

Methanolic extract of *Pereskia bleo* (Kunth) DC. (Cactaceae) induces apoptosis in breast carcinoma, T47-D cell line

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Abstract

Currently, breast cancer is the leading cause of cancer-related death in women. Therefore, there is an urgent need to develop alternative therapeutic measures against this deadly disease. Here, we report the cytotoxicity activity and the mechanism of cell death exhibited by the methanol extract prepared from *Pereskia bleo* (Kunth) DC. (Cactaceae) plant against human breast carcinoma cell line, T-47D. In vitro cytotoxicity screening of methanol extract of *Pereskia bleo* plant indicated the presence of cytotoxicity activity of the extract against T-47D cells with EC₅₀ of 2.0 µg/ml. T-47D cell death elicited by the extract was found to be apoptotic in nature based a clear indication of DNA fragmentation which is a hallmark of apoptosis. In addition, ultrastructural analysis also revealed apoptotic characteristics (the presence of chromatin margination and apoptotic bodies) in the extract-treated cells. RT-PCR analysis showed the mRNA expression levels of c-myc, and caspase 3 were markedly increased in the cells treated with the plant extract. However, p53 expression was only slightly increased as compared to caspase 3 and c-myc. Thus, the results from this study strongly suggest that the methanol extract of *Pereskia bleo* may contain bioactive compound(s) that caused breast carcinoma, T-47D cell death by apoptosis mechanism via the activation of caspase-3 and c-myc pathways.

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Keywords: *Pereskia bleo*; Cytotoxicity; T-47D cell line; Anticancer; Medicinal plants; Apoptosis; DNA fragmentation

1. Introduction

Breast cancer is the most common cancer in women in most parts of the world today. In the year 2000, there were 1,050,346 cases reported with 372,969 deaths from breast cancer worldwide. The incidence ranged from an average of 95 per 100,000 in more developed countries to 20 per 100,000 in less developed countries (Ferlay et al., 2001). In the USA alone, 184,000 cases of breast cancer are detected annually. The National Cancer Institute (USA) estimates that one in every eight women in the USA will develop breast cancer over their lifetime. Thus, breast cancer is a worldwide disease and needs to be addressed seriously.

For many years, the cytotoxic actions of the chemotherapeutic drugs were ascribed solely to their ability to induce

genotoxic death (Kamesaki, 1998). However, there were accumulating evidences that these agents exert their cytotoxic effects mainly by inducing apoptosis in tumor cells. Impairment of apoptosis is known to be related to cell immortality and carcinogenesis and the induction of apoptosis in neoplastic cells, therefore, is vital in cancer treatment. The chemotherapeutic drugs that have been observed to induce apoptosis in vitro include etoposide, camptothecin, VM26, vincristine, *cis*-platinum, cyclophosphamide, paclitaxel, 5-fluorouracil and doxorubicin (Kaufman, 1989; Walker et al., 1991; Shinomiya et al., 1994; Havrilesky et al., 1995; Huschtscha et al., 1996). In accordance with these in vitro studies, other studies also provide evidences that chemotherapeutic agents induce apoptotic tumor cell death in vivo. Experimental studies of murine tumors have demonstrated that *cis*-paltinum, cyclophosphamide and other chemotherapeutic agents induced apoptosis in various tumors in vivo (Meyn et al., 1994, 1995). Several clinical studies have also shown

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that chemotherapy triggers apoptotic cell death in patients undergoing chemotherapy (Gorczyca et al., 1993; Moreira et al., 1995). Thus, identification of potential chemotherapeutic agents using mechanism-based studies holds great promise for elucidating mechanisms and devising more specific and effective treatments for cancer-related diseases.

One of the approaches used in drug discovery, is the ethnomedical data approach, in which the selection of a plant is based on the prior information on the folk medicine use of the plant. It is generally known that ethnomedical data provides substantially increased chance of finding active plants relative to random approach (Chapuis et al., 1988; Cordell et al., 1991). Thus, *Pereskia bleo* (Kunth) DC. (Cactaceae), a medicinal plant used as a remedy in cancer-related diseases was evaluated for its properties. *Pereskia bleo*, commonly known as the 'jarum tujuh bilah' by the locals, belongs to the botanical family Cactaceae. It is a spiny scrub with distinct orange-coloured flowers. Some species have purple coloured flowers (*Pereskia grandifolia*). Both have been used as a natural remedy either eaten raw (leaves) or taken as a concoction brewed from dried plant. The leaves were also taken as a vegetable by some natives. This medicinal plant is believed to have anti-tumour, anti-rheumatic, anti-ulcer and anti-inflammatory activities (Goh, 2000).

Therefore, the main aims of this study were to evaluate the *Pereskia bleo* extract cytotoxic properties and to determine the possible mechanisms of cell death elicited by the extract on breast carcinoma cancer cells.

2. Materials and methods

2.1. Preparation of extracts

Pereskia bleo plant was collected from the Penang Botanicals Garden, Penang, Malaysia. The plant was identified by Dr. Shaida Fariza Sulaiman of the School of Biological Sciences, Universiti Sains Malaysia, Malaysia and the voucher specimen (No. USM-TML-001) was preserved and deposited in lab106, herbal library of School of Biological Sciences, Universiti Sains Malaysia. The leaves and stems were washed, dried and chopped finely using a blender. Sixty grams of dried material were exhaustively extracted with methanol by soxhlet extraction. The methanol extract was filtered and concentrated using a rotary evaporator and then evaporated to dryness. The recovery weight was about 20% from dried material. The extract was dissolved in DMSO (Sigma) and subsequently diluted to appropriate working concentrations.

2.2. Cell lines and culture medium

T-47D cells (human breast carcinoma cell line) were purchased from American Type Culture Collection (ATCC, Rockville). T-47D cells were cultured in RPMI 1640 (Hyclone), supplemented with 0.01 mg/ml bovine insulin

(Sigma), 10% (v/v) Fetal Calf Serum (FCS) [Hyclone], 100 U/ml penicillin (Hyclone) and 100 mg/ml streptomycin (Hyclone), 2 mM L-Glutamine (Hyclone) and 1 mM sodium pyruvate (Hyclone).

2.3. In vitro cytotoxicity assay

In vitro cytotoxicity assay was carried out using methylene blue assay as previously described (Li and Hwang, 1991). Briefly, the cells were plated onto 96-well plates (Costar) at a density of approximately 6000 cells/plate and incubated at 37 °C in a humidified incubator supplemented with 5% (v/v) CO₂ for 24–48 h. When the cells reached a confluency between 80 and 90%, the medium was replaced with medium containing only 0.5% (v/v) FCS and the cells were incubated for further 4 h. Subsequently, the cells were treated with different concentrations of methanolic extract from *Pereskia bleo*. Control cells were cultured in 0.5% (v/v) FCS-containing medium alone. DMSO was used to dilute the extract and the final concentration of DMSO in test wells and control wells used was not in excess of 1% (v/v).

After treatment, the plates were incubated for 72 h. After incubation, glutaraldehyde was added to each well to a final concentration of 2.5% (v/v) and the surviving cells were fixed for 15 min. Then, the cells were washed with 0.15 M sodium chloride and the dead cells were carefully removed. The fixed cells subsequently were stained with 0.1 ml 0.05% (w/v) methylene blue solution for 15 min. After washing off the excess dye with 0.15 M sodium chloride solution, dye elution was carried out with 0.2 ml 0.33 M HCl. Absorbance was read at 650 nm using Vmax Kinetic Microplate Reader (Molecular Devices, USA). The number of surviving cells was determined from the absorbance.

2.4. Detection of DNA fragmentation (apoptosis)

DNA fragmentation was detected using Deadend™ Colometric Apoptosis Detection System as described by the manufacturer (Promega). Briefly, T-47D cells were subcultured into Labtek® Chamber Slides and incubated for 24–48 h. When the cells reached confluency between 80–90%, the medium was replaced with fresh medium containing only 0.5% (v/v) FCS. The cells were then incubated for a further 4 h. Subsequently, the cells were stimulated with *Pereskia bleo* methanol extract at concentration required for 50% inhibition of growth of T-47D cells (EC₅₀) for 24 h. Control cells were treated with the same final concentration of DMSO present in treated wells [1% (v/v)]. Positive control cells were treated with DNase I.

After stimulation, the cells were fixed with 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 25 min at room temperature and rinsed twice with PBS. Then, the cells were immersed in 0.2% (v/v) Triton X-100 solution for 5 min and rinsed with PBS. The cells were then incubated in 100 µl equilibration buffer (supplied with the kit) for 5–10 min. Subsequently, 100 µl of reaction buffer containing TdT enzyme

and biotinylated nucleotide mix was added to the cells, covered with coverslips and incubated for 1 h at 37 °C. The reaction was terminated with by immersing the slide in 2× SSC for 15 min followed by washing with PBS and 0.3% (v/v) hydrogen peroxide for 5 min. The slides were then incubated with 100 µl streptavidin HRP solution for 30 min, rinsed with PBS and finally incubated with DAB components until a light brown background developed. The stained cells were immediately observed under the light microscope.

2.5. Determination of the expression level of apoptotic-related genes

The mRNA expression levels of widely established apoptotic-related genes, i.e., c-myc, p53 and caspase 3 were carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) as described (Kousteni et al., 1999; Tengku Muhammad et al., 2000). Briefly, the cells were cultured in T-25 flasks and starved in medium with 0.5% (v/v) FCS for 4 h before stimulation. Concentration of *Pereskia bleo* methanol extract needed to achieve 50% growth inhibition was used to stimulate the cells over the period of 6 h. Total cellular RNA was isolated from the untreated and treated cells using Tri Reagent LS according to manufacturer's protocol. Subsequently, 1 µg RNA was reverse transcribed into cDNA and used as the template for PCR amplification.

PCR was carried out in a final volume of 50 µl containing 1× PCR buffer and 1 U Taq-polymerase (Promega), 2.5 mM MgCl₂, 200 µM of each dNTP and 10 pmol of each primer. The template was denatured for 2 min at 94 °C, followed by amplification cycles at 94 °C for 45 s, 55 °C for 1 min and 72 °C for 2 min, and terminated with an additional extension step for 10 min at 72 °C. The oligonucleotide sequences of the PCR primers used herein were designed based on the human mRNA encoding the respective genes (Table 1). The PCR conditions including the quantity of RNA and cDNA samples used to amplify c-myc, caspase-3, p53 and β-actin genes were in the exponential phase of amplification (data not shown) indicating that the conditions were optimised to be utilised for semi-quantitative studies (Kousteni et al., 1999; Tengku Muhammad et al., 2000). The mRNA level of β-actin was used as an internal control for template levels. The PCR products were electrophoresed on a

1.2% (w/v) agarose gel and visualized with ethidium bromide staining.

2.6. Ultra structural analysis of T-47D cells using transmission electron micrograph (TEM)

Cells were treated with *Pereskia bleo* methanol extract (EC₅₀ concentration) for 24 h. Subsequently, the treated cells were washed and resuspended in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.3 at 4 °C for 24 h. Cells were then pelleted and rinsed in 0.1 M phosphate buffer for 10 min and repeated three times. The cells were fixed with 1% (w/v) osmium tetroxide prepared in 0.1 M phosphate buffer for 1–2 h at room temperature and washed twice with distilled water for 10 min. Dehydration was conducted as follows; the cells were washed with 50% (v/v) ethanol for 15 min, followed by 75% (v/v) ethanol for 15 min, 95% (v/v) ethanol for 15 min and repeated, 100% (v/v) ethanol for 30 min and repeated, and, finally with 100% (v/v) acetone for 100 min and repeated. Infiltration was carried out using the mixture of acetone:Spurr's resin mix (1:1) in a rotator for 2–3 days with daily change of Spurr's mix of each sample specimen. Finally, the cells were embedded and cured at 60 °C for 12–48 h. Sectioning (Reichert Supernova Ultra Microtome) was generally preceded by the analysis of semi-thin sections (1 µm), stained at 40 °C with 1% (v/v) toluidine blue, followed by sectioning of thin sections (<1 µm). The thin sections were initially stained with uranyl acetate for 15 min, washed with distilled water and dried on blotting paper. The sections were subsequently stained with lead citrate, rinsed, dried and examined under a Philips CM 12 Transmission Electron Microscope.

2.7. Calculations and statistics

Cytotoxicity experiments were performed in triplicate and results were expressed as percentage growth inhibition of control. EC₅₀ values for growth inhibition was derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable) and computed using GraphPadPrism (Graphpad). Data are given as mean ± S.E.M.

Gene expression signals at each point of time were determined by densitometric scanning using the Gel Analysis Software Genetools (Syngene). The signals from c-myc, p53 and caspase 3 were normalized to that from β-actin and the ratio in unstimulated samples were assigned as 1.

Table 1
The sequence of primers used in RT-PCR

Primer	Sequence (5'–3')
c-myc Forward	GAACAAGAAGATGAGGAAGA
c-myc Reverse	AGTTTGTGTTTCAACTGTTC
p53 Forward	TGTGGAGTATTTGGATGACA
p53 Reverse	GAACATGAGTTTTTTATGGC
Caspase-3 forward	TCACAGCAAAGGAGCAGTTT
Caspase-3 reverse	CGTCAAAGGAAAAGGACTCAA
β-Actin forward	TCACCCTGAAGTACCCCATC
β-Actin reverse	CCATCTCTGCTGCAAGTCC

3. Results

3.1. Cytotoxicity activity of the methanolic extract of *Pereskia bleo* on T-47D cells

As shown in Fig. 1, in vitro screening of the methanolic extract of *Pereskia bleo* on breast carcinoma, T-47D

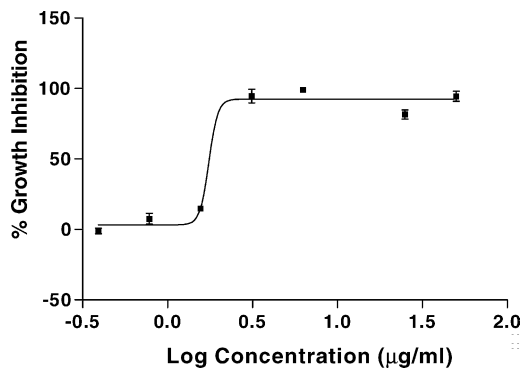


Fig. 1. Cytotoxicity effects of methanolic extract of *Pereskia bleo* on breast carcinoma T-47D cells. The cells were treated with various concentrations of the extracts for 24 h. Each value represents the mean \pm standard error mean (S.E.M.). The 100% growth inhibition corresponds to total lethality compared to control and -100% growth inhibition corresponds to 100% growth compared to controls.

cell line produced a dose-dependent inhibition on the cell growth at lower concentrations (0.391–3.125 $\mu\text{g/ml}$). Interestingly, the level of inhibition appeared to be relatively unchanged at higher concentrations (3.125–50 $\mu\text{g/ml}$). Thus, the growth inhibitory rate was $-1.0 \pm 3.4\%$ as compared to control when the cells were incubated with 0.391 $\mu\text{g/ml}$ of the extract and the inhibitory level was increased to $7.5 \pm 6.6\%$, $14.9 \pm 1.9\%$ and $94.6 \pm 8.4\%$ when the concentration of the extract was increased to 0.781, 1.563 and

3.125 $\mu\text{g/ml}$, respectively. At concentrations 3.125 $\mu\text{g/ml}$ and above, the extract killed more than 90% of the cells.

The EC_{50} value deduced from the graph for the methanolic extract of *Pereskia bleo* on T-47D cells was 2.00 $\mu\text{g/ml}$, indicating that the extract was cytotoxic as judged by the criterion set by the National Cancer Institute (Geran et al., 1972) which stated that the extracts with $\text{EC}_{50} < 20 \mu\text{g/ml}$ were considered to be cytotoxic against the treated cells.

3.2. Induction of apoptosis by the methanol extract of *Pereskia bleo* on T-47D cells

In order to determine apoptosis may play an important in mediating the cell death of T-47D cells elicited by the methanol extract of *Pereskia bleo*, a modified TUNEL using Deadend Apoptosis Detection System (Promega) was carried out. As shown in Fig. 2A and B, the cells treated with the extract produced dark brown stained nuclei with similar observation was also found in the positive control cells treated with DNase I (Fig. 2C) indicating the presence of DNA fragmentation which is the biological hallmark of apoptosis (Bowen et al., 1998). However, none of the cell nucleus was stained in the negative control cells (Fig. 2D). Thus, it is tempting to speculate that T-47D cell death elicited by the extract was mediated via apoptotic mechanism.

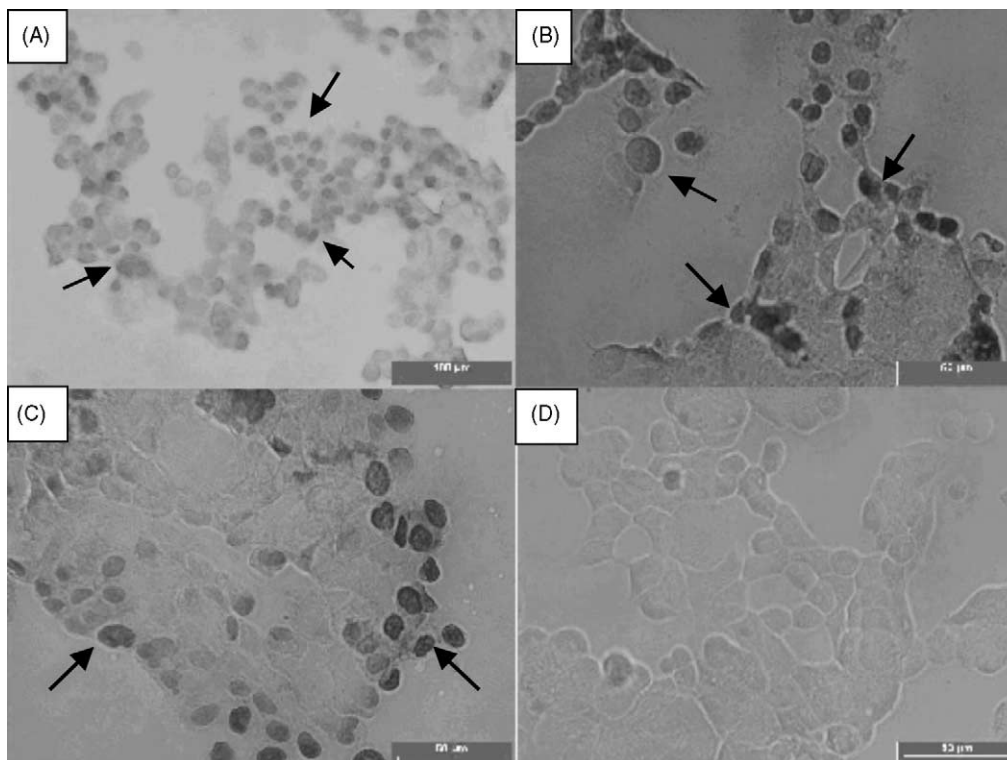


Fig. 2. Breast carcinoma T-47 cells were treated with either the methanol extract of *Pereskia bleo* (A and B) or DNase I (positive control, C) or DMSO (negative control, D) for 24 h and subjected to Deadend Apoptosis Detection System (Promega). Dark stained nuclei (arrows) of the T-47D cells were observed after cell treatment with the extract and DNase I whereas no stained nucleus in cells treated with DMSO was detected. A: 100 \times magnification; B–D: 200 \times magnification.

3.3. Expression level of apoptosis-related genes in methanol extract of *Pereskia bleo*-treated T-47D cells

In order to determine the expression level of apoptosis-related genes induced in the *Pereskia bleo* methanolic extract-treated T-47D cells, the mRNA levels of c-myc, p53 and caspase 3 were evaluated by RT-PCR. Fig. 3A and B show that the steady state mRNA levels of caspase-3 and c-myc were increased drastically when the cells were treated with the extract at 30 min (six-fold increase as compared to untreated cells) and 15 min (10-fold increase as compared to untreated cells), respectively. However, the expression levels of caspase-3 and c-myc were reduced to two- and four-fold, respectively, after 3 h incubation and increased again to six- and seven-fold, respectively, at 6 h. Interestingly, the expression level of p53 mRNA was slightly increased at 30 min and decreasing thereafter, to the level below baseline (Fig. 3A and B). Thus, the results strongly indicate that the extract killed T-47D cells through apoptosis mechanism mainly via the activation of caspase-3 and c-myc.

3.4. Ultrastructural analysis in *Pereskia bleo* methanolic extract-treated T-47D cells

Fig. 4 is a representative image of T-47D cells treated with *Pereskia bleo* methanolic extract for 24 h. It was demonstrated in Cell A, a clear morphological change was observed in the nucleus with the formation of sharply, uniformly and finely granular masses which marginated against the nuclear envelope (Fig. 4, thick arrow). A white or transparent background of the nucleus (N1) was also evident as compared to the nucleus of other cells (N2 and N3). The presence of partly degraded apoptotic bodies (A) around the cells and the phagocytosed fragments in the cell cytoplasm (F) was also evident. All these morphological characteristics are the properties of the apoptotic cells further confirming the involvement of apoptosis cell death mechanism elicited by the extract on T-47D cells. Interestingly, Cell B showed a highly vacuolated cytoplasm (V), suggesting the presence of type II non-apoptotic programmed cell death (Bowen et al., 1998; Amin et al., 2000) as a minor mode of cell death produced by the extract on the cells.

4. Discussion

Ethnopharmacological data has been one of the common useful ways for the discovery of biological active compounds from plants (Cordell et al., 1991; Cragg et al., 1994). Methanolic extracts were normally used for anticancer screening because traditional practitioners believed that mostly the polar compounds were responsible for the claimed anticancer properties. In this study, the methanolic extract of *Pereskia bleo* showed significant cytotoxic activity on T-47D cell line. The activities of this plant may be due to the presence of highly complex glycosides and

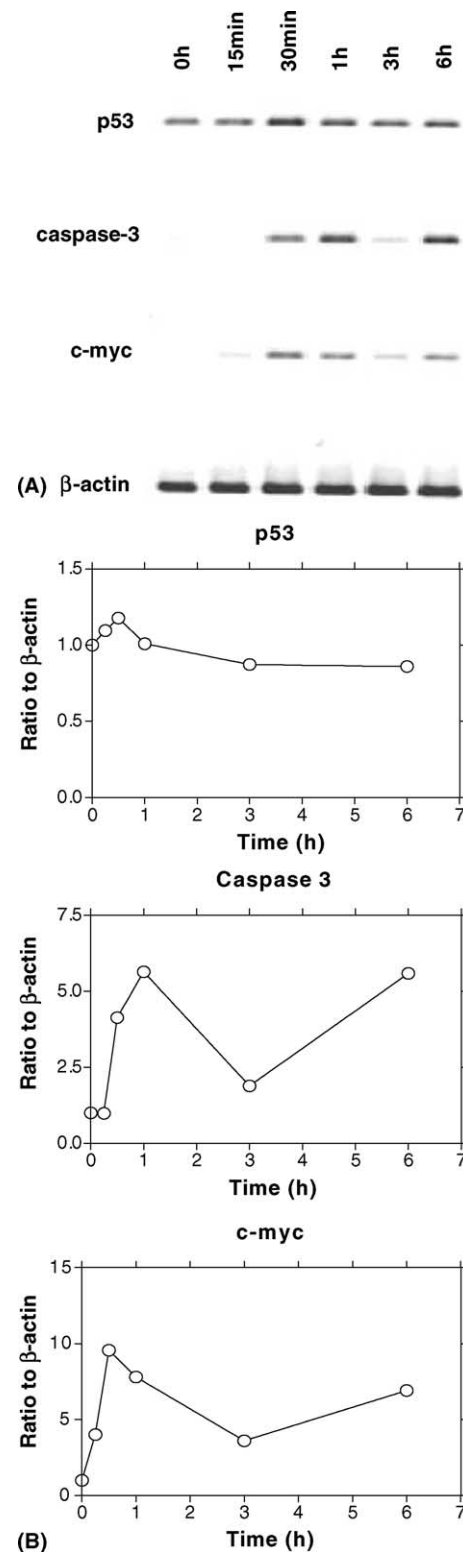


Fig. 3. (A) Time dependency effects of the p53, caspase 3 and c-myc mRNA levels in human breast cancer cell line, T-47D, incubated in the absence or presence of *Pereskia bleo* methanolic extract. β -Actin was used as an internal control for integrity and equal amount of cDNA used in each PCR reaction. (B) Graphical representation of the expression profile of p53, caspase 3 and c-myc mRNA in T-47D cells following treatment with the extract.

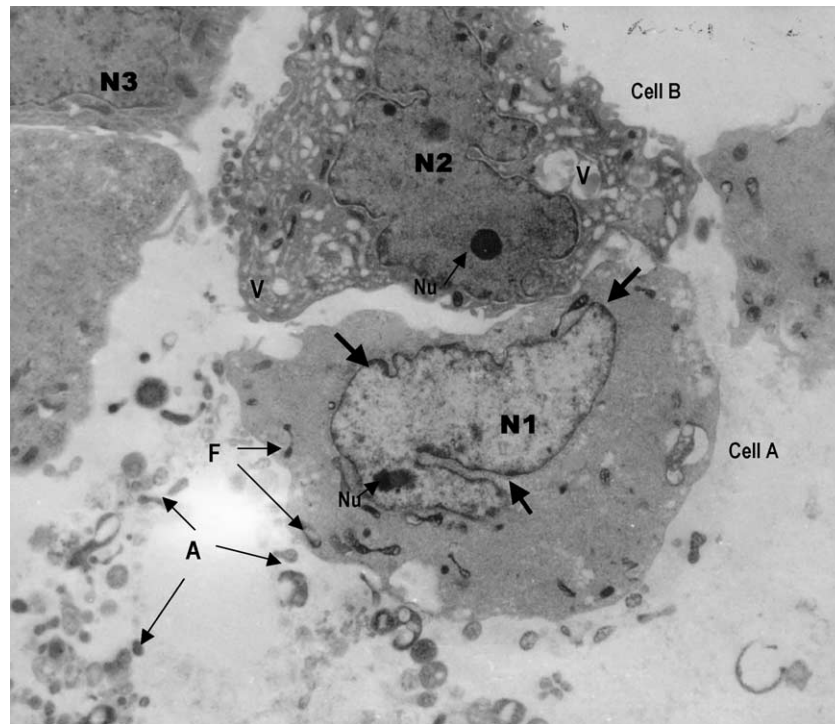


Fig. 4. Morphological characteristics of apoptosis in T-47D cells after treatment with the methanol extract of *Pereskia corrugata* for 24 h. Note the marked margination of nuclear chromatin in cell A (thick arrows) and the presence of apoptotic bodies (A) and the phagocytosed fragments in the cell cytoplasm (F). Note the highly vacuolated cytoplasm of cell B indicating the presence of type II non-apoptotic programmed cell death (7500 \times magnification). N1: cell nucleus showed chromatin margination; N2: cell nucleus showed normal chromatin distribution; N3: cell nucleus showed normal chromatin distribution; Nu: nucleolus; A: apoptotic bodies; F: phagocytosed fragments of the apoptotic bodies; V: vacuoles.

saponins that occurred in genus *Pereskia*. Some plants owed their medicinal or other uses largely, if not entirely, to the saponins of which mostly were toxic. For example, saponins of oleonolic acid were isolated from *Pereskia grandifolia* (Niranjan et al., 1974). In addition to that, some other compounds such as sitosterols and stigmaterols were found to be present in *Pereskia aculeata* (Thomas et al., 1987).

It is well known that in apoptosis, the earliest recognised morphological changes are compaction and segregation of the nuclear chromatin, with the result of chromatin margination and condensation of the cytoplasm (Kerr et al., 1972). Progression of the condensation is accompanied by convolution of the nuclear and cell outlines followed by breaking up of the nucleus into discrete fragments and by budding of the cell as a whole to produce membrane-bounded apoptotic bodies. The apoptotic bodies are quickly ingested by nearby cells and degraded within their lysosomes (Kerr et al., 1972; Kerr and Harmon, 1994). The cellular events in apoptosis are accomplished quickly, with only a few minutes elapsing between onset of the process and the formation of cluster of apoptotic bodies. T-47D cells treated with the *Pereskia bleo* methanolic extract clearly demonstrated DNA fragmentation (Fig. 3) and apoptotic cell morphology (Fig. 4) indicating apoptotic cell death as the major mechanism involved. However, the presence of type II non-apoptotic programmed cell death detected using TEM (Fig. 4) may suggest possible combination of death modes elicited by this plant.

In this study, the mRNA expression levels of three apoptotic-related genes, p53, c-myc and caspase 3, in T-47D cells treated with the extract were investigated. From Fig. 2, it was found that the apoptosis elicited by the extract on T-47D cells was mediated largely via c-myc and caspase-3 although the role of p53 cannot be totally ruled out. These findings were in agreement with many studies that demonstrated the role of caspase-3 and c-myc in inducing apoptosis.

The family of caspases formed an executionary arm that implement the apoptotic cell death processes. Caspases are normally present in the cell as proenzymes that require limited proteolysis for activation of enzymatic activity (Nunez et al., 1998). It was well documented that once activated, caspases cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery and a number of protein kinases. Collectively, these scissions disrupt survival pathways and disassemble important architectural components of the cell, contributing to the stereotypic morphological and biochemical changes that characterise apoptotic cell death (Earnshaw et al., 1999). Among the caspases, caspase-3 is the most commonly activated caspase in the apoptosis process (Janicke et al., 1998). Caspase-3 has been widely shown to mediate the limited proteolysis of the structural protein gelsolin, p21-activated kinase 2 (PAK2), focal adhesion kinase (FAK) and rabaptin 5 (Cosulich et al., 1997; Kothakota et al., 1997; Rudel and Bokoch, 1997; Wen et

al., 1997) and cleavage inactivation of DNA fragmentation factor such as DFF45 and ICAD (Liu et al., 1997; Enari et al., 1998; Sakahira et al., 1998). Evidences of caspase-3 involvement in the apoptosis process are well documented, for example, caspase-3^{-/-} mice (caspase-3-knockout mice) can survive to birth, but they exhibit perinatal mortality as a result of defects in brain development that correlate with a decrease in levels of apoptosis (Kuida et al., 1996; Woo et al., 1998). Caspase-3 was also reported to be required for apoptosis in neutrophils and activated T-cells (Woo et al., 1998).

c-myc, a member of the Myc-family of transcription factors, is a regulator of cell cycle progression and can also cause cells to undergo apoptosis (Askew et al., 1991; Evan et al., 1992). An increased expression of c-myc has been demonstrated to lead cells into apoptotic route (Wurm et al., 1986; Wyllie et al., 1987; Askew et al., 1991; Evan et al., 1992).

In conclusion, the results strongly suggest that the methanol of *Pereskia bleo* may contain bioactive compound that kill breast carcinoma cell, T-47D by apoptosis mechanism mainly via the activation of caspase-3 and c-myc pathways.

Acknowledgements

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