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ANTITUMOR EFFECTS OF CHRYSANTHEMIN IN PC-3 HUMAN PROSTATE CANCER CELLS ARE MEDIATED VIA APOPTOSIS INDUCTION, CASPASE SIGNALLING PATHWAY AND LOSS OF MITOCHONDRIAL MEMBRANE POTENTIAL.

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Abstract

Background: The main objective of the current research work was to investigate the antitumor and apoptotic effects of chrysanthemin in PC-3 human prostate cancer cells.
Materials and Methods: MTT assay was used to evaluate the effects of chrysanthemin on cell viability whereas flow cytometry along with fluorescence microscopy were used to study apoptotic induction in these cells. Effects on caspase activation were detected through western blot assay.
Results: Results showed that chrysanthemin inhibited cancer cell growth in PC-3 cancer cells in a time-dependent as well as concentration-dependent manner. Chrysanthemin-treated cells at 10, 50 and 150 µM doses led to 34.2%, 56.7% and 69.1% apoptosis in these cells respectively.The percentage of cells with depolarized mitochondria increased from 5.3% in untreated control cells to 27.2%, 57.6% and 86.9% in cells treated with 10, 50 and 150 µM dose of chrysanthemin respectively. Chrysanthemin also enhanced the activity of all three caspases viz., caspase-3, 8 and 9 in a dose-dependent fashion.
Conclusions: The study concluded that chrysanthemin ledanticancer effects in PC-3 prostate cancer cells by inducing apoptosis, activating caspasesignaling pathway and loss of mitochondrial membrane potential.

Key words: Chrysanthemin, anthocyanin, prostate cancer, apoptosis, flow cytometry, caspases

Introduction

Prostate cancer is a leading cause of cancer-related mortalitywithin the United States and many other Western countries. This disease is one of the most common health problem affecting men across the globe (Cooperberg., 2014). It is usually diagnosed after 60 or 70 years of age and this allows greater opportunity for its prevention or slowing its progression.It has been reported that 50% of prostate cancer patients ultimately develop metastatic prostate carcinoma due to non-availability of effective treatment plan(Greenlee et al., 2001; Whittemore et al., 1995). Existing treatment plan include:surgical resection, chemotherapy or radiotherapy for localized tumor. Androgen ablation constitutes one of the basic treatment regimen for metastatic prostate cancer patients because of its ability to induce cell cycle arrest and apoptotic cell death in prostate tumor cells. Most of these therapies are only effective at the initial stages of the disease but in the advanced stages of prostate cancer, these treatment strategies fail and the patients ultimately die (Chen & Zhao., 2013; Nobili et al., 2009; Karantanos et al., 2013).

According to World Health Organization (WHO), about 80% of the global population relies heavily on traditional medicines for meeting their primary healthcare needs. There are ample evidences that phytochemicals have the ability to provide significant protection against various kinds of cancers including prostate cancer. Natural products can be developed into promising anticancer drugs because of their relatively less toxicity and inhibiting the development of different types of carcinomas. There are various biochemical mechanisms through which these natural products and other plant extracts exert their anticancer effects and these include apoptosis induction, cell cycle arrest, disruption of mitochondria, DNA fragmentation, caspase activation, modulation of PI3K/Akt signalling pathways (Reed., 2002; Etiana et al., 2016; Shaista et al., 2015; Mahmoud et al., 2014).
The aim of the present research work was to study the antitumor effects of chrysanthemin against PC-3 human prostate cancer cells by assessing its effects on apoptosis, caspase signalling pathway and loss of mitochondrial membrane potential.

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Materials and Methods

Chemicals

Chrysanthemin (≥ 95% purity as determined by HPLC), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide, 5% heat-inactivated fetal calf serum, penicillin, streptomycin were purchased from Thomas Scientific, High Hill Road, Swedesboro, U.S.A. Acridine orange (AO)/ethidium bromide (ETBR), propidium iodide and rhodamine-123 were purchased from Wuhan Boster Biological Technology Ltd. (Wuhan, China).

Cell line and cell culture medium

The PC-3 human prostate cancer cell line was purchased from Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. The cell culture conditions comprised of Minimum Essential Medium (MEM) and RPMI supplemented with 10% (v/v) fetal bovine serum (FBS) under humidified atmosphere of 5% CO_2 at 37 ^{o}C.

Cell proliferation assay

Human prostate cancer cells were plated at cell density of 2x10^6 cells/well in a 96-well culture plate. Different concentrations (0, 10, 50, 75, 100, 150 and 200 µM) of chrysanthemin in dimethyl sulfoxide (DMSO) were prepared. The cells were treated with the doses as stated above, and then a 10 µl solution of MTT was added to each well. The cells were then incubated for different time intervals including 24, 48 and 72 h. Absorbance was measured on a microplate reader (ELX 800; Bio-tek Instruments, Inc., Winooski, VT, USA) at a wavelength of 490 nm and the growth inhibition ratio was calculated. The half-maximal inhibitory concentration values (IC_{50}) were obtained from the MTT viability curves.

Annexin V-FITC assay for cell apoptosis

In brief, PC-3 human prostate cancer cells at a density of 2x10^6 cells per ml were seeded into a 12-well plate and then put on incubation for 12 h. The cells were treated with 0, 10, 50 and 150 µM dose of chrysanthemin for 48 h, washed with PBS and then resuspended in binding buffer comprising of Annexin V-fluorescein isothiocyanate and propidium iodide for 20 min. DMSO (0.50 %) dissolved in cell media served as control. Finally, the cells were analyzed by FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software.

Fluorescence microscopic study of apoptosis

The fact that chrysanthemin induced apoptosis in PC-3 human prostate cancer cells was further evaluated by using fluorescence microscopy involving acridine orange/propidium iodide double dye. In short, PC-3 human prostate cancer cells at a density of 2x10^6 cells/well were plated and then treated with 0, 10, 50 and 150 µM dose of chrysanthemin for 48 h. Then both groups of cells (untreated and treated cells) were incubated with 5 µg/ml each of acridine orange and propidium iodide for 1 h. Then using a fluorescent microscope (400 x magnification, Nikon, Tokyo, Japan), the apoptotic features induced by the drug were evaluated.

Measurement of Mitochondrial Membrane Potential (ΛΨm) loss

The fact that chrysanthemin induced loss of mitochondrial membrane potential was evaluated by using flow cytometer combined with rhodamine-123 dye. In brief, PC-3 human prostate cancer cells were seeded at a density of 2x10^6 cells/ml, treated with 0, 10, 50 and 150 µM dose of chrysanthemin for 48 h and then washed with PBS and then harvested. A 10 µl solution of rhodamine-123 was added to the cell suspension and then analyzed by flow cytometry (FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software).

Western blot analysis

In order to evaluate the changes in the expression levels of caspase-related proteins, western blot assay was used as already described previously (Xu et al., 2013). The cells were treated with varying doses of chrysanthemin for 24 h and then the treated cells were collected and then lysed using lysis buffer. The cells were then centrifuged at 15,000 g for 20 min. Subsequently the supernatant was collected and then using BCA method, the protein content was estimated. The protein was then mixed with loading buffer and incubated for 10 min at 90 ^{o}C. The cell lysates were analyzed with primary

antibodies of caspase-3, caspase-8 and caspase-9. Using ECL Western detection reagents, protein expressions were evaluated using Image Lab analysis software (Bio-Rad).

Statistical analysis

Data are presented as the mean ± SEM of the control. All experiments were repeated at least three times. The differences between groups were analyzed by one-way ANOVA with Tukey's posthoc tests, significance of difference was indicated as *$P < 0.05$, **$P < 0.01$.

Results

Chrysanthemin exerts potent antitumor effects in PC-3 human prostate cancer cells

Chrysanthemin, which is the 3-glucoside of cyanidin belongs to the anthocyanin class of natural products and its chemical structure is shown in Fig.1. MTT assay showed that chrysanthemin has the tendency to exert growth inhibitory effects in PC-3 cancer cells. The cytotoxic effects of chrysanthemin against PC-3 human prostate cancer cells were evaluated at varying doses as well as different time intervals. Results indicated that chrysanthemin induces dose-dependent and time-dependent cytotoxic effects in PC-3 cancer cells. The MTT results are shown in figure 2 and indicate that 150 and 200 µM doses of chrysanthemin exhibited 65.3 and 92.5% inhibitory effects respectively.

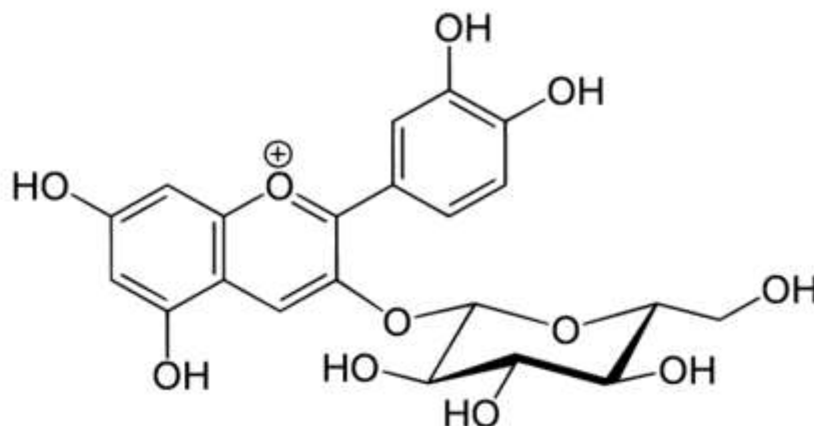

Figure 1: Chemical structure of chrysanthemin (also known as Glucocyanidin).

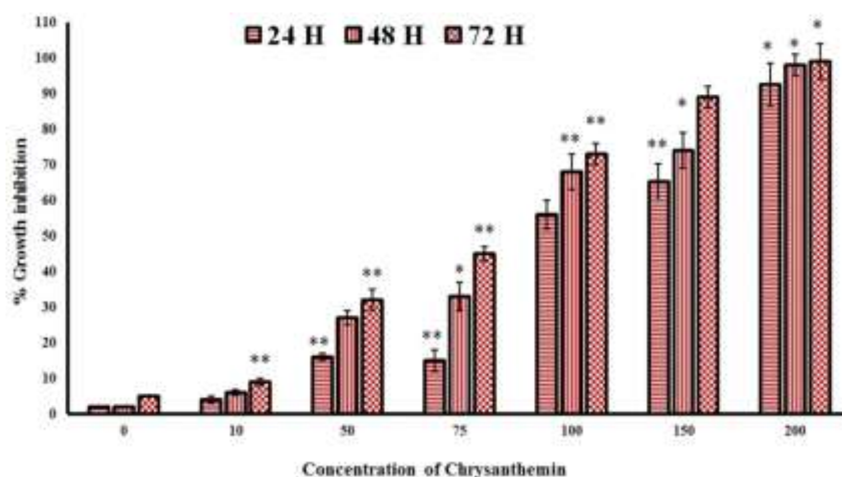

Figure 2: Cytotoxic effect of chrysanthemin in PC-3 human prostate cancer cells. The cells were exposed to varying doses of the drug at different time intervals. Data are shown as the mean ± SD of three independent experiments. *, $P < 0.05$, **, $P < 0.01$, vs 0 µM (control).

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Chrysanthemin induces apoptosis in PC-3 human prostate cancer cells

The fact that chrysanthemin induces early and late apoptosis in PC-3 human prostate cancer cells was evaluated by flow cytometry using annexin V as a probe. The results which are shown in figure 3 indicate that in comparison to the untreated control cells which do not show any signs of apoptosis, chrysanthemin-treated cells at 10, 50 and 150 µM doses led to 34.2%, 56.7% and 69.1% apoptosis in these cells respectively. R1, R2, R3 and R4 in figure 3 indicate necrotic cells, late apoptotic cells, viable cells and early apoptotic cells respectively.

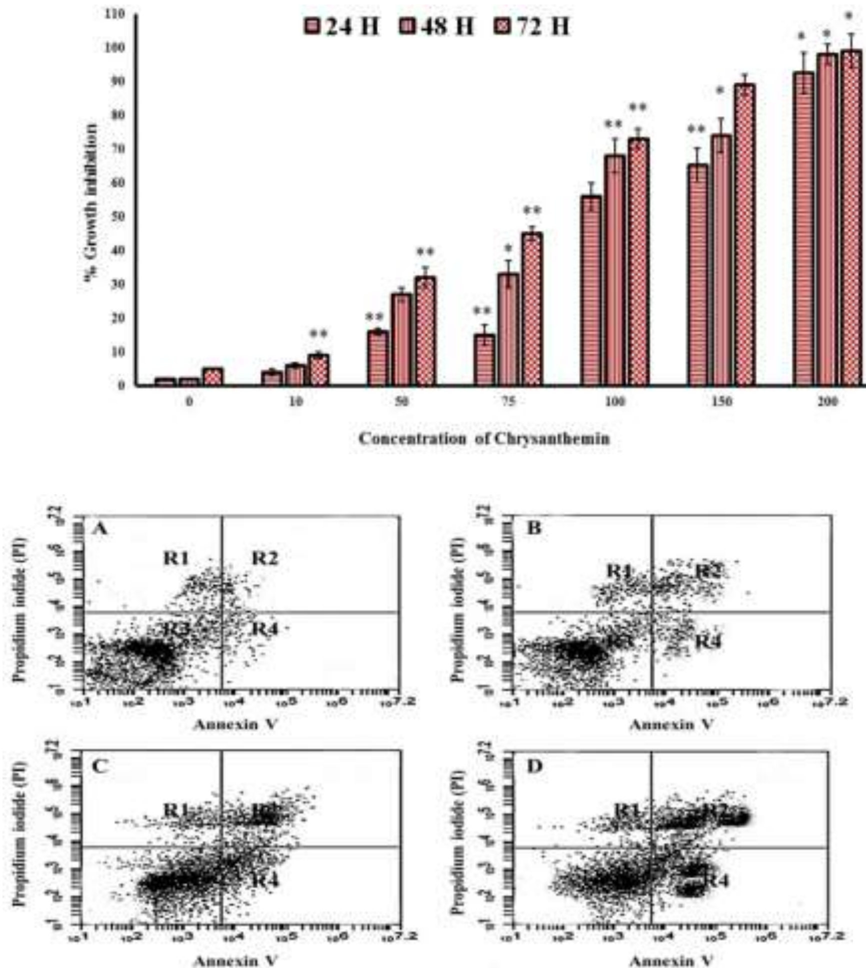

Figure 3: Evaluation of apoptosis induction by chrysanthemin in PC-3 human prostate cancer cells by annexin v assay. The cells were treated with 0, 10, 50 and 150 µM dose of chrysanthemin for 48 h and then analyzed by flow cytometry. R1, R2, R3 and R4 indicate necrotic cells, late apoptotic cells, viable cells and early apoptotic cells respectively.

Fluorescence microscopy study of apoptosis induction by chrysanthemin in PC-3 cells

Further experiments using fluorescence microscopy in combination with acridine orange and propidium iodide were carried out in order to confirm apoptosis induced by chrysanthemin in human prostate cancer cells. The results of this assay are shown in figure 4 and reveal that untreated control cells showed normal cellular morphology with no obvious signs of morphological features of apoptosis (Fig.4 A). However, on treating PC-3 cells with 10, 50 and 150 µM dose of chrysanthemin, significant and divulging signs of apoptosis were seen including chromatin condensation and cell shrinkage.

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Viable cells emit completely green fluorescence in this assay while as apoptotic cells emit orange/red fluorescence. As is evident, the number of cells emitting red fluorescence increased with increasing concentration of chrysanthemin.

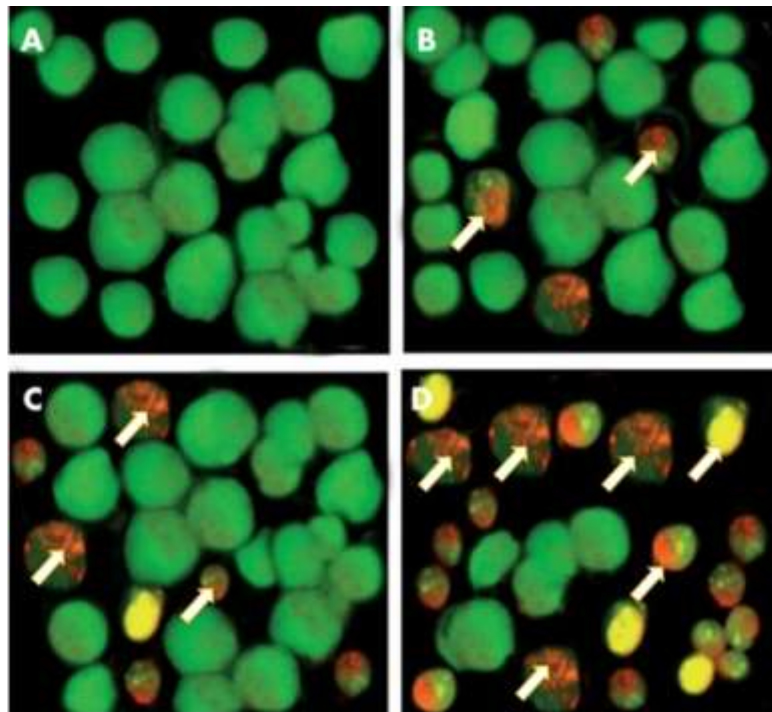

Figure 4: Apoptotic effects of chrysanthemin on the PC-3 human prostate cancer cells were evaluated by fluorescence microscopy using acridine orange/propidium iodide double dye. The cells were treated with 0, 10, 50 and 150 µM dose of chrysanthemin for 48 h and then analyzed under the microscope at a magnification of x400. White arrows indicate the cells which have undergone early and late apoptosis.

Chrysanthemin induced loss of mitochondrial membrane potential in PC-3 cells

As mitochondrial membrane potential is a key factor in regulating cellular metabolism in all cells, next we observed the effect of chrysanthemin on the mitochondrial transmembrane potential in human prostate cancer cells. For this purpose, rh-123 which is a fluorescent dye was used in combination with flow cytometer. Results revealed that chrysanthemin led to a dose-dependent loss of mitochondrial membrane potential. The percentage of cells with depolarized mitochondria increased from 5.3% in untreated control cells to 27.2%, 57.6% and 86.9% in cells treated with 10, 50 and 150 µM dose of chrysanthemin respectively (Figure 5 A-D).

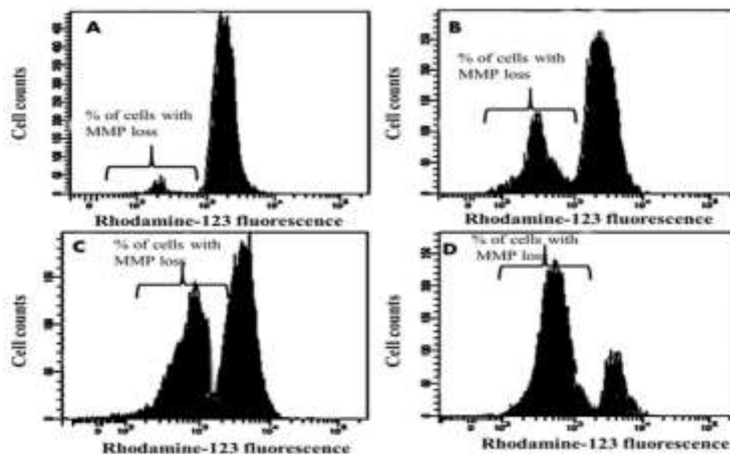

Figure 5: Chrysanthemin induced loss of mitochondrial membrane potential in PC-3 human prostate cancer cells. The cells

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after treatment with 0 (A), 10 (B), 50 (C) and 150 (D) μM dose of chrysanthemin for 48 hr were incubated with rh-123 fluorescent dye and then analyzed by flow cytometer.

Chrysanthemin induced upregulation of caspase-3, caspase-8 and caspase-9

Caspases play an important role in apoptosis induction in cells. Further, we designed our experiment in order to evaluate the effects of chrysanthemin on the expression levels of caspase-3, caspase-8 and caspase-9 using western blot assay. The results are shown in figure 6 indicating that chrysanthemin enhanced the activity of all three caspases viz., caspase-3, 8 and 9. The upregulating effect of chrysanthemin was found to be dose-dependent. As compared to the control, drug-treated groups showed higher protein expression levels which increased further as the dose increased. Thus, the apoptotic effect of chrysanthemin in human prostate PC-3 cancer cells could be thought to be mediated via involvement of the different caspases.

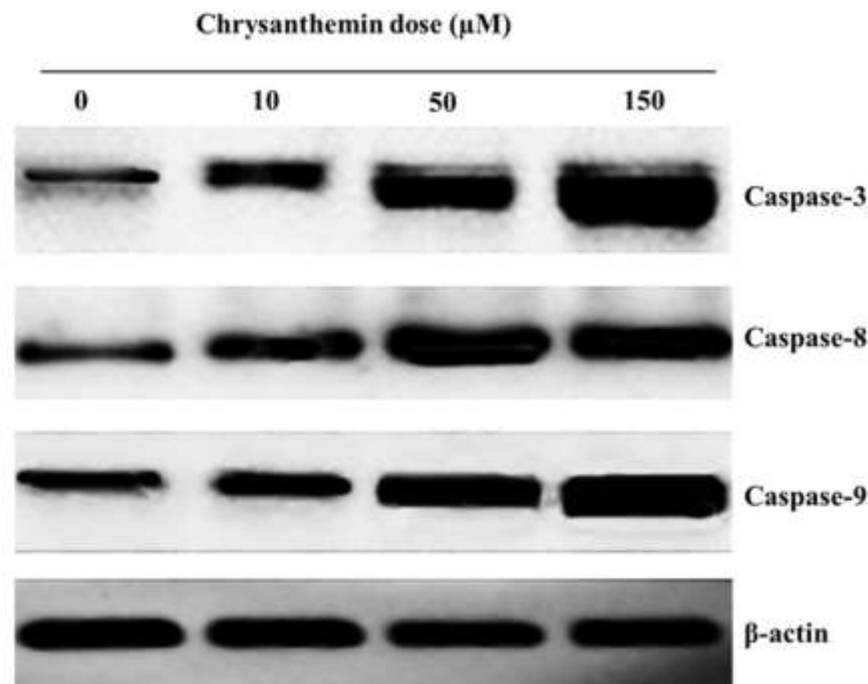

Figure 6: Chrysanthemin led to upregulation of different caspases in PC-3 cancer cells. Western blot method was used to analyze the changes in caspase protein expressions. The cells were treated with 0, 10, 50 and 150 μM dose of chrysanthemin for 48 h. β-actin was used as an internal control.

Discussion

The main objective of the present research work was to demonstrate the antitumor properties of chrysanthemin in PC-3 human prostate cancer cells using MTT cell viability assay. The effects on apoptosis induction, mitochondrial membrane potential and caspase activity were also examined. Chrysanthemin was revealed to exert growth inhibitory effects in PC-3 human prostate cancer cells in a time-dependent as well as dose-dependent fashion. Furthermore, it was observedthat chrysanthemin has the propensity to induce early and late apoptosis in PC-3 human prostate cancer cells. The percentage of cells which emitted red/orange fluorescence was shownto increase with increasing doses of chrysanthemin. As comparedto the untreated control cells which do not exhibit any signs of apoptosis, chrysanthemin-treated cells at 10, 50 and 150 μM doses led to 34.2%, 56.7% and 69.1% apoptosis in these cells respectively. Chrysanthemin also led to depolarization of the mitochondria by inducing loss of mitochondrial membrane potential. Chrysanthemin also led to increase inthe activity of all three caspases viz., caspase-3, 8 and 9 in a dose-dependent fashion.

Apoptosis, also known as programmed cell death is a highly efficient, systematized and well regulated biochemical process involved in the eradication of unwanted dead cells from the body (Wang et al., 2014; Jie-Jie et al.,

2015). Apoptosis process is regulated by a set of closely related enzymes including caspases and proteins of Bcl-2 family. It has been reported earlier that caspases including caspase-3, caspase-8 and caspase-9 play central roles in executing the process of apoptosis (Wang and Lenardo., 2000). Whereas caspase-9 was involved in the initiation of the apoptotic cascade, caspase-3 as the actual executioner of the apoptotic process leading to mostly mitochondrial mediated apoptosis. As such, with this in mind, the process of apoptosis induction in cancer cells can be readily demonstrated by evaluating the protein expressions of these caspase enzymes (Adams & Cory., 2002; Slee et al., 2001; Zhang et al., 2000). Chrysanthemin, which is the 3-glucoside of cyanidin belongs to the anthocyanin class of natural products. It can be isolated from various plants including *Hibiscus sabdariffa*, *Rhaponticum* species and some *Viburnum* species. It also occurs in various food plants including European elderberry, peach, lychee and soybean seed coats (Yoshitama et al., 1972; Vereskovskii and Chekalinskaya., 1978; Choung et al., 2001). No previous published reports could be seen documenting the anticancer effects of this naturally occurring anthocyanin in human prostate cancer cells. As such, the present research work is the first of such report on the antitumor effects of chrysanthemin.

In conclusion, it may be summarized that anticancer effects of chrysanthemin are induced in PC-3 human prostate cancer cells via the apoptosis induction, loss of mitochondrial membrane potential and activation of caspase-3, caspase-8 and caspase-9 enzymes.

Conflict of interest

The authors declare that there is no conflict of interest to reveal.

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