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ORIGINAL ARTICLE

Antiproliferative activity of Farnesol in HeLa cervical cancer cells is mediated via apoptosis induction, loss of mitochondrial membrane potential (ΔΨm) and PI3K/Akt signalling pathway

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Summary

Purpose: *Cervical cancer remains the most gruesome health problem in women worldwide as it ranks third in incidence. Despite recent developments in the treatment options of cervical cancer, the survival of patients not fit for surgical treatment rather remains poor. The main purpose of the current research was to determine the anticancer effect of farnesol in HeLa human cervical cancer cells together with studying its impact on apoptosis induction, mitochondrial membrane potential (MMP) and PI3K/Akt signalling cascade.*

Methods: *Cell viability was estimated by MTT assay while clonogenic assay was used to assess the effects on colony formation tendency in these cells. Fluorescence microscopy indicated apoptosis induction while flow cytometry showed the farnesol effects on the loss of MMP.*

Results: *Farnesol exerted both dose and time-dependent antiproliferative effects on cervical cancer cells with IC_{50} values of 33.5, 23.8 and 17.6 μM at 24, 48 and 72 hrs time intervals, respectively. Colony formation of HeLa cells was considerably affected in a dose-dependent manner with the addition of farnesol to the cell culture. Farnesol-treated cells mostly emitted orange fluorescence indicating apoptotic cell death and this effect increased with increasing dose of the compound. Furthermore, farnesol induced considerable reduction in the number of cells with depolarized mitochondria corresponding to a reduction of MMP. With increase in the dosage of farnesol, there was a noticeable decrease in the expression levels of PI3K, p-PI3K and p-Akt proteins.*

Conclusions: *In brief, this study showed that farnesol -a naturally occurring sesquiterpene- exerts powerful antiproliferative activity via apoptosis induction, loss of MMP and downregulation of the expression levels of PI3K, p-PI3K and p-Akt proteins.*

Key words: *apoptosis, cervical cancer, farnesol, flow cytometry, malignancy*

Introduction

Cervical cancer is the third most prevalent malignancy in women globally. Cervical neoplasms emerge from pre-malignant precursor lesions that gradually transform to cervical cancer. Each year around 550,000 new cases of cervical cancer are reported globally and more than 75% of these cases are registered in developing countries [1,2]. Owing to the absence of a national organized cervical cancer screening program in China, cervical cancer remains one of the main public health issues. According to the China Cancer Registration Annual Report 2004, cervical cancer was ranking as the 8th most prevalent cancer in women, with a crude incidence rate of 8.55 per 100,000 women [3].

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Over 99% of cervical cancers are linked with human papillomavirus (HPV), however HPV infection alone is not a prerequisite for cervical carcinoma development. Primary prevention of cervical cancer involves prophylactic vaccination but recombinant human papillomavirus vaccine (Gardasil) is not available in many parts of China [4]. Treatment of cervical cancer includes surgical resection, radiotherapy and chemotherapy. The amalgamation of external beam radiotherapy and brachytherapy is considered to be the typical treatment for cervical carcinoma. However, in case of locally advanced cervical cancer patients, the survival rate is very poor [5-7]. As such novel and effective treatment strategies are required.

The main goal of this investigation was to evaluate the antiproliferative activity of farnesol in HeLa cervical cancer cells and also to demonstrate its mode of action by determining its effects on apoptosis induction, mitochondrial membrane potential (MMP) and PI3K/Akt signalling cascade. Farnesol is produced by a large number of plant species including *Lamium purpureum* and has antimicrobial and insecticidal properties. The antiproliferative activity of farnesol against HeLa cervical cancer cells has not been explored up until today, although many essential oils containing farnesol have been shown to exhibit anticancer properties [8-11].

Methods

Chemicals and reagents

Farnesol (≥95% purity) was obtained from Sigma-Aldrich (St. Louis. Co, SUA). The culture media (MEM/RPMI), fetal bovine serum (FBS), antibiotics (penicillin, streptomycin), trypsin and DMSO were procured from Hangzhou Sijiqing Biological Products Co. Ltd, China. All chemicals and solvents used were procured from Merck (Mainland, China) and Sigma-Aldrich. 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide (MTT), propidium iodide (PI), Hoechst 33258, acridine orange/PI (AO/PI) were procured from Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.A. Phosphorylated p-PI3K, PI3K, p-Akt and Akt were procured from Cell Signalling Technology, MA, USA, while X-ray film for chemiluminescent system was obtained from Fuji Photo Film, Japan.

Cell line and cell culture conditions

Human cervical cancer cells (HeLa) were procured from American Type Culture Collection (ATCC) Manassas, VA, USA. Cells were grown in Minimum Essential Medium (MEM) and RPMI containing 10% FBS and antibiotics (penicillin and streptomycin (100U/ml and 100 mg/ml, respectively) at 37°C. The cells were grown in incubator with 95% air and 5% CO_2 and the cell line was kept at 37°C for 24 hrs.

MTT and clonogenic assays for cell viability

The fact that farnesol induces cytotoxic effects in HeLa cancer cells was assessed by MTT and clonogenic assays. For MTT assay, HeLa cells were cultured on a 96-well plate at a density of 2×10^5 cells per well. The cells were then exposed to farnesol at varied doses (0, 5, 10, 20, 50, 100, 150 μM) in 3 time periods of 24, 48 and 72 hrs. The plates with the treated HeLa cells were then administered 20 μl MTT solution for 4 hrs in PBS. The formazan crystals so developed were dissolved with the help of DMSO and the optical density (OD) was taken on a microplate reader. The impact of farnesol on cell viability was reported as inhibition ratio (I%).

For clonogenic assay, HeLa cells at the exponential growth phase were harvested and counted with a hemocytometer. The cells were seeded at 200 cells per well, incubated for 48 hrs to allow the cells to attach and then different doses (0, 10, 50 and 150 μM) of farnesol were added to the cell culture. After treatment, the cells were again kept for incubation for 6 days, washed with PBS, colonies were methanol-fixed and then subjected to eosin staining for 30 min before being counted under light microscope.

Acridine orange (AO)/Propidium iodide (PI) staining assay for cell apoptosis

The fact that farnesol induces apoptotic cell death in HeLa human cervical carcinoma cells was assessed by AO/PI staining assay using fluorescence microscopy. HeLa cells were seeded in 6-well plates and then administered with 0, 10, 50 and 150 μM of farnesol for 48 hrs. Then, both untreated control cells as well as farnesol-treated cells were incubated with AO/PI (50 μg/ml each) and analyzed under a fluorescent microscope (200x, Nikon, Tokyo, Japan). The cells were classified as living or dead and also the type of cell death was evaluated along with determining the percentage of necrotic and apoptotic cells.

Mitochondrial membrane potential (ΔΨm) measurement

Farnesol-induced loss of MMP was evaluated by using flow cytometry and rhodamine-123 (Rh-123) fluorescent dye (Sigma-Aldrich). Briefly, HeLa cervical cancer cells at a density of 1×10^5 cells per ml were administered increasing doses of farnesol (0, 10, 50 and 150 μM) for 48 hrs at 37°C. Rh-123 (5 mM) was added 2 hrs before the end of the experiment. The cells were collected, washed in PBS and stained with PI (20 μg/ml) for 20 min at room temperature. MMP was then determined using flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA, USA).

Western blot assay

The effect of farnesol on various apoptosis-related proteins was evaluated by western blotting. HeLa cancer cells were administered varied doses of farnesol (0, 10, 50 and 150 μM) for 48 hrs. The cells were then collected and subjected to PBS washing two twice and then lysis was done in RIPA buffer and protease inhibitor for 30 min. This was followed by centrifugation and then the protein content was estimated by bicinchoninic acid

(BCA) assay. The protein extracts (10 µg/lane) were run on 12% SDS-PAGE and blotted onto nitrocellulose membranes (Millipore, MA, USA). Each of the membranes was blocked with 5% skim milk, and then treated with the designated primary antibodies at 4°C for 24 hrs.

Statistics

All results were presented as mean ± standard error (S.E.) of three independent experiments. The differences between groups were analyzed by one-way ANOVA, and p value was considered significant at *p<0.05 and **p<0.01.

Results

Effect of farnesol on HeLa cells viability

The chemical structure of farnesol is shown in Figure 1 while the cytotoxic effect of this compound on HeLa cell viability is shown in Figure 2. The results indicate that farnesol is a strong cytotoxic agent which exerts both concentration as well as time-dependent antiproliferative effects on cervical cancer cells. Exposure of the HeLa cervical cancer cells to 24, 48 and 72-h incubation with the drug resulted in higher growth inhibitory effects. The IC_{50} values of the compound at 24, 48 and 72 hrs time intervals were calculated to be 33.5, 23.8 and 17.6 µM, respectively.

Farnesol inhibited the colony formation tendency of HeLa cells

The effect of farnesol on colony formation tendency of HeLa cervical cancer cells was evaluated by clonogenic assay using crystal violet staining and light microscopy. The results of this assay are presented in Figure 3A-D and reveal that colony formation efficacy of HeLa cancer cells was significantly affected in a dose-dependent manner upon addition of farnesol to the cell culture. In comparison to the untreated control cells (Figure 3A)

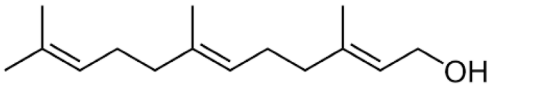

Figure 1. Chemical structure of farnesol.

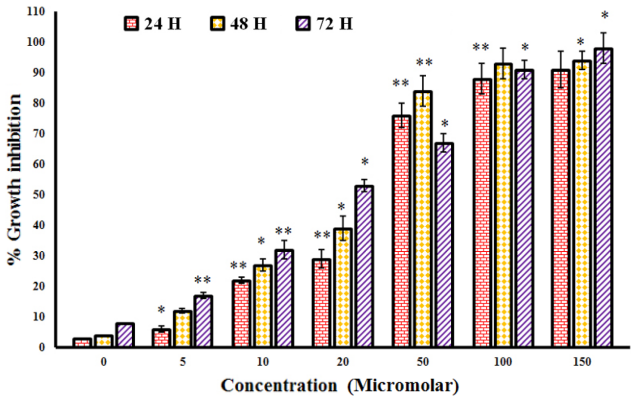

Figure 2. Dose and time-dependent growth inhibitory effects of farnesol in human cervical cancer cells (HeLa). Data are shown as the mean ± SD of three independent experiments. *p<0.05, **p<0.01 vs 0 µM (control).

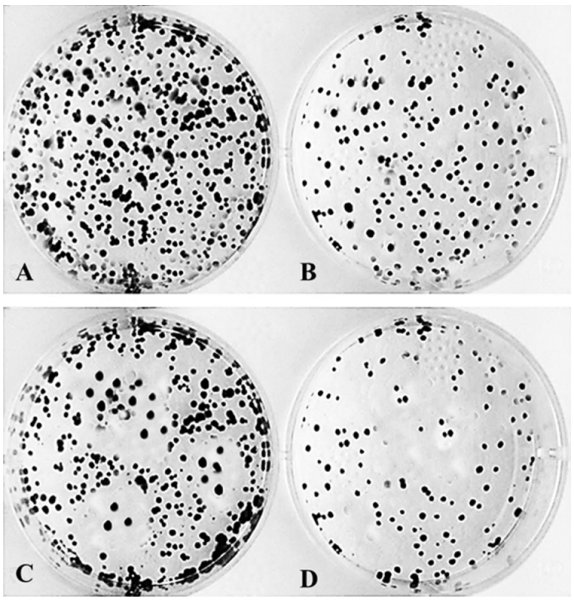

Figure 3. Farnesol suppresses the colony formation tendency of HeLa cervical cancer cells. The cells were treated with 0 **(A)**, 10 **(B)**, 50 **(C)** and 150 **(D)** µM dose of farnesol for 48 hrs and then analyzed by using a light microscope. Increasing doses of farnesol led to decrease in cancer cell colonies.

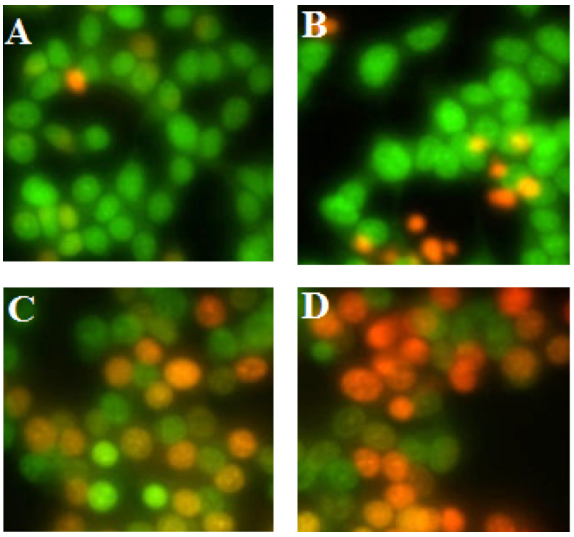

Figure 4. Effect of farnesol on the apoptosis induction in human cervical (HeLa) cancer cells using AO/PI staining along with fluorescence microscopy. The cells were treated with 0 **(A)**, 10 **(B)**, 50 **(C)** and 150 **(D)** µM dose of farnesol for 48 hrs and then analyzed by fluorescent microscope at a magnification of 200×. Green fluorescence indicates viable cells while orange fluorescence is an indication of apoptotic cell death. Increasing doses of farnesol led to early apoptosis initially, and after that to late apoptosis at even higher doses of farnesol.

which showed higher fraction of cell colonies, the farnesol-treated cells exhibited a significant decrease in the number of cell colonies (Figure 3B-D). Thus taking together MTT and clonogenic experiments, it appears that farnesol induces both anchorage-dependent as well as anchorage-independent cytotoxic effects in these cells.

Apoptotic evaluation by fluorescence microscopy using AO/PI staining

This assay is based on the fact that viable cells appear as green while as apoptotic dead cells emit orange fluorescence. As shown in Figure 4A-D, in comparison to the untreated control cells (Figure 4A) which emitted green fluorescence, farnesol-treated cells mostly emitted orange fluorescence indicating an apoptotic cell death and this effect increased with increasing dose of farnesol (Figure 4B-D). This confirms that farnesol induces dose-dependent apoptotic effects in HeLa cervical cancer cells. Low doses of farnesol were shown to induce early apoptotic features including chromatin condensation, cell shrinkage and membrane blebbing.

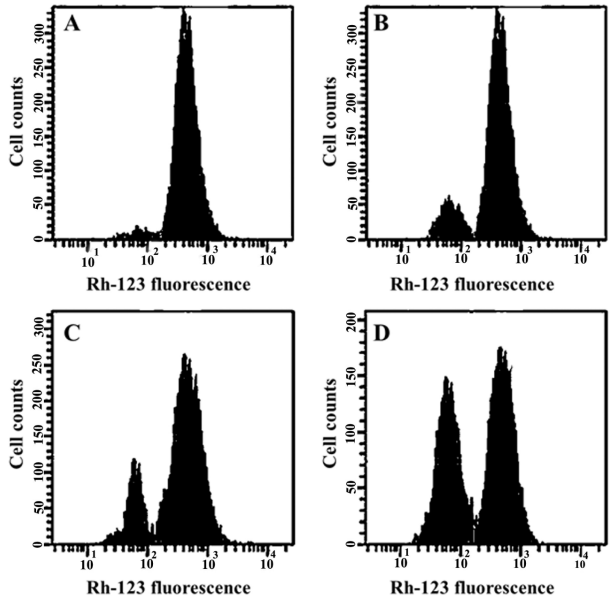

Figure 5. Effect of farnesol on the mitochondrial membrane potential in human cervical (HeLa) cancer cells. The cells were treated with 0 **(A)**, 10 **(B)**, 50 **(C)** and 150 **(D)** μM dose of farnesol for 48 hrs and then analyzed by flow cytometry. Farnesol led to a dose-dependent decrease of mitochondrial membrane potential.

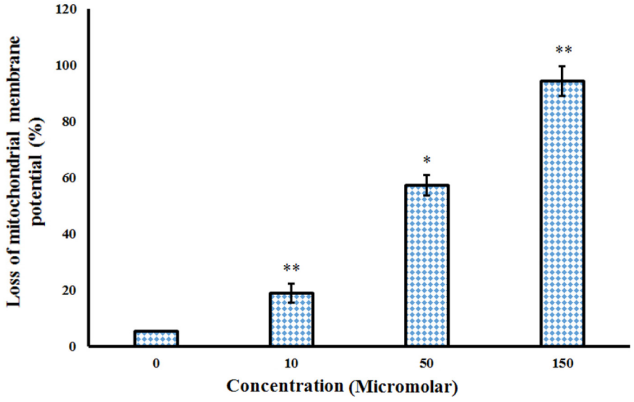

Figure 6. Graphical representation of the increase in the mitochondrial membrane potential loss with increased dose of farnesol in HeLa human cervical cancer cells. Data are shown as mean ± SD of three independent experiments. *p<0.05, **p<0.01 vs 0 μM (control).

Farnesol induced loss of MMP (ΔΨm) in HeLa cervical cancer cells

The effect of farnesol on MMP is shown in Figure 5A-D. The results indicated that farnesol induced a considerable reduction in the number of cells with a depolarized mitochondria, corresponding to loss of MMP. As compared to the untreated control cells (Figure 5A) which showed no loss of MMP, the farnesol-treated cells (Figure 5B-D) indicated a considerable proportion of HeLa cervical cancer cells with decreased MMP which showed strong dose-dependence. Figure 6 shows the graphical representation of the loss of MMP with increasing doses of farnesol.

Farnesol induced downregulation of PI3K/Akt pathway

The fact that farnesol does have an effect on the PI3K/Akt signalling pathway, western blot assay was carried out and the expression levels of the indicated proteins was evaluated at different

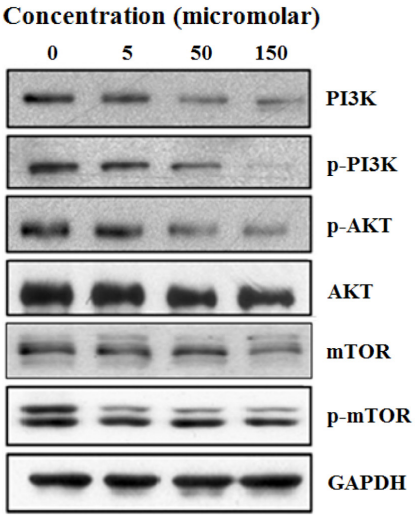

Figure 7. Effect of farnesol on the expression of PI3K/Akt proteins in HeLa human cervical cancer cells. After treating the cells with 0, 10, 50 and 150 μM dose of farnesol, the expression levels of different proteins were evaluated by western blot assay. Farnesol-treated cells showed that with increase in the dosage of the compound there was a noticeable reduction in the expression levels of PI3K, p-PI3K and P-Akt proteins.

doses of farnesol . The western blots prepared from the nuclear extracts of the farnesol -treated cells showed that with increase in the dosage of the compound, there was a noticeable reduction in the expression levels of PI3K, p-PI3K, p-Akt proteins (Figure 7). Thus it seems obvious that the anticancer effects of farnesol in HeLa cervical cancer cells are mediated via PI3K/Akt pathway.

Discussion

Worldwide, cancer remains the second leading cause of death after cardiovascular diseases and as such remains a hard public health problem. Cancer cells are characterized by uncontrolled division and deregulation of other biochemical pathways. Most of the cancers show no symptom till the disease is diagnosed and in most cases the disease has already reached advanced stages [12]. Chemoprevention of cancer has been suggested as an easy and economical alternative for cancer treatment. Chemoprevention of various cancers by natural product-derived drugs, especially plant-based compounds, has been reported in a number of previously published studies. Most of the currently used clinical anticancer drugs have either been isolated from natural sources or are semisynthetics or analogues of some naturally occurring compounds. More than half of the currently accessible drugs are natural compounds or are associated with them, and in the case of cancer this percentage surpasses 60% [13-16]. Apoptosis constitutes a well-organized biological process characterized by several biochemical and morphological features. The morphological features associated with apoptosis include but are not limited to cell shrinkage, membrane blebbing, chromatin condensation and cell membrane rupture. Apoptosis of cancer cells is one of the important mechanisms which can be utilized for the treatment of different types of tumors.

Apoptotic dysfunction is associated with numerous diseases, cancer in particular. Apoptosis may occur via two main pathways, the intrinsic pathway and the extrinsic pathway. While the intrinsic pathway is initiated by various external factors like chemical exposure, UV light, reactive oxygen species, the extrinsic pathway is triggered by activation of caspases like caspase 8 and caspase 10 [17,18]. It has also been reported that deregulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has been shown to have crucial role in the beginning and progression of several human malignancies [19].

The results of the present study indicate that farnesol exhibits potent antitumor effects in HeLa human cervical cancer cells. Farnesol was shown to inhibit colony formation tendency in these tumor cells. Further using fluorescence microscopy, it was shown that farnesol led to induction of early and late apoptosis in these cells. In addition, it was also shown that farnesol could also induce loss of MMP in a dose-dependent manner. Using western blot assay, it was revealed that farnesol can also lead to downregulation of PI3K/Akt signalling pathway in a dose-dependent manner.

In conclusion, farnesol induces antitumor effects in HeLa human cervical cancer cells via induction of apoptosis and loss of MMP along with the downregulation of PI3K/Akt signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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