EJGO European Journal of Gynaecological Oncology

Pterostilbene induces apoptosis through caspase activation in ovarian cancer cells

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Summary

Aim: Pterostilbene, an analog of resveratrol increasing bioavailability has shown to offer antioxidant and anticancer properties *in vitro* and *in vivo*. Dietary compounds with anti-oxidant properties have been shown to gain importance due to therapeutic applications. In addition, compounds with higher bioavailability levels show great interest in present scenario. Thus, the present study aimed at investigating the cytotoxic role of pterostilbene and its mechanism of cell death in ovarian cancer cells line. *Materials and Methods:* The effect of pterostilbene was determined on SKOV-3 cells, by cytotoxicity assays, oxidative stress levels, $[Ca^{2+}]i$ levels, mitochondrial depolarization, cell cycle analysis and caspase 3, 8, and 9 activities. *Results:* The study revealed that pterostilbene offered cytotoxic effect at a concentration of IC_{50}-55 uM. Further, pterostilbene induced reactive oxygen species (ROS) mediated intrinsic pathway of apoptosis through enhancing oxidative stress, $[Ca^{2+}]i$ levels, mitochondrial depolarization, Sub G1 accumulation, and activation of caspase 3 and 9. *Conclusion:* The study demonstrates for the first time the cytotoxic potential of pterostilbene against ovarian cancer cells.

Key words: Anticancer; ROS; Oxidative stress; Caspase.

Introduction

Pterostilbene is a natural dietary compound present in blueberries. It is a methylated analog of resveratrol with increased bioavailability and lipophilicity compared to that of resveratrol [1]. So, recent research has been into much focus on this compound. Pterostilbene is an excellent antioxidant compound [2], which has been reported for various pharmacological properties like antifungal, anti-inflammatory, antidiabetic and anti-cancer properties [3-5]. The anti-cancer role of pterostilbene has been reported in different cancers such as breast, gastric, prostate, hepatic etc., by both *in vitro* and *in vivo* studies [6-12]. The mechanism through which pterostilbene exerts anti-cancer potential has been reported to include both apoptosis and autophagy [13, 14]. Although it has been studied for its anti-cancer property in different cancers, its role in ovarian cancer has not been explored.

Thus the present study was designed with the aim to elucidate the mechanism of action of pterostilbene against ovarian cancer cells. The study evaluated its cytotoxic potential and analyses its mechanism of cell death through oxidative stress parameters, ROS generation, mitochondrial membrane potential, caspase activation, and cell cycle analysis.

Materials and Methods

Chemicals

Pterostilbene (> 97%), Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum, trypsin, antibiotics (penicillin, streptomycin), Fura-2 AM, ortho-pthalaldehyde (OPT), 3, 3'- dihexyloxacarbocyanine iodide (DiOC6), and carbonyl cyanide 4-(trifluoromethoxy phenylhydrazone (CCCP).

Cell culture and conditions

SKOV-3 (human ovarian adenocarcinoma) cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics 100 units/ml penicillin, and 30 µg/ml streptomycin at 37ºC in a CO_2 incubator with 5% CO_2. Cells at 80% confluency were used for all the assays.

Cytotoxicity assays

3-[4, 5-dimethylthiazol-2-yl] - 2, 5-diphenyltetrazolium bromide (MTT) assays were included. Cell viability was determined by 3-[4, 5-dimethylthiazol-2-yl] - 2, 5-diphenyltetrazolium bromide (MTT) assay [15]. Cells were trypsinised and seeded at a density of 10,000 cells/well. Following attachment, the cells were with different concentrations of pterostilbene (20, 40, 60, 80, 100, 120 µM) for 24 and 48 hours. After the incubation period, 20 µl of MTT (five mg/ml) was added to each well and incubated for further for five hours. At the end of the treatment period, DMSO (dimethyl sulphoxide) was added to dissolve the purple formazon crystals and read at 570 nm using a microplate reader. The percentage cell viability was calculated by taking viability of the control cells as 100%.

Lactate dehydrogenase (LDH) release

The lactate dehydrogenase assay was determined as described in the Cayman's LDH Cytotoxicity Assay Kit according to the manufacturer's instructions. The principle of the kit measures the LDH activity in the supernatant using a coupled two-step reaction (First Step - LDH catalyzes the reduction of NAD^+ to NADH and H^+ by oxidation of lactate to pyruvate. Second step-

Revised manuscript accepted for publication April 2, 2015

Diaphorase uses the newly-formed NADH and H^+ to catalyze the reduction of a tetrazolium salt (INT) to highly-colored formazon). The absorbance was measured spectrophometrically at 520 nm.

Nitric oxide assay

The amount of nitrite released was measured using Griess reagent at 540 nm as described by Stuehr and Marletta [16].

Measurement of oxidative stress

Reactive oxygen species generation

Reactive oxygen species was determined using fluorescent probe DCF-DA [17]. The cells were seeded at a density of $2x10^5$ cells/well. After attachment, the cells were incubated with 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) for 30 minutes. Following this, pterostilbene was added to the cells and incubated at 24 and 48 hours. At the end of the treatment period, the cells were washed with PBS and centrifuged at 1,500 rpm for 15 minutes. The supernatant was discarded and resuspended in PBS and read in a spectrofluorimeter (excitation wavelength 480 nm, emission wavelength 520 nm). The entire experiment was carried out in dark conditions. The results were expressed as % DCF fluorescence.

Total antioxidant capacity (TAC)

Total antioxidant capacity **(**TAC) in the cells treated with pterostilbene was determined by the method described by Erel 2004 [18]. After an appropriate treatment period, the cells were sonicated and the cell lysate was used for the assay. The principle involves measurement of hydroxyl radical formation between the antioxidants in the sample against free radicals. The results are expressed as mmol of Trolox equiv./L.

Cellular uptake assay

The cells were incubated with different concentrations of pterostilbene for 30 minutes and for one to five hours in 5% CO_2. After the treatment period, the cells were centrifuged at 1,500 rpm for 10 minutes. The pellet was washed with PBS and re-suspended in one ml of buffer (10 mM-Tris-HCl, 1mMEDTA, 1mM MgCl 2, pH 7.5). The cell suspension was probe-sonicated and centrifuged to remove the cytosolic fraction. To the fraction menthol was added with the final concentration of 40%. To this, ethyl acetate was added in equal amounts and the pooled ethyl acetate fractions were evaporated [19]. As the final step reconstitution is done with methanol and analyzed by high performance liquid chromatography (HPLC) [20].

Lipid peroxidation

Lipid peroxidation was determined as described by Okhawa *et al.*, 1979 [21]. To the cell extract 8% SDS; 0.8% TBA in 20% acetic acid was added and the final volume was made up to four ml with distilled water and heated for 60 minutes at 90°C. After cooling to room temperature, two ml of butanol/ pyridine mixture was added and shaken vigorously. Centrifuged at 4,000 rpm for ten min and the organic layer were read at 532 nm. The lipid peroxide content was expressed as n moles of TBA reactants/mg of protein.

Measurement of intracellular calcium

The cells at a density of $1x10^5$ cells/well were treated with pterostilbene and incubated for 24 and 48 hours. Following the treatment period, Fura - 2 AM was added at 37°C in CO_2 incubator for 30 minutes. The intracellular calcium levels were monitored at excitation wavelength: 510 nm and emission wavelength between 340 and 380 nm using fluorescence spectrophotometer. The values are expressed as % relative fluorescence [22].

Mitochondrial membrane potential (Δψm)

The cells were treated with pterostilbene and incubated for 24 and 48 hours for apoptotic induction. After the treatment period, Dioc6 (3) (3, 3'- dihexyloxacarbocyanine iodide) at was added and incubated for one hour. The readings were further recorded in a fluorescence spectrophotometer at excitation wavelength of 488 nm and emission wavelength at 500 nm.

Cell cycle analysis

Cells seeded at a density of 5 × 106 cells/well were treated with Pterostilbene for 24 and 48 hours. Following this the cells were fixed with 70% (ice-cold) ethanol at 4°C overnight. The cells were further washed with PBS and stained with PI/RNase staining solution for 45 minutes. The DNA content was analyzed on FACS Calibur flow cytometer.

Caspase activity determination

The cells were seeded at a density of $4X10^6$ cells/well. After attachment, cells were treated with pterostilbene in the presence/absence of caspase inhibitors (caspase 9 - Z-LEHD-FMK, caspase 8- Z-IETD-FMK, and caspase 3- Z-DEVD-FMK). After treatment, 100 µg of protein was analysed for caspase activities using caspase activity kits. Absorbance was measured at 405 nm.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. All biochemical experiments were performed thrice in triplicates to ensure reproducibility.

Results

Cytotoxicity of pterostilbene against ovarian cancer: MTT assay, LDH, NO, and LPO levels

Dose and time dependent study revealed that pterostilbene caused concentration dependent cell death at 48 hours compared to 24 hours. IC_{50} value was found to be 55 µM, which was a statistically significant ($p < 0.001$) different from control cells (Figure 1A). Further studies of cytotoxicity revealed a time dependent lactate dehydrogenase (LDH) release in cells treated with pterostilbene (Figure 1B). LDH is a cytosolic enzyme released into the cell culture supernatant in response to altered membrane integrity. Thus the measurement serves as an important indicator of the cytotoxicity. LDH release was time dependent with the increase release of 12%, 21%, and 34 % at time point of 12, 24, and 48 hours respectively. Figure 1C shows time dependent nitrite release. The results show that pterostilbene caused the significant nitrite release from 12 hours (< 0.01) with the maximum release observed at 48 hours ($p < 0.001$). Figure 1D shows time dependent lipid peroxide levels during pterostilbene treatment; the results were statistically significant for 12 ($p < 0.01$), 24 ($p < 0.01$), and 48 hours ($p < 0.001$) when compared to control cells.

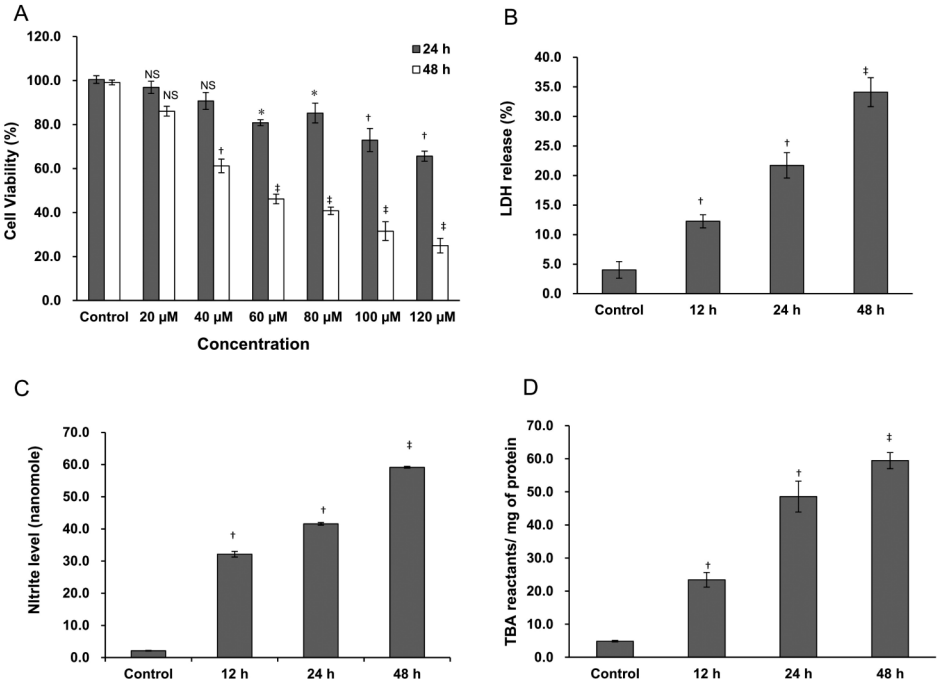

Figure 1. — Pterostilbene inhibits cell proliferation and induces cytotoxic effects in SKOV-3 cells. A) Cells were treated with pterostilbene (20-120 µM) for 24 and 48 hours and IC_{50} values were calculated B) Pterostilbene increases LDH release. LDH release was assessed in cells treated with pterostilbene. Results were expressed as % LDH leakage when compared to control. C) Pterostilbene increases no release. The no release was assessed in the cells treated with pterostilbene at different time points D) Pterostilbene increases lipid peroxide levels. Results were expressed as nanomoles of TBA reactants/mg of protein (results shown as mean ± SEM). *$p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$; NS – non-significant when compared to control.

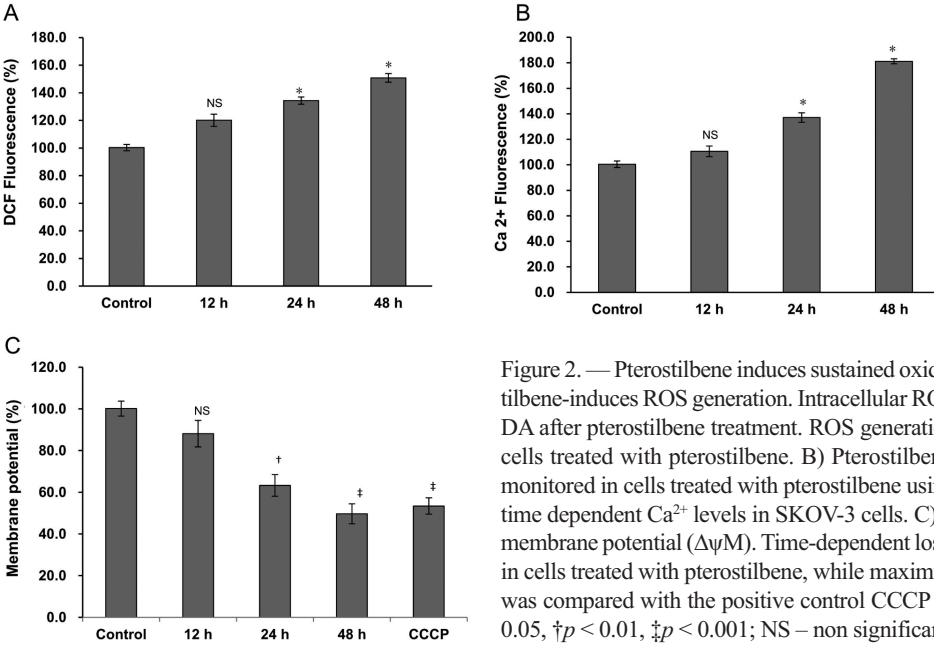

Figure 2. — Pterostilbene induces sustained oxidative stress in SKOV-3 cells A) Pterostilbene-induces ROS generation. Intracellular ROS generation was determined by DCF-DA after pterostilbene treatment. ROS generation was maximum at 45 minutes in the cells treated with pterostilbene. B) Pterostilbene- increased [Ca2+]i. Ca levels were monitored in cells treated with pterostilbene using FURA- 2 AM. Pterostilbene caused time dependent Ca^{2+} levels in SKOV-3 cells. C) Pterostilbene disrupted mitochondrial membrane potential (ΔψM). Time-dependent loss of membrane potential was observed in cells treated with pterostilbene, while maximum was observed at 48 hours. The loss was compared with the positive control CCCP (results shown as mean ± SEM). *$p <$ 0.05, †$p < 0.01$, ‡$p < 0.001$; NS – non significant when compared to control.

Pterostilbene induces reactive oxygen species (ROS) and intracellular calcium levels

Pterostilbene-induced ROS generation was measured by DCF-DA. The results showed that pterostilbene-induced ROS generation at 120%, 134% and 157% in 12, 24, and 48 hours, respectively (Figure 2A). Further results from Ca^{2+} levels also showed that pterostilbene caused significant levels of calcium release with maximum levels up to 180% ($p < 0.001$) when compared to control cells (Figure 2B).

Cellular uptake of pterostilbene in ovarian cancer cells

High performance liquid chromatography (HPLC) analysis of intracellular levels of pterostilbene at different time points showed significant levels of increase from 15 minutes which increased gradually which peaked at 45 minutes

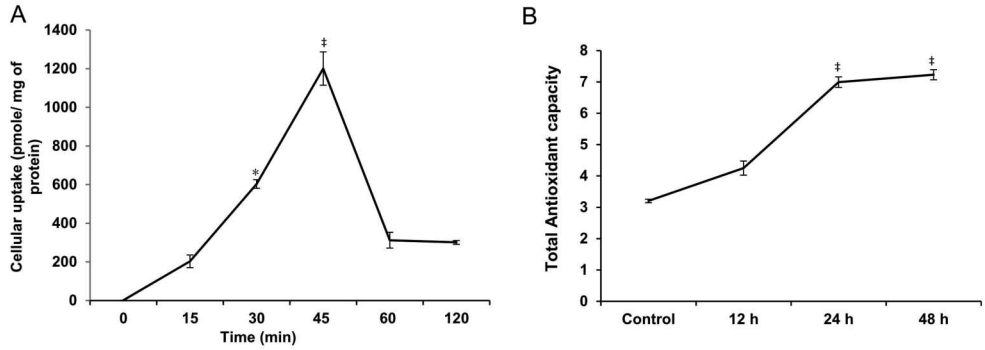

Figure 3. — Pterostilbene enhances antioxidant status A) Cellular uptake of pterostilbene in SKOV-3 cells. Time-dependent study (15, 30, and 45 minutes, and one and two hours) on pterostilbene uptake showed maximum levels at 45 minutes when compared to control cells. B) Pterostilbene-enhanced TAC. Cells treated with pterostilbene at different time points showed time dependent increase in antioxidant levels when compared to control cells (results shown as mean ± SEM). *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$; NS – non-significant when compared to control.

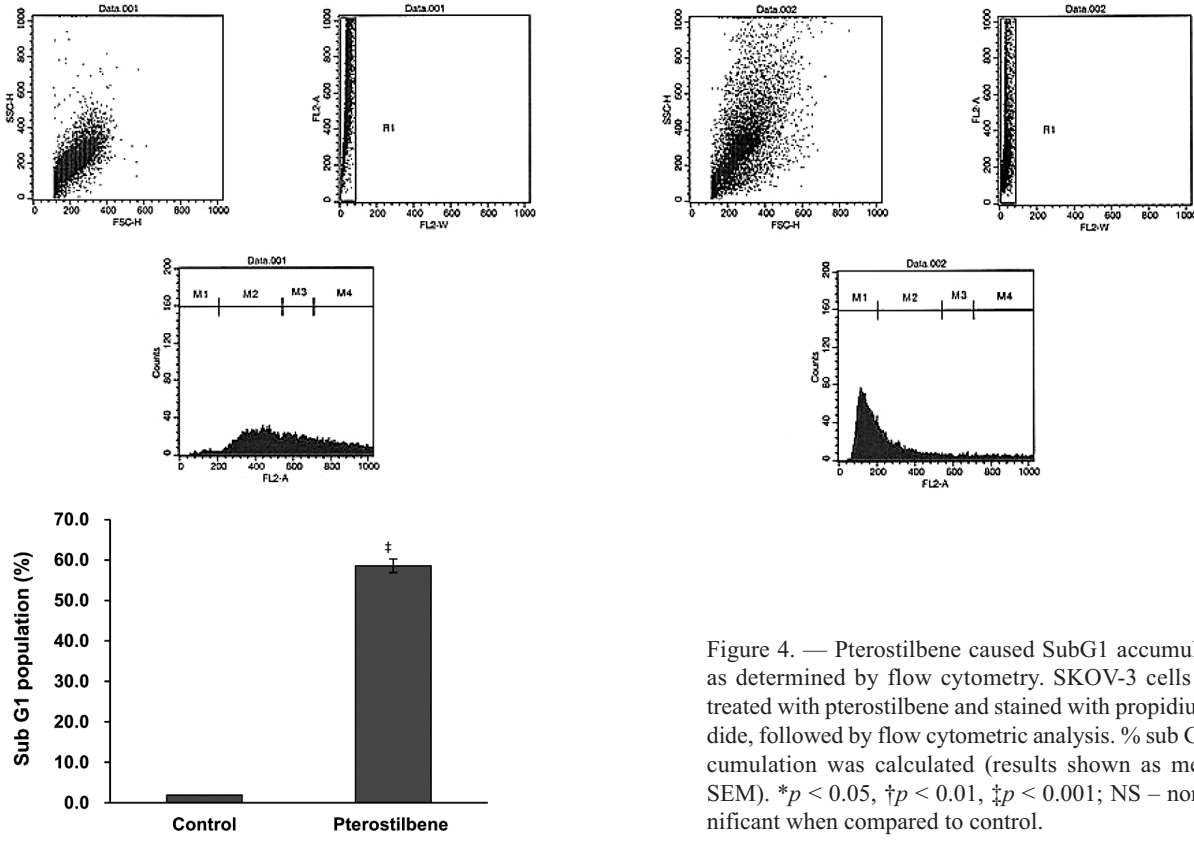

Figure 4. — Pterostilbene caused SubG1 accumulation as determined by flow cytometry. SKOV-3 cells were treated with pterostilbene and stained with propidium iodide, followed by flow cytometric analysis. % sub G1 accumulation was calculated (results shown as mean ± SEM). *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$; NS – non-significant when compared to control.

($p < 0.001$). The levels subsequently dropped at one hour and it was stable until two hours (Figure 3A).

Pterostilbene increased the antioxidant status

The total antioxidant capacity of cell treated with pterostilbene revealed an increase in antioxidant activity in a time dependent manner with maximum effect was observed at 48 hours ($p < 0.001$) (Figure 3B).

Pterostilbene induces apoptosis through the intrinsic pathway of apoptosis

Pterostilbene induced loss of membrane potential in ovarian cancer cells in timely dependent fashion. The results showed a maximum decrease at 48 hours ($p < 0.001$) when compared to control cells; the effect was comparable to that of the positive control (CCCP) (Figure 2C). Cell cycle analysis revealed that pterostilbene induced cell cycle ac-

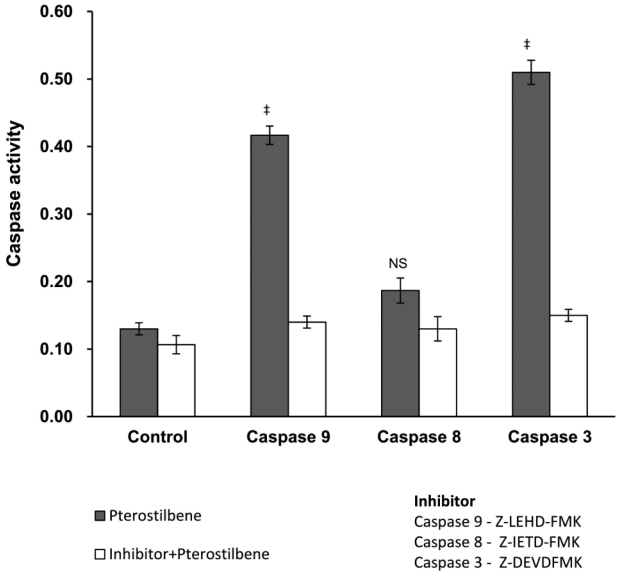

Figure 5. — Pterostilbene-induces caspase activation. Cells were treated with pterostilbene for 48 hours and analysed for caspase (9, 8, and 3 activities). Pterostilbene caused significant induction of caspase 9 and 3 activities when compared to control cells (results shown as mean ± SEM). *$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$; NS – non-significant when compared to control.

cumulation at subG1 phase (Figure 4). The results showed that pterostilbene caused 58% of cells at subG1 phase when compared to control (1.88%). Further, the authors examined the involvement of caspase 9, 8, and 3 activities in pterostilbene-induced cell death (Figure 5). As a positive control, cells were treated with their specific inhibitor and pterostilbene. It was observed that pterostilbene caused a significant increase in activities of caspase 9 and 3 levels ($p < 0.001$); however the levels of caspase 8 was not significantly increased.

Discussion

Ovarian cancer is one of the common and lethal gynaecologic tumours worldwide. Chemoresistance plays major roadblocks for the treatment of ovarian cancer. Reports suggest that compounds which sensitize these cells to cell death could act as a therapeutic strategy [23]. The present study showed the cytotoxic effect of pterostilbene against ovarian cancer at a level of 55 uM. Furthermore, increase in LDH release during pterostilbene treatment reveals that it causes membrane damage and subsequent cell death. These results are in corroboration with previous study conducted by Mena *et al.* [24] where pterostilbene increased LDH levels in A375, A549, HT-29, and MCF7 cancer cell line.

It has been well known that reactive oxygen species mediates cell death mechanisms [25]. The present study evidenced that pterostilbene caused a significant increase in ROS generation in a time dependent manner. However, these increases in ROS, did not affect the antioxidant status; as the present authors observed a consistent increase in antioxidant status. Thus, this level of antioxidant activity could be explained as cellular stress mechanisms evoked to combat the cell death. It is observed in many cases, where the antioxidant enzymes are upregulated during cellular stress responses [26]. The present results are consistent with the previous study conducted by Hasiah *et al.*, [27] where pterostilbene mediated cell death in HepG2 by increasing levels of ROS and antioxidant status. ROS plays a major role in cell death by inducing oxidative stress and thereby destructs the macromolecular structure and induce cell damage [25]. The present authors also observed that ROS levels concomitantly increased oxidative stress markers like nitrite levels and lipid peroxide during pterostilbene treatment.

Alterations in mitochondrial membrane potential and intracellular calcium levels play a key role in the induction of apoptosis [28]. In addition involvement of ROS and intracellular Ca^{2+} levels play a major role in dissipation in mitochondrial membrane potential [29]. The present study results show that pterostilbene resulted in a significant increase in ROS, Ca^{2+} levels and subsequent loss of membrane potential. Reports by Chakraborty *et al.* [30] suggest that pterostilbene caused cell death in prostate cancer cells through ROS induction and mitochondrial membrane depolarization, which are consistent with the present observations. Downstream to these effects the present authors determined that pterostilbene-induced cell death in ovarian cancer cells through sub G1 accumulation, an event which is hallmark to apoptosis. Similar results of prevention of cell cycle regulation at SubG1 phase and induction of apoptosis has been reported by Lin *et al.* [31] in prostate cancer cells. Furthermore, the present authors examined the activity of caspase, which plays a central role in regulating the apoptotic mechanisms. These proteases activate and transducer various cellular targets and thus execute cell death mechanisms [32]. In order to determine the mechanism of cell death, the authors next analyzed the expression levels of caspase during pterostilbene treatment. A significant enhancement in activity of caspase 9 and 3 levels with no significant levels in caspase 7 levels was observed, suggesting that pterostilbene-induced cell death occurs through mitochondrial mediated apoptotic pathway. Similar role of pterostilbene by inducing intrinsic pathway of cell death has been reported earlier [33-35].

The present study demonstrates for the first time that pterostilbene induces apoptosis in ovarian cancer cell line, through ROS generation, mitochondrial depolarization, activation of caspase 9 and 3. Dietary phenols and antioxidants play a major role in cancer prevention. However, compounds with increased bioavailability have

gained much importance. Owing to its higher bioavailability and apoptosis inducing capability, pterostilbene might act as promising dietary intervention in preventing ovarian cancer.

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