

Induction of programmed cell death by *Prangos uloptera*, a medicinal plant

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ABSTRACT

Inhibition of the cell growth or induction of cell death is the most promising area in cancer therapy. The induction of apoptosis by dichloromethane extract of *Prangos uloptera* was evaluated on the McCoy cell line. This plant's roots, aerial parts and fruit have medicinal value. Cell growth inhibitory and cell cytotoxicity effects of the extract were assayed by MTT and Trypan-blue tests, respectively. Morphological changes and DNA fragmentation were also evaluated. The viability tests showed 0.49 and 0.3 mg/ml as 50% inhibition concentration and 50% cytotoxicity concentration after 24 hours of treatment, respectively. Fluorescent microscopy analysis revealed chromatin fragmentation and scanning electron microscopy showed cell shrinkage and cytoplasmic blebbing. These findings were confirmed by DNA fragmentation analysis. The results demonstrated efficient induction of apoptosis by the plant extract in moderate concentrations, but administration of higher concentrations showed that the primary manner of cell death was necrosis.

Key terms: Apoptosis, cytotoxicity, McCoy, *Prangos uloptera*.

INTRODUCTION

Programmed cell death or apoptosis is a genetically controlled operation leading to cell death in response to environmental or developmental signals. Cytoplasmic blebbing, cell shrinkage, chromatin condensation and nucleosomal fragmentation are the outstanding morphological characteristics of apoptosis (Wyllie *et al*, 1980a). Regulation of apoptosis induction is critical for normal development and tissue homeostasis, physiological processes in maturation, embryogenesis, and normal tissue turnover, as well as cancer therapy. Disruption of this process can have severe consequences (Jacobson *et al*, 1997). Too many cells dying may produce neurodegenerative diseases and impaired development, while insufficient cell death can lead to increased susceptibility to cancer and sustained viral infection (Watters & Lavin, 2005). The mechanisms of killing and

degrading cells via apoptosis are expressed constitutively and is activated through various stimuli (Rodriguez-Nieto & Zhivotovsky, 2006). Many antitumor agents, such as cisplatin, mitomycin, and actinomycin D have been reported to induce an apoptotic type of death in susceptible cells, thus apoptosis in tumor cells plays a critical role in killing tumor cells during cancer therapy (Adjei & Rowinsky, 2003).

In a previous report, we described the antiproliferative potential of some *P. uloptera* roots (Razavi *et al*, 2009a). We also revealed that the plant's roots were dominated by a coumarin compound that could be responsible for the observed biological activity of plant (Razavi *et al*, 2009b) The present study investigated the conditions and morphological and molecular properties of apoptosis induction using *P. uloptera* root extract on the McCoy cell line. This is the first report of apoptosis induction by extracts of *Prangos uloptera*.

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MATERIALS AND METHODS

Cell line and culture conditions

The McCoy cell line was obtained from the national cell bank of Iran (NCBI: C123). The cell culture was maintained in an RPMI 1640 (Gibco) medium supplemented with 10% fetal bovine serum, 100U/ml penicillin and 100 mg/ml streptomycin in 25 cm² culture flasks at 37°C in a humidified atmosphere with 5% CO₂. In the following assays, all cells had a passage number of 3- 5.

Plant materials

Plant material preparation and the plant root extraction with dichloromethane (DCM) were carried out exactly as described by Razavi *et al.*, (2009a).

Analysis of cell viability

Cell viability and antiproliferative effects of the plant extracts were evaluated by the reduction of MTT and trypan blue exclusion assay (Freshney, 2005; Hansen *et al.*, 1989; Vian *et al.*, 1995).

McCoy cells in exponential growth stage were harvested from culture and resuspended in a fresh medium. The cell suspension was dispensed into 24-well microplates at 3×10⁵ cell/well. After being cultured for 24h, the cells were immediately treated with various doses (1, 0.5, 0.4, 0.3, 0.2 and 0 mg/ml) of the plant extract for another 4, 8 and 24h. The experiments were performed in triplicate to confirm the accuracy of the results.

Cell viability was evaluated by MTT (Sigma cat. 2128) colorimetric technique and OD₅₇₀ was measured using a spectrophotometer (Doyle & Griffith, 1998; Zhang *et al.*, 2004). The wells containing only RPMI-FCS and MTT were used as the control. The IC₅₀ values (50% inhibition concentration) were defined as the concentration of an agent to induce 50% reduction in the absorbance. Viability percentage was evaluated as $OD_{\text{treatment}} / OD_{\text{control}}$ (Yeldjou, 2006).

In order to evaluate cell membrane integrity and direct counting of living and

dead cells, trypan blue assay was performed. In this method, 300 dead and living cells per sample were counted and cytotoxicity was calculated as follow: Cytotoxicity (%) = [(dead cell number/total cells) × 100]. The CC₅₀ values (50% Cytotoxicity Concentration) were defined as the concentration of an agent to induce 50% cell death.

Morphological assessment of apoptotic cells

An inverted phase contrast microscope was used to observe the morphological changes of the cells. For this purpose, cells were inoculated at 3×10⁵ cell/well in 24 well microplates and treated the same as in above-mentioned manner. Other culture wells were treated by Actinomycin D (50μM) and H₂O₂ (100 μM) as positive control of apoptosis and necrosis, respectively, and some culture wells were prepared without any treatment as negative control. After being cultured for 16h, the culture media was removed and cells were fixed and stained by the standard hematoxylin-eosin method. The prepared samples were photographed at × 100.

Scanning electron microscopy (SEM)

McCoy cells grown on glass coverslips were incubated with the above mentioned concentration of the plant extract for 16 h. The treated and untreated cells were fixed according to Qi, *et al.* (2005). The coverslips were photographed directly without coating, under fixed aperture and by a Quarter backscatter detector on a Leo- 1430VP Scanning electron microscope.

Morphological assessment of apoptotic cell nucleus

For assessment of the cell nucleus change in apoptotic cells, the above mentioned treated cells were fixed according to (Hong *et al.*, 2000) and stained by ethidium bromide/ Acridin orange (EtB/AO) according to Ribble *et al.* (2005). Stained cells were washed in PBS and observed under a fluorescent microscope.

DNA fragmentation analysis

The cells in the culture flask were treated with DCM extract of *P. uloptera* roots during exponential growth for 16h. The DNA was extracted from the cells using a DNP- DNA isolation kit (Cinagene, Iran) according to the supplier's manual, and then 10 μ l of the extract was loaded onto a 1.5% agarose gel. Actinomycin D-treated cells were used as positive control of apoptosis.

RESULTS

In this study, the exponentially growing McCoy cell line was treated with different concentrations of the DCM-extract of *P. uloptera* roots ranging from 0.2 to 1 mg/ml, and cell viability was measured by MTT and trypan blue assay. The results showed that the viability of McCoy cells was clearly inhibited in a time and dose-dependent manner. MTT assay indicated that different concentrations of the extract reduced the viability of McCoy cells after 4, 8 and 24 h of incubation with IC₅₀ value of 1.08, 0.59 and 0.49 mg/ml, respectively. The cells treated with 1 mgmL⁻¹ DCM-

extract at 4, 8 and 24 h exhibited the viability of 60, 30 and 20%, respectively. A 100% cytotoxicity was found when the extract concentrations were increased to above 1 mg/ml (Figure 1).

Interestingly, direct counting for dead and living cells using the trypan-blue exclusion test depicted that 100% of treated cells at the concentration of 1 mg/ml absorbed the dye. The results showed a CC₅₀ value of 0.4 and 0.3 mg mL⁻¹ for 8h and 24h of treatment, respectively.

Morphological study of cell shape changes was performed by direct microscopy, hematoxylin and eosin staining, AO/Etb staining and scanning electron microscopy analysis. Using an inverted phase-contrast microscope (400X), it was found that the untreated cells exhibited normal shapes, with clear outline. However, the growth of the DCM-extract-treated cells was obviously inhibited. The extract-treated cells were round, inter cellular connection became loose, proliferation was inhibited and slowed and the granules in cytoplasm increased (Figure 2, 1A-1C).

To further examine morphological changes, the treated cells were stained with haematoxylin and eosin. The shape of control cells was normal and the nuclei

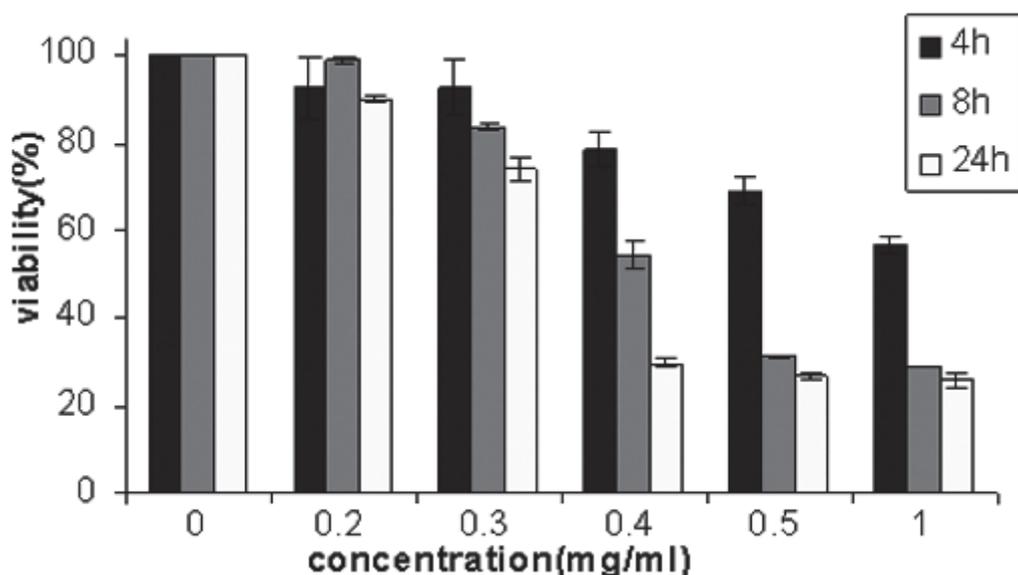


Fig. 1: The effects of DCM-extract of *P. uloptera* on McCoy cell lines that were measured using MTT cell viability assay. Bars indicate standard errors of the mean.

were round, homogeneous and dark blue, while the cells treated with 0.4 and 0.5 mg/ml after 24 h exhibited typical characteristic of apoptosis, such as nuclear condensation and fragmentation that was stained dark blue, as shown in positive control of apoptotic cells (Figure 2, 2A-2C).

In order to evaluate nucleus morphology alteration and chromatin fragmentation, treated and control cells were stained using EtB/AO and visualized by a fluorescent microscope. At the control cultures, it was found that the cell nuclei were sharp, shiny and integrated, but at a concentration of 0.5

mg/ml of the extract, treated cells contained condensed and fragmented chromatin. Fragmented chromatin was observed in all treated cell cultures.

The treated cells were studied by SEM under Fixed Aperture to reveal cell blebbing and fragmentation. The results showed normal shapes for controls, but in the concentration of 0.5mg/ml of the DCM-extract, the cells were globular and fragmented with condensed pieces. Furthermore, at 1mg/ml, the treated cells were perceived as shrunken, with irregular outlines and broken surfaces (Figure 2, 3A-3C)

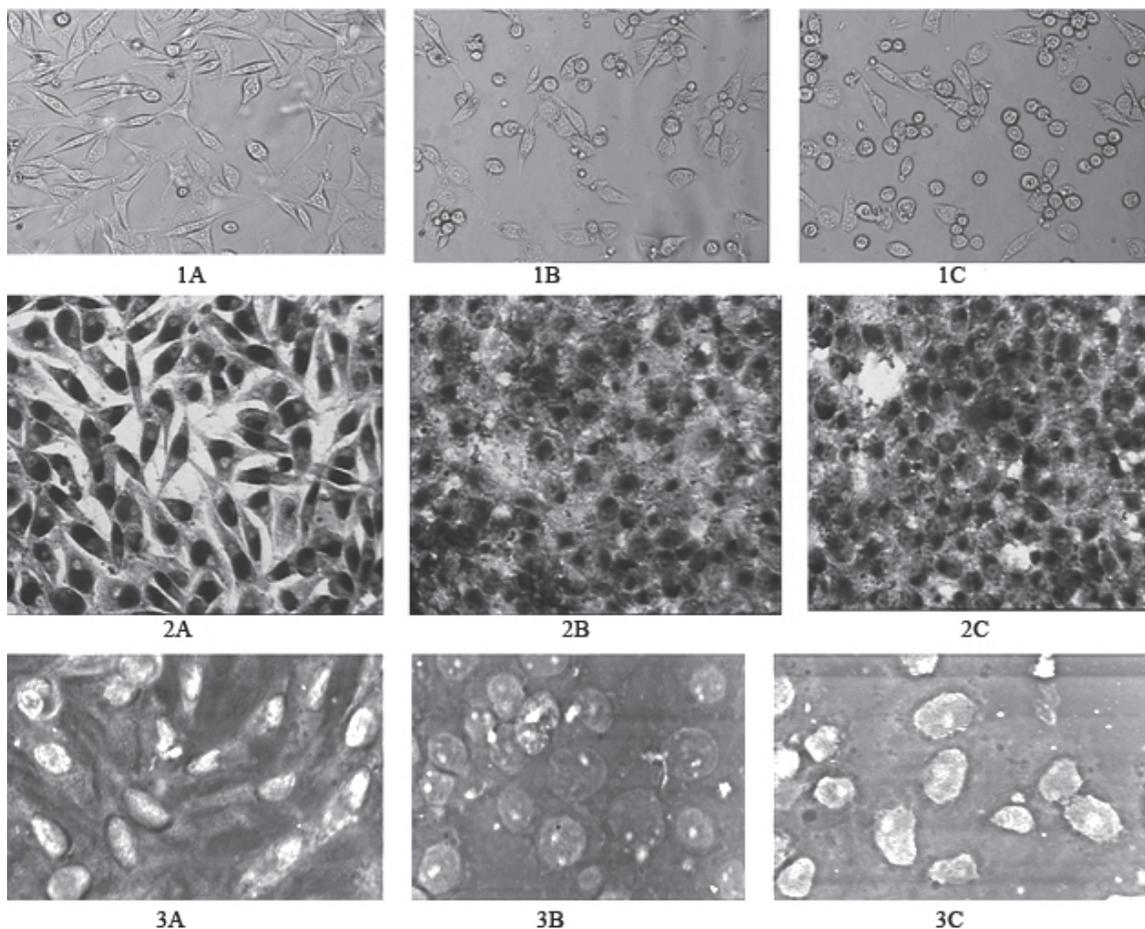


Fig. 2: The effects of DCM extract of *P. uloptera* on the morphology of McCoy cells cultured in RPMI 1640 for 24 h. Upper row: Direct microscopy study, (1A)effect of 0 mg/ml, (1B) 0.5 mg/ml and (1C) 1 mg/ml of the extract. Middle row: haematoxylin - eosin staining of treated cells, (2A) effect of 0 mg/ml and (2B) 0.5 mg/ml of plant extract and (C) effect of 50 μ M of Actinomycin D as positive control of apoptosis. Lower row: Scanning electron microscopy analysis, (A) Control, (B) Apoptotic cells -0.5mg/ml, (C) Necrotic cells -1mg/ml

Detection of DNA fragmentation was performed by agarose gel electrophoresis within 16h after treatment with the DCM-extract. The results showed that DNA fragmentation induction did not occur in the concentrations of 0.2 mg/ml, but was significantly present at 0.5 mg/ml. The intensity of fragmented DNA at 1mg/ml was less than 0.5 mg/ml (Figure 3).

DISCUSSION

Using natural products to inhibit or repress malignant growth is one the goals of cancer chemoprevention. Previous studies have reported that defects in apoptotic pathways or repression of apoptosis contribute to expansion of cancer development (Korsmeyer, 1992; Miyashita & Reed, 1993). Thus, agents that can eliminate abnormal clones by induction of apoptosis rather than merely slowing down their proliferation may have chemopreventive potential (Sun, 2001). It seems likely that rational strategies to manipulate cell suicide programs will produce new therapies that are less toxic and mutagenic than current treatment regimens (Lowe & Lin, 2000).

Our results show that the DCM-extract of *P. uloptera* has significant antiproliferative and cytotoxic effects on McCoy cells. The concentration of 1mg/ml of the extract showed 20% and 0% viability by MTT and trypan-blue exclusion test, respectively. In other words, this assay showed that 100% of cells absorbed the trypan-blue dye. This test is based on the principle that living cells possess intact cell membranes that exclude the dye, whereas dead cells do not (Strober, 2003). Rupture of the cellular membrane is one of the crucial criteria used to distinguish necrosis from apoptosis (Kim *et al*, 2001). Thus, it means that the cell membrane was disrupted and the cells died by necrosis at higher concentrations of the extract. A number of methods have been used by researchers to distinguish between apoptosis and necrosis. Assessments of membrane integrity with trypan blue dye exclusion are nonspecific unless morphological traits can be directly analyzed with the dyes (Allen, 1997). Direct microscopic study of the treated cell cultures noted changes in cell shape, discontinuity in cell-cell adhesion and slowing down of the growth rate in a dose dependent manner. Detailed studies using

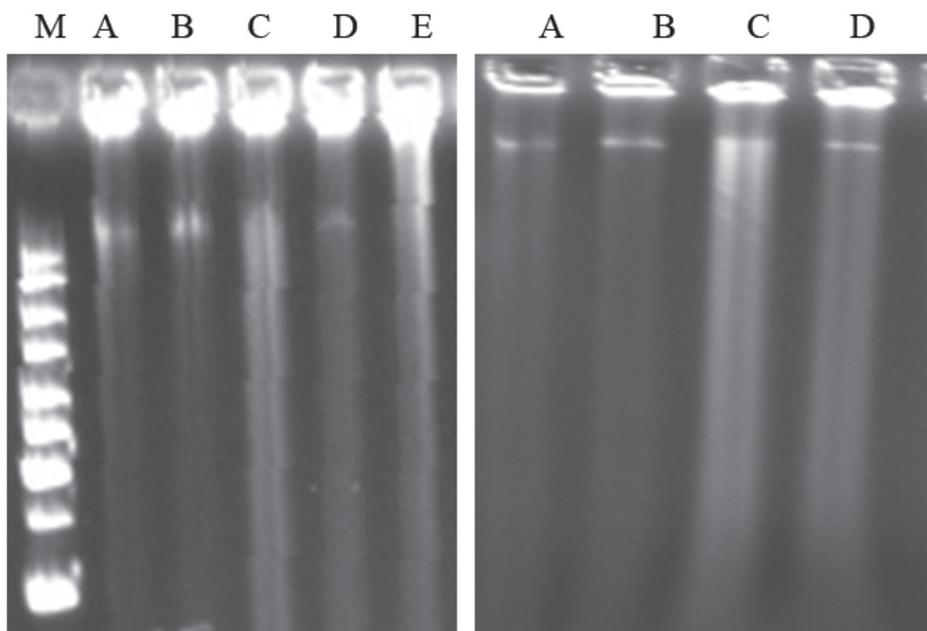


Fig. 3: An example of DNA fragmentation pattern: DNA size marker - 1kb standard (M), Negative control (A), 0.2 mg/ml (B), 0.5 mg/ml (C), 1 mg/ml (D), Positive control of apoptosis (E).

hematoxylin -eosin staining showed that the morphology of the treated cultures with 0.5mg/ml of the extract and Actinomycin D were alike. Actinomycin D is a classical anti-neoplastic drug that promotes induction of apoptosis. As well, following administration of 0.5 mg/ml of the extract, the changes in treated cells shapes, nucleus fragmentation and failure in absorption of EtB were observed by using fluorescent staining. As well, membrane blebbing and cell shrinkage were found by using SEM analysis. Thus, these findings clarified induction of an apoptosis pathway. Finally, because internucleosomal DNA crushing by undefined endonuclease activities is a highly distinguishable event and evidence of the early stage of apoptosis (Wyllie, 1980b), observation of significant and typical DNA fragmentation in the concentration of 0.5 mg/ml of the extract accurately confirmed induction of apoptosis in McCoy cell lines. Thus, these findings clearly confirmed that the DCM extract of *P. uloptera* effectively induces apoptosis and necrosis in moderate and higher concentrations, respectively. Identification and isolation of natural chemical agents from this extract and evaluation of their probable effects on cancerous cell lines is in progress.

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