

Sericin Preparation from Cocoons of Oak Tasar Silkworm *Antheraea proylei* J. Induces Apoptosis in a Caspase-dependent Manner in A549 and HeLa Cells and Caspase-independent Manner in PC3 Cells

Potsangbam Jolly Devi

Manipur University

Asem Robinson Singh

Manipur University

Lisam Shanjukumar Singh (✉ shanju.lisam@manipuruniv.ac.in)

Manipur University <https://orcid.org/0000-0001-5755-2234>

Laishram Rupachandra Singh

Manipur University

Sanjenbam Kunjeshwori Devi

Manipur University

Research article

Keywords: Sericin, *Antheraea proylei* J., silk cocoons, Caspase-3, PARP, MAPK

Posted Date: August 26th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-56346/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background

Despite much progress in understanding the biology of cancer disease, advancement in technology for early diagnosis, the expanding array of anticancer drugs, and treatment modalities, the global cancer burden is still significant and increasing. It is estimated that the new cases of cancer in the year 2040 will be 29.4 million per year globally. Sericin, an adhesive protein of silk cocoon, is a potential protein in various biomedical applications including cancer therapeutics. The present study evaluates the anticancer property of sericin prepared from cocoons of *Antheraea proylei* J. (*A. proylei*) against human lung cancer (A549), cervical cancer (HeLa), and prostate cancer (PC3) cell lines. This is the first report of the anti-cancer activity of the non-mulberry silkworm *A. proylei*.

Methods

Sericin preparation (SP) was prepared from cocoons of *A. proylei* J. by the process of the degumming method. The amino acid composition of the SP was determined by HPLC. Cytotoxicity activity was assessed by MTT assay and genotoxicity activity was assessed by comet assay. Cleavage of caspase and PARP proteins and phosphorylation of MAPK pathway members were analyzed by Western blotting. Cell cycle analysis was done by FACS flow cytometry.

Results

SP causes cytotoxicity to A549, HeLa, and PC3 cell lines with the IC_{50} values ranging from 3.4-3.9 $\mu\text{g}/\mu\text{l}$. SP induces apoptosis in a dose-dependent manner through caspase-3 and p38/SAPK/ERK pathways in A549 and HeLa cells whereas in PC3 cells SP induces apoptosis independent of caspase but through p38 pathway. Moreover, in the case of A549 and HeLa cells, SP induces cell cycle arrest at the S phase whereas at the G_0 phase in the case of PC3 cells in a dose-dependent manner.

Conclusion

The difference in the molecular mechanisms of apoptosis induced by SP in A549 and HeLa cell lines, and in PC3 cell lines may be due to the difference in the genotypes of the cancer cell lines where A549 and HeLa cells are being non-malignant and p53 positive whereas PC3 cell is being malignant and p53 negative. The overall results of the present study envisage the possibility of using SP as an anti-tumorigenic agent.

Background:

Although we have made much progress in understanding the biology of cancer disease, advancement in technology for early diagnosis, the expanding array of anticancer drugs, and treatment modalities, the global cancer burden is still significant and increasing. It is estimated that new cases of cancer have risen to 18.1 million and deaths due to cancer disease to 9.6 million in 2018 (1). Sadly, the estimated new

cases of cancer in the year 2040 is 29.4 million per year globally (2). The increasing trend of cancer burden is due to several factors, including population growth, aging as well as the changing prevalence of certain causes of cancer linked to social and economic development particularly in rapidly growing economies. In contrast to other world regions, the proportions of cancer deaths in Asia and Africa (57.3% and 7.3%, respectively) are higher than the proportions of incident cases (48.4% and 5.8%, respectively), because these regions have a higher frequency of certain cancer types associated with poorer prognosis and higher mortality rates, in addition to limited access to timely diagnosis and treatment (2). According to the World Health Organization (WHO), the total number of cases of cancer diagnosed in India between the years 2017 and 2018 is 7,84,821 (increased by 324%) out of which 4,13,519 are men and 3,71,302 are women (1).

In view of the above facts, there is a need for urgent and serious attention towards finding new anticancer therapeutic drugs to prevent the increasing number of cancer cases in the coming decades in addition to other strategies. One such potential anti-cancer agent is sericin, a silk protein that binds together the silk fibroin fibers to form the cocoon (3). Silk textile industry targets only the silk fiber obtained after the process of sericin removal, through degumming (4). Depending upon the species from where the sericin is obtained, the amino acid composition varies considerably. Sericin from wild silkworms has a higher content of threonine, glutamic acid, cysteine and phenylalanine and a lower content of serine, proline, methionine, glucosamine, galactosamine, and histidine (5). Owing to the difference in the proportions of amino acid compositions in the domesticated silkworm, *Bombyx .mori* and the wild type silkworm, *Antheraea* sp., there may be variation in the bioactivity.

Sericin stands as a promising anti-cancer agent that inhibits the growth of cancer cells. The effect of sericin was studied in the colon cancer mice models induced by 1,2-Dimethylhydrazine (DMH). The studies concluded that sericin supplemented diet reduced the formation of colonic aberrant crypt foci (6). Further Zhaorigetu et al., (7) 2001 reported that sericin suppresses the development of colonic tumors by reducing oxidative stress, cell proliferation, and nitric oxide production. The strong antioxidant activity of sericin and its resistance to intestinal proteases prolongs its sustainability in the colon thereby lowering oxidative stress and tumorigenesis in the colon (7). Yet in another study, sericin was reported to suppress skin tumorigenesis in a mice cancer model induced by 7,12-dimethylbenz (α) anthracene (DMBA) and 12-O-tetradecanoylphorbol 13-acetate (TPA) by reducing oxidative stress, inflammatory responses and endogenous tumor promoter (TNF-α) (8). It was also observed that sericin from *B. mori* induced apoptosis through the caspase pathway and downregulation of Bcl-2 expression in human colorectal cancer cells (SW480) (9). Most of the previous reports related to the prevention and treatment of colon cancer are concerned with sericin obtained from the commonly domesticated silkworm, *Bombyx mori* which has been extensively studied as compared to the wild silkworms including *Antheraea* sp. The oak Tasar silkworm, *A. proylei* is reared in several sericulture farms in Manipur, India and adjoining states and feeds on leaves of oak (*Quercus* sp.) which are naturally grown in the region. The sericin from *A. proylei* silkworm has yet to be explored for its prospective anti-cancer properties and health benefits.

Kumar et. al. 2019 (10) reported anticancer activities of sericin from non-mulberry silkworm *Antheraea assamensis* on MCF-7 and A431 cells through induction of oxidative stress and reduction of mitochondrial membrane potential. Further Zhang et al. 2003 (11), studied the potential of cecropins from *Antheraea pernyi* on inducing apoptosis in human colon adenocarcinoma cell lines. However, detailed studies including molecular mechanisms of inducing apoptosis have not been reported yet. Till now, the sericin from *A. proylei* has not been evaluated for its anticancer potential. The present study investigates the anticancer activity of the SP from the non-mulberry cocoon.

Methods:

Extraction of SP

A. proylei cocoons were collected from Uyumpok Tasar Silk Farm, Imphal East, Manipur, India. Five grams of fresh cocoon cut (~ 1 cm² pieces) were added to 100 mL distilled water and subjected to heat treatment at 121°C under pressure for 1h. The resulting suspension was filtered through Whatmann filter paper No.1 and centrifuged at 21,000 g for 30 mins. The process was repeated for 2 times with the same cocoon shell sample. The supernatants obtained were pooled and then lyophilized. The lyophilized SP powder was stored at -20°C until use.

HPLC analysis of predominant amino acids of SP

SP prepared as above was subjected to acid hydrolysis by dissolving in 6N HCl in boiling water bath for 24 h and mixed every hour for proper hydrolysis. It was then centrifuged at 42,000 g for 15 mins. The supernatant was filtered and neutralized with 1N NaOH. The filtered solution was then diluted to 1:1000 of the volume with milli-Q water and then analyzed for amino acids in HPLC (Agilent 1100 HP), C18, 4.5 X 150, 5µm column using mobile phase A (20 mM sodium acetate + 0.018% triethylamine, pH to 7.20 ± 0.05) and mobile phase B (20% of 100 mM sodium acetate + 40% methanol and + 40% acetonitrile, pH 7.20 ± 0.05). The flow rate was maintained at 0.5mL/min and the column temperature was kept at 40°C and detected at 338nm.

Cell lines and culture conditions

Three human cancer cell lines, namely, lung cancer (A549), cervical cancer (HeLa), and prostate cancer (PC3) were purchased from NCCS, Pune. All the cell lines were cultured in RPMI 1640 (Gibco, USA) media with 10% fetal bovine serum (Gibco, USA) and 1% PenStrep (Gibco, USA) and incubated with 5% CO₂ at 37°C.

Cell treatment with SP

SP was dissolved in RPMI culture media and centrifuged at 15,700g for 30 mins. The supernatant was sterilized through a syringe filter (0.2 μm pore size) for the treatment to the above three cancer cells with different doses (final concentration; 0.2, 0.3, 1.0, 1.5, 3.0, 5, and 10.0 $\mu\text{g}/\mu\text{L}$.)

Cell viability assay

Cell viability assay was carried out as per the manufacturer's protocol provided with MTT assay kit, Vybrant MTT assay Kit (Invitrogen Life Technologies). A549, HeLa, and PC3 cells were cultured with a density of 1×10^4 cells per well in 100 μL RPMI (without phenol red), 10% FBS, and incubated with 5% CO_2 at 37°C in a 96-well tissue culture plate. Various doses of SP were treated to the cells in triplicates. After 24 h, cell viability was assessed by adding 10 μL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) to all the wells followed by incubation at 37°C for 4 h. The formazan crystals formed were dissolved by adding 50 μL of DMSO after removal of the culture media and further incubated for 10 mins at room temperature. The numbers of viable cells were then quantified by measuring absorbance at 540 nm. The experiment was conducted three times.

Comet assay

Comet assay was carried out according to the protocol described by Olive and Banath, 2006 (12) Olive & Banáth, 2006). A549, HeLa, or PC3 cells were treated with various doses of SP for 24 h, and cells were detached by trypsinization. The number of cells was adjusted at 2×10^4 cells/ml and suspended in PBS. About 400 μL of the cell suspension was mixed with 1.2 ml of low-melting agarose at 40°C by gentle pipetting followed by pouring onto agarose pre-coated microscope slides. After proper solidification of the agarose, the slides were submerged in a neutral lysis solution containing 2% SDS, 0.5 mg/ml Proteinase K, 0.5M EDTA, and incubated at 37°C for 16 h in the dark. The slides were then washed three times with a neutral rinse buffer followed by electrophoresis at 0.6 V/cm for 25 mins. Slides were then stained with 10 $\mu\text{g}/\text{ml}$ propidium iodide (PI) and observed under a fluorescent microscope and photographed. The tail lengths of at least 100 comets in each slide were scored for analysis.

Western blots

A549, HeLa, and PC3 cells were seeded in a 60 mm culture dish, grown overnight, and treated with various doses of SP. The cells were lysed using RIPA buffer and the protein concentration of the cell lysate was estimated using the BCA protein assay kit (Thermo Scientific) and 20 μg of proteins were separated by 12% SDS-PAGE. After transferring the separated proteins on PVDF membrane, it was blocked with 5% (w/v) skimmed milk in 1X Tris-buffered saline (TBS) with Tween-20 for 1 h at room temperature. The blocked membranes were then incubated with the primary antibodies (1:1000 dilution) with gentle shaking on a rocker at 4°C overnight. The membrane was probed with anti-PARP, anti-caspase 3 (both

total and cleaved), anti-ERK, anti-P38 (both total and phosphorylated), or anti-JNK antibodies. The membranes were then washed with 1X Tris-buffered saline (TBS) with Tween-20 three times for 10 mins each with changes of buffer. After washing the membrane, secondary antibodies conjugated with horseradish peroxidase (HRP) were added at 1:1000 dilutions and kept with gentle shaking for 1h at room temperature. The membranes were re-blotted with an anti- β actin antibody to normalize the total protein loaded. The antibodies were obtained from the Cell Signaling Technology, MA, USA. The blots were then developed using ECL (GE Amersham) and visualized under BioRad Gel Doc.

Cell cycle analysis

A549, HeLa, and PC3 cells were cultured in a 6-well culture plate, grown overnight, and treated with various doses of SP. After 12 h, cells were harvested after trypsinization and washed with PBS. The cells were then fixed with ice-cold 70% ethanol added dropwise with regular vortexing to avoid cell clumps and kept at 4°C for 30 mins. The fixed cells were washed twice with PBS by centrifuging at 850 g for 5 mins. Then the cells were treated with 50 μ g of RNaseA (100 μ g/mL) and 200 μ L of propidium iodide (50 μ g/mL) was added. Cell cycle distribution was analyzed in a FACS flow cytometer (BD Biosciences, USA).

Statistical Analysis

Significant variance between groups was performed for all groups using Student's *t*-test. Data are expressed as means \pm SD. Differences with $P < 0.05$ were considered statistically significant.

Results:

Amino acid composition of SP:

The HPLC analysis of SP for constituent amino acids revealed the presence of aspartic acid, serine, glutamic acid, and histidine in the majority with other essential and non-essential amino acids. The free amino acid composition of sericin from *Antheraea proylei* and comparison with previous reports of sericin from *Bombyx mori* and *Antheraea pernyi* (12, 13) is shown in Table

Table 1
 Amino acid analysis of sericin preparation (SP) from *Antheraea proylei* by HPLC and comparison with previous reports of sericin from *Bombyx mori* and *Antheraea pernyi*

Amino acids	<i>Antheraea proylei</i> (mol %)	<i>Bombyx mori</i> (mol %)	<i>Antheraea pernyi</i> (mol %)
Glycine	33.01	14.48	38.85
Alanine	1.64	4.05	24.25
Valine	0.54	3.22	0.4
Leucine	0.55	0.99	0.5
Isoleucine	0.44	0.76	0.22
Serine	19.25	35.63	13.00
Threonine	0.97	8.14	0.38
Aspartic acid	14.04	15.65	6.69
Glutamic acid	7.33	4.74	1.22
Lysine	1.37	2.72	1.15
Arginine	2.08	3.10	3.17
Histidine	15.76	1.55	1.11
Tyrosine	2.52	3.39	0.89
Phenylalanine	0.20	0.56	7.51
Proline	NA	0.57	0.52
Methionine	0.24	0.14	0.00
Cysteine	NA	0.29	0.12

The free amino acid composition of SP from *Antheraea proylei* was determined by acid hydrolysis with 6N HCl in boiling water bath and analysed using HPLC (Agilent 1100 HP). The amino acid content of sericin from *Bombyx mori*, and *Antheraea pernyi* is adapted from Yang et al., (12, 13). The percentage of amino acid contents of sericin varies according to species. NA indicates Not Available

SP induces cytotoxicity to A549, HeLa, and PC3 cells in a dose-dependent manner.

A549, PC3, or HeLa cells were treated with various doses of SP for 24h. Morphological changes (rounding up), cell detachment, cell death, and decrease of cell viability were observed after treatment of SP for 24

hrs [Figure 1(a-f)]. The results showed that SP induces cytotoxicity in all the cancer cell lines tested in a dose-dependent manner. Further, cells were treated with various doses of SP (final concentration; 0, 2, 0.3, 1.0, 1.5, 3.0, 5.0, and 10.0 $\mu\text{g}/\mu\text{L}$.) for 24 h and MTT assay was performed to determine the IC_{50} (half maximal inhibitory concentration). The results showed that IC_{50} values of SP for inhibiting the viability of A549, HeLa and PC3 cells were 3.8 $\mu\text{g}/\mu\text{L}$, 3.9 $\mu\text{g}/\mu\text{L}$ and 3.4 $\mu\text{g}/\mu\text{L}$ respectively [Figure 1(g)]. Therefore, the results indicate that SP induces cytotoxicity.

SP induces genotoxicity

To ascertain the cytotoxic activity induced by SP on A549, HeLa, and PC3 cells is due to induction of apoptosis, genotoxicity was assessed by comet assay. Cells were treated with different doses of SP (final concentration of 2, 4, and 8 $\mu\text{g}/\mu\text{L}$) for 24 h and comet assays were performed as described above. The number of apoptotic cells and tail length were assessed under a fluorescent microscope. A dose-dependent increase in the number of apoptotic cells and the average tail length of comets was observed in all the three cancer cell lines tested. In A549, the average tail lengths of the apoptotic cells at the dose of 0 $\mu\text{g}/\mu\text{L}$ (control), 2 $\mu\text{g}/\mu\text{L}$, 4 $\mu\text{g}/\mu\text{L}$ and 8 $\mu\text{g}/\mu\text{L}$ were $65.48 \pm 11.49 \mu\text{m}$, $139.90 \pm 6.03 \mu\text{m}$, $217.26 \pm 6.84 \mu\text{m}$ and $259.57 \pm 16.27 \mu\text{m}$ [Figure 2 (a-d)] while in HeLa, the tail lengths were $50.34 \pm 5.10 \mu\text{m}$, $91.60 \pm 17.09 \mu\text{m}$, $176.45 \pm 12.58 \mu\text{m}$ and $162.69 \pm 3.54 \mu\text{m}$ [Figure 2 (e-h)] and in PC3 the tail lengths were $64.33 \pm 12.26 \mu\text{m}$, $112.83 \pm 10.54 \mu\text{m}$, $225.67 \pm 19.84 \mu\text{m}$ and $241.64 \pm 61.13 \mu\text{m}$ [Figure 2 (i-l)] respectively. Unpaired t-tests of average tail lengths between groups of the three cell lines showed a strong statistically significant difference ($p < 0.0001$ at 95% CI) [Figure 2 (m)]. The results showed that SP induces genotoxicity, which is the hallmark of apoptosis, in all the cell lines tested a dose-dependent manner suggesting that SP induces cell apoptosis.

SP induces A549 and HeLa cells apoptosis through caspase-3 activation and PARP deactivation while in PC3 independent of caspase and PARP

To determine the molecular mechanism of the apoptosis induced by SP, the activation of executioner caspase-3 and deactivation of PARP in A549, PC3, and HeLa cells were assessed by Western blotting. The three cell lines were treated with various doses of SP for 24 hrs and immunoblotted against caspase-3 or PARP antibodies. The results showed that the treatment of SP to A549 and HeLa cells leads to the cleavage of caspase-3 and PARP proteins [Figure 3 (right and left panels)]. On the other hand, in PC3 cells no cleavage of caspase-3 and PARP proteins was observed [Figure 3 (middle panel)]. When assessed the cleavage of caspase-7 and caspase-9, no cleavage of none was observed in the case of PC3 (result not shown). The results suggest that SP induces cell apoptosis in a caspase and PARP dependent manner in A549 and HeLa cells but induces apoptosis in PC3 cells in a caspase and PARP independent manner.

SP induces apoptosis through MAPK pathways

To determine the possible pathways of the apoptosis induced by SP, activation of MAPK pathways were assessed as it plays important roles in cell survival and death. Cells were treated with different doses of SP for 24 h and immunoblotted against the total as well as phosphorylated p38, p44, or SAPK/JNK antibodies. The results showed that SP leads to phosphorylation of p38, p44, and SAPK/JNK proteins in a dose-dependent manner in A549 and HeLa cells whereas in PC3 cells significant increase in phosphorylation in a dose-dependent manner is observed only in p38 protein. Phosphorylation of SAPK/JNK and ERK proteins in PC3 were observed only in the highest dose of SP [Figure 4]. Therefore, SAPK/JNK and ERK pathway may not be directly involved in the SP induced apoptosis in PC3. The overall results indicate the involvement of p38, SAPK/JNK, and ERK pathways in A549 and HeLa cells whereas the p38 pathway but neither SAPK/JNK nor ERK pathways in PC3 are observed in the apoptosis induced by SP.

SP promotes cell cycle arrest at S phase in A549 and HeLa cells.

As seen with anticancer drugs, which arrests cell cycle at specific points and thereby inducing apoptosis by destabilizing the normal biochemical processes of the cell, the anti-cancer activity of SP on A549, HeLa, and PC3 cells may affect the normal cell cycle. To determine whether the mechanism of action of SP for inducing apoptosis is due to the arrest of the cell cycle, cell cycle analysis was performed. Cells treated with varying doses of the SP for 12 h were subjected to flow cytometry analysis after staining with propidium iodide. An increase in the cell population at the S phase was observed in A549 as well as HeLa cells treated with SP. Although unpaired t-test between control and cells treated with 8 μ g/ μ L showed a statistically significant increase in cells at S phase in both HeLa and A549 cells ($p < 0.05$), there was no statistically significant change in the population of cells at G0/G1 stage in both the cell lines. Reduction in the cell population at the G2/M phase was also observed in both the cell lines with a statistically significant difference observed between control and 8 μ g/ μ L. Contrastingly, cell cycle analysis of PC3 cells treated with SP showed cell arrest at the G1 phase in a statistically significant manner between control and treated cells ($p < 0.05$), and also a consistent population was seen in S phase. We also observed a reduction of the number of cells at the G2M phase in a dose-dependent manner with statistical significance compared to untreated cells ($p < 0.00$) [Figure 5].

Discussion:

Sericin from *B. mori* has been reported to have anti-proliferative effects against colon cancer by downregulating the expression of Bcl-2, an anti-apoptotic protein, and activating the caspase-3 pathway (9). The chemopreventive effect of sericin in colon tumors was demonstrated by Zhaorigetu et al., 2007 (15) indicating that consumption of sericin reduces colonic oxidative stress and development of aberrant crypt foci due to its high content of serine (hydroxyl group) and the protease-resistant property which

makes it readily absorbed by the large intestine and therefore can be used as a dietary supplement (15). However, the amino acid composition SP of *A. proylei* is different from those reported earlier in mulberry and non-mulberry silkworms [Table 1]. This corroborates with earlier studies of sericin from different species of silkworms [5, 16]

The present study aims to evaluate the anti-cancer potential of SP on three types of cancers namely lung cancer, cervical cancer, and prostate cancer using A549, HeLa, and PC3 cell lines. Though such properties were observed in sericin of *B. mori*, it is the first report on SP from the cocoon of *A. proylei*. Initially, dose-dependent inhibition of cell proliferation of A549, PC3 and HeLa cell lines was observed as assessed by MTT assays and morphological changes of these cell lines were also observed under a simple microscope after SP treatments. Morphological changes of cells like rounding up of cells and bleb like structures which are the signs of possible programmed cell death were induced by SP [Figure 1]. However, certain cell death pathways such as autophagy and apoptosis share similar morphological changes (17). To negate the possibility of undergoing cell autophagy, expression of autophagy-related genes; ATG1, ATG-5, DRAM, and LC3 were assessed by semi-qPCR after SP treatments. No change in the expression of the autophagy-related genes was observed (data not shown). However, comet assay revealed that SP significantly induces genotoxicity in a dose-dependent manner suggesting induction of cell apoptosis [Fig. 2]. Further molecular events leading to genotoxicity were investigated using Western blot analysis in which a caspase-3 and PARP dependent cell death was observed in A549 and HeLa cells whereas caspase-3 and PARP independent mechanism was observed in PC3 cells [Figure 3]. The difference in the dependent of caspase-3 and PARP may be due to differences in the genotypes of the cancer cell lines where PC3 cells are highly malignant and p53 negative, whereas HeLa and A549 cells are non-malignant and p53 positive. Cancer cells have evaded normal cell death through a plethora of molecular changes and evading caspase-dependent cell death is one of the mechanisms observed in many cell lines. The finding in the present studies that SP induces PC3 apoptosis independent of caspase and PARP is parallel with the findings of previous studies on plant extracts (18, 19, 20). Therefore, the findings of caspase and PARP involvement in the case of A549 and HeLa cells but not in the case of PC3 cells suggest following different pathways for inducing cell apoptosis.

To investigate the signaling pathways of the apoptosis induced by SP, MAPK pathways were selected since it plays important roles in cell survival and cell death. The findings in our study indicate that SP induces apoptosis in A549 and HeLa cells through activation of p38, SAPK/JNK, and ERK pathways. However, SP induces cell apoptosis through p38 pathways activation but not SAPK/JNK and ERK pathways in the case of PC3 [Figure 4]. Our observations are in agreement with earlier studies in which p38 and JNK are stress-activated involved in apoptosis of A549 cells (21, 22), HeLa (11) and PC3 cells (23, 24). Although the role of ERK in apoptosis remains controversial it is observed that DNA damage can induce ERK phosphorylation and further leading to cell death (25). Moreover, the role of ERK in cell death is dependent on cell lineage and intensity as well as the duration of pro- or anti-apoptotic signals of ERK1/2. Our observation of high-level phosphorylation in PC3 cells in the highest dose may be a result of extensive DNA damage activating ERK for its pro-apoptotic signal in a p53 independent manner (26).

Arrest of cell cycle at specific points is a complex molecular mechanism and our observations of blockage of cell cycle progression induced by SP at G0/G1 in PC3 and S phase in A549 and HeLa reveal an interesting phenomenon suggesting different mechanisms of cell cycle arrests for different cell lineages [Figure 5]. Further investigation is warranted to explain the molecular mechanisms of different cell cycle arrest induced by SP in prostate cancer.

Conclusion:

SP induces apoptosis in lung, cervical, and prostate cancer cell lines as observed in the assessment of cell death and genotoxicity with IC_{50} values of 3.4 to 3.9 $\mu\text{g}/\mu\text{l}$ through activation of MAPK pathways. However, A549 and HeLa cells follow a molecular mechanism of caspase and PARP dependent while in PC3, it is a caspase and PARP independent mechanism. Further SP induces apoptosis in A549 and HeLa cells through activation of p38, SAPK/JNK, and ERK pathways but in PC3 SP induces cell apoptosis through p38 pathways activation. The difference in the molecular mechanisms of apoptosis induced by SP in A549, HeLa, and PC3 cell lines may be due to the difference in the genotypes of the cancer cell lines where A549 and HeLa cells are being non-malignant and p53 positive whereas PC3 cells are being highly malignant and p53 negative.

The overall results of the present study envisage the possibility of using SP as an anti-tumorogenic agent. The study is limited to the fact that the cellular protein that used for sericin binding needs to be determined. Further, the study opens up avenues for use of peptides that can act as anti-cancer agents which can increase the specificity and efficacy of drug designs in the future.

Abbreviations:

PARP: Poly (ADP-ribose) polymerase

MAPK: Mitogen-activated protein kinase

JNK: Jun N-terminal kinase

mins: Minutes

h: Hour

DMSO: Dimethyl sulfoxide

EDTA :Ethylenediaminetetraacetic acid

SDS: Sodium dodecyl sulfate

BCA: Bicinchoninic Acid

PAGE : Polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

SD: Standard deviation

SAPK/JNK: Stress-activated protein kinases/Jun amino-terminal kinases

ATG1: AuTophagy related 1

ATG-5: AuTophagy related 5

DRAM: DNA Damage Regulated Autophagy Modulator

LC3: Microtubule Associated Protein 1 Light Chain 3 Alpha

Declarations:

Availability of data and materials

All data generated or analysed during this study are included in this published article

Ethics approval and consent to participate

Not applicable

Consent to publish

Not applicable

Funding

Not applicable

Authors' Contributions

PJD and ARS performed experiments. PJD, ARS and LSS analysed the data. PJD and LSS wrote the manuscript. LRS and SKD reviewed the systematic review. All authors read and approved the final manuscript.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

The authors are grateful to the Directorate of Sericulture, Government of Manipur, Manipur, India and Mr. P.Ibohal (Farm Overseer, Uyumpok Tasar Silk Farm) for providing the silkworm cocoons.

References:

1. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *68*(6), 394–424.
2. Ferlay J, Ervik M, Lam F, et al. Global Cancer Observatory: Cancer Today. Lyon, France: IARC, 2018. Available from: <https://gco.iarc.fr/today>.
3. Yun, H., Oh, H., Kim, M. K., Kwak, H. W., Lee, J. Y., Um, I. C., Vootla, S. K., & Lee, K. H. (2013). Extraction conditions of *Antheraea mylitta* sericin with high yields and minimum molecular weight degradation. *International Journal of Biological Macromolecules*, *52*, 59–65.
4. Kunz, R. I., Brancalhão, R. M. C., Ribeiro, L. de F. C., & Natali, M. R. M. (2016). Silkworm sericin: properties and biomedical applications. *BioMed Research International*, *2016*.
5. Silva, S. S., Kundu, B., Lu, S., Reis, R. L., & Kundu, S. C. (2019). Chinese oak tasar silkworm *Antheraea pernyi* silk proteins: Current strategies and future perspectives for biomedical applications. *Macromolecular Bioscience*, *19*(3), 1800252.
6. Sasaki, M., Kato, N., Watanabe, H., & Yamada, H. (2000). Silk protein, sericin, suppresses colon carcinogenesis induced by 1, 2-dimethylhydrazine in mice. *Oncology Reports*, *7*(5), 1049–1101.
7. Zhaorigetu, Siqin, Sasaki, M., Watanabe, H., & KATO, N. (2001). Supplemental silk protein, sericin, suppresses colon tumorigenesis in 1, 2-dimethylhydrazine-treated mice by reducing oxidative stress and cell proliferation. *Bioscience, Biotechnology, and Biochemistry*, *65*(10), 2181–2186.
8. Zhaorigetu, Siqin, Yanaka, N., Sasaki, M., Watanabe, H., & Kato, N. (2003). Silk protein, sericin, suppresses DMBA-TPA-induced mouse skin tumorigenesis by reducing oxidative stress, inflammatory responses and endogenous tumor promoter TNF- α . *Oncology Reports*, *10*(3), 537–543.
9. Kaewkorn, W., Limpeanchob, N., Tiyaboonchai, W., Pongcharoen, S., & Sutheerawattananonda, M. (2012). Effects of silk sericin on the proliferation and apoptosis of colon cancer cells. *Biological Research*, *45*(1), 45–50.
10. Kumar, J. P., & Mandal, B. B. (2019). Silk sericin induced pro-oxidative stress leads to apoptosis in human cancer cells. *Food and Chemical Toxicology*, *123*, 275–287.
11. Zhang, W. M., Lai, Z. S., He, M. R., Xu, G., Huang, W., & Zhou, D. Y. (2003). Effects of the antibacterial peptide cecropins from Chinese oak silkworm, *Antheraea pernyi* on 1, 2-dimethylhydrazine-induced

- colon carcinogenesis in rats. *Di 1 Jun Yi Da Xue Xue Bao= Academic Journal of the First Medical College of PLA*, 23(10), 1066–1068.
12. Yang M, Zhou G, Shuai Y, Wang J, Zhu L, Mao C. Ca²⁺-induced self-assembly of Bombyx mori silk sericin into a nanofibrous network-like protein matrix for directing controlled nucleation of hydroxylapatite nano-needles. *J Mater Chem B. Royal Society of Chemistry*; 2015;3:2455–62.
 13. Yang M, Shuai Y, Zhang C, Chen Y, Zhu L, Mao C, et al. Biomimetic nucleation of hydroxyapatite crystals mediated by Antheraea pernyi silk sericin promotes osteogenic differentiation of human bone marrow derived mesenchymal stem cells. *Biomacromolecules. ACS Publications*; 2014;15:1185–93.
 14. Olive, P. L., & Banáth, J. P. (2006). The comet assay: A method to measure DNA damage in individual cells. *Nature Protocols*. <https://doi.org/10.1038/nprot.2006.5>
 15. Zhaorigetu, Siquin, Sasaki, M., & Kato, N. (2007). Consumption of sericin suppresses colon oxidative stress and aberrant crypt foci in 1,2-dimethylhydrazine-treated rats by colon undigested sericin. *Journal of Nutritional Science and Vitaminology*. <https://doi.org/10.3177/jnsv.53.297>
 16. Malay, A. D., Sato, R., Yazawa, K., Watanabe, H., Ifuku, N., Masunaga, H., Hikima, T., Guan, J., Mandal, B. B., & Damrongsakkul, S. (2016). Relationships between physical properties and sequence in silkworm silks. *Scientific Reports*, 6, 27573.
 17. Cooper, K. F. (2018). Till death do us part: the marriage of autophagy and apoptosis. *Oxidative Medicine and Cellular Longevity*, 2018.
 18. DebRoy, S., Kramarenko, I. I., Ghose, S., Oleinik, N. V, Krupenko, S. A., & Krupenko, N. I. (2013). A novel tumor suppressor function of glycine N-methyltransferase is independent of its catalytic activity but requires nuclear localization. *PloS One*, 8(7), e70062.
 19. Reddivari, L., Vanamala, J., Chintharlapalli, S., Safe, S. H., & Miller Jr, J. C. (2007). Anthocyanin fraction from potato extracts is cytotoxic to prostate cancer cells through activation of caspase-dependent and caspase-independent pathways. *Carcinogenesis*, 28(10), 2227–2235.
 20. Zhang, M., Liu, H., Tian, Z., Griffith, B. N., Ji, M., & Li, Q. Q. (2007). Gossypol induces apoptosis in human PC-3 prostate cancer cells by modulating caspase-dependent and caspase-independent cell death pathways. *Life Sciences*, 80(8), 767–774.
 21. Taylor, C. A., Zheng, Q., Liu, Z., & Thompson, J. E. (2013). Role of p38 and JNK MAPK signaling pathways and tumor suppressor p53 on induction of apoptosis in response to Ad-*elF5A1* in A549 lung cancer cells. *Molecular Cancer*. <https://doi.org/10.1186/1476-4598-12-35>
 22. Zhang, L., Yang, X., Li, X., Li, C., Zhao, L., Zhou, Y., & Hou, H. (2015). Butein sensitizes HeLa cells to cisplatin through the AKT and ERK/p38 MAPK pathways by targeting FoxO3a. *International Journal of Molecular Medicine*, 36(4), 957–966.
 23. Hilchie, A. L., Furlong, S. J., Sutton, K., Richardson, A., Robichaud, M. R. J., Giacomantonio, C. A., Ridgway, N. D., & Hoskin, D. W. (2010). Curcumin-induced apoptosis in PC3 prostate carcinoma cells is caspase-independent and involves cellular ceramide accumulation and damage to mitochondria. *Nutrition and Cancer*, 62(3), 379–389.

24. Rodríguez-Berriguete, G., Fraile, B., Martínez-Onsurbe, P., Olmedilla, G., Paniagua, R., & Royuela, M. (2012). MAP kinases and prostate cancer. *Journal of Signal Transduction*, 2012.
25. Mebratu, Y., & Tesfaigzi, Y. (2009). How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle*, 8(8), 1168–1175.
26. Tang, D., Wu, D., Hirao, A., Lahti, J. M., Liu, L., Mazza, B., Kidd, V. J., Mak, T. W., & Ingram, A. J. (2002). ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. *Journal of Biological Chemistry*. <https://doi.org/10.1074/jbc.M111598200>

Figures

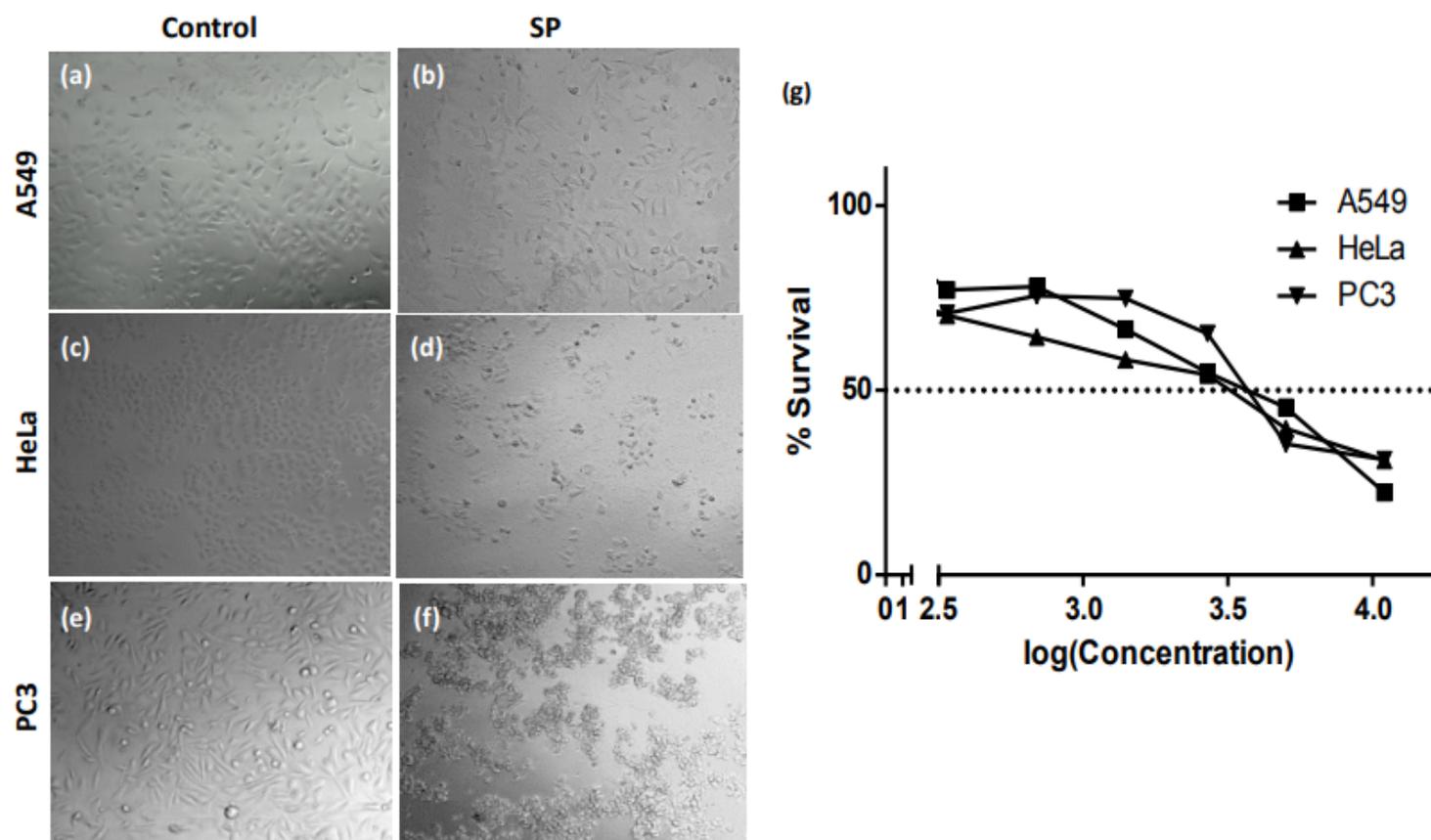
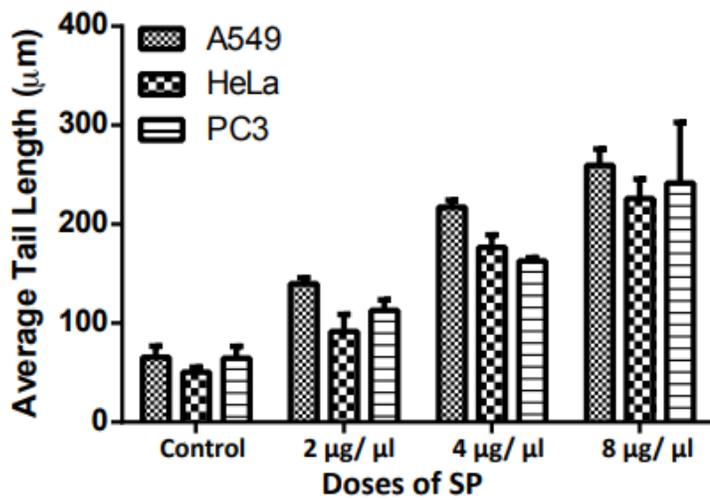
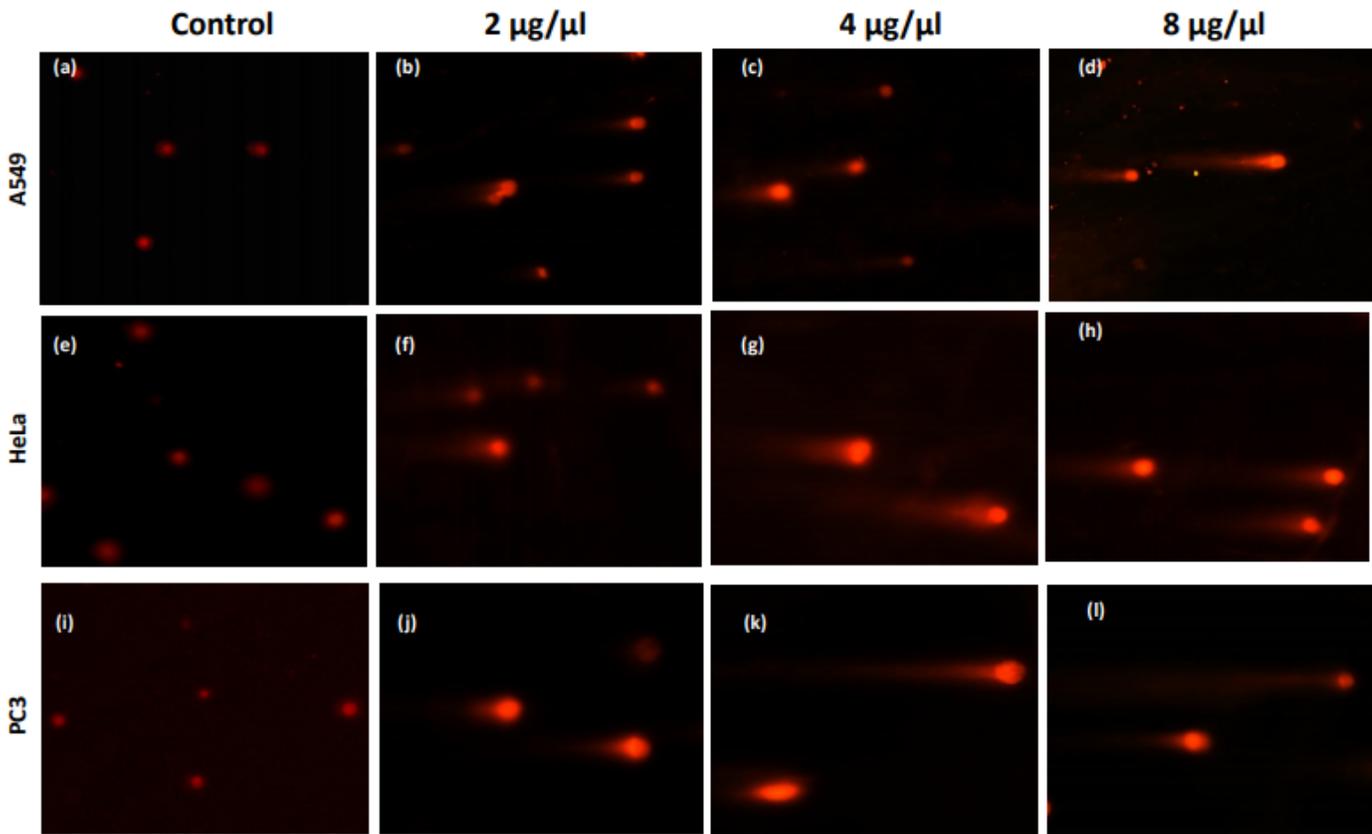


Figure 1

SP (SP) induces cytotoxicity in A549, HeLa, and PC3 cells: Morphological changes were observed under a simple microscope and acquired pictures; (a-b) A549, (c-d) HeLa, and (e-f) PC3 cells. (g) survival curves for inhibition of cell proliferation after treatment with different doses of SP were analyzed by MTT assay and IC50 values were determined.



(m)

Figure 2

SP induces genotoxicity: (a-l) gDNA fragmentation was assessed by comet assay after SP treatment to A549 (a-d), HeLa (e-h), and PC3 (i-l) for 24 h. Negative controls were treated with PBS (a, e, and i) and different doses of SP; 2µg/µl (b,f. and i), 4µg/µl (c, g and k) and 8µg/µl (d, h and l). Pictures were captured under an inverted fluorescence microscope. (m) the average tail lengths of the 100 apoptotic

cells each were measured and the graph between the dose of SP and the average tail lengths of each cell line is shown.

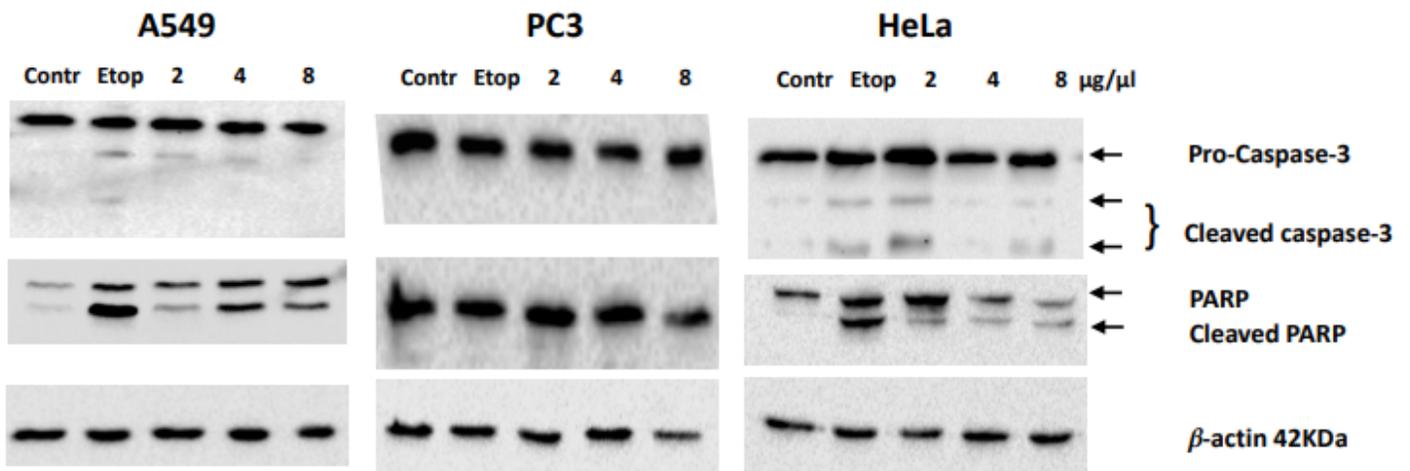


Figure 3

SP induces cleavage of caspase-3 and PARP proteins in A549 and HeLa cells but not in PC3 cells. Cells were treated with PBS (Contr) as the negative control, Etoposide ($59.2\mu\text{mol}$) (Etop) as positive control and different doses of SP (2,4, and 8 $\mu\text{g}/\mu\text{l}$ final concentration) for 24 h. Western blots were performed against anti-pro-caspase-3, anti-cleaved caspase-3, anti-PARP, and anti-cleaved PARP antibodies. Western blots against anti- β -actin antibody were also performed on the same respective membranes to normalize the proteins loading.

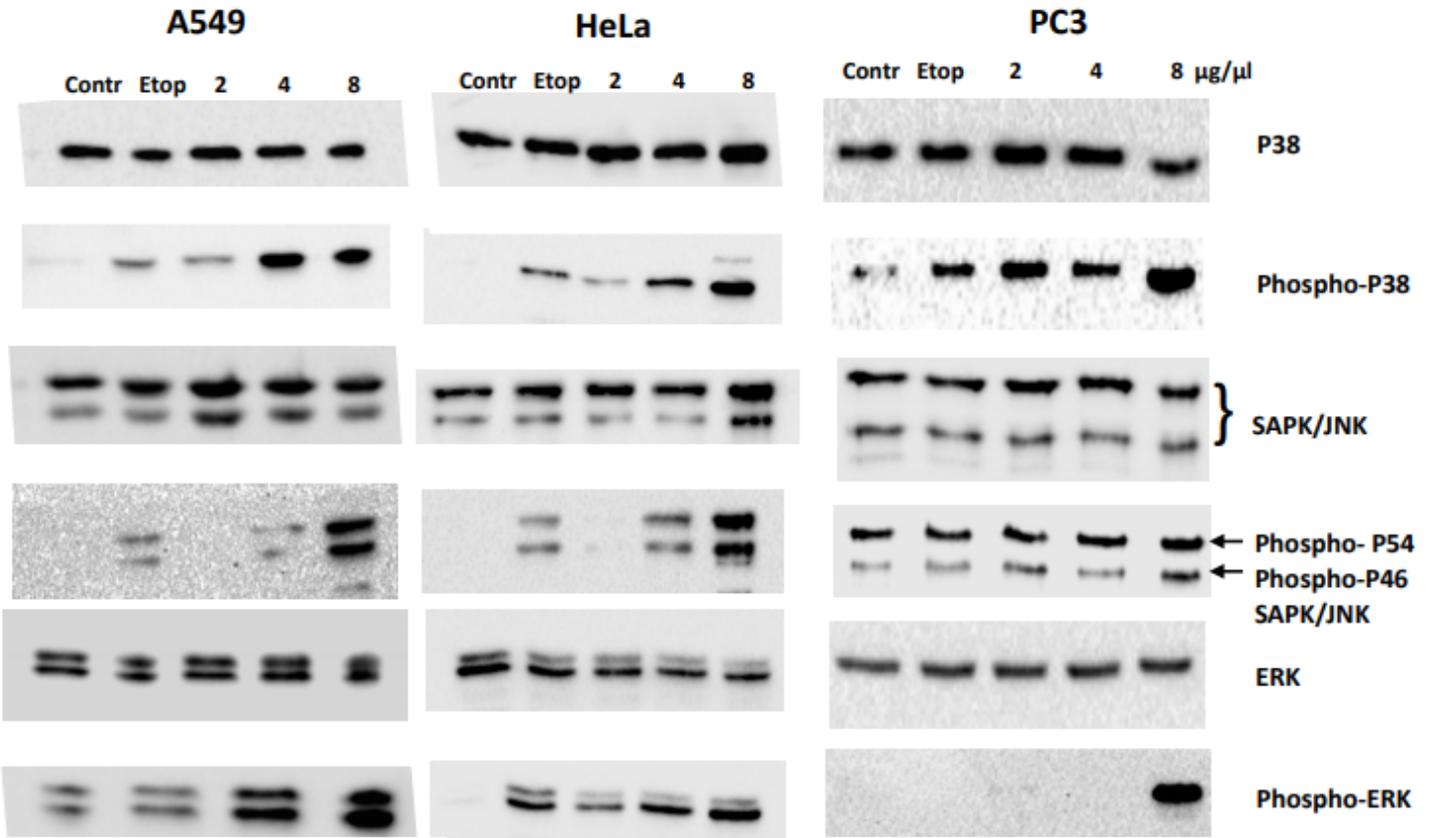


Figure 4

SP induces MAPK pathways: Cells were treated with PBS (Contr) as the negative control, Etoposide (59.2 μmol) (Etop) as the positive control, and different doses of SP (2,4, and 8 μg/μl final concentration) for 24 h. Western blots were performed against anti-p38, anti-phospho-p38, anti-SAPK/JNK, anti-phospho-SAPK/JNK, anti-ERK, and anti-phospho-ERK.

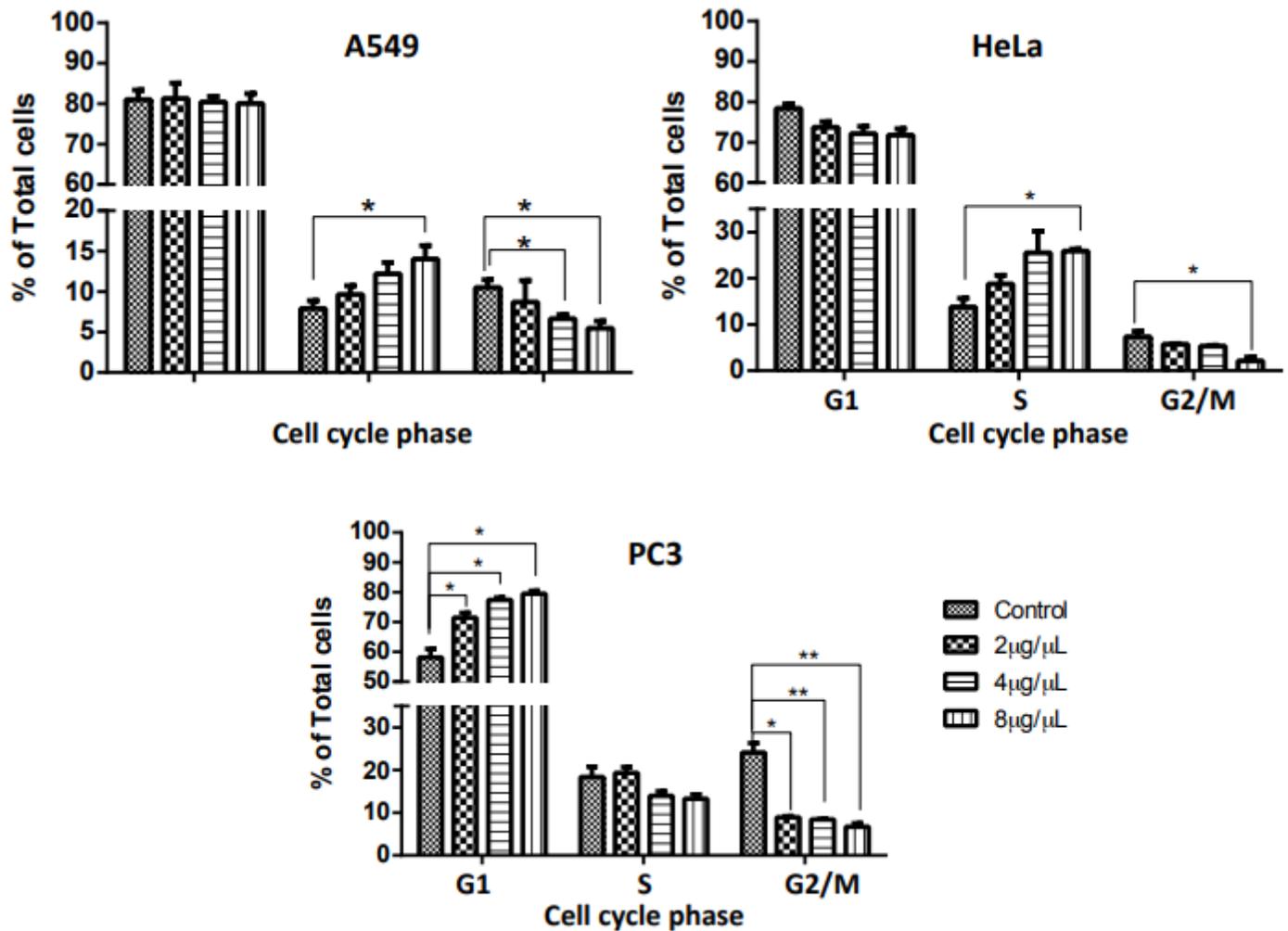


Figure 5

SP induces cell cycle arrest: Cells were treated with PBS (Contr) as the negative control and different doses of SP (2,4, and 8 µg/µl final concentration) for 24 h. Cell cycles were analyzed by flow cytometry. The percentages (%) of total cells at each phase, G1, S, or G2M are indicated against each cell line.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [uncroppedimages.pdf](#)