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# Anti-proliferative and mutagenic activities of aqueous and methanol extracts of leaves from *Pereskia bleo* (Kunth) DC (Cactaceae)

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**Abstract**

The anti-proliferative effects of the aqueous and methanol extracts of leaves of *Pereskia bleo* (Kunth) DC (Cactaceae) against a mouse mammary cancer cell line (4T1) and a normal mouse fibroblast cell line (NIH/3T3) were evaluated under an optimal (in culture medium containing 10% foetal bovine serum (FBS)) and a sub-optimal (in culture medium containing 0.5% FBS) conditions. Under the optimal condition, the aqueous extract showed a significant ($p < 0.05$) anti-proliferative effect at 200 μg/mL and 300 μg/mL in 4T1 cells and 300 μg/mL in NIH/3T3 cells, whereas the methanol extract did not show any notable anti-proliferative effect in these cell lines, at any of the concentrations tested. Under the sub-optimal condition, the aqueous extract showed a significant ($p < 0.05$) anti-proliferative effect at 200 μg/mL and 300 μg/mL in NIH/3T3 cells, whilst the methanol extract showed a significant ($p < 0.05$) anti-proliferative effect at 200 μg/mL and 300 μg/mL in both cell lines. An upward trend of apoptosis was observed in both 4T1 and NIH/3T3 cells treated with increasing concentrations of the aqueous extract. The level of apoptosis observed at all the concentrations of the aqueous extract tested was consistently higher than necrosis. There was a significant ($p < 0.05$) increase in the level of necrosis observed in the 4T1 cells treated with 300 μg/mL of the methanol extract. Generally, the level of necrosis was noted to be higher than that of apoptosis in the methanol extract-treated cells. The mutagenicity assay performed showed that in the absence of S-9 liver metabolic activation, the extract was not mutagenic up to the concentration of 165 μg/mL. However, in the presence of S-9 liver metabolic activation, the aqueous extract was mutagenic at all the concentrations tested. This study shows that both the aqueous and methanol extracts of the leaves from *Pereskia bleo* (Kunth) DC (Cactaceae) do not have appreciable anti-proliferative effect on the 4T1 and NIH/3T3 cells as the $EC_{50}$ values obtained are greater than 50 μg/mL when tested under optimal culture condition. Moreover, the aqueous extract may form mutagenic compound(s) upon the metabolisation by liver enzymes.


*Keywords:* *Pereskia bleo* (Kunth) DC (Cactaceae); Apoptosis; Methanol extracts; Aqueous extracts; Mutagenic; Anti-proliferative

## 1. Introduction

Despite the advancement of medical research and technology, the complete cure for cancer remains elusive. Current cancer therapies include radical surgery, chemotherapy and radiotherapy, which can bring about undesired physical and psychological distress to the patients. Therefore, continual global efforts in the search for novel anti-cancer compounds that possess high therapeutic efficacy and less side-effect compared to the existing anti-cancer drugs in the market are necessary.

*Pereskia bleo* (Kunth) DC (Cactaceae) (*Pereskia bleo*) is a plant commonly used by the local community in Malaysia for its medicinal properties. This plant is claimed to have medicinal properties to treat a variety of illnesses including diabetes and hypertension (Goh, 2000). There are also claims that *Pereskia bleo* can be used to prevent and treat cancer, which resulted in its popularity among certain ethnic groups in Malaysia. The local ethnic groups in Malaysia who believed in the medicinal properties of this plant generally consumed the leaves of this plant either in the raw form or by drinking the soup made by boiling the leaves of this plant in water.

In a previous study, the methanolic extract of *Pereskia bleo* was shown to be cytotoxic against a human mammary cancer cell line, T47-D (Tan et al., 2005), thus suggesting it might be effective for the treatment of cancer. However, the study was carried out under sub-optimal culture conditions. In the current

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study, the anti-proliferative effects of the water and methanolic extracts of the leaves of *Pereskia bleo* against a mouse mammary cancer cell line, 4T1, and a normal mouse fibroblast cell line, NIH/3T3 and the major mechanism of cell death, i.e. apoptosis or necrosis were evaluated.

To date, the mutagenic effect of the plant extracts has not yet been evaluated for its safety for consumption. As some of the local ethnic groups in Malaysia consume the leaves of this plant either in the raw form or by drinking the soup made by boiling the leaves of this plant in water, it was important that the mutagenic effect of the aqueous extract was examined to evaluate its safety for consumption. In addition, the aqueous extract from the leaves of *Pereskia bleo* exhibited a higher anti-proliferative effect compared to the methanol extracts of this plant.

## 2. Materials and methods

### 2.1. Preparation of extracts

The *Pereskia bleo* plant was collected from Taman Pertanian of the Universiti Putra Malaysia (UPM), Serdang, Malaysia. The plant was authenticated by Dr. E. Soepadmo from the Forest Research Institute of Malaysia (FRIM), Selayang, Malaysia. The voucher specimen was retained at FRIM for future reference.

The leaves of the plant were dried at 40 °C in a convection oven till consistent weights were obtained. Following this, the dried leaves were blended into a powder form. The powder was then extracted sequentially with hexane, chloroform, methanol and water using a soxhlet extractor. The solvents in the extracts were removed under reduced pressure at 40 °C until consistent weights were obtained. The aqueous extract was further freeze-dried until a consistent weight was achieved.

### 2.2. Cell lines and culture medium

The NIH/3T3 (normal mouse fibroblast cell line) and 4T1 (mouse mammary cancer cell line) cells were purchased from the American Type Culture Collection (ATCC, Rockville). They were cultured in RPMI 1640 medium containing L-glutamine and supplemented with 10% FBS, 1% HEPES buffer solution, 1% sodium pyruvate (100 mM) and 0.5% penicillin-streptomycin (GIBCO, Invitrogen Corp., New Zealand) in a humidified 5% $CO_2$ incubator at 37 °C.

### 2.3. Cell proliferative assay

The NIH/3T3 or 4T1 cells were plated in 96-well flat-bottomed tissue culture plates (Nunc, Denmark) at a concentration of $5 \times 10^3$ cells/well under optimal (when 10% FBS was used) or sub-optimal (when 0.5% FBS was used) conditions. Following this, various concentrations of the extracts (filtered through a 0.22 μm membrane filter) were added to the respective wells of the plate. The final volume in each well was 200 μL. The final cell concentration of $5 \times 10^3$ cells/well was chosen based on preliminary studies (data not shown) carried out to determine the optimum cell concentration for the cell proliferation assays with these.

A dose–response study was first carried out using extract concentrations of 6.25, 12.5, 25, 50, 100, 200 and 300 μg/mL (data not shown). Difficulty with solubility was encountered at extract concentrations higher than 300 μg/mL. Besides, 300 μg/mL was chosen as the maximum concentration tested as this was well above 20 μg/mL, which is the maximum $EC_{50}$ concentration for a crude extract to be considered cytotoxic according to the National Cancer Institute (Geran et al., 1972). For the objective of the current study supported by statistical analysis, the cell proliferative assay was carried out at fixed concentrations, i.e. 10 μg/mL, 50 μg/mL, 200 μg/mL and 300 μg/mL for the extracts and 1.56 μg/mL, 3.125 μg/mL, 6.25 μg/mL and 12.5 μg/mL for cisplatin.

Triplicates for each extract concentration (10–300 μg/mL) were performed and the assays were repeated at least twice. Wells containing cells only in culture medium were included as the negative controls, i.e. no anti-proliferative effect while wells containing cells and cisplatin (1.56–12.5 μg/mL) were included as the positive control. The plates were incubated in a humidified 5% $CO_2$ incubator at 37 °C for 72 h. At the end of the 72 h incubation period, the anti-proliferative effect was evaluated using the XTT kit (Roche Applied Science, Germany) as recommended by the manufacturer. The data are expressed as the mean percentages of cell survival ± standard deviation (S.D.).

The XTT assay is based on the ability of viable, metabolic active cells to cleave yellow tetrazolium salt, XTT (sodium 3′-[1-phenylaminocarbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) to form an orange formazan dye using the mitochondrial dehydrogenase. Dead cells or tissue culture medium do not participate in the reduction. The formazan product that is soluble in aqueous solution can be analysed spectrophotometrically and the percentage of cell survival can then be quantified.

### 2.4. Cell death detection by enzyme-linked immunosorbent assay (ELISA)

The mechanism of cell death, i.e. apoptosis or necrosis, was determined using the Cell Death Detection ELISA$^{PLUS}$ assay (Roche Applied Science, Germany) as recommended by the manufacturer. The assay is based on quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies which are directed against DNA and histones associated DNA fragments produced as a result of apoptosis and necrosis in cells. The kit is able to detect both apoptosis and necrosis based on the different morphological changes. In apoptosis, the DNA fragments remain within the cell membrane or apoptotic body. However, in necrosis, lysosomes containing lysosomal enzymes are released and these enzymes digest the lipid bi-layer of the cell membrane. This causes cell lysis and the release of DNA fragments from the cells.

After incubating the cells (4T1 or NIH/3T3) with the extracts, cisplatin (the positive control) or culture medium (negative control) for 72 h, the plates containing both treated and untreated cells were removed from the incubator and centrifuged. The DNA fragments released from the cells due to necrosis will remain in the supernatant layer. The supernatant was carefully