Food and Chemical Toxicology 123 (2019) 275–287

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Silk sericin induced pro-oxidative stress leads to apoptosis in human cancer cells

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ARTICLE INFO

Keywords:
Silk sericin
Pro-oxidative effect
Apoptosis
p53 upregulation
Bax/Bcl-2 dysregulation

ABSTRACT

Pro-oxidative stress induced by dietary polyphenols elevates reactive oxygen species (ROS) production in cancer cells, which subsequently leads to oxidative stress-mediated apoptosis. Sericin, a principal component of silk is associated with a mixture of polyphenols and flavonoids, possesses various biomedical attributes including anticancer activity. In the present study, we have evaluated the pro-oxidative effect of *Bombyx mori* sericin (BMS), *Antheraea assamensis* sericin (AAS), and *Philosamia ricini* sericin (PRS) against different cancer cells. Cytotoxicity of silk sericin (SS) evaluated using A431, SAS, and MCF-7 cells showed ≥50% reduction in their viability at 4 mg/mL. Intracellular ROS levels, cell cycle arrest, and apoptosis assessed using flow cytometry corroborated that SS treatment elevated the intracellular ROS levels, caused cell cycle arrest at the sub-G1 phase and resulted in apoptotic cell death. SS treated A431 and SAS cells showed upregulation of p53 and dysregulation of Bax and Bcl-2 gene expression. Whereas, AAS treated MCF-7 cells showed upregulation of Bax and downregulation of Bcl-2 gene expression. AAS treated MCF-7 and SAS cells showed downregulation of Bcl-2 protein expression in comparison to their control cells. Thus, the present study demonstrates that the pro-oxidative effect induced by SS suppresses the cancer growth indicating its potential anticancer activity.

1. Introduction

Reactive oxygen species (ROS) are produced in mitochondria as a by-product of aerobic metabolism. They act as secondary messengers in various biological processes and cell signaling pathways (Glasauer and Chandel, 2014). Regulation of ROS levels plays an important role in maintaining the health and longevity of the organism (McCord and Fridovich, 1988). Alteration in the redox balance by endogenous or exogenous factors lead to elevation or depletion of ROS compared to basal levels. Depletion of ROS levels disrupts cell signaling and biological process that affects cellular homeostasis (Cairns et al., 2011). Elevated ROS levels induce oxidative stress that damages cellular biomacromolecules and leads to carcinogenesis (Trachootham et al., 2009), diabetes, atherosclerosis (Paravicini and Touyz, 2006), aging (Haigis and Yankner, 2010), and neurodegeneration (Shukla et al., 2011).

Cancer cells bypass senescence and uncontrollably proliferate by hijacking the mechanism of normal cells (Hanahan and Weinberg, 2011). Hyper-metabolism of cancer cells elevates their intracellular ROS levels which play a key role in cancer progression, spreading and regulating signaling pathways; like angiogenesis and metastasis (Weinberg and Chandel, 2009; Weinberg et al., 2010). However, the elevation of ROS levels beyond a critical threshold induces oxidative stress that causes cell cycle arrest and apoptotic cell death. In order to maintain the redox balance, cancer cells elevate the production of endogenous antioxidants and scavenge the elevated levels of ROS (Gorrini et al., 2013). Therefore, elevation of ROS levels in cancer cells than their basal levels induces apoptotic/necrotic cell death.

In recent years, the pro-oxidant strategy also known as oxidant therapy is gaining much interest in cancer treatment (León-González et al., 2015). Dietary agents (polyphenols, flavonoids, resveratrol, and curcumin) and drug molecules (paclitaxel, doxorubicin, and cisplatin) that elevates the ROS levels could efficiently kill cancer cells (Fang et al., 2009; Lee et al., 2013). Any compound which can disrupt redox balance could affect both normal and cancer cells. Thus, a potential pro-oxidant compound must ideally elevate oxidative stress that could kill cancer cells and affect normal cells marginally (León-González et al., 2015). Conventional drug molecules are non-specific and induce oxidative stress that kills both normal and cancer cells. Dietary polyphenols, flavonoids, vitamin E, and vitamin C act as antioxidants and scavenge the elevated levels of intracellular ROS (León-González et al., 2015; Padayatty et al., 2003; Traber and Atkinson, 2007). However, at

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https://doi.org/10.1016/j.fct.2018.10.063
Received 30 August 2018; Received in revised form 29 October 2018; Accepted 30 October 2018
Available online 31 October 2018

Table 1
Description of primer sequences.

Gene	Primer	Sequence	Accession number
p53	Forward	5′-GCCCAACAACACCAGCTCCT-3′	NM_001126118.1
	Reverse	5′-CCTGGGCATCCTTGAGTTCC-3′	
Bcl-2	Forward	5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′	NM_000657.2
	Reverse	5′-GGTGCCGGTTCAGGTACTCAGTCA-3′	
Bax	Forward	5′-CCTGTGCACCAAGGTGCCGGAACT-3′	NM_001291431.1
	Reverse	5′- CCACCCTGGTCTTGGATCCAGCCC-3′	
Caspase-8	Forward	5′-CTGCTGGGGATGGCCACTGTG-3′	NM_001080125.1
	Reverse	5′-TCGCCTCGAGGACATCGCTCTC-3′	
Caspase-9	Forward	5′-AGTTCCCGGGTGCTGTCTAT-3′	NM_032996.3
	Reverse	5′-GCCATGGTCTTTCTGCTCAC-3′	
Caspase-3	Forward	5′-ACATGGCGTGTCATAAAATACC-3′	NM_001354779.1
	Reverse	5′-CACAAAGCGACTGGATGAAC-3′	
Cyt *c*	Forward	5′-ATGGTCTCTTTGGGCGGAAG-3′	NM_018947.5
	Reverse	5′-GGCAGTGGCCAATTATTACTCA-3′	
β-actin	Forward	5′-CCCTGGCACCCAGCAC -3′	NM_001101.4
	Reverse	5′-GCCGATCCACACGG AGTAC-3′	

higher concentrations, they also act as pro-oxidants, elevates ROS levels in the cells and leads to oxidative stress-mediated apoptotic/necrotic cell death (León-González et al., 2015; Pearson et al., 2006). The concentration of dietary polyphenols and flavonoids used should strategically trigger a marginal or minimal side effect on normal cells while proving fatal for cancer cells.

Silk sericin (SS) is a natural protein polymer produced by the silkworms (Dash et al., 2007). It possesses various biological activities such as anti-oxidation, suppression of elastase and tyrosinase activity (Chlapanidas et al., 2013; Kumar and Mandal, 2017). SS reduces ROS, protect the cells from H_2O_2 and UVB radiation-induced oxidative damage, and prevents cancer progression (Kumar and Mandal, 2017; Zhaorigetu et al., 2003). Kaewkorn et al. reported that SS extracted from the cocoons of *Bombyx mori* suppressed the proliferation of colon cancer and induced apoptotic cell death (Kaewkorn et al., 2012). The effect of SS on the redox balance of cancer cells not yet explored. The biological properties of SS are attributed to its amino acid compositions and associated secondary metabolites (polyphenols and flavonoids) (Kumar and Mandal, 2017). Mulberry silk varieties are enriched with quercetin, catechin, epicatechin, *trans*-resveratrol, rutin, procyanidin B1, kaempferol, procyanidin B2, myricetin, luteolin, naringenin, and Σ phenolics, however, their content varies based on their source (Butkhup et al., 2012). Whereas, Eri silk lacks quercetin, luteolin, naringenin, and kaempferol (Butkhup et al., 2012). It has been reported that polyphenols and flavonoids of fruits and vegetables (mixture of quercetin, kaempferol, myricetin, catechin, epicatechin, rutin, isorhamnetin, malvidin-3-glucoside, caffeic acid, chrysin, galangin, apigenin, fisetin, luteolin, morin, and several anthocyanidins) significantly suppressed the growth of human breast carcinoma (MCF-7) by arresting the cell cycle at G1 phase (Kubatka et al., 2016). They also induced apoptosis in MCF-7 cells and caused DNA fragmentation (Kubatka et al., 2016). A mixture of polyphenols and flavonoids associated with SS vary based on their source as well as extraction methods. Therefore, exploring the pro-oxidant activity of SS against cancer cells would be beneficial in understanding the impact of SS and associated secondary metabolites (polyphenols and flavonoids) on controlling the cancer growth.

In the present study, we aimed to explore the effect of SS extracted from the cocoons of *Bombyx mori* (BM), *Antheraea assamensis* (AA) (endemic to Assam, India), and *Philosamia ricini* (PR) on the redox balance of cancer cells. Further, we tried to understand the influence of redox imbalance on the endogenous antioxidant activity, mitochondrial membrane potential and their role in suppressing the cancer growth. To elucidate these reservations we have investigated the anticancer activity of BM sericin (BMS), AA sericin (AAS) and PR sericin (PRS) using human breast carcinoma (MCF-7), oral carcinoma (SAS) and squamous carcinoma (A431) cells. Human cancer (A431, SAS, and MCF-7) cells were treated with SS and thereafter, assessed for anticancer activity in the context of cytotoxicity, ROS levels, cell cycle arrest, and mitochondrial membrane potential. The effect of SS on the expression of p53, cytochrome *c* (cyt *c*), Bax and Bcl-2 genes and apoptotic/anti-apoptotic proteins in cancer cells have been studied.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO) and sodium carbonate were sourced from SRL, India; Antibiotic-antimycotic solution, Tris-base, and sodium chloride was obtained from Himedia, India. Thiazolyl blue tetrazolium bromide (MTT), Bradford's reagent, epidermal growth factor (EGF), cholera toxin, insulin, RNase A, superoxide dismutase (SOD) detection kit, nonidet P-40 (NP-40), sodium orthovanadate, sodium deoxycholate, sodium dodecyl sulfate (SDS), phenylmethylsulfonyl fluoride (PMSF), aprotinin and hydrocortisone were obtained from Sigma-Aldrich, USA. Propidium iodide (PI), Annexin V-FITC/PI assay kit, JC-1 assay kit, Amplex red catalase assay kit, and horse serum were supplied by Invitrogen, USA. Dulbecco's modified Eagle's medium (DMEM), DMEM/F12 and fetal bovine serum (FBS) were procured from Gibco, USA.

2.2. Extraction of silk sericin using alkali-degradation

SS was extracted from the cocoons of AA, BM, and PR using our previously established protocol (Kumar and Mandal, 2017). In brief, cocoons were chopped and boiled in 0.5 M Na_2CO_3 for 30 min. Post boiling, silk fibers were removed; the protein solution was centrifuged and filtered to remove the residual solid particles. Further, the filtrate was dialyzed against MiliQ H_2O for 48 h. Dialyzed protein solution was freeze-dried and stored at −20 °C.

2.3. In vitro cell culture studies

2.3.1. Cell culture

Human squamous carcinoma (A431), breast adenocarcinoma (MCF-7) and keratinocyte (HaCaT) cell lines were sourced from the National Centre for Cell Science (NCCS), Pune, India. Human tongue carcinoma (SAS), HaCaT, MCF-7, and A431 cells were cultured in high glucose DMEM, supplemented with 10% FBS and 1X antibiotic-antimycotic solution. Whereas, non-tumorigenic epithelial (MCF-10) cells were cultured in DMEM/F12 media supplemented with 5% horse serum, 0.5 mg/mL hydrocortisone, 20 ng/mL EGF, 10 μg/mL insulin and 100 ng/mL cholera toxin.

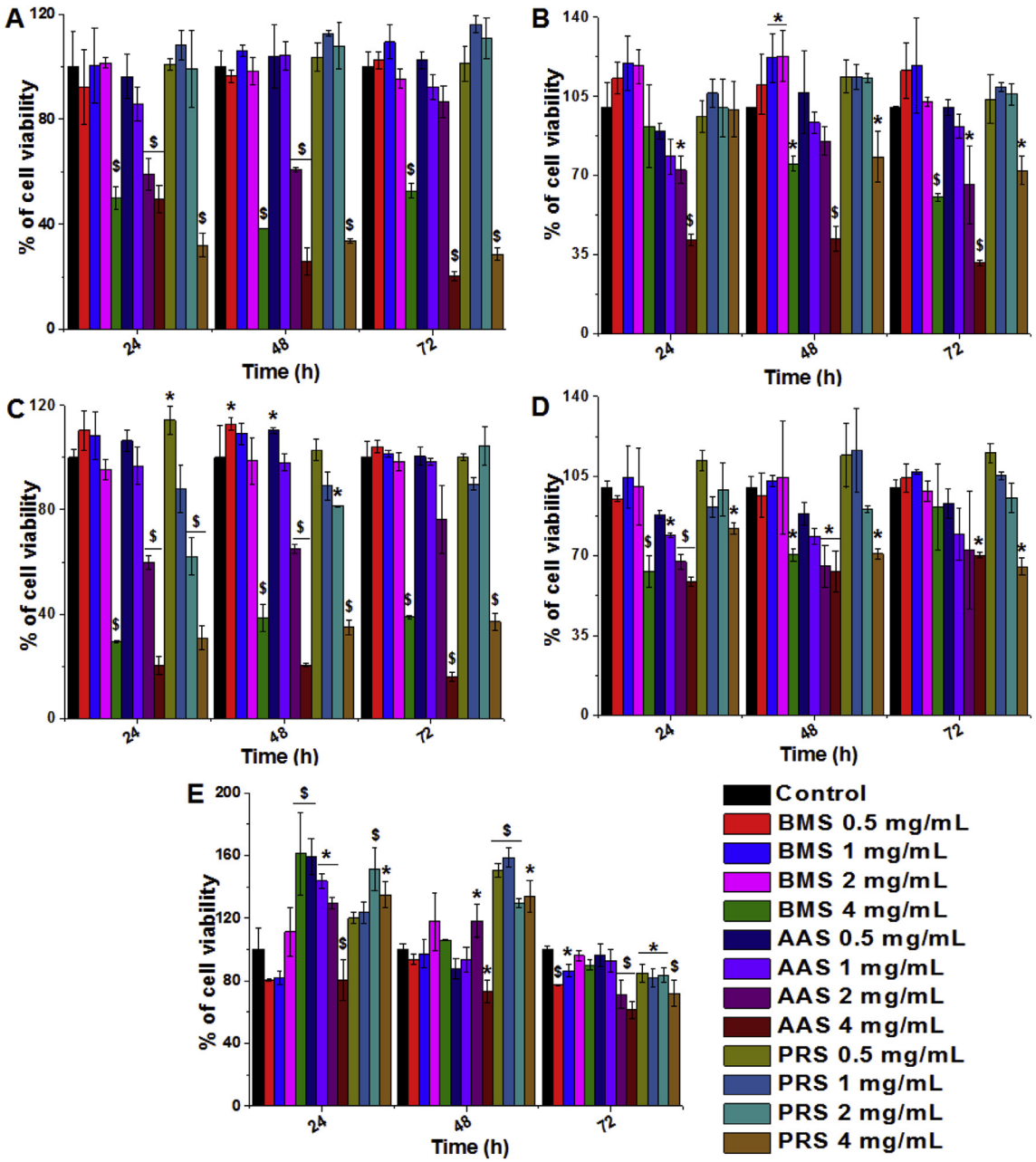

Fig. 1. The effect of silk sericin on the viability of (A) A431, (B) MCF-7, (C) SAS, (D) HaCaT, and (E) MCF-10 cells was assessed using MTT assay. ($p ≤ 0.001 and *p ≤ 0.01 in comparison with control).

2.3.2. Cytotoxicity study of silk sericin

Cytotoxicity of SS was assessed using the MTT assay. Briefly, 1×10^4 cells per well were seeded in 48 well plates, cultured for 24 h at 37 °C and treated with different (500, 1000, 2000 and 4000 μg/mL) concentration of SS. After 24, 48 and 72 h of treatment, cells were incubated with 200 μL of MTT (1: 9 ratio of MTT and phosphate buffer saline) solution for 4 h. Formazan crystals formed by mitochondrial dehydrogenase were dissolved in DMSO and absorbance was measured at 570 nm by using a multiplate reader (Tecan, Infinite 200).

2.3.3. Lactate dehydrogenase (LDH) assay

The effect of SS on the cellular membrane integrity of cancer cells was assessed using the LDH assay. In brief, cancer cells (2×10^4) were cultured in 48 well plates for 24 h and treated with 4 mg/mL of SS for 24 h. Post-treatment, 50 μL of spent media was collected and analyzed for LDH activity according to the manufacturer's protocol.

2.3.4. Determination of intracellular reactive oxygen species levels

The change in the intracellular ROS levels of SS treated cancer cells were assessed using DCFH-DA (Kumar et al., 2017). In brief, a density of 3×10^5 cells per well was seeded in 6 well plates, incubated for 24 h and treated with 4 mg/mL of SS. After 24 h, cells were incubated with 10 μM DCFH-DA for 1 h at 37 °C. Fluorescence generated by the hydrolysis of DCFH-DA to DCF due to cellular esterases and intracellular ROS was measured using flow cytometer (FACS Calibur, BD). For each sample, 50,000 events were collected and the results were analyzed using FCS Express 6.

2.3.5. Determination of endogenous antioxidant activity

2.3.5.1. Superoxide dismutase (SOD) activity. SOD activity in SS treated cancer cells was assessed using the SOD assay kit. Briefly, 3×10^5 cells per well were seeded in 6 well plates, cultured for 24 h and treated with 4 mg/mL of SS. Cells were scraped and lysed in 50 μL of cell lysis buffer

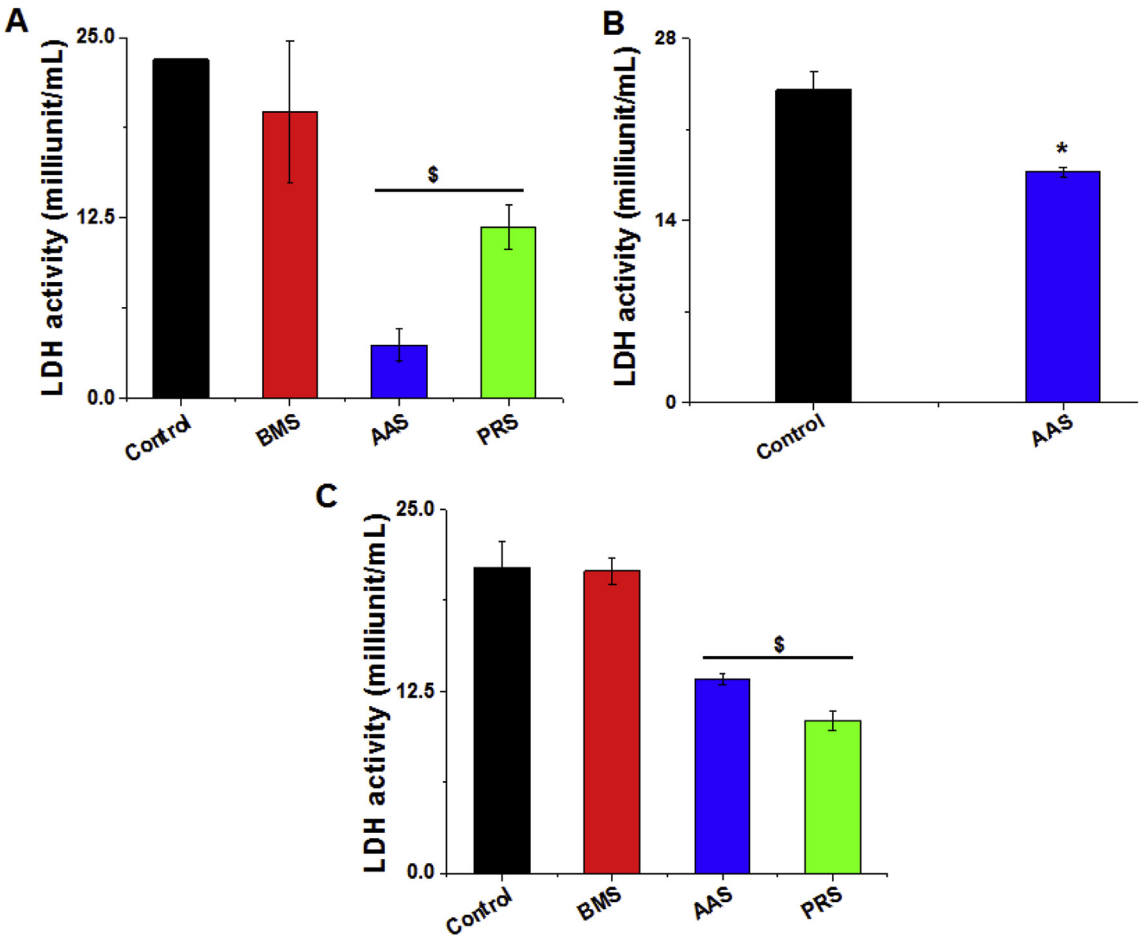

Fig. 2. The effect of silk sericin on the cellular membrane integrity of (A) A431, (B) MCF-7, and (C) SAS cells were assessed by determining their LDH activity. ($p ≤ 0.001 and *p ≤ 0.01 in comparison with control).

[20 mM HEPES-KOH (pH-7.4), 2 mM EDTA, 250 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 μg/mL of pepstatin A and 2 μg/mL of aprotinin] for 30 min on ice. Cell lysates were centrifuged and the aliquots of the supernatants were analyzed for SOD activity according to the manufacturer's protocol. The assay kit utilized water-soluble tetrazolium (WST-1) salt, which converted to water-soluble formazan upon reduction of superoxide anion (O_2^-). Conversion of WST-1 to formazan was recorded at 450 nm using the multiplate reader.

2.3.5.2. Catalase (CAT) activity. The effect of SS on the CAT activity was assessed using the Amplex red catalase assay kit. Cancer cells (3×10^5 cells) were seeded in 6 well plate and cultured for 24 h followed by SS (4 mg/mL) treatment. After 24 h of treatment, cells were scraped and lysed in 50 μL of cell lysis buffer for 30 min followed by centrifugation. Aliquots of the supernatant were used to analyze catalase activity according to the manufacturer's protocol.

2.3.6. Cell cycle analysis

Cancer cells (3×10^5 cells per well) were cultured in 6 well plates for 24 h and treated with 4 mg/mL of SS. After 24 h of treatment, cells were harvested and assessed for cell cycle arrest by PI using our previously established protocol (Kumar et al., 2016). For each sample, 50,000 events were collected and the results were analyzed using FCS Express 6.

2.3.7. Mitochondrial membrane potential (ψm) analysis

Change in inner mitochondrial membrane potential of the cancer cells after SS treatment was analyzed by flow cytometer using the JC-1 assay kit. In brief, cancer cells were seeded in 6 well plates at a density of 3×10^5 cells per well and cultured for 24 h. Cells were treated with 4 mg/mL of SS for 24 h, harvested, labeled with JC-1 dye and analyzed using flow cytometer. For each sample, 50,000 events were collected and the results were analyzed using FCS Express 6.

2.3.8. Annexin V/PI assay

Apoptotic cell death was determined using the Annexin V-FITC/PI assay kit. Cancer cells were seeded in 6 well plates at a density of 3×10^5 cells per well and cultured for 24 h. Cells were treated with SS (4 mg/mL) for 24 h, harvested and labeled with FITC conjugated Annexin V and PI, and analyzed using flow cytometer. For each sample, 50,000 events were collected and the results were analyzed using FCS Express 6.

2.3.9. Gene expression studies

A density of 5×10^5 cancer cells was cultured in 60 mm Petri dishes for 24 h at 37 °C and treated with 4 mg/mL of SS. After 24 h of treatment, RNA was extracted from the cancer cells using TRIzol reagent. The concentration and purity of the RNA were measured using NanoDrop (Eppendorf, Germany) spectrophotometer. 1 μg of RNA was reverse transcribed to cDNA using High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Thermo Fisher Scientific, USA) in a thermal cycler (Takara, Japan). Expression level of p53, Bcl-2, Bax, cytochrome *c* (cyt *c*), β-actin, caspase-3, caspase-8, and 9 genes was quantified using Power SYBR Green PCR master mix (Applied Biosystems, Life Technologies) in a real-time-polymerase chain reaction (RT-PCR) machine (Applied Biosystems 7500) with the sequences

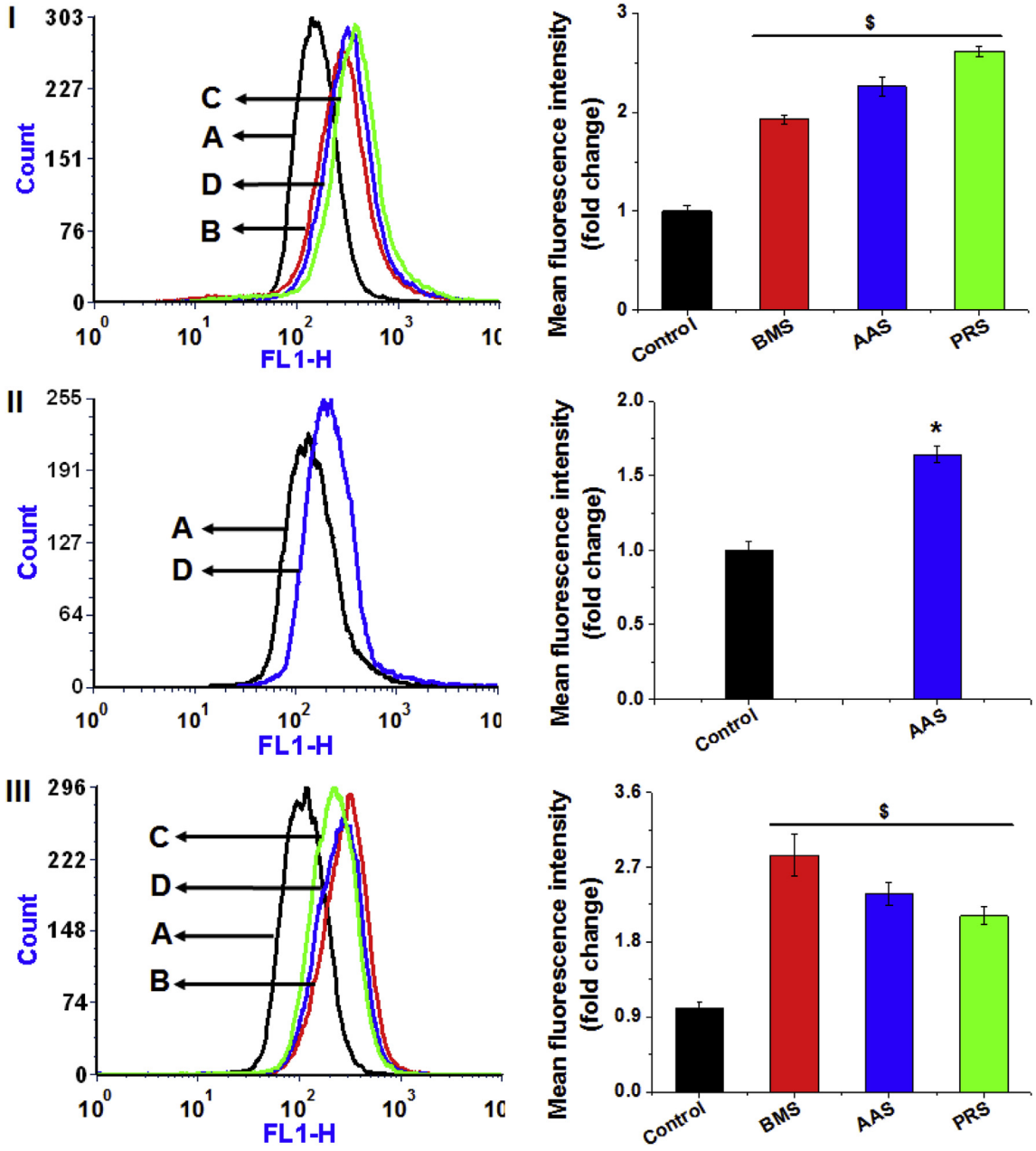

Fig. 3. The effect of silk sericin on the intracellular ROS levels of (I) A431, (II) MCF-7, and (III) SAS cells was assessed using DCFH-DA; where (A) control, (B) BMS, (C) PRS, and (D) AAS treated cells. ($p ≤ 0.001 and *p ≤ 0.01 in comparison with control).

shown in Table 1.

2.3.10. Western blotting

Cancer cells (3×10^5) were cultured in 6 well plates for 24 h and treated with 4 mg/mL of SS. Post-treatment, cells were washed with PBS, trypsinized and lysed in 50 μL of RIPA buffer [10 mM Tris (pH 8), 0.1% NP-40, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 0.1% SDS, 1 mM PMSF and 4 μg/mL of aprotinin] (Liang et al., 2001). The cell lysates were centrifuged and the total protein concentration in the supernatants was determined using the Bradford assay (Sigma, USA). An equal amount of protein (30 μg) was separated using SDS-PAGE (12% separation gel and 5% stacking gel) and transferred to Immobilon-P PVDF membrane (Merck Millipore, USA) using a western blot apparatus. The blot was blocked with Tris-buffered saline-Tween (TBST) blocking buffer containing 5% skim milk for 1 h followed by incubation in specific primary antibodies; anti-Bcl-2 (mouse monoclonal, Invitrogen, USA) (1:500), anti-Bax (mouse monoclonal, Invitrogen, USA), and anti-GAPDH (mouse monoclonal, Abcam, UK) (1:1000). The blot was washed with TBST wash buffer and incubated for 1 h in goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Abcam, USA) (1:5000). Using ECL kit (Biorad, USA), the protein expression levels were found using an image analyzer (Biorad, USA).

2.4. Statistical analysis

All quantitative experiments were carried out in triplicate and data were analyzed to attain mean ± standard deviation. One-way analysis of variance (ANOVA) was examined with the Holm-Sidak method using Sigma-plot software.

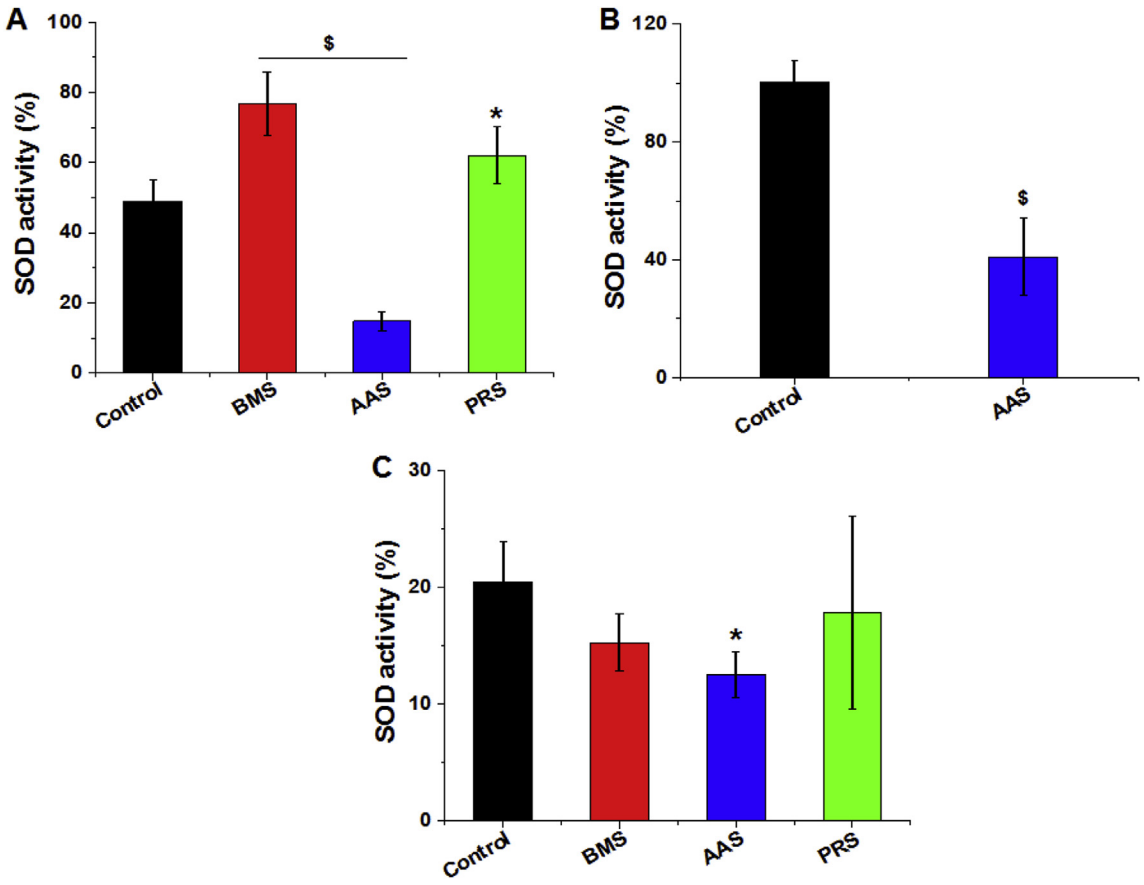

Fig. 4. The effect of silk sericin on the activity of endogenous SOD activity of (A) A431, (B) MCF-7, and (C) SAS cells were assessed by the percentage inhibition of the formazan formation from WST-1 using the superoxide anion produced by xanthine oxidase. ($p ≤ 0.001 and *p ≤ 0.01 in comparison with control).

3. Results

3.1. Cytotoxicity study of silk sericin

Metabolically active viable cells convert water-soluble thiazolyl blue tetrazolium bromide to water-insoluble formazan in the presence of mitochondrial dehydrogenases. The viability of A431, MCF-7, SAS, HaCaT and MCF-10 cells after SS treatment was assessed by measuring their mitochondrial activity using the MTT assay. The viability of cancer (A431, SAS, and MCF-7 cells) and normal (HaCaT and MCF-10 cells) cells after SS treatment for 24, 48 and 72 h are depicted in Fig. 1. The viability of both cancer and normal cells remained unaffected after treating with SS up to 1 mg/mL; however, with increasing SS concentration the viability of cells was decreased. In comparison with control cells, A431 and SAS cells treated with 4 mg/mL of BMS, PRS, and AAS showed < 50% cell viability. Whereas, MCF-7 cells treated with AAS (4 mg/mL) exhibited > 50% growth inhibition. HaCaT and MCF-10 cell lines treated with 4 mg/mL of SS showed ~70% viable cells. This indicated that SS is more toxic to cancer cells than normal cell lines. 4 mg/mL of AAS (A431, MCF-7, and SAS), BMS (A431 and SAS cells), and PRS (A431 and SAS cells) were selected for further evaluation of their anticancer activity against specific cancer cells based on their IC_{50} value.

3.2. Lactate dehydrogenase (LDH) assay

Pro-oxidant compounds might also induce cytotoxicity by disrupting cellular membrane integrity. The release of cytoplasmic LDH into surrounding media is an early marker for cellular membrane disruption. The effect of SS on the membrane integrity of cancer cells was assessed using the LDH assay kit. Fig. 2 illustrates the LDH activity of SS treated cancer cells. In comparison with control cells, AAS and PRS treated cancer cells showed significantly low LDH activity ($p ≤ 0.01$), however, LDH activity of BMS treated cancer cells remain unchanged.

3.3. Determination of intracellular reactive oxygen species levels

Pro-oxidant compounds induce oxidative stress in cancer cells by elevating their intracellular ROS levels. The change in the intracellular ROS levels was determined using DCFH-DA. The intracellular ROS levels of cancer cells are illustrated in Fig. 3. SS treatment significantly enhanced ROS levels of cancer cells in comparison with their perspective control cells.

3.4. Superoxide dismutase (SOD) activity

SOD, an endogenous antioxidant enzyme converts highly unstable superoxide (O_2^-) to stable hydrogen peroxide and molecular oxygen (Gough and Cotter, 2011). Loss of SOD activity, in turn, enhances ROS production in the cells resulting in oxidative damage. SOD activity was assessed by the inhibition of formazan formation from WST-1 using the superoxide anion produced by xanthine oxidase utilizing xanthine and molecular oxygen. Fig. 4 depicts endogenous SOD activity of SS treated cancer cells. In comparison with control cells, SOD activity was significantly reduced in AAS treated cancer cells ($p ≤ 0.01$). Whereas, SOD activity of A431 cells was increased by BMS and PRS treatment.

3.5. Catalase (CAT) activity

CAT is a cytoplasmic antioxidant enzyme that converts hydrogen peroxide to water and molecular oxygen (Gough and Cotter, 2011). CAT activity was evaluated using Amplex red, which reacts with

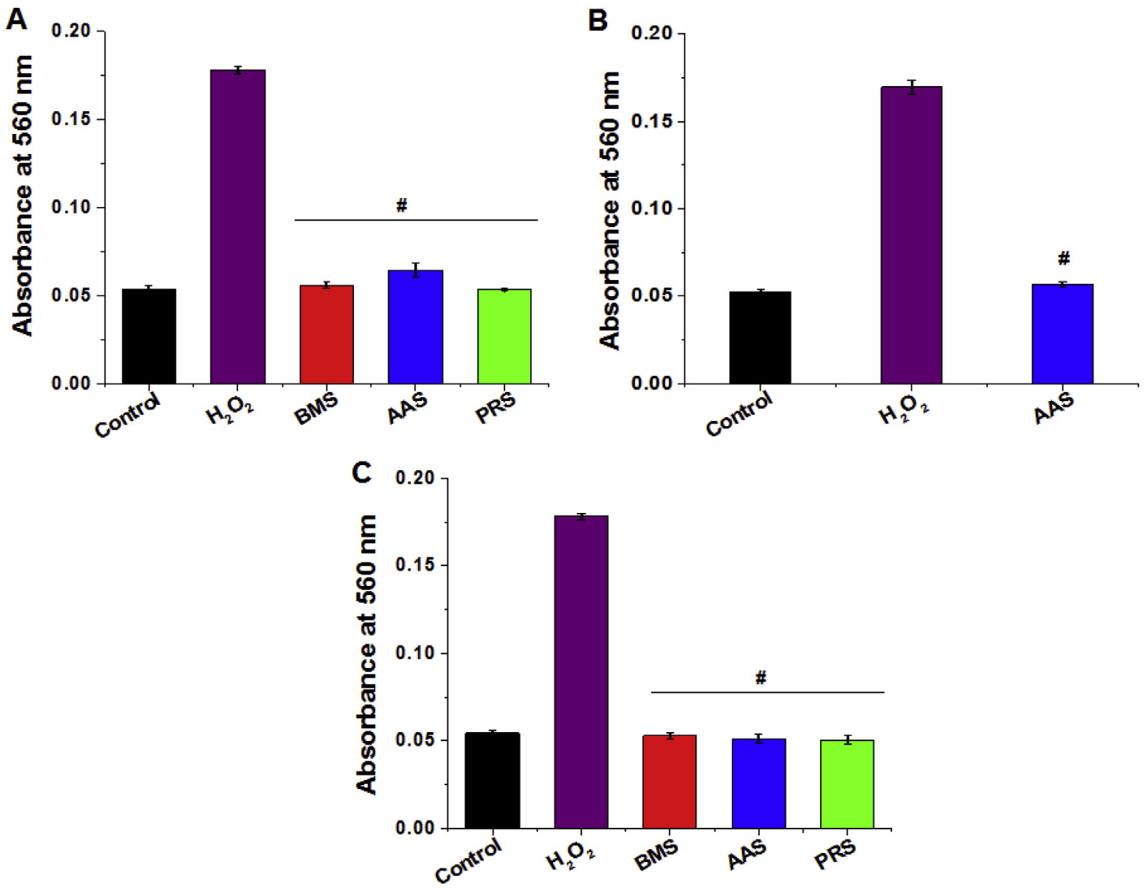

Fig. 5. The effect of silk sericin on the endogenous CAT activity of (A) A431, (B) MCF-7, and (C) SAS cells were assessed using the Amplex red assay kit. (#p ≤ 0.001 in comparison with only hydrogen peroxide). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

hydrogen peroxide in 1:1 ratio and emits fluorescence. Fig. 5 illustrates the absorbance of remaining hydrogen peroxide present after breakdown to water and molecular oxygen by the endogenous CAT of cancer cells. In comparison with control, the CAT activity of SS treated cancer cells remained unaffected.

3.6. Cell cycle analysis

Most of the anticancer/pro-oxidant compounds induce cell cycle arrest and cause apoptotic cell death. The effect of SS on the cell cycle of cancer cells was analyzed by flow cytometry using PI. Fig. 6 depicts the percentage of the gated population of cancer cells present in different phases of the cell cycle. Gated cell population represented in sub-G1 and G1 phase correspond to cell death by apoptosis/necrosis and cell cycle arrest, respectively. In comparison with A431 control cells, AAS and PRS treated cells showed a significantly high percentage of gated cell population at the sub-G1 phase and reduced gated population at G1 phase ($p \leq 0.01$). As compared with MCF-7 control cells, AAS treatment increased the percentage of gated cell population at sub-G1 phase ($p \leq 0.001$), without changing the population at G1 phase. Whereas, the gated population of SS treated MCF-7 and A431 cells remained unchanged at S and G2 phases. SS treated SAS showed a high percentage of the gated population at the sub-G1 phase and reduced gated population at G1 and G2 phases than control cells ($p \leq 0.01$).

3.7. Mitochondrial membrane potential (ψm) analysis

Depletion of inner mitochondrial membrane potential is an early marker during apoptotic cell death (Garg and Chang, 2004). Changes in inner mitochondrial membrane potential were monitored by flow cytometer using JC-1 cationic fluorescent dye. Fig. 7 depicts the change in the inner mitochondrial membrane potential of SS treated cancer cells. The degree of mitochondrial membrane potential change varied with the source of SS. In comparison with control, the mitochondrial membrane potential of SS treated A431 and SAS cells remain unchanged, however, AAS treated MCF-7 cells showed 35.2 fold depletion of inner mitochondrial membrane potential.

3.8. Annexin V/PI assay

Apoptotic cell death caused by SS treatment was assessed by flow cytometer using Annexin V/PI staining kit. Fig. 8 depicts the quadrat plots, which represents the percentage of cell population present at early (positive for Annexin V only), late (positive for both Annexin V and PI) apoptosis and dead (positive for PI only) phase. SS treatment induced the apoptotic death in cancer cells. BMS, PRS, and AAS treated A431 cells enhanced percentages of cell population at early (6.3, 3, and 3.6 folds, respectively) and late (3.7, 3.1, and 4.47 folds, respectively) apoptotic phase. AAS treated MCF-7 cells showed 9.6 and 9.2 fold enhanced cell population at early and late apoptotic phase, respectively. When compared to SAS control cells, BMS, PRS, and AAS treated cells showed enhanced cell population at early (3.9, 1.5 and 2.9 fold, respectively) and late (24.3, 5.6, 13.33 fold, respectively) apoptotic phase.

3.9. Gene expression studies

Pro-oxidants/anticancer compounds induce redox imbalance, which

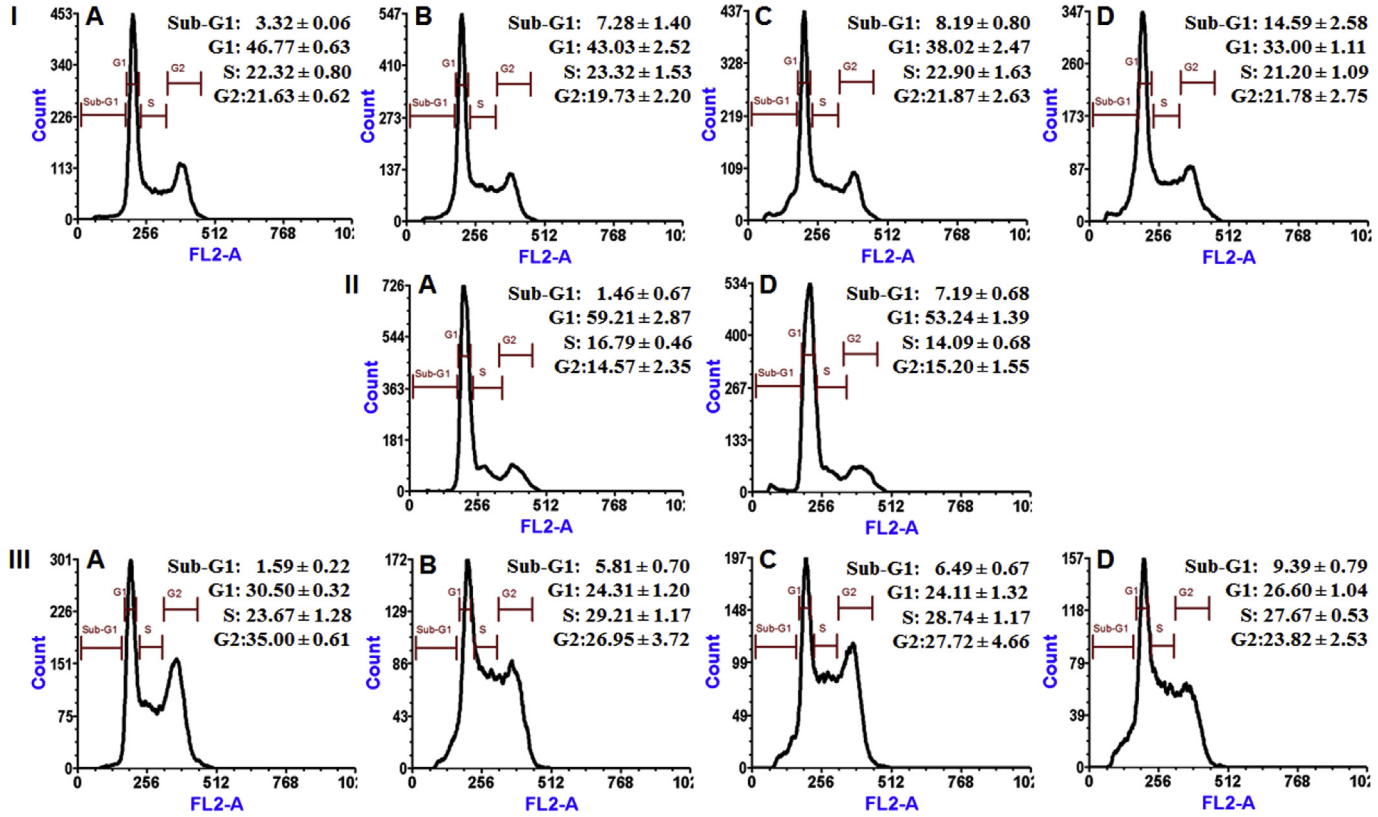

Fig. 6. The effect of silk sericin on the cell cycle analysis of (I) A431, (II) MCF-7, and (III) SAS cells was assessed by flow cytometer using PI. Where (A) control, (B) BMS, (C) PRS and (D) AAS treated cancer cells. Data are expressed as mean ± S.D (n = 3).

leads to the upregulation of tumor suppressor, pro-apoptotic, and caspase gene expression along with downregulation of anti-apoptotic genes and results in apoptotic death. The effect of SS on the expression of a tumor suppressor (p53), cytochrome *c* (cyt *c*), caspase-3, caspase-8, caspase-9, Bax and Bcl-2 genes was evaluated using quantitative RT-PCR. Fig. 9 depicts the expression of p53, cyt *c*, caspase-3, caspase-8, caspase-9, Bax, and Bcl-2 genes in SS treated cancer cells. In comparison with control cells, SS treated A431 and SAS cells showed ~1.5 and 2 fold high expression of p53 gene, respectively, whereas, in MCF-7 cells p53 gene expression remain unaffected. A431 cells treated with BMS and PRS showed upregulation of cyt *c*, caspase-3, caspase-8, and caspase-9 gene expression than control. While AAS treated A431 cells showed 9.8 fold upregulation of caspase-9 expression, however, cyt *c*, caspase-3, and caspase-8 gene expression remained unchanged. When compared to control cells, SS treated A431 cells exhibited dysregulation of Bax/Bcl-2 genes. AAS treated MCF-7 cells showed upregulation of cyt *c* (2 fold), caspase-9 (1.3 fold) and Bax (4.2 fold) expression, whereas, downregulation of Bcl-2 and caspase-8 expression. SAS cells showed upregulation of cyt *c*, caspase-3 (for BMS, AAS, and PRS treated cells), caspase-9 (for AAS and PRS treated cells), caspase-8, and Bcl-2 (for BMS and PRS treated cells) expression after SS treatment than control cells. In SS treated SAS cells, Bax expression remained unchanged.

3.10. Western blotting

Bax and Bcl-2 protein expression play an important role in regulating apoptotic cell death. Redox imbalance induced by pro-oxidants/anticancer compounds upregulate Bax and downregulates Bcl-2 protein expression that leads to apoptotic death. The effect of redox imbalance induced by the various source of SS on the expression of Bax and Bcl-2 proteins was assessed using western blotting. Bax and Bcl-2 expression in A431, SAS, and MCF-7 cells post-treatment with SS are depicted in Fig. 10. The Bax expression was upregulated in AAS treated A431 cells when compared to control cells, however, its expression was unchanged in BMS and PRS treated cells. Similar Bcl-2 expression was observed in SS treated A431 cells and its counterparts. In comparison with their control cells, AAS treatment significantly downregulated Bcl-2 expression in MCF-7 and SAS cells, while BMS and PRS treatment did not change its expression. The Bax expression in SS treated MCF-7 and SAS remained similar with control cells.

4. Discussion

Reactive oxygen species (ROS) play a key role in cancer initiation, progression, and metastasis (Weinberg and Chandel, 2009; Weinberg et al., 2010). Endogenous antioxidants protect the cancer cells from ROS induced oxidative damage by maintaining them at basal levels (Gorrini et al., 2013). However, insufficient endogenous antioxidant mechanism elevates intracellular ROS levels that lead to oxidative stress-mediated apoptosis in cancer cells (Palanivel et al., 2014). Accumulation of intracellular ROS in cancer cells due to chemotherapeutics (doxorubicin, cisplatin etc.) and pro-oxidants (polyphenols, flavonoids, curcumin, and resveratrol) is one of the major strategies for inducing apoptosis (Fang et al., 2009; Lee et al., 2013).

Dietary polyphenols and flavonoids protect the cells from oxidative stress-induced damage by reducing the ROS and modulating the activity/expression of endogenous antioxidants enzymes (Pietta, 2000; Tadić et al., 2008). Further, they reduce the risk of occurrence of various diseases like cancer, neurodegeneration, metabolic and cardiovascular diseases (Cotelle, 2001; Dai and Mumper, 2010; León-González et al., 2015; Mink et al., 2007). In certain conditions like high polyphenol concentrations and the presence of redox active transition metals cations, they can act as pro-oxidants and induce oxidative stress (Park and M Pezzuto, 2012). The pro-oxidant properties of polyphenols depend on the formation of labile aroxil radical or redox complex with transition metal ion. The labile aroxil radicals react with molecular O_2

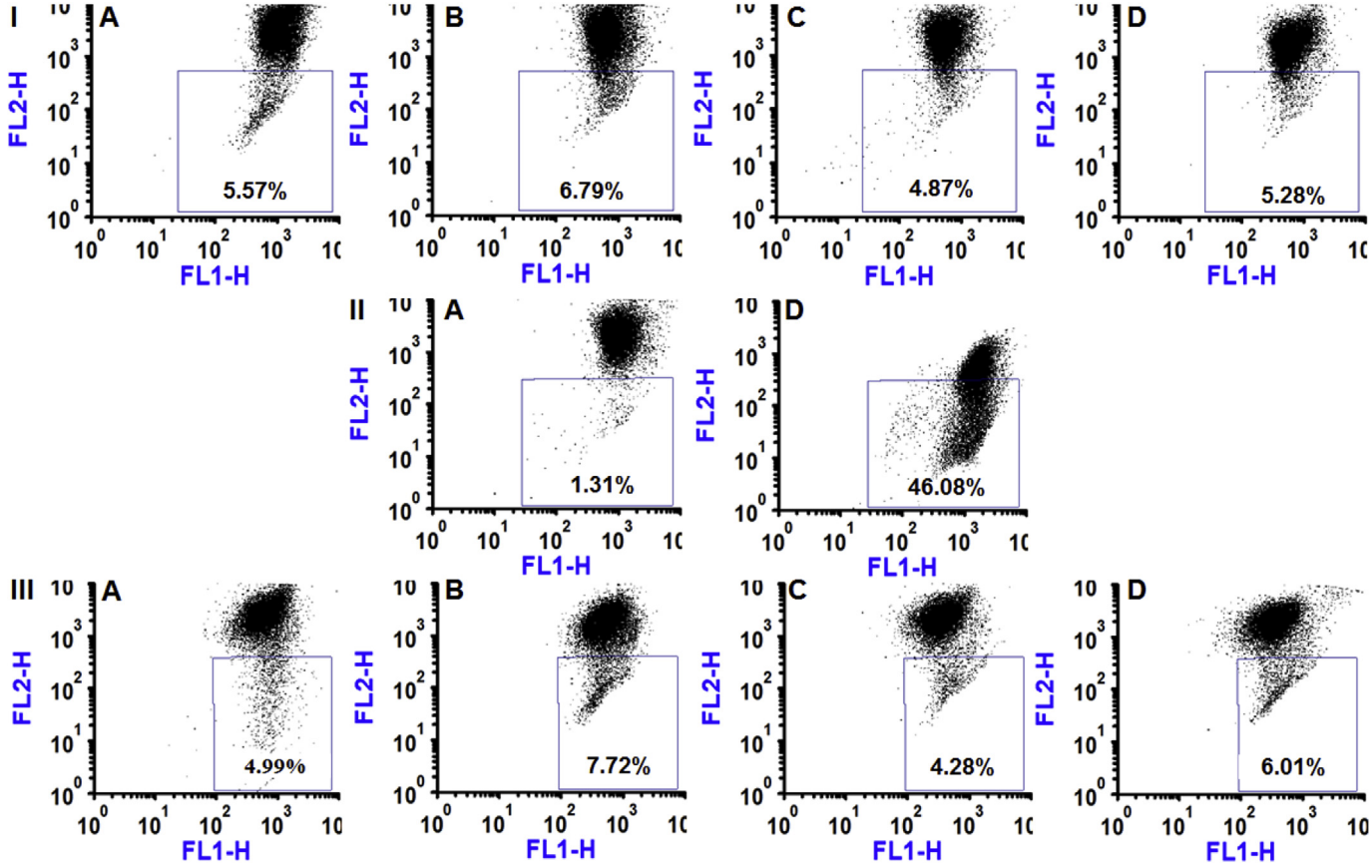

Fig. 7. The change in the inner mitochondrial membrane potential of silk sericin treated (I) A431, (II) MCF-7, and (III) SAS cells was assessed using the JC-1 assay kit. Where (A) control, (B) BMS, (C) PRS, and (D) AAS treated cancer cells.

and results in the formation of superoxide anions (O_2^-) (Hodnick et al., 1988). O_2^- induce redox imbalance in cancer cells that leads to oxidative stress mediated apoptotic cell death.

Silk sericin (SS), a glycoprotein known to possess antioxidant activity along with other biological properties (Chlapanidas et al., 2013; Kumar and Mandal, 2017). The antioxidant properties of SS are attributed to the amino acids (hydroxyl group of serine and threonine; electron donating groups of aromatic amino acids) and secondary metabolites (polyphenols and flavonoids) associated with it (Kumar and Mandal, 2017). Their composition varies with the source of sericin as well as with extraction methods (Kumar and Mandal, 2017). The amino acids of SS and associated secondary metabolites, which contribute to antioxidant activity, may act as pro-oxidants, elevate intracellular ROS production and lead to oxidative stress-mediated apoptotic death in cancer cells. Therefore, in the present study, the pro-oxidant activity of SS isolated from the *B. mori* (BM), *A. assamensis* (AA) and *P. ricini* (PR) was investigated using human squamous carcinoma (A431), breast adenocarcinoma (MCF-7) and tongue carcinoma (SAS).

The properties of SS alters with respect to the extraction methods (Kumar and Mandal, 2017). In our previous study, alkali-degraded BM sericin (BMS), AA sericin (AAS) and PR sericin (PRS) showed antioxidant activity and protected the murine fibroblast (L929) cells against H_2O_2 induced oxidative stress (Kumar and Mandal, 2017). Alkali-degraded BMS, AAS, and PRS were selected in the current study to evaluate the anticancer activity by inducing oxidative stress. Cytotoxicity of SS evaluated by MTT and LDH assay revealed that 4 mg/mL of SS inhibited > 50% cancer cells proliferation (Fig. 1) without disrupting their cellular membrane integrity (Fig. 2 and Fig. S1); whereas, ~30% decrease in cell viability was observed in SS treated normal cells. Cancer cells exhibit higher endogenous ROS levels than normal cells and disruption of their ROS levels induces cell death (Zhu et al., 2014). At low concentrations, SS acts as an antioxidant and maintain intracellular ROS levels (Terada et al., 2002). Whereas, at high concentration, the mixture of polyphenols and flavonoids associated with SS might have interacted with O_2 and induced the formation of superoxide anion (O_2^-) that elevated ROS levels and leads to the death of the cancer cells (Hodnick et al., 1988; Park and M Pezzuto, 2012). Intracellular ROS levels determined using DCFH-DA also showed that SS (4 mg/mL) treatment enhanced ROS production in both cancer cells (Fig. 3) and normal cells (Fig. S2).

Endogenous antioxidants protect the cells from oxidative damage by scavenging the elevated ROS levels (Birben et al., 2012). O_2^- is a primary ROS produced by the aerobic organisms (Brand, 2010). SOD present in mitochondria and cytoplasm convert O_2^- to H_2O_2 and O_2 in the presence of metal ion co-factors such as Zinc (Zn), copper (Cu) and manganese (Mn) (Gough and Cotter, 2011). H_2O_2 is converted to H_2O and O_2 by the catalytic activity of CAT (Gough and Cotter, 2011). AAS treated cancer cells showed a significant reduction of SOD activity, whereas, significantly high SOD activity was seen in BMS and PRS treated A431 cells (Fig. 4). Co-factors of the SOD play an important role in the stability of enzyme and its activity (Al-Naama et al., 2015; Chan et al., 1999), however, depletion of co-factors decreases the SODs activity (Al-Naama et al., 2015). Hydroxyl groups of serine and threonine of SS chelate the trace elements such as copper and iron (Kato et al., 1998). Serine and threonine of AAS might have chelated the copper ion of SOD and reduced its activity. Whereas in BMS and PRS treated A431 cells, SOD activity was upregulated to reduce the elevated levels of ROS. Amino acid and secondary metabolites present in BMS, PRS, and AAS were unable to disturb the co-factor of CAT due to which there was no loss in its activity (Fig. 5).

Oxidative stress induced by redox imbalance activates the tumor suppressor genes and inhibits the cyclin-dependent kinases (CDK) that

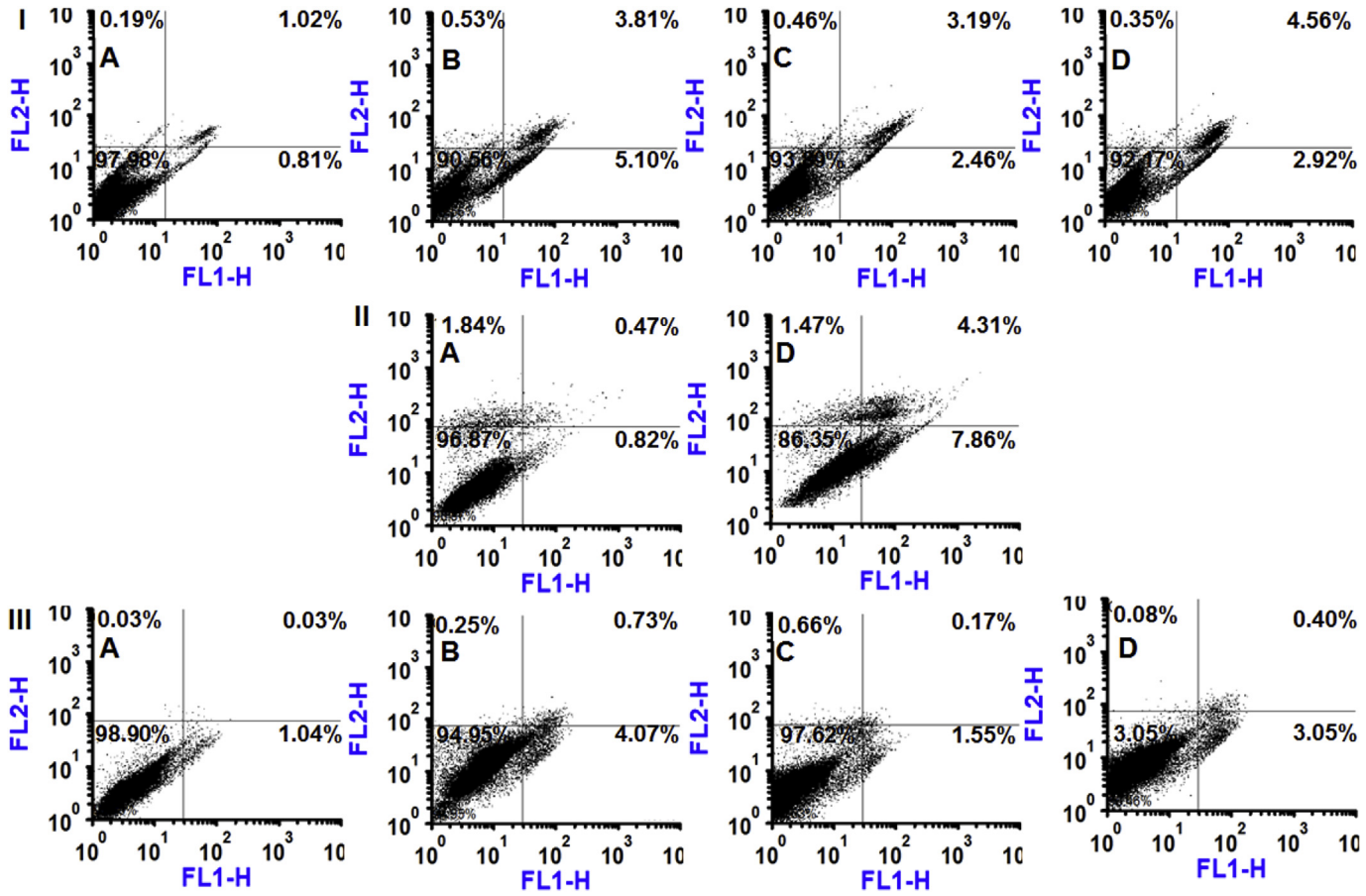

Fig. 8. Apoptotic death induced by oxidative stress-mediated with silk sericin treatment in (I) A431, (II) MCF-7, and (III) SAS cells was assessed using Annexin V/PI. Where (A) control, (B) BMS, (C) PRS, and (D) AAS treated cells.

result in cell cycle arrest at Go/G1 or G2/M phases (Habold et al., 2008). Redox imbalance induced by SS in cancer cells might have activated tumor suppressor genes and inhibited the CDK that resulted in cell cycle arrest at G1 phases followed by cell death (sub-G1 phase). These changes resulted in an increased and decreased percentage of gated cell population at the sub-G1 phase and G1 phase, respectively (Fig. 6). Whereas, redox imbalance induced by SS in HaCaT cells resulted in cell cycle arrest at G1 phases, however, they did not enter into death phase (sub-G1) (Fig. S3). Redox imbalance also destabilizes the inner mitochondrial potential of cells and initiates apoptosis by releasing the cytochrome *c* (cyt *c*) into the cytoplasm (Li et al., 1999). AAS treated A431, SAS, and MCF-7 cells showed redox imbalance and low SOD's activity. These changes caused the depletion of inner mitochondrial membrane potential in MCF-7, whereas, the inner mitochondrial membrane potential was retained in A431 and SAS cells (Fig. 7). Elevation of ROS levels, cell cycle arrest and change in inner mitochondrial membrane potential (MCF-7 cells) occurred by the redox imbalance after treating with SS triggered the apoptosis in cancer (A431, SAS, and MCF-7) cells (Fig. 8).

The p53 (tumor suppressor) gene is an important regulator of apoptosis (Wolff et al., 2008). p53 proteins translocate to mitochondria, where it interacts with the Bax and causes depletion of mitochondrial membrane potential that release cyt *c*, activates caspase and results in apoptotic cell death (Chipuk et al., 2004). p53 also induces apoptosis by downregulating the expression of the Bcl-2 protein (Hemann and Lowe, 2006). Redox imbalance induced in cancer cells activates the p53 gene and protein expression that leads to apoptosis (Habold et al., 2008). Redox imbalance induced in cancer cells by SS treatment upregulated the expression of the p53 gene in A431 and SAS (Fig. 9 I and IIIA), whereas in AAS treated MCF-7, its expression remained unchanged (Fig. 9 IIA). Polyphenols extracted from strawberry induced apoptosis in MCF-7 cells by activating p73 (tumor suppressor gene, structurally similar to p53 protein) gene without upregulating p53 (Somasagara et al., 2012). Redox imbalance induced in MCF-7 due to AAS treatment might have upregulated p73 gene expression and initiate apoptotic pathway. In BMS and PRS treated A431 cells, p53 gene upregulated the expression of cyt *c*, Bax, caspase-3, caspase-8, and caspase-9 gene (Fig. 9 IB, C, D, E, and F). However, the p53 gene activated by AAS treatment induced the upregulation of caspase-9 and downregulation of Bcl-2 without altering the expression of cyt *c*, Bax, caspase-3, and 9 genes (Fig. 9 I). Activated p53 gene induces the upregulation of cyt *c* and caspase-3, caspase-8 and caspase-9 gene expression in SAS (Fig. 9 IIIB, C, D, and E). While BMS and PRS treated SAS cells tried to overcome the oxidative stress-mediated cell death through upregulated Bcl-2 gene expression (Fig. 9 IIIG). MCF-7 cells are negative to caspase-3 activity (Liang et al., 2001), we have studied the effect of AAS induced oxidative on the expression of caspase-3, caspase-8, caspase-9 along with cyt *c*, Bax, and Bcl-2 expression. AAS mediated oxidative stress induced the upregulation of cyt *c*, Bax, and caspase-9, downregulation of Bcl-2 and caspase-3 expression was undetermined (Fig. 9 II).

In addition to tumor suppressor gene activation, elevated levels of ROS also activates the cJun-terminal kinases (JNKs), which catalyzes the downregulation of Bcl-2 and Bcl-XL (Cadenas, 2004; Habold et al., 2008). p53 protein activation leads to the downregulation of Bcl-2 expression and upregulates the Bax expression that causes the cell senescence (Adams and Cory, 1998). AAS treatment upregulated Bax expression in A431 cells and downregulated Bcl-2 expression in MCF-7 and SAS cells (Fig. 10). Redox imbalance induced in cancer cells by AAS treatment might have activated the JNK pathway along with p53 protein expression that upregulated Bax protein expression in A431 cells

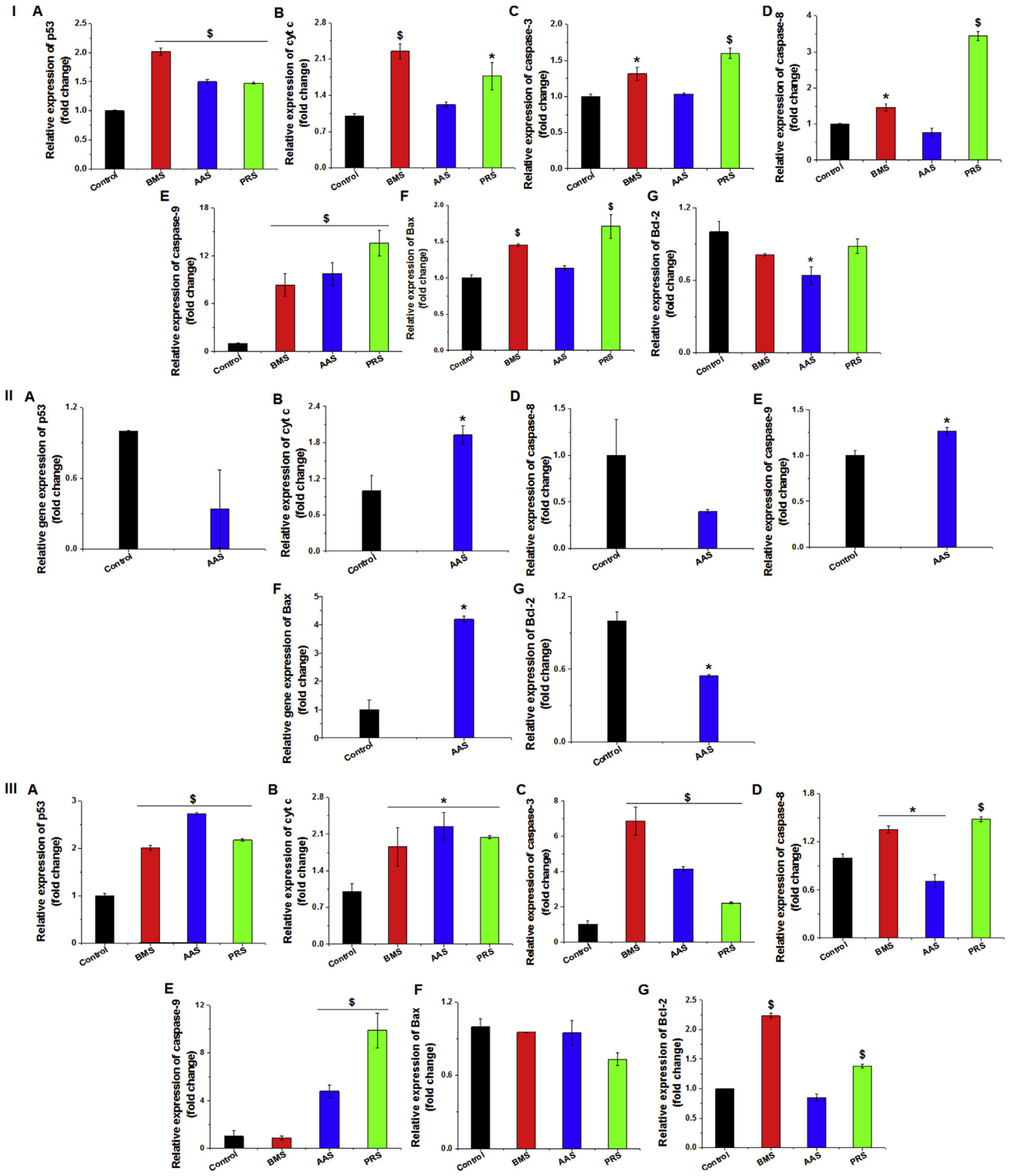

Fig. 9. The effect of silk sericin on the relative expression of (A) p53, (B) cyt *c*, (C) caspase-3, (D) caspase-8, (E) caspase-9, (F) Bax, and (G) Bcl-2 genes in (I) A431, (II) MCF-7, and (III) SAS cells was assessed using RT-PCR. ($p ≤ 0.001 and *p ≤ 0.01 in comparison with control).

and downregulated the Bcl-2 expression in MCF-7 and SAS cancer cells. Upregulation of Bax or downregulation of Bcl-2 expression inhibits the dimerization of Bax/Bcl-2 and leads to the death of cancer cells (Adams and Cory, 1998). Amino acids of SS and mixture of polyphenols and flavonoids associated with it induced oxidative stress in cancer cells and leads to cell cycle arrest, depleted mitochondrial membrane potential (MCF-7), upregulated the tumor suppressor gene and downregulated the anti-apoptotic gene expression, which in turn inhibited the proliferation of cancer cells.

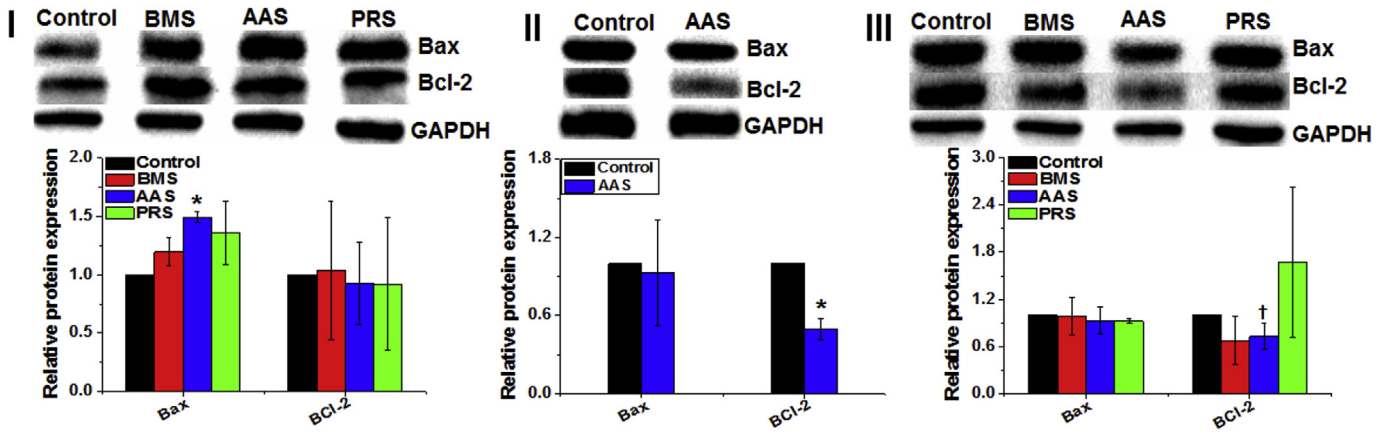

Fig. 10. The effect of silk sericin on the expression of Bax and Bcl-2 protein in (I) A431, (II) MCF-7, and (III) SAS cells was determined by western blotting. (*p ≤ 0.01, and †p ≤ 0.05 in comparison with control).

5. Conclusion

In conclusion, silk sericin extracted from the cocoons of *A. assamensis, B. mori,* and *P. ricini* suppressed the growth of human cancer cells by inducing the redox imbalance. SS peptides generated (amino acid composition) and a mixture of polyphenols and flavonoids obtained during alkali-degradation played a critical role in their anti-tumor activity. The combination of protein and secondary metabolites killed the cancer cells with a marginal effect on normal cells. AAS efficiently suppressed the growth of all three different types of human cancer cells by suppressing their endogenous SOD activity and elevating ROS production. Thus, suppressing the cancer growth with marginal effect towards normal cells by SS treatment would be important for its clinical relevance. However, *in vivo* assessment of SS is necessary to validate it as a potential anti-tumor molecule.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgment

B.B.M thankfully acknowledges the funding support from the Department of Biotechnology (DBT) and the Department of Science & Technology (DST), Govt. of India. The assistance received from the Department of Biosciences and Bioengineering (BSBE), IIT Guwahati is thankfully acknowledged. We also acknowledge Dr. Ajaikumar B. Kunnumakkara, IIT Guwahati, and Dr. Pradeep Kumar Sharma, CSIR-IITR, Lucknow for providing SAS and MCF-10 cell lines, respectively.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2018.10.063.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.10.063.

References

Adams, J.M., Cory, S., 1998. The Bcl-2 protein family: arbiters of cell survival. Science 281, 1322–1326.
Al-Naama, L.M., Hassan, M.a.K., Mehdi, J.K., 2015. Association of erythrocytes anti-oxidant enzymes and their cofactors with markers of oxidative stress in patients with sickle cell anemia. Qatar Med. J. 2015, 14.
Birben, E., Sahiner, U.M., Sackesen, C., Erzurum, S., Kalayci, O., 2012. Oxidative stress and antioxidant defense. World Allergy Org. J. 5, 9–19.
Brand, M.D., 2010. The sites and topology of mitochondrial superoxide production. Exp. Gerontol. 45, 466–472.
Butkhup, L., Jeenphakdee, M., Jorjong, S., Samappito, S., Samappito, W., Butimal, J., 2012. Phenolic composition and antioxidant activity of Thai and Eri silk sericins. Food Sci. Biotech. 21, 389–398.
Cadenas, E., 2004. Mitochondrial free radical production and cell signaling. Mol. Aspect. Med. 25, 17–26.
Cairns, R.A., Harris, I.S., Mak, T.W., 2011. Regulation of cancer cell metabolism. Nat. Rev. Canc. 11, 85–95.
Chan, A.C., Chow, C.K., Chiu, D., 1999. Interaction of antioxidants and their implication in genetic anemia. PSEBM (Proc. Soc. Exp. Biol. Med.) 222, 274–282.
Chipuk, J.E., Kuwana, T., Bouchier-Hayes, L., Droin, N.M., Newmeyer, D.D., Schuler, M., Green, D.R., 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science 303, 1010–1014.
Chlapanidas, T., Faragò, S., Lucconi, G., Perteghella, S., Galuzzi, M., Mantelli, M., Avanzini, M.A., Tosca, M.C., Marazzi, M., Vigo, D., 2013. Sericins exhibit ROS-scavenging, anti-tyrosinase, anti-elastase, and in vitro immunomodulatory activities. Int. J. Biol. Macromol. 58, 47–56.
Cotelle, N., 2001. Role of flavonoids in oxidative stress. Curr. Top. Med. Chem. 1, 569–590.
Dai, J., Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313–7352.
Dash, R., Ghosh, S.K., Kaplan, D.L., Kundu, S., 2007. Purification and biochemical characterization of a 70 kDa sericin from tropical tasar silkworm, Antheraea mylitta. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 147, 129–134.
Fang, J., Seki, T., Maeda, H., 2009. Therapeutic strategies by modulating oxygen stress in cancer and inflammation. Adv. Drug Deliv. Rev. 61, 290–302.
Garg, T.K., Chang, J.Y., 2004. 15-deoxy-delta 12, 14-Prostaglandin J2 prevents reactive oxygen species generation and mitochondrial membrane depolarization induced by oxidative stress. BMC Pharmacol. 4, 6.
Glasauer, A., Chandel, N.S., 2014. Targeting antioxidants for cancer therapy. Biochem. Pharmacol. 92, 90–101.
Gorrini, C., Harris, I.S., Mak, T.W., 2013. Modulation of oxidative stress as an anticancer strategy. Nat. Rev. Drug Discov. 12, 931–947.
Gough, D., Cotter, T., 2011. Hydrogen peroxide: a Jekyll and Hyde signalling molecule. Cell Death Dis. 2, e213.
Habold, C., Poehlmann, A., Bajbouj, K., Hartig, R., Korkmaz, K.S., Roessner, A., Schneider-Stock, R., 2008. Trichostatin A causes p53 to switch oxidative-damaged colorectal cancer cells from cell cycle arrest into apoptosis. J. Cell Mol. Med. 12, 607–621.
Haigis, M.C., Yankner, B.A., 2010. The aging stress response. Mol. Cell. 40, 333–344.
Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674.
Hemann, M.T., Lowe, S.W., 2006. The p53-Bcl-2 connection. Cell Death Differ. 13, 1256–1259.
Hodnick, W.F., MllosavljeviĆ, E.B., Nelson, J.H., Pardini, R.S., 1988. Electrochemistry of flavonoids: relationships between redox potentials, inhibition of mitochondrial respiration, and production of oxygen radicals by flavonoids. Biochem. Pharmacol. 37, 2607–2611.
Kaewkorn, W., Limpeanchob, N., Tiyaboonchai, W., Pongcharoen, S., Sutheerawattananonda, M., 2012. Effects of silk sericin on the proliferation and apoptosis of colon cancer cells. Biol. Res. 45, 45–50.
Kato, N., Sato, S., Yamanaka, A., Yamada, H., FUWA, N., NOMURA, M., 1998. Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. Biosci. Biotechnol. Biochem. 62, 145–147.
Kubatka, P., Kapinová, A., Kello, M., Kruzliak, P., Kajo, K., Výbohová, D., Mahmood, S., Murin, R., Viera, T., Mojžiš, J., 2016. Fruit peel polyphenols demonstrate substantial anti-tumour effects in the model of breast cancer. Eur. J. Nutr. 55, 955–965.
Kumar, J.P., Bhardwaj, N., Mandal, B.B., 2016. Cross-linked silk sericin–gelatin 2D and 3D matrices for prospective tissue engineering applications. RSC Adv. 6,

105125–105136.
Kumar, J.P., Konwarh, R., Kumar, M., Gangrade, A., Mandal, B.B., 2017. Potential nanomedicine applications of multifunctional carbon nanoparticles developed using green Technology. ACS Sustain. Chem. Eng. 6, 1235–1245.
Kumar, J.P., Mandal, B.B., 2017. Antioxidant potential of mulberry and non-mulberry silk sericin and its implications in biomedicine. Free Radic. Biol. Med. 108, 803–818.
Lee, W.-L., Huang, J.-Y., Shyur, L.-F., 2013. Phytoagents for cancer management: regulation of nucleic acid oxidation, ROS, and related mechanisms. Oxid. Med. Cell. Longevity 2013, 925804.
León-González, A.J., Auger, C., Schini-Kerth, V.B., 2015. Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. Biochem. Pharmacol. 98, 371–380.
Li, P.F., Dietz, R., Von Harsdorf, R., 1999. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J. 18, 6027–6036.
Liang, Y., Yan, C., Schor, N.F., 2001. Apoptosis in the absence of caspase 3. Oncogene 20, 6570–6578.
McCord, J.M., Fridovich, I., 1988. Superoxide dismutase: the first twenty years (1968–1988). Free Radic. Biol. Med. 5, 363–369.
Mink, P.J., Scrafford, C.G., Barraj, L.M., Harnack, L., Hong, C.-P., Nettleton, J.A., Jacobs, D.R., 2007. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. Am. J. Clin. Nutr. 85, 895–909.
Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., Levine, M., 2003. Vitamin C as an antioxidant: evaluation of its role in disease prevention. J. Am. Coll. Nutr. 22, 18–35.
Palanivel, K., Kanimozhi, V., Kadalmani, B., Akbarsha, M.A., 2014. Verrucarin a induces apoptosis through ROS-mediated EGFR/MAPK/Akt signaling pathways in MDA-MB-231 breast cancer cells. J. Cell. Biochem. 115, 2022–2032.
Paravicini, T.M., Touyz, R.M., 2006. Redox signaling in hypertension. Cardiovasc. Res. 71, 247–258.
Park, E.-J., M Pezzuto, J., 2012. Flavonoids in cancer prevention. Anti Cancer Agents Med. Chem. 12, 836–851.
Pearson, P., Lewis, S.A., Britton, J., Young, I.S., Fogarty, A., 2006. The pro-oxidant activity of high-dose vitamin E supplements in vivo. BioDrugs 20, 271–273.
Pietta, P.-G., 2000. Flavonoids as antioxidants. J. Nat. Prod. 63, 1035–1042.
Shukla, V., Mishra, S.K., Pant, H.C., 2011. Oxidative stress in neurodegeneration. Advances in pharmacological sciences 2011, 572634.
Somasagara, R.R., Hegde, M., Chiruvella, K.K., Musini, A., Choudhary, B., Raghavan, S.C., 2012. Extracts of strawberry fruits induce intrinsic pathway of apoptosis in breast cancer cells and inhibits tumor progression in mice. PLoS One 7, e47021.
Tadić, V.M., Dobrić, S., Marković, G.M., Đorđević, S.M., Arsić, I.A., Menković, N.a.R., Stević, T., 2008. Anti-inflammatory, gastroprotective, free-radical-scavenging, and antimicrobial activities of hawthorn berries ethanol extract. J. Agric. Food Chem. 56, 7700–7709.
Terada, S., Nishimura, T., Sasaki, M., Yamada, H., Miki, M., 2002. Sericin, a protein derived from silkworms, accelerates the proliferation of several mammalian cell lines including a hybridoma. Cytotechnology 40, 3–12.
Traber, M.G., Atkinson, J., 2007. Vitamin E, antioxidant and nothing more. Free Radical Biol. Med. 43, 4–15.
Trachootham, D., Alexandre, J., Huang, P., 2009. Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. 8, 579–591.
Weinberg, F., Chandel, N.S., 2009. Reactive oxygen species-dependent signaling regulates cancer. Cell. Mol. Life Sci. 66, 3663.
Weinberg, F., Hamanaka, R., Wheaton, W.W., Weinberg, S., Joseph, J., Lopez, M., Kalyanaraman, B., Mutlu, G.M., Budinger, G.S., Chandel, N.S., 2010. Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. Proc. Natl. Acad. Sci. Unit. States Am. 107, 8788–8793.
Wolff, S., Erster, S., Palacios, G., Moll, U.M., 2008. p53's mitochondrial translocation and MOMP action is independent of Puma and Bax and severely disrupts mitochondrial membrane integrity. Cell Res. 18, 733–744.
Zhaorigetu, S., Yanaka, N., Sasaki, M., Watanabe, H., Kato, N., 2003. Inhibitory effects of silk protein, sericin on UVB-induced acute damage and tumor promotion by reducing oxidative stress in the skin of hairless mouse. J. Photochem. Photobiol. B Biol. 71, 11–17.
Zhu, C., Hu, W., Wu, H., Hu, X., 2014. No evident dose-response relationship between cellular ROS level and its cytotoxicity–a paradoxical issue in ROS-based cancer therapy. Sci. Rep. 4.