# Pterostilbene (3',5'-dimethoxy-resveratrol) exerts potent antitumor effects in HeLa human cervical cancer cells via disruption of mitochondrial membrane potential, apoptosis induction and targeting m-TOR/PI3K/Akt signalling pathway

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## Summary

**Purpose:** To examine the molecular mechanism of action behind the anticancer effects of pterostilbene in HeLa human cervical cancer cells.

**Methods:** MTS assay was used to study the pterostilbene cytotoxic effects, while inverted phase contrast and fluorescence microscopy was used to study the effects of the drug on the cell apoptosis and changes in cell morphology. Flow cytometry was used to study changes in mitochondrial membrane potential (MMP), while immunoblotting assay was used to demonstrate its effects on m-TOR/PI3K/Akt protein signalling pathway.

**Results:** Pterostilbene induced potent, dose-dependent and time-dependent cytotoxic effects in HeLa cancer cells exhibiting $IC_{50}$ of 101.2 μM and 65.9 μM at 24 and 48 hrs time intervals, respectively. As compared to the untreated control cells which revealed normal cell morphology, pterostilbene-treated cells exhibited significant cellular shrinkage which increased with increasing doses of the drug. Untreated con-

trol cells showed complete green fluorescence corresponding to absence of apoptosis. However, pterostilbene-treated cells with 25, 100 and 200 μM showed increasing emission of red/orange fluorescence corresponding to apoptotic induction. Pterostilbene-treated cells showed evident signs of DNA ladder formation and the effect increased with increasing concentrations of the drug. The percentage of cells with depolarized mitochondria (loss of MMP) increased from 2.3% in the control group to 24.5, 43.2 and 65.8% in cells treated with 0, 25, 100 and 200 μM concentration of pterostilbene, respectively.

**Conclusion:** Pterostilbene exerts potent anticancer effects in HeLa human cervical cancer cells via disruption of MMP, apoptosis induction and targeting m-TOR/PI3K/Akt signalling pathway.

**Key words:** apoptosis, cervical cancer, chromatin condensation, fluorescence microscopy, pterostilbene

## Introduction

Cervical cancer is one among the most common malignancies affecting the genital tract of females, and ranks second in incidence after breast cancer. Every year, about half a million new cervical cancer cases are reported globally. This cancer is more prevalent in developing countries as compared to developed countries [1]. Cervical cancer remains the most prevalent cause of cancer-related deaths in low-income countries. In China the prevalence and mortality have shown lately an impressive increase [2]. Several contributing factors have been identified which lead to cervical cancer develop-

ment in women, including human papillomavirus infection, which alone accounts for around 80% of the cervical cancer cases. Other risk factors include compromised immune system, smoking, contraceptive, etc [3,4]. Currently available treatments of this disease include surgical resection, radiotherapy, chemotherapy or a combination of these three. However, severe side-effects have been reported with the use of these treatment modalities, coupled with the fact that one-third of patients will fail and will develop advanced or recurrent disease making its treatment more cumbersome [5,6]. Keeping this in mind, there is an urgent need for effective, novel and much safer treatment for this malignancy. Several anticancer drugs are known which selectively target cancer cells without causing too much damage to the normal cells. These naturally occurring compounds exert their effects via a unique process known as apoptosis. Apoptosis is a systematic biological process involved in the eradication of unwanted and damaged cells, keeping control of the homeostasis of the body. This specialized process is characterized by several biochemical and morphological indicators like membrane blebbing, chromatin condensation, cellular shrinkage along with apoptotic body formation [7,8].

The main purpose of this study was to demonstrate the anticancer effects of pterostilbene in HeLa human cervical cancer cells along with evaluating its mode of action by studying its effects on apoptotic induction, MMP and m-TOR/PI3K/Akt signalling pathway.

## Methods

### Chemicals, cell lines and cell culture conditions

All chemicals and reagents were obtained from Sigma-Aldrich, USA, unless otherwise specified. HeLa human cervical cancer cells were purchased from the cell bank of the Basic Medical College of Huazhong University of Science and Technology (HUST). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) along with 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere with 5% $CO_2$.

### MTS assay for cell proliferation

The antiproliferative effects triggered by pterostilbene in HeLa cancer cells were evaluated by MTS assay. HeLa cells at a density of $2 \times 10^6$ cells/well were incubated in 96-well plates for 24 hrs. The cells were then treated with various doses (0, 5, 25, 50, 100, 200 and 400 µM) of pterostilbene for 24 and 48 hrs. The cells were again incubated for another 12 hrs and MTS solution (10 µM) was added to the cells according to the manufacturer instructions. Finally the optical density (OD) was taken at 490 nm using ELISA plate reader.

### Cell morphological evaluation by inverted phase contrast microscopy

HeLa cancer cells were grown at a density of $2 \times 10^6$ cells/well in a 6-well plate (Guangzhou Jet Biofil, Guangzhou, People's Republic of China) and subjected to treatment with various doses (0, 25, 100 and 200 µM) of pterostilbene for 48 hrs. The cells treated with pure DMSO only served as vehicle control. Finally, the cells were examined under an inverted phase contrast microscope at 100 x magnification (Nikon, Tokyo, Japan).

### Morphological cell evaluation by fluorescence microscopy

In this assay, HeLa cells were analyzed by fluorescence microscopy using a staining method involving acridine orange (AO) and ethidium bromide (EB). In brief, HeLa cells were grown at a density of $2 \times 10^6$ cells/well in a 6-well plate and then subjected to treatment with varying doses (0, 25, 100 and 200 µM) of pterostilbene for 48 hrs. The cells treated with pure DMSO served as vehicle control. The cells were then collected and washed twice with phosphate buffered saline (PBS) and finally subjected to AO/EB (50 µg/ml each) double staining before being analyzed by a fluorescence microscope (Nikon, Tokyo, Japan).

### DNA fragmentation analysis

To investigate whether pterostilbene induces DNA damage in HeLa cells we used gel electrophoresis. Briefly, HeLa cells were seeded in a 60-mm cell culture plate and kept for incubation for 24 hrs. After incubation, the cells were treated with different doses (0, 25, 100 and 200 µM) of pterostilbene for 48 hrs and were then harvested using trypsinization followed by washing two times with PBS. The pellets were then lysed with a DNA lysis buffer for 2 hrs and then centrifuged at 10,000 rpm. The supernatant was prepared in an equal amount of 5.0% sodium-dodecyl sulphate, incubated with 20 mg/ml RNase A for 1 hr. Finally, DNA was dissolved in gel loading buffer and separated using electrophoresis in agarose gel (2.5%).

### Measurement of changes in mitochondrial membrane potential loss (Δψm)

Rhodamine-123 fluorescence has been used to determine variations in membrane polarization in live cells and within mitochondria. The principle is based on the fact that Rhodamine-123 gets accumulated within membranes in a way which is directly dependent on transmembrane potential. In brief, HeLa cells at a density of $2 \times 10^6$ cells/well were grown into a 6-well plate and incubated for 24 hrs. After incubation, the cells were treated with 0, 25, 100 and 200 µM of pterostilbene for 48 hrs. The cells treated with DMSO served only as vehicle control. Both treated and untreated control cells were collected using trypsinization and washed with PBS twice. Subsequently, the cell pellets were resuspended in fresh medium comprising of 5.0 µM rhodamine 123 and further incubated for 20 min and then examined under flow cytometer (BD FACSCalibur, San Jose, CA, USA).

*Western blot assay*

Pterostilbene–administered cells were washed three times in ice-cold PBS and then extracted with a RIPA buffer containing a mixture of 5% each of phosphate inhibitor and protease inhibitor. The cell lysates were centrifuged at 12,000× g for 20 min and using Bio-Rad protein assay, protein concentrations were determined. The proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Millipore Corporation, MA, USA). The membranes were then probed with specific antibodies at 4°C overnight, then washed with PBS and incubated with the appropriate secondary antibody for 1 hr. The bands were observed using an ECL chemiluminescent detection kit (Perkin Elmer Cetus, Foster City, CA, USA).

*Statistics*

The experiments were repeated at least three times and shown as mean ± SD. One way ANOVA was used for statistical analyses. GraphPad Prism 7 software was used for carrying out the statistical analyses. Statistical significance was considered at *p<0.05, and **p<0.01.

## Results

*Pterostilbene exerts significant growth inhibitory effects in HeLa human cervical cancer cells*

Pterostilbene or 3',5'-dimethoxy-resveratrol is a stilbenoid and is chemically related to resveratrol
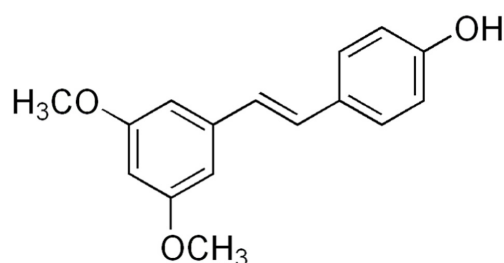


**Figure 1.** Chemical structure of pterostilbene (3',5'-dimethoxy-resveratrol).
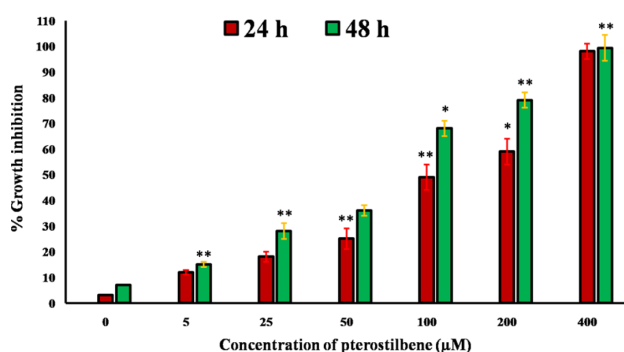


**Figure 2.** Growth inhibitory effects induced by pterostilbene in HeLa cancer cells. The cells were treated with 0, 5, 25, 50, 100, 200 and 400 μM dose of pterostilbene for 24 and 48 hrs. Data are shown as the mean ± SD of three independent experiments. *p <0.05, **p <0.01, vs 0 μM (control).

- a naturally occurring phenolic compound present in grapes and blueberries. The chemical structure of pterostilbene is shown in Figure 1 while its growth inhibitory effects in HeLa cells are shown in Figure 2. The cytotoxic effects of pterostilbene evaluated by MTS assay revealed that the compound induces potent, dose-dependent and time-dependent cyto-
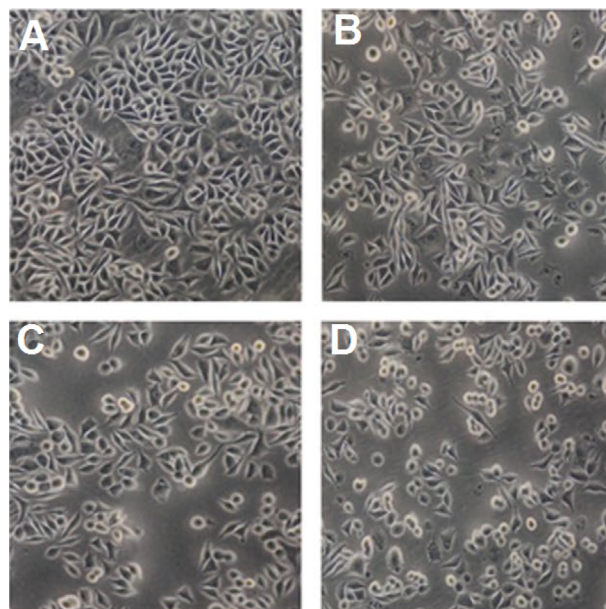


**Figure 3.** Inverted phase contrast microscopic study of the morphological effects induced by pterostilbene in HeLa cancer cells. The cells were treated with 0 **(A)**, 25 **(B)**, 100 **(C)** and 200 **(D)** μM for 48 hrs and then examined under phase contrast microscope at 100× magnification. Cellular shrinkage could be seen with increasing doses of pterostilbene.
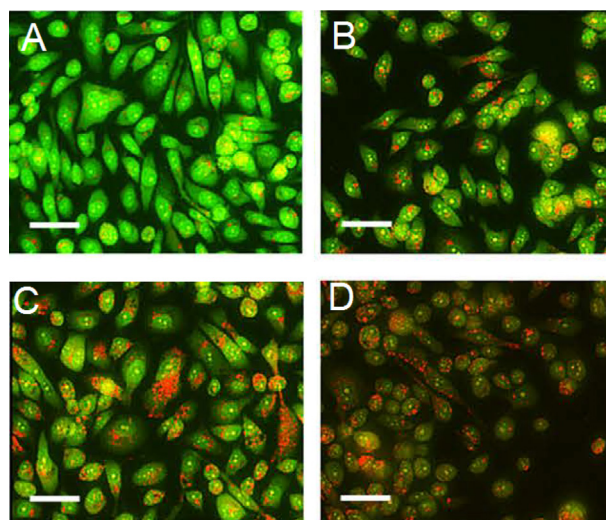


**Figure 4.** Fluorescence microscopic evaluation of the apoptotic induction by pterostilbene in HeLa cells. HeLa cells were treated with 0 **(A)**, 25 **(B)**, 100 **(C)** and 200 **(D)** μM of pterostilbene, incubated for 48 hrs and then stained with acridine orange/ethidium bromide double stain before being examined under fluorescence microscope (magnification ×200).

toxic effects in these cancer cells. The IC$_{50}$ (the half maximal inhibitory concentration) values of the compound which gives an indication of the potency of the drug were calculated graphically and were found to be 101.2 µM and 65.9 µM, respectively at 24 and 48 hrs, respectively.
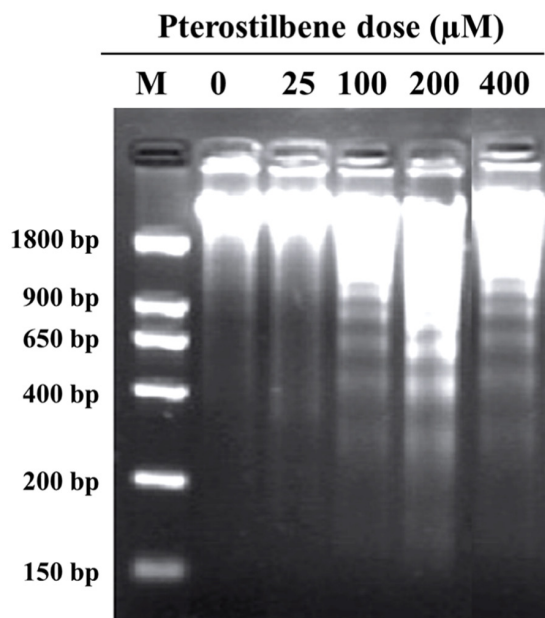


**Figure 5.** Pterostilbene induced DNA fragmentation in HeLa cancer cells. HeLa cells were treated with increasing doses of the drug, harvested using trypsinization, DNA was extracted and then analyzed using 1.5% agarose gel electrophoresis. The Figure proves that pterostilbene triggers DNA damage in HeLa cells.
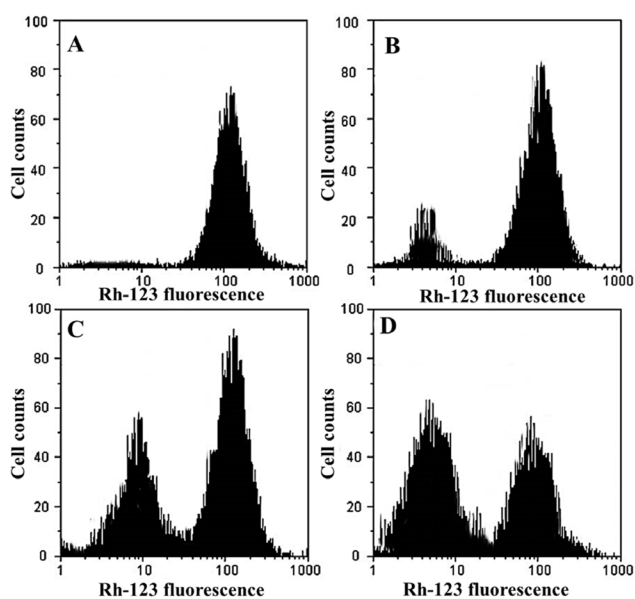


**Figure 6.** Pterostilbene induced mitochondrial membrane potential loss in HeLa cells. HeLa cells were treated with increasing doses of the drug [0 **(A)**, 25 **(B)**, 100 **(C)** and 200 **(D)**] and then analyzed using flow cytometry. The Figure shows that pterostilbene triggers cell cycle arrest of HeLa cells in a dose-dependent manner.

*Morphological changes induced by pterostilbene in HeLa human cervical cancer cells*

The effect of pterostilbene on the cellular morphology in HeLa cells was evaluated by inverted phase contrast microscope. As compared to the untreated control cells (Figure 3 A) which showed normal cell morphology, pterostilbene-treated cells exhibited significant cellular shrinkage which increased with increasing doses of the compound. Furthermore, it was observed that as the pterostilbene dose increased, higher number of HeLa cells got detached from one another and from the substratum. It appeared that pterostilbene induced apoptosis in these cells as phase contrast microscopy showed membrane blebbing, fragmentation of the nuclei and chromatin condensation on drug treatment (Figure 3 A-D).

*Fluorescence microscopic evaluation of the pterostilbene-induced apoptosis*

Fluorescence microscopy using AO/EB double staining is an excellent method of determining the apoptotic induction in cancer cells. In the current study this assay was used to study the apoptotic effects of pterostilbene in HeLa cancer cells. The results, which are depicted in Figure 4 A-D, indi-
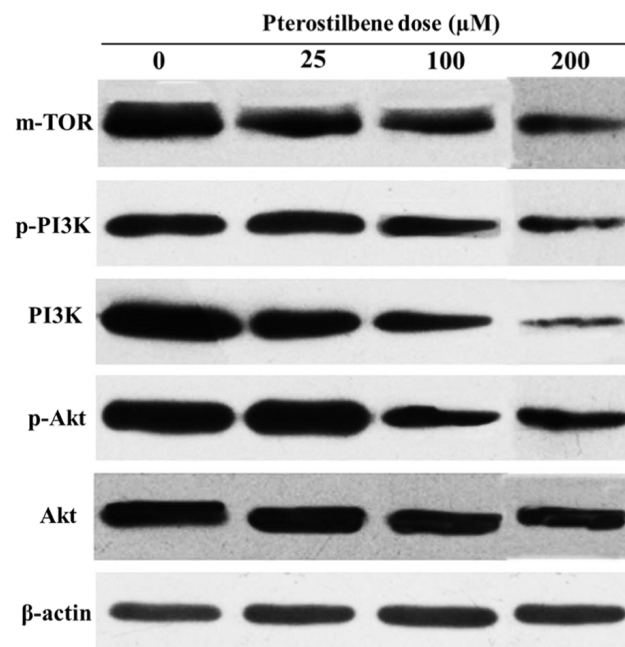


**Figure 7.** Pterostilbene led to downregulation of m-TOR and PI3K/Akt pathway protein expressions. The cells were treated with 0, 25, 100 and 200 µM dose of pterostilbene for 48 hrs. The cell lysates were centrifuged and using Bio-Rad protein assay and protein concentrations were determined. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

cate that the untreated control cells showed complete green fluorescence corresponding to no signs of apoptosis. However, pterostilbene-treated cells with 25, 100 and 200 μM dose led to increasing emission of red/orange fluorescence corresponding to apoptotic induction. With increase in drug dose, the percentage of apoptotic cells also showed a significant increase indicating that pterostilbene exhibited dose-dependent apoptotic induction in HeLa cells.

*Pterostilbene induced DNA fragmentation*

As DNA fragmentation is a crucial stage in the apoptotic process, it was examined in HeLa cells treated with pterostilbene. The results of this assay are shown in Figure 5 and reveal that in contrast to the untreated control group which did not reveal any DNA ladder formation, pterostilbene-treated cells showed evident signs of DNA ladder formation and the effect increased with increasing concentrations of the drug. Thus it can be concluded that pterostilbene induces anticancer effects in HeLa cancer cells by inducing apoptosis and DNA damage.

*Pterostilbene led to mitochondrial membrane potential loss (Δψm)*

Using rhodamine-123 dye in combination with flow cytometry, the effects of pterostilbene on the MMP were evaluated. As shown in Figure 6 A, the untreated control cells did not show any loss of MMP, however, on increasing pterostilbene concentration, MMP was significantly decreased in a dose-dependent manner. The percentage of cells with depolarized mitochondria (loss of MMP) increased from 2.3% in the control group to 24.5, 43.2 and 65.8% respectively in cells treated with 0, 25, 100 and 200 μM dose of pterostilbene respectively (Figure 6 B-D).

*Pterostilbene led to downregulation of m-TOR/PI3K/Akt pathway-related protein expressions*

In order to confirm which kind of biochemical pathway is affected by treating cells with pterostilbene, western blot assay was performed. The results of this assay are shown in Figure 7 and reveal that pterostilbene led to a considerable and dose-dependent downregulation of proteins of m-TOR, PI3K/Akt biochemical pathway. Compared to the control group, pterostilbene-treated groups showed a gradual decrease in the expression levels of m-TOR as well as PI3K/Akt proteins. Thus it can be concluded that pterostilbene exerts anticancer effects in HeLa cells by targeting m-TOR/PI3K/Akt signalling pathway.

## Discussion

Cancer which is characterized with uncontrolled growth of cells within the body is now considered a major human health problem and the death rates caused due to this disease are consistently increasing. The currently used treatment regimens against cancer are having lots of serious side-effects coupled with the fact that many solid cancers are acquiring multidrug resistance. Thus it seems imperative that new and effective treatment strategies are adopted to combat this deadly disease. One of the important strategies in the management and therapy of cancer involves controlling cancer cell survival and death. An effective and safe anticancer agent should selectively kill cancer cells without causing too much damage to the normal cells. Such an effect can be achieved through induction of apoptosis. Apoptosis involves programmed cell death in both cancer and normal tissues [9-11]. This process is characterized by various biochemical and morphological changes including chromatin condensation, membrane blebbing and formation of apoptotic bodies. Many of the natural product-based cytotoxic agents induce apoptosis in cancer cells. Around 3,000 species of plants have been found to be used for cancer treatment and several natural plant compounds have reached clinical trials [12,13]. Other modes of cell death induced by plant-derived anticancer agents include necrosis, autophagy and senescence leading to cell death. Herbal medicinal agents have been shown to offer a huge pharmaceutical potential and various herbal agents have been used to treat different forms of human malignancies [14].

The aim of the present investigation was to study the anticancer effects of pterostilbene in HeLa cervical cancer cells. Pterostilbene is a natural dietary molecule mainly isolated from blueberries and heartwood [15-17]. It is the main antioxidant constituent of blueberries and has been reported to exhibit several therapeutic and preventive effects in a wide range of human diseases including hematological, cardiovascular, metabolic and neurological. The anticancer properties of pterostilbene against various malignancies have been reported in several preclinical trials [18,19]. Several studies have indicated that pterostilbene has all the trademarks of an effective and safe anticancer drug based on its ability to inhibit the growth of various malignant tumors. As for its mode of action, it has been reported that pterostilbene shows anticancer activity by disturbing cell cycle phase distribution, apoptosis induction and inhibition of metastatic potential [18]. In the current study it was shown that pterostilbene showed potent growth inhibition

on HeLa cells. The compound exerted cytotoxic effects time- and concentration-dependently in these cancer cells with an $IC_{50}$ value of 101.2 µM and 65.9 µM respectively at 24 and 48 hrs , respectively. Pterostilbene induced morphological changes in these cells including cell shrinking and detachment from the substratum. Untreated cells showed no signs of apoptosis while pterostilbene-treated cells with 25, 100 and 200 µM led to increasing emission of red/orange fluorescence corresponding to apoptotic induction. Pterostilbene-treated cells showed evident signs of DNA ladder formation corresponding to DNA damage and the compound

led to dose-dependently decreased MMP. Immunoblotting analysis indicated that pterostilbene led to dose-dependent downregulation of m-TOR, PI3K/Akt proteins.

In conclusion, the current research work indicated that pterostilbene induced anticancer activity against the HeLa cancer cells by triggering apoptosis, DNA fragmentation, loss of MMP and downregulation of m-TOR, PI3K/Akt pathway proteins.

## Conflict of interests

The authors declare no conflict of interests.

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