

Prodigiosin induced the caspase-dependent apoptosis in human chronic myelogenous leukemia K562 cell

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Abstract

Background and purpose: Chronic myeloid leukemia (CML) as a myeloproliferative disease is characterized by increased cellularity of bone marrow. Implementing the latest treatment protocols is currently accompanied by serious and life-threatening side effects. There are worldwide attempts to find new effective and potent therapeutic agents with minimal side effects on CML patients. This *in vitro* study was carried out to discover the potential antiproliferative and apoptotic effects of naturally produced prodigiosin (PDG) on K562 cells as an accepted model of CML.

Experimental approach: The anti-proliferative effect of PDG was measured by MTT assay. To highlight the mechanism of cytotoxicity, the apoptotic cell death pathway was investigated by morphological and biochemical assessments. The dual acridine orange/ethidium bromide staining technique and western blotting method were applied to assess the mechanism of the potential apoptotic impact of PDG on K562 cells.

Findings/Results: PDG-induced time- and concentration-dependent anti-proliferative effects were revealed with an estimated IC₅₀ value of 54.06 µM. The highest cell viability reduction (60%) was recorded in cells, which were exposed to 100 µM concentration. Further assays demonstrated that in the dual acridine orange/ethidium bromide staining method the cell population in the late apoptosis phase was increased in a concentration-dependent manner, which was confirmed with remarkable DNA fragmentation.

Conclusion and implications: We found that the PDG-induced apoptosis in K562 cells is mediated through the caspase-3 activation both in mRNA and protein levels. Our results suggest that PDG could be a potent compound for further pharmacokinetic and pharmacodynamics studies in the *in vivo* model of CML.

Keywords: Apoptosis; Caspase-3; DNA fragmentation; Prodigiosin.

INTRODUCTION

Leukemia is the most fatal type of cancer that often occurs in the bone marrow due to the abnormal increase in the number of blood cells (1-3). These blood cells are called blasts or leukemia cells (4). Chronic myeloid leukemia (CML) is among the chronic proliferative diseases that originate from the exclusive mutation in the multipotent stem cells of the bone marrow. The main cause of CML is the two-way translocation of the BCR gene

(chromosome 22) and ABL gene (chromosome 9). The BCR-ABL proto-oncogene is the result of this translocation, which produces a tyrosine kinase protein. Tyrosine kinase causes the unregulated increase in the number of myeloid progenitor cells, thereby impairing apoptosis (i.e. programmed cell death) (5-7).

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The average age of the CML patients is 50-60 years but it has been reported from all age groups including children. CML accounts for almost 15% of all leukemia cases and 7-20% of the adult leukemia cases (8).

Leukemia is currently managed using therapeutic methods such as radiotherapy and chemotherapy. These methods are challenged by many problems including the side effects of chemical compounds and drug resistance. Therefore, researchers are currently searching for other compounds with minimal side effects and greater anticancer effects for the treatment of leukemia (9). Today, most of the available and commercial anticancer drugs are chemically synthesized agents that considerably affect the patients. In addition to the cancer cells, they affect the healthy and natural cells and unfortunately lose their effect after a while. Hence, it is extremely important to find new and effective therapeutic agents with minimum side effects that can eliminate the targeted cancer cells.

Among the other natural substances with therapeutic potency secondary-bacterial metabolites have garnered the attention of researchers. An example of these compounds is 3-methyl-6-pentyl-6-methoxy (prodigiosin, PDG), which is extracted from the cell wall of *Serratia marcescens* bacterium (8,10). The anti-proliferative effect of this bioactive red pigment has been demonstrated in different types of malignant cells. The molecular studies showed that PDG-induced apoptosis cell death through down-regulation of survivin in human breast carcinoma cells (11).

Apoptosis is a natural cellular process that removes old, damaged cells and prevents cells from acquiring tumorigenic potential. Any disorder in the apoptotic pathway will lead to disease and the growth of abnormal cells such as cancer (12). Caspases as a class of cysteine proteases are crucial mediators of programmed cell death (apoptosis). Caspases are mainly divided into two groups according to their functions in apoptosis (caspase-3/6/7/8/9) and inflammation (caspase-1/4/5/12) (13). The intrinsic and extrinsic pathways of apoptosis involve two major caspase cascades leading to apoptosis. Caspase-3 is cleaved and activated by both caspase-8 and caspase-9 initiator

caspases. To form the active enzyme caspase 3, it must be cleaved at an aspartate residue to p12 and p17 subunit (14). Activated caspase-3 is responsible for morphological changes and DNA fragmentation in cells during apoptosis (15).

The anti-proliferative and apoptotic effects of PDG on some cancer cells have been documented, while the data regarding its effects and molecular mechanism of action on K562 cells are scarce. The goal of this research was to study the effect of PDG on the proliferation and possible cell death mechanism in the K562 cell line as an accepted model of CML in humans.

MATERIALS AND METHODS

Chemicals and kits

PDG (Sigma-Aldrich, CAS 82-89-3) was dissolved in ethanol and the 10 mg/mL primary stock solution was prepared and stored at -20 °C. For all the tests, the PDG stock solution was used to prepare 5 to 100 μ M concentrations. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, Phosphate-buffered saline (PBS) buffer, Tris-ethylenediaminetetraacetic acid (Tris-EDTA) buffer, and proteinase K were bought from Sigma-Aldrich Company and all the other chemicals and solutions were bought from Merck (Germany).

Cancer cells culture and preparation

The K562 cancer cell line was obtained from the cell bank of the Pasteur Institute of Iran. The cells were cultured in the RPMI1640 culture medium, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, England), in an incubator (Memert, Germany) with 5% of CO₂ at 37 °C temperature. The cell morphologies were examined constantly with an inverted microscope during the culturing period.

Cell viability measurement

The K562 cells were cultured in a cell culture flask to obtain a density of 90-100%. Thereafter, the cells were plated in a 96-well (1×10^4 cells/well). The cells were treated with PDG at 5, 50, and 100 μ M in triplicates. Next, the plates were incubated for 24, 48, and 72 h at

37 °C. After the incubation process, 20 µL of the MTT solution (5 mg of MTT powder in 1 mL of PBS) was added to each well, and incubation was performed for 4 h and thereafter formazan crystals were dissolved with pure dimethyl sulfoxide (DMSO). After 15 min of incubation at room temperature and dissolution of the formazan particles, the absorbance of the control and treated cells was recorded at 570 nm wavelength using an ELISA reader (Biotek, USA). The viability rate of the cells was calculated using the following equation:

$$\text{Viability rate (\%)} = \frac{\text{Sample OD}}{\text{Control OD}} \times 100$$

DNA fragmentation test

The K562 cells were removed from the culture flask and were cultured with a density of 1×10^6 cells/well in a 6-well plate. Thereafter, the cells were treated with PDG at 5, 50, and 100 µM. The control cells were treated with the solvent of PDG (maximum concentration of ethanol as the solvent of PDG was not exceeded from 1% as the treated cells). These plates were incubated for 48 h at 37 °C and 5% CO₂. After 48 h, the cells in each group were separately collected for DNA extraction. To extract DNA, 600 µL of a lysis buffer (containing 100 µM of sodium chloride, 10 mM of EDTA, 50 mM of Tris, and 0.5% sodium dodecyl sulfate at pH 8) was added to the cells and was placed at the 56 °C for 2 h with 5 µL of proteinase K. The DNA of the cells was extracted using the phenol:chloroform:isoamyl alcohol method (25:24:1). The upper solution was carefully removed and transferred into clean microtubes. Thereafter, 30 µL of 6 M NaCl solution and two times the solution volume of absolute ethanol were added to each sample to settle the DNA in the samples. For the settlement of the extracted DNAs, the samples were stored overnight in a freezer at -20 °C. Next, the DNA deposit was dissolved in a Tris-EDTA buffer (10 mM of Tris and 10 mM of EDTA, pH 8) and was prepared for electrophoresis. The prepared DNA was isolated using an electrophoresis device (Bio Rad, USA) at the 80 V voltage for an hour in 1% agarose gel containing ethidium bromide. Finally, images were obtained using a gel imaging device (SYNGENE, UK) (16).

Cell death analyses by dual acridine orange/ethidium bromide staining

After treating the cells with PDG at 5, 50, and 100 µM for 48 h, 1000 µL of the cell suspension and 20 µL of the acridine orange (AO)/ethidium bromide (Eb) solution was mixed with a volumetric ratio of 1:1 and 20 µL of the mixture were mounted on a slide. Next, the cells with different stained features were photographed using a fluorescence microscope (Ecolab, USA).

RNA isolation and cDNA synthesis

K562 cells were grown in 6-well plates (1×10^6 /well) and exposed to various concentrations of PDG (5, 50, and 100 µM) for 48 h. Thereafter, cells were harvested into 100 µL TRIzol[®] solution. The total RNA was extracted from the PDG-treated and non-treated cells using the TRIzol[®] method. It was carefully tried to prevent genomic DNA contamination. The RNA level was measured through spectrophotometry at 260 nm wavelength ($260/280 = 1.8-2$). The samples were stored at -70 °C. The cDNA synthesis was carried out in accordance with the manufacturer's instructions (Fermentas, Germany) using 20 µL of the reaction mixture containing 1 µg of RNA, 1 µL of the oligo primer, 4 µL of the reaction buffer (5×), 1 µL of the RNase inhibitor, 2 µL of the 10 mM dNTP mixture, and 1 µL of reverse transcriptase. For 20 µL of the reaction mixture, the reaction lasted for 5 min at the 60 °C, and then 60 min at the 42 °C, and finally 5 min at the 70 °C temperature.

Real-time polymerase chain reaction analyses

The real-time polymerase chain reaction (RT-PCR) reaction was conducted with a total volume of 25 µL containing 12.5 µL of PCR master mix, the reverse primer (0.5 µL), and the forward primer (0.5 µL), cDNA as the pattern (1 µL), and 10.5 µL of nuclease-free water. Thereafter, PCR was run in the following condition: 15 min at 95 °C, 40 cycles at 95 °C for 20 s, 57 °C annealing temperature for caspase-3, and 57 °C temperature for β-actin for 60 s, and general elongation for 1 min at the 72 °C temperature and 5 min at the 72 °C temperature.

Table 1. Nucleotide sequence and product size for used primers in quantitative polymerase chain reaction.

Genes	Primer sequences (5'-3')	Product size (bp)
Caspase-3	Forward: GAACTGGACTGTGGCATTGAG Reverse: GGCACAAAGCGACTGGATG	165
β -Actin	Forward: CTGGAACGGTGAAGGTGACA Reverse: TGGGGTGGCTTTTAGGATGG	161

Primer pairs shown in Table 1 were designed according to the melting temperature and primer-dimer formation and were blasted in ncbi/primer-blast (<http://blast.ncbi.nlm.nih.gov/blast.cgi>). Primers were checked for primer-dimer and hairpin formation again with Generunner software (<http://www.generunner.net>). Primers manufactured by Genfanavarán (Genfanavarán. Co. Tehran, Iran).

Western blotting for the caspase-3 expression

K562 cells were grown in 6-well plates (1×10^6 /well) and treated with various concentrations of PDG (5, 50, and 100 μ M) for 48 h. Total protein concentrations were assessed by a BCA protein assay kit (Thermo Fisher Scientific, MA, USA) and equal protein amounts of boiled samples (20 μ g) were separated by electrophoresis (Criterion TM Gel, 4-20 % Tris-HCL, Bio-Rad Laboratories Inc., USA) and electro-transferred onto polyvinylidene difluoride membranes, facilitated by Turbo Trans-Blot Transfer Pack (Bio-Rad Laboratories Inc., USA). The membrane was blocked with tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) supplemented with 5% bovine serum albumin (BSA) for 1 h. Afterward, the membrane was incubated overnight at 4 °C with monoclonal antibodies against caspase-3 (1:1000) or β actin (1:1000) (Santa Cruz, Dallas, Texas, USA). The membrane was washed with TBST and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:5000, Dako, Glostrup, Denmark) for 2 h at room temperature. Eventually, the blot was washed in TBST and incubated with ECL prime western blotting detection reagent (Amersham Biosciences, Roosendaal, The Netherlands). Digital images were obtained with the Chemi Doc TMMP imager (Bio-Rad Laboratories Inc., USA). Signal intensity was quantified using the Image J 1.52 software and

the protein expression was normalized with β -actin and expressed as mean fold change in relation to the control group.

Statistical analysis

The results were analyzed in Graph Pad Prism software (version 8.01, Graph Pad software Inc., San Diego, CA) using the One Way ANOVA statistical test to compare the effects of different concentrations of PDG with the control group, $P < 0.05$ was considered significant. Data were expressed as mean \pm SD.

RESULTS

PDG reduced the viability of the K562 cells

The results of the MTT assay revealed that PDG reduced cell viability as compared to the control group. It was found that PDG reduced the cell viability in K562 cells in a concentration- and time-dependent manner. The highest cell viability reduction (60%) was recorded in cells, which were exposed to 100 μ M of PDG for 72 h (Fig. 1).

The data was analyzed with a specific software of Compusyn and the IC₅₀ value was calculated. The calculated IC₅₀ value for the test compound was 54.06 μ M for 72 h exposure time.

DNA fragmentation enhanced by PDG treatment in K562 Cells

The results indicated that the 48-h treatment of K562 cells with different PDG concentrations changed the DNA pattern of the treated cells as compared to untreated cells (Fig. 2). The DNA of the treated K562 cells was smeared with an increase in concentration, which is the marker of DNA fragmentation into smaller segments. However, this was not observed in the control cells. These results showed the induction of apoptosis by PDG on the K562 cancer cells.

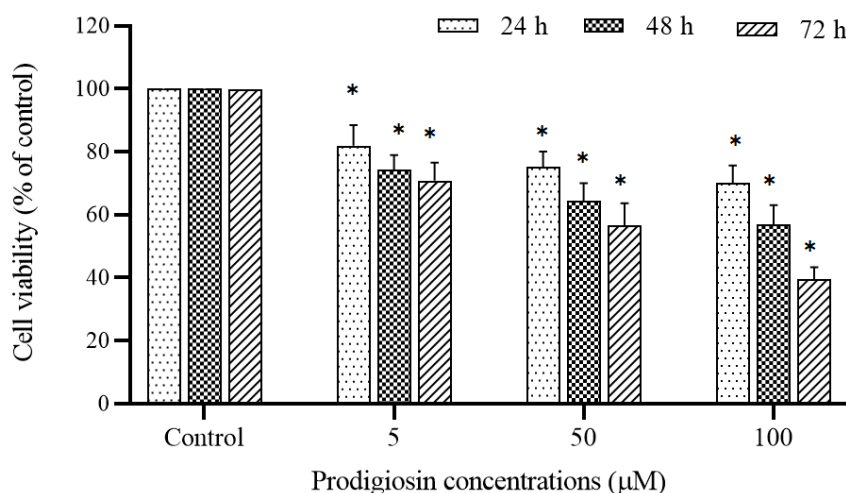


Fig. 1. Effects of prodigiosin after 24, 48, and 72 h treatment on K562 cancer cell viability. Data are expressed as mean \pm SD for each concentration in triplicates. * $P < 0.05$ Represents significant differences compared with the corresponding control group.

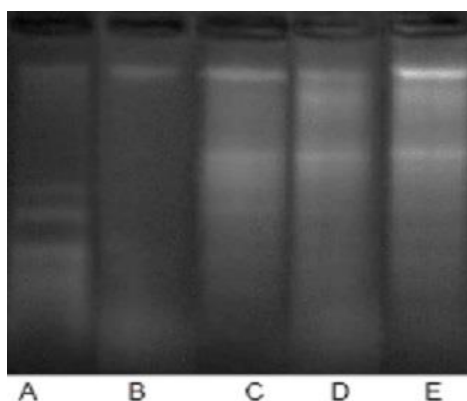


Fig. 2. Effect of prodigiosin on DNA fragmentation in K562 cells. A, DNA ladder marker; B, untreated cells; C-E, cells treated with 5, 50, and 100 μ M of prodigiosin for 48 h.

PDG-induced apoptotic cell death was screened by dual AO/Eb staining

The dual AO/Eb staining technique was applied to estimate the potential apoptotic impact of PDG on K562 cells. This method demonstrated that in the control group the majority of the cell were in green color indicating their healthy condition, while in PDG-exposed groups of cells, the number of yellow, orange and red-stained cells were increased exponentially to concentration increase, indicating the elevation of early and late apoptosis and necrotic cells, respectively (Fig. 3).

PDG up-regulated the expression of caspase-3 in K562 cells

The expression of the caspase-3 gene at both mRNA and protein level, as one of the important genes involved in apoptosis induction, was analyzed using the RT-PCR and western blotting techniques. A concentration-dependent up-regulation of caspase-3 at mRNA level was obtained, which corresponds to the cell viability assay results (Fig. 4A). The same profile of up-regulation was observed in the western blot analyses as presented with concentration-dependent up-regulation of cleaved caspase-3 protein (Fig. 4B-C).

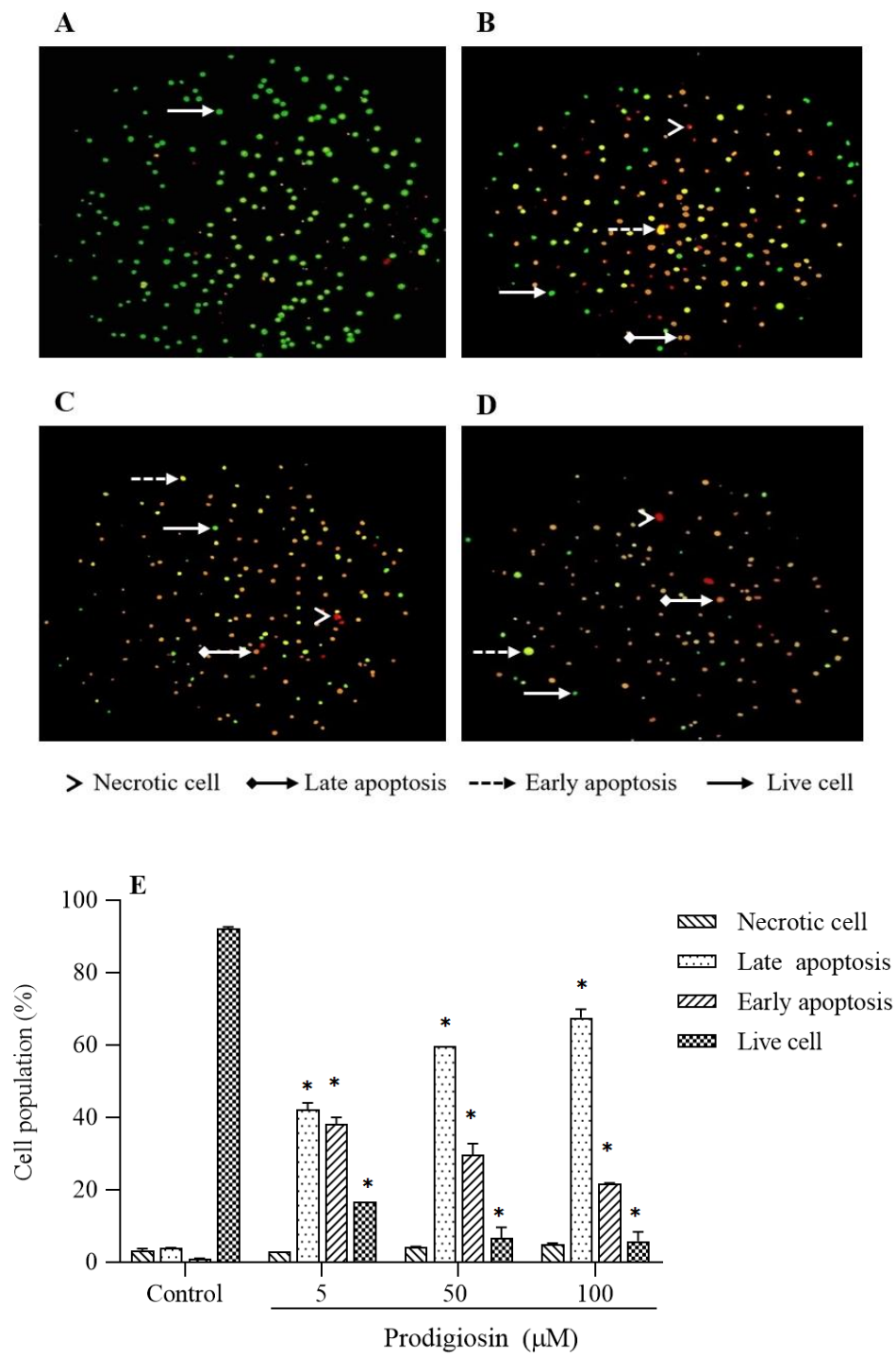


Fig. 3. Effects of PDG on K562 cell death processes. The dual acridine orange/ethidium bromide staining method was conducted on K562 cells. The distribution of cells undergoing early and late apoptosis and/or necrosis are monitored by green cells for healthy and yellow, orange, and red cells are representing the early and late apoptotic and/or necrotic cells, respectively. (A) Control cells and (B-D) the cells were exposed against 5, 50, and 100 μM of PDG for 48 h, respectively. (E) The density of each color was quantified by MATLAB R2019b. * $P < 0.05$ Represents significant differences compared with the corresponding control group. PDG, prodigiosin.

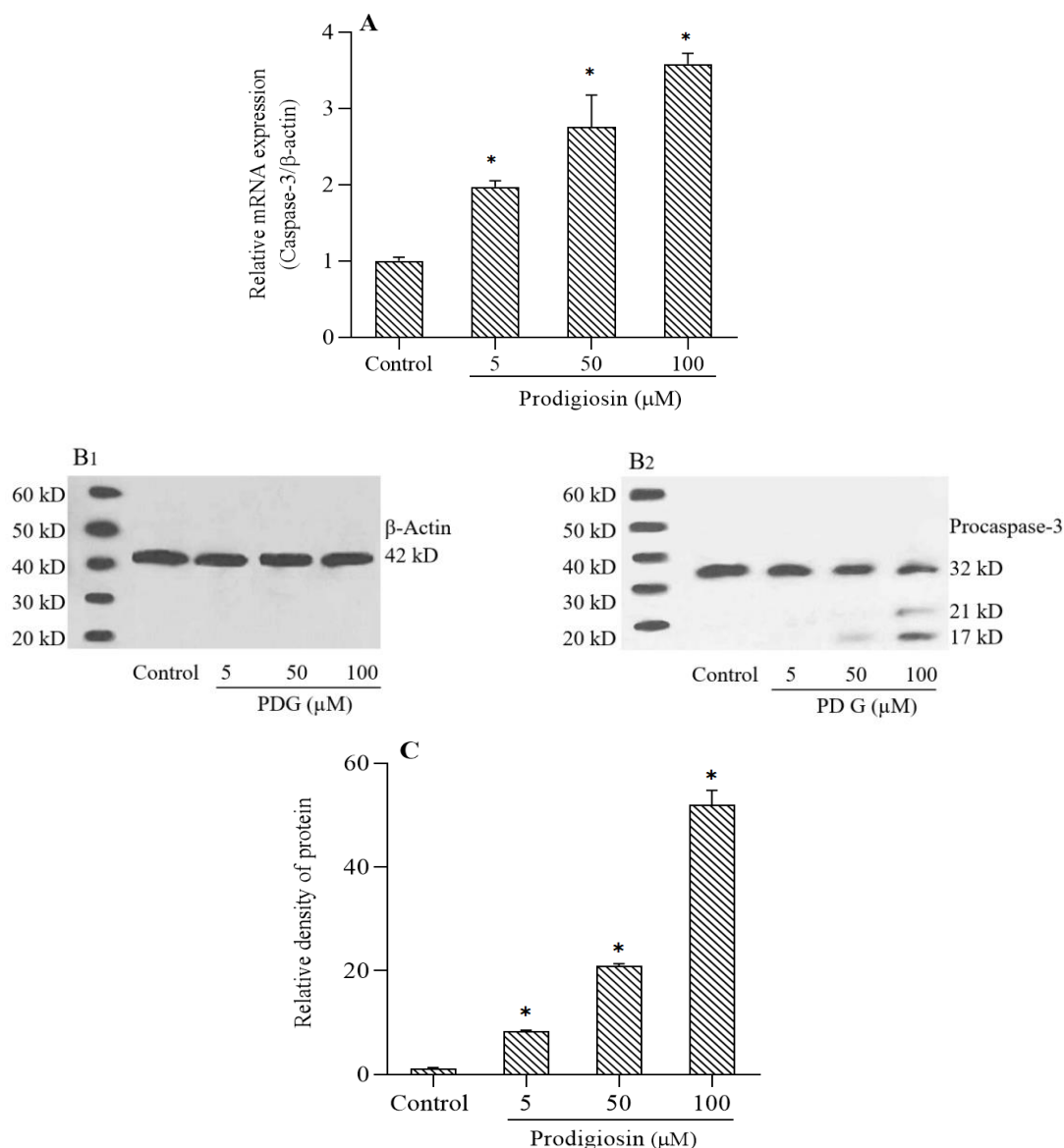


Fig. 4. Effect of PDG on (A) the expression of caspase-3 at mRNA Level, (B) the expression of cleaved caspase-3 in the K652 cells which exposed against various concentrations of PDG for 48 h; the expression of cleaved caspase-3 was normalized based on the expression of β-actin, and (C) densitometry analyses of cleaved caspase-3. **P* < 0.05 Indicates significant differences compared with the control group. PDG, prodigiosin.

DISCUSSION

In the current *in vitro* study, we discovered that PDG as a naturally produced compound is able to inhibit cell proliferation in K562 cells as a proven model of CML. We further highlighted the possible cytotoxic pathways such as apoptosis induction, which was characterized by DNA fragmentation in PDG-treated cells. Ultimately the expression of caspase-3 as one of the crucial genes involving in the apoptosis induction was down-regulated following PDG treatment both in mRNA and also in protein levels. One of the key steps in the preclinical phase of new drug development

is an investigation of the proposed compound's pharmacodynamics containing the mechanism of action (therapeutic and toxic) followed by efficacy studies in animal models. The current study aimed to uncover the potential anticancer effects of PDG in K562 cells. In the very first step, we found that PDG at 100 μM in a time-dependent fashion reduced remarkably the cell viability. The calculated IC₅₀ value (54.06 μM) indicates its acceptable potency. Previous reports indicated a 5 μM concentration as calculated IC₅₀ value for imatinib as a proven first choice chemical agent against the CML on K562 cells. Comparing the anti-proliferation potency of PDG with other natural compounds

including apigenin ($IC_{50} = 140 \mu M$) and luteolin ($IC_{50} = 100 \mu M$) also supports this idea that PDG might be a potent agent in the inhibition of K562 cells proliferation (17).

To find the molecular mechanism of PDG-induced cytotoxicity on K562 cells, type I cell death was investigated. There are few morphological and biochemical characters, which describe apoptosis including cell shrinkage, chromatin condensation, apoptotic body formation, and DNA degradation. We in this study using various methods tried to uncover the molecular mechanism of the PDG-induced cytotoxicity. Firstly, the effect of PDG on one of the well-documented (if not absolute) markers of apoptotic cell death were examined and indeed we found that PDG in a concentration-dependent fashion resulted in DNA fragmentation. DNA laddering indicates an internucleosomal cleavage of DNA as the biochemical event following PDG treatment in K562 cells. Following DNA damage, in our study due to PDG-treatment, the integrity of DNA is enzymatically interrupted and initial cleavage at chromatin loop domains produce 50-300 kbp fragment and in second stage cleavage of loose parts of internucleosomal DNA generally generates ~180-200 bp multiples, which is commonly referred as "DNA ladder" (18). It is worth to be noticed that sticking on just DNA fragmentation as the sole feature of apoptosis determination would not be essentially recommended and both morphological and biochemical characteristics collectively should be examined (19).

To further highlight the upstream role player in the DNA fragmentation, any alteration in the expression of caspase-3 at both mRNA and protein levels was studied. Our findings demonstrated that the PDG-induced DNA fragmentation is mediated most likely through caspase-activated DNase (CAD). Caspase 3 activates CAD by proteolytic inactivation of the inhibitor of CAD and consequently potentiates DNA fragmentation by inactivating DNA repair elements to prevent the repair of the fragmented genome in the nucleus (20). Additionally, caspases and in particular caspase-3 cleaves poly (ADP-ribose) polymerase, which is involved in DNA repairment and maintenance of genomic stability (21). There is evidence indicating a

close relationship between caspase-3 and -7 and has also been documented that their pro-caspase forms are localized in the cytosolic part of cells. During apoptosis, although both caspases are activated only caspase-3 translocated from the cytosol into the nucleus (22). Previous reports have demonstrated the caspase-3-dependent apoptotic efficacy of PDG in various cells including human oral squamous cell carcinoma HSC-2 cells and human colorectal cancer HT-29 cells (23,24). To confirm the apoptosis induction by PDG treatment, another morphological assay was performed. The method of dual AO/Eb staining gives the primary clue about the portion of cells, which morphologically demonstrate feature of normal, early, or late apoptotic and/or necrotic cells. Our results clearly demonstrated the higher concentration of PDG, the higher number of orange stained cells indicating the higher number of apoptotic cells.

CONCLUSION

Taken together, our findings showed that PDG as a natural substance-induced anti-proliferative effect against K562 cells in a time- and concentration-dependent manner. Moreover, molecular analyses revealed that PDG induced apoptosis in K562 cells through caspase-3 activation that resulted in DNA fragmentation. Certainly, further studies are warranted to highlight all pharmacokinetic and pharmacodynamics properties of PDG. Additionally, risk assessment analyses would be another crucial point, which should be taken into account.

Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

All authors participated in the acquisition, analysis, and interpretation of data. M. Niakani contributed to the experiment design and performance along with the first draft writing of the manuscript. H. Malekinejad contributed to the study design, experiments performance, manuscript drafting, revising, and discussing. A. Majd and P. Pakzad contributed to the discussion and data analyses.

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