>[105-107]</sup> whereas arrest at the  $G_2/M$  phase was induced in prostate,<sup>[105]</sup> hepatoma,<sup>[108]</sup> and bladder<sup>[109]</sup> cancer. Commercial chemotherapeutic drug such as metformin or irinotecan triggered cell cycle arrest at G1 and S phases of HCT116 and SW480 (colorectal cancer cell lines).<sup>[110]</sup> On the other hand, Dudhgaonkar *et al.*<sup>[111]</sup> isolated tri-terpenes from *G. lucidum*,

and observed that they induced cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase, their study comprising specific biologically active compounds, as well as particular cells.

Colony formation is a key attribute of cancer cells in acquiring metastatic potential. Ganoderic acids from *Ganoderma lucidum* led to an inhibition of cell proliferation, as well as colony formation with invasive breast cancer cells.<sup>[112]</sup> *P. ostreatus* aqueous extract exhibited a greater reduction in the number of colonies of COLO-205 (oral cancer) cells (43.8 ± 3.5% [*P* < 0.01] compared with 100% proliferation of untreated cells), whereas aqueous extracts from *Auricularia polytricha* and *Macrolepiota procera* also exhibited a reduction in colony formation potential, to 59.9 ± 2.6% (*P* < 0.01) and 47.7% (*P* < 0.01) colony formation, respectively.<sup>[91]</sup> In the present study, PFEE inhibited the colony formation of HeLa cells significantly compared with the negative control (DMEM vehicle), in agreement with other groups' studies. Similarly, migration of cancer cells is also an important factor associated with the metastatic behavior of cancer, and likewise, in the present study, PFEE's treatment inhibited the migration of cells, as demonstrated in the scratch wound assay experiment.

A study of mice *in vivo* with implanted BGC-823 cancer cells revealed that the weight and volume of the tumors were reduced after 15 days' treatment with *P. ostreatus* mycelium polysaccharide 2 (POMP2).<sup>[113]</sup> In another study, polysaccharides were extracted from *Lepista sordida*, and when applied against laryngo carcinoma, these inhibited cell growths *in vitro* and caused a reduction in tumor size *in vivo*.<sup>[114]</sup> The compound ergosterol was isolated from *Agaricus brasiliensis*, and led to a retardation in tumor growth in sarcoma 180-bearing mice. In studies *in vivo*, ergosterol was shown to inhibit neovascularization, as well as being potentially an inhibitor of angiogenesis.<sup>[9,115]</sup> A marked reduction in tumor fluid volume, and changes in various other properties, were identified in Dalton's lymphoma ascites-bearing mice following *C. indica* (milky mushroom) treatment, in comparison with negative control group mice.<sup>[116]</sup> In the present study, 50 mg PFEE/kg body weight of mice also led to a marked reduction in tumor size and volume in HeLa cell-implanted mice.

## Conclusions

Considering the various points included in the above discussion, it may be concluded that PFEE containing high antioxidant content and activity, inhibited the proliferation of HeLa cells, induced mitochondrial apoptosis, and arrested cell cycle at the G<sub>0</sub>/G<sub>1</sub> phase. Furthermore, it inhibited colony formation and migration of these cancer cells, blocking metastasis and markedly reducing the tumor size and weight of HeLa-implanted mice. Taken together, these results suggest that the *P. florida* mushroom may be a novel promising agent for the treatment of cervical cancer.

## Limitations

This study limited to extraction of mushroom fruit body using ethanolic solvent and EE was tested for antioxidant content, activity and anticancer activity against HeLa cell line and also as antitumor activity against HeLa implanted mice.

## Recommendations for Future Studies

Future analytical chemistry studies for the active chemicals responsible for the anticancer potentials of PFEE and human trial of PFEE are likely recommended to identify a novel class of mushroom species that have broad-spectrum anti-tumor activity for cervical cancer management.

## Authors' Declaration Statements

### Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Animal Ethics Committee, Government of India (approval no:12/P/S/IAEC/2018).

### Availability of data and material

The data used in this study are available and will be provided by the corresponding author on a reasonable request.

### Competing interests

The authors declare that there are no conflicts of interest.

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### Authors' Contributions

SKG contributed in the design of the study as well as interpretation of the data. KP, TS, TB, and SP participated in data collection and analysis, and manuscript writing

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