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## Cytotoxic Activity of *Pereskia bleo* (Cactaceae) Against Selected Human Cell Lines

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**Abstract:** *Pereskia bleo* (Cactaceae) was investigated for its cytotoxic effect against selected human cell lines, namely the Human Nasopharyngeal Epidermoid Carcinoma Cell Line (KB), Human Cervical Carcinoma Cell Line (CasKi), Human Colon Carcinoma Cell Line (HCT 116), Hormone-Dependent Breast Carcinoma Cell Line (MCF7) and non-cancer Human Fibroblast Cell Line (MRC-5) by using an *in vitro* neutral red cytotoxicity assay. All the crude methanolic and its fractionated extracts (hexane, ethyl acetate and water) exert no damage to the MRC-5 normal cells. The crude methanol extract and the ethyl acetate fraction of *Pereskia bleo* possessed notably high cytotoxic effect against KB cells with IC<sub>50</sub> values of 6.5 and 4.5 µg mL<sup>-1</sup>, respectively. Four compounds isolated from the ethyl acetate fraction of *Pereskia bleo* were identified as phytol, β-sitosterol, 2,4-di-tert-butylphenol and vitamin E. All four compounds have not been reported for this plant.

**Key words:** Cytotoxic activity, *Pereskia bleo*

## INTRODUCTION

*Pereskia bleo*, commonly known as Jarum Tujuh Bilah (in Malay) and Cak Sing Cam (in Chinese) by the locals, belongs to the family Cactaceae. *Pereskia bleo* is a spiny shrub with distinct orange-red flowers and has been used as natural remedy in cancer-related diseases, either eaten raw or taken as a concoction brewed from fresh plant. The leaves were also consumed as vegetables by some people. *Pereskia bleo* is believed to have anti-cancer, anti-tumor, anti-rheumatic, anti-ulcer and anti-inflammatory properties. They are also used as remedy for the relief of gastric pain and for revitalising the body (Goh, 2000).

There were only two phytochemical and biological studies reported for this plant. The earliest phytochemical study was by Doetsch *et al.* (1980) who reported the isolation of four alkaloids, namely 3,4-dimethoxy-β-phenethylamine, mescaline, 3-methoxytyramine and tyramine from *Pereskia bleo*. A more recent investigation by Tan *et al.* (2005) reported that the methanol extract of *Pereskia bleo* possessed cytotoxic effect against T-47D cells and cell death was found to be apoptotic in nature mainly *via* the activation of caspase-3 and c-myc pathways.

In view of the traditional use of *Pereskia bleo* in cancer-related diseases and the initial investigation by Tan *et al.* (2005) which suggested the potential use of *Pereskia bleo* in the treatment of breast cancer, it was thus necessary to further expand this area of research to

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other cancer cell lines. An investigation on the cytotoxic effects of the methanol and fractionated extracts of *Pereskia bleo* against selected human cancer cell lines, namely the Human Nasopharyngeal Epidermoid Carcinoma Cell Line (KB), Human Cervical Carcinoma Cell Line (CasKi), Human Colon Carcinoma Cell Line (HCT 116), Hormone-Dependent Breast Carcinoma Cell Line (MCF7) and non-cancer Human Fibroblast Cell Line (MRC-5) using an *in vitro* neutral red cytotoxicity assay was thus pursued and the findings is reported in this communication. In addition, identification of isolated compounds present in the active fraction was also performed.

## MATERIALS AND METHODS

### Plant Material

The fresh leaves of *Pereskia bleo* were collected from Petaling Jaya, Selangor, Malaysia in July to September 2006. They were identified by Professor Dr. Halijah Ibrahim of Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia and voucher specimens were deposited at the herbarium of the Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

### Instrumentation

NMR spectra were recorded on a JEOL 400 MHZ FT NMR spectrometer at 400 MHZ for  $^1\text{H}$  NMR and at 100.40 MHZ for  $^{13}\text{C}$  NMR. Internal standards used in  $^1\text{H}$  NMR spectra was TMS ( $\delta$ : 0.00) for  $\text{CDCl}_3$ ; in  $^{13}\text{C}$  NMR was  $\text{CDCl}_3$  ( $\delta$ : 77.0).

GCMS analysis was performed using a Agilent Technologies 6980 N gas chromatograph equipped with a 5979 Mass Selective Detector (70 eV direct inlet); a HP-5ms (5% phenyl methyl siloxane) capillary column (30.0 m  $\times$  250  $\mu\text{m}$   $\times$  0.25  $\mu\text{m}$ ) initially set at 60°C for 10 min, then programmed to 230°C at 3°C min $^{-1}$  and held for 1 min at 230°C using helium as the carrier gas at a flow rate of 1 mL min $^{-1}$ .

The GC analysis was performed on a Shidmadzu GC 14A equipped with a FID detector using fused-silica capillary HP-5 ms column (30.0 m length  $\times$  250  $\mu\text{m}$ ; 0.25  $\mu\text{m}$  film thickness) with helium as carrier gas at a flow rate of 1 mL min $^{-1}$ . Column temperature was programmed initially at 60°C for 10 min, then programmed to 230°C at 3°C min $^{-1}$  and held for 1 min at 230°C.

### Thin Layer Chromatography

Thin Layer Chromatography (TLC) analyses were carried out using precoated TLC plates 60 F $_{254}$  (20.25 mm thickness) purchased from Merck and were visualized in UV light (254 and/or 343 nm) and/or iodine vapour.

### Preparation of Plant Extracts for Cytotoxic Investigation

The leaves of *Pereskia bleo* (4526.79 g) were washed, dried and ground to a fine powder (752.92 g) using a blender. The dried, ground leaves (752.92 g) were then soaked in methanol (1.5 L) for 3 days at room temperature. The solvent-containing extract was then decanted and filtered. The extraction of the ground leaves were further repeated (2x) with methanol (1.5 L each time). The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator to give a dark green crude methanol extract (79.81 g, 10.60%). The methanol extract (33.58 g) was further extracted with hexane to give a hexane-soluble fraction (2.19 g, 6.52%) and a hexane insoluble residue. The hexane-insoluble residue was further partitioned between ethyl acetate-water (1:1, 100 mL : 100 mL) to give an ethyl acetate-soluble fraction (0.99 g, 2.95%). The

water layer was freeze-dried to give a brown coloured water fraction (18.14 g, 54.02%). The crude methanol and fractionated extracts were dissolved in Dimethylsulfoxide (DMSO) with the exception of the water fraction, to form stock solutions 20 mg mL<sup>-1</sup> before testing. The final concentration of DMSO in test wells was not in excess of 1 v/v%.

### ***In vitro* Cytotoxic Assays**

#### **Human Cell Lines**

Human cancer cell lines used were the nasopharyngeal epidermoid carcinoma KB, the cervical carcinoma CasKi, colon carcinoma HCT 116 and hormone-dependent breast carcinoma MCF7. The normal fibroblast cell line MRC-5 was also used. The cells were purchased from the American Tissue Culture Collection (ATCC, USA). KB cells were maintained in Medium 199 (Sigma), CasKi cells and MCF7 cells in RPMI 1640 medium (Sigma), HCT 116 in McCoy's 5A Medium (Sigma) and MRC-5 cells in EMEM (Eagle Minimum Essential Medium) (Sigma), supplemented with 10% foetal bovine serum (FBS, PAA Lab, Austria), 100 µg mL<sup>-1</sup> penicillin or streptomycin (PAA Lab, Austria) and 50 µg mL<sup>-1</sup> of fungizone (PAA Lab, Austria). The media were filter sterilized using a 0.22 µm filter membrane (Schleicher and Schuell). The cells were cultured in a 5% CO<sub>2</sub> incubator (Shel Lab water-jacketed) kept at 37°C in a humidified atmosphere. The culture was further subcultured every 2 or 3 days and routinely checked under an inverted microscope (IMT-2 Olympus, Japan) for any contamination.

#### **Neutral Red Cytotoxicity Assay**

The neutral red cytotoxicity assay was used as described previously by Borenfreund and Puerner (1984). The IC<sub>50</sub> is the concentration of extract that causes 50% inhibition or cell death and was obtained by plotting the percentage of inhibition versus concentrations of plant samples. The extract that gave an IC<sub>50</sub> value of 20 µg mL<sup>-1</sup> or less was considered active (Swanson and Pezzuto, 1990).

#### **Extraction and Isolation of Compounds 1-4**

Dried, ground leaves *Pereskia bleo* (1050.56 g) were extracted (3x) with methanol (1.5 L each time). The methanol-containing extract obtained was initially treated with charcoal, then filtered over celite and the filtrate was evaporated under reduced pressure to give a crude methanolic extract (99.44 g). Treatment with charcoal was necessary to remove the high content of chlorophyll present in the extract. The presence of chlorophyll interfered with efforts at chromatographic separation. The crude methanol extract was then further partitioned with ethyl acetate and water using a separating funnel. The solvent in the ethyl acetate-soluble layer was then removed under reduced pressure to give the ethyl acetate fraction (18.34 g). The ethyl acetate fraction was subjected to flash column chromatography over Merck kieselgel 60 (0.063-0.200 mm size); elution starting with chloroform with stepwise increment of methanol. The chloroform fraction (3.47 g) obtained was then purified over a column (3×49 cm) packed with 180 g of silica gel 60 (0.063-0.200 mm) and the components were separated by successive elution with hexane followed by hexane enriched with increasing percentages of dichloromethane, monitoring with TLC.

Compound 1 was isolated from the fraction obtained upon elution with dichloromethane-hexane (1.6:8.4). Further elution with dichloromethane-hexane (3.5: 6.5) yielded a fraction (39.6 mg) from which compound 2 was obtained. Preparative-TLC on the dichloromethane-hexane (1:9) fraction (192.7 mg) using ethyl acetate: hexane (1:9) as developing solvent yielded compound 3 (21.0 mg). Repeated prep-TLC on the dichloromethane-hexane

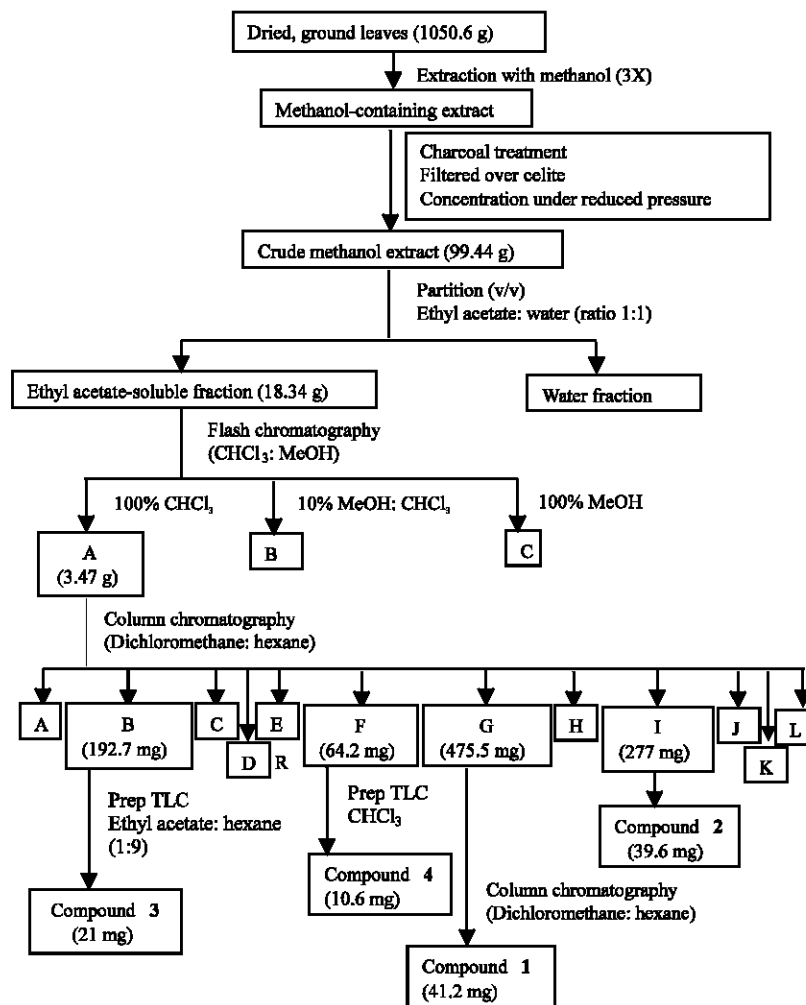


Fig. 1: Fractionation and isolation of bioactive compounds of *Pereskia belo*

(1.6: 8.4) fraction (64.2 mg) using chloroform as the developing solvent yielded 4 (10.6 mg). The extraction and fractionation procedures leading to the isolation of compounds is shown in Fig. 1.

## RESULTS AND DISCUSSION

### Cytotoxic Activity of the Crude Methanol and Fractionated Extracts

The results of preliminary cytotoxicity screening of the methanol and fractionated extracts are summarized in Table 1. The methanol extract of *Pereskia bleo* demonstrated some cytotoxic effect when screened against KB, CaSki, HCT 116 and MCF7 cancer cell lines, the highest inhibitory effect was shown by KB cells ( $IC_{50}$  6.5  $\mu\text{g mL}^{-1}$ ). The water fraction was found to have no effect on all the cancer cell lines ( $IC_{50}$  >100  $\mu\text{g mL}^{-1}$  in all cases). The ethyl acetate fraction was the only fraction that possessed high inhibitory effect against KB cells

Table 1: *In vitro* cytotoxic activity ( $IC_{50}$   $\mu$ g mL<sup>-1</sup>) of crude methanolic and fractionated extracts of *Pereskia bleo* against various cancer and non-cancer cell lines

Extracts/fractions	$IC_{50}$ ( $\mu$ g mL <sup>-1</sup> )				
	KB	CaSki	HCT 116	MCF7	MRC-5
Methanol extract	6.5	40.5	41.0	39.0	61.0
Hexane fraction	28.0	89.5	67.5	25.0	>100.0
Ethyl acetate fraction	4.5	58.0	22.0	28.0	>100.0
Water fraction	>100.0	>100.0	>100.0	>100.0	>100.0
Doxorubicin	$1.25 \times 10^{-2}$	$6.00 \times 10^{-3}$	$3.60 \times 10^{-1}$	$7.55 \times 10^{-2}$	$5.5 \times 10^{-1}$

with an  $IC_{50}$  value of 4.5  $\mu$ g mL<sup>-1</sup>. It can thus be concluded that the ethyl acetate fraction was selectively toxic against KB cells. Even though the mechanism of action has not been investigated in the present study, DNA fragmentation studies are now being planned. Chemical investigation was also directed to the ethyl acetate fraction. The active ingredients may lead to valuable compounds that may have the ability to kill cancer cells but exert no damage to normal cells ( $IC_{50}$  >100.0  $\mu$ g mL<sup>-1</sup> against normal cells, MRC-5). At this stage, no conclusion can be drawn regarding damage to normal cells as the effect of over-use of *Pereskia bleo* would need chronic toxicity studies involving experiments on animals.

The ethyl acetate fraction also displayed a slightly lower cytotoxic activity against HCT 116 and MCF7 cells ( $IC_{50}$  22.0 and 28.0  $\mu$ g mL<sup>-1</sup>, respectively). The hexane fraction was found to exert a moderate effect on KB and MCF cells ( $IC_{50}$  28.0 and 25.0  $\mu$ g mL<sup>-1</sup>, respectively). The cytotoxic effect on MCF7 was not consistent to that reported by Tan *et al.* (2005) on the effect of methanol extract of *Pereskia bleo* against T-47D cells. It is not possible to make a direct comparison of different cell lines. It would be advantageous to repeat the investigation to see the effect of *Pereskia bleo* on T-47D cells. All the fractions demonstrated weak cytotoxic activity against CaSki cells compared to other cell lines.

#### Structural Determination of Isolated Compounds 1-4

Compound 1 was identified as phytol, 2 as  $\beta$ -sitosterol, 3 as 2,4-di-tert-butylphenol and 4 as Vitamin E. All four compounds have not been reported for this plant. Further studies are in progress on the effect of the isolated compounds on KB cells and to explore its mode of action.

Compound 1 (Phytol). Yellow viscous oil; EI-MS  $m/z$  (%): 278 (2, [M<sup>+</sup>-H<sub>2</sub>O]), 137 (5, [C<sub>10</sub>H<sub>18</sub>]<sup>+</sup>), 123 (40, [M-CH<sub>3</sub>]<sup>+</sup>), 109 (15, [M-CH<sub>2</sub>]<sup>+</sup>), 95 (36, [M-CH<sub>2</sub>]<sup>+</sup>), 81 (38, [M-CH<sub>2</sub>]<sup>+</sup>), 71 (100, (CH<sub>3</sub>)<sub>2</sub>CHCH<sub>2</sub>CH<sub>2</sub><sup>+</sup>), 57 (62, (CH<sub>3</sub>)<sub>2</sub>CHCH<sub>2</sub><sup>+</sup>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 399.65 MHz) $\delta$ : 0.82 (12H, H-16, H-17, H-18, H-19, methyls), 0.95-1.61 (19H, m, methyls and methylene protons), 1.68 (3H, s, H-2), 2.00 (2H, t, J = 7.6 Hz, H-4), 4.15 (2H, d, J = 7.1 Hz, H-1), 5.42 (1H, t, J = 7.1 Hz, H-2); <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100.40 MHz) $\delta$ : 59.4 (C-1), 123.1 (C-2), 140.3 (C-3), 39.9 (C-4), 25.1 (C-5), 36.6 (C-6), 32.8 (C-7), 37.3 (C-8), 24.5 (C-9), 37.4 (C-10), 32.7 (C-11), 37.3 (C-12), 24.8 (C-13), 39.4 (C-14), 28.0 (C-15), 22.6 (C-16), 22.7 (C-17), 19.7 (C-18), 19.7 (C-19), 16.2 (C-20).

Compound 1, present as the major component in this plant, was identified by comparison of its spectral data as written above with that reported by Ming *et al.* (2004). Phytol (1) showed significant anti-tumor activity against P388 mouse lymphocytic leukemia cells (Phutdhawong *et al.*, 2004), molt 4B lymphoid leukemia cells (Hibasami *et al.*, 2002), HT-29 human colon cancer cells, MG-63 osteosarcoma cells and AZ-521 gastric cancer cells (Lee *et al.*, 1999). The cytotoxic activity of phytol was due to an induction of apoptosis (Komiya *et al.*, 1999). Based on these reported studies, it can be concluded that phytol (1) maybe responsible for the remarkable cytotoxic effect of the ethyl acetate fraction against the KB cancer cell lines.

Compound 2 ( $\beta$ -sitosterol). White coloured needles; EI-MS  $m/z$  (%): 414 (100, M<sup>+</sup>), 396 (57, [M-H<sub>2</sub>O]<sup>+</sup>), 381 (43, [M-H<sub>2</sub>O-CH<sub>3</sub>]<sup>+</sup>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 399.65 MHz) $\delta$ : 0.69 (3H, s, H-18), 0.82 (3H, s, H-27), 0.83 (3H, s, H-26), 0.85 (3H, s, H-29), 0.94 (3H, br, H-19), 1.02 (3H, s, H-21), 0.97-2.28

(m, CH<sub>2</sub>, CH), 3.54 (H-3), 5.36 (br, H-6); <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100.40 MHz)δ: 37.2 (C-1), 31.7 (C-2), 71.8 (C-3), 42.3 (C-4), 140.7 (C-5), 121.7 (C-6), 31.9 (C-7), 32.2 (C-8), 50.1 (C-9), 36.5 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.8 (C-14), 24.3 (C-15), 28.2 (C-16), 56.0 (C-17), 11.8 (C-18), 19.4 (C-19), 36.1 (C-20), 18.8 (C-21), 33.9 (C-22), 26.0 (C-23), 45.8 (C-24), 29.1 (C-25), 19.8 (C-26), 19.2 (C-27), 23.1 (C-28), 12.0 (C-29). These data were in fair agreement to that reported by Nes *et al.* (1992). In agreement with literature data (Block *et al.* (2004), Chaturvedula *et al.* (2002), Awad *et al.* (2000), Moghadasian (2000) and Jackson *et al.* (2000), β-sitosterol (2) was not known to be cytotoxic to several cell lines.

Compound 3 (2,4-ditert-butylphenol). Yellow coloured crystalline powder; EI-MS m/z (%): 206 (15, M<sup>+</sup>), 191 (100, [M-CH<sub>3</sub>]<sup>+</sup>); <sup>1</sup>H-NMR (CDCl<sub>3</sub>, 399.65 MHz)δ: 1.24 (9H, s, three methyls), 1.36 (9H, s, three methyls), 6.53 (d, J = 8.39 Hz, H-6), 7.02 (dd, J = 8.39, 2.40 Hz, H-5), 7.24 (d, J = 2.40 Hz, H-3); <sup>13</sup>C-NMR (CDCl<sub>3</sub>, 100.40 MHz)δ: 151.7 (C-1), 135.2 (C-2), 123.5 (C-3), 143.0 (C-4), 124.0 (C-5), 115.9 (C-6), 30.0 (C-7), 34.7 (C-8), 31.6 (C-9, 10, 11), 31.8 (C-12,13,14). The spectral data was in agreement with that reported by Kamitori *et al.* (1984). Compound 3 is structurally related to the well known antioxidant Butylated Hydroxyanisole (BHA). It is highly probable that compound 3 has antioxidant properties too. Phenolic antioxidants were reported to exert anticarcinogenic activity, presumably through the induction of phase II detoxifying enzymes such as glutathione S-transferases and quinone reductase, which provides prevention of tumour initiation (Yoshioka *et al.*, 1995; Yu *et al.*, 1997). They can also decrease oxidative stress-induced carcinogenesis by a direct scavenging of Reactive Oxygen Species (ROS) (Tanaka *et al.*, 1997) and by inhibiting cell proliferation secondary to the inhibition of protein phosphorylation (Schreck *et al.*, 1992).

Compound 4 (Vitamin E). Yellow viscous oil; EI-MS m/z (%): 430 (34, [M<sup>+</sup>]), 205 (8, C<sub>13</sub>H<sub>17</sub>O<sub>2</sub>), 165 (100, C<sub>10</sub>H<sub>13</sub>O<sub>2</sub>). Compound 4 was identified by comparison of its mass spectral data with that reported by Pereira *et al.* (2002).

Vitamin E (4) is a dietary anti-oxidant capable of eliminating free radical damage, inducing apoptosis and decreasing oncogene expression (Bermudez *et al.*, 2007). Previous studies indicated that Vitamin E decrease the toxicity of chemotherapy without reducing its effectiveness (Lamson and Brignall, 1999; Drisko *et al.*, 2003). Bermudez *et al.* (2007) suggested that Vitamin E might be an important protective agent against ovarian cancer cell growth as well as potentially effective therapeutic adjuvant. Structures of compounds 1-4 are shown in Fig. 2.

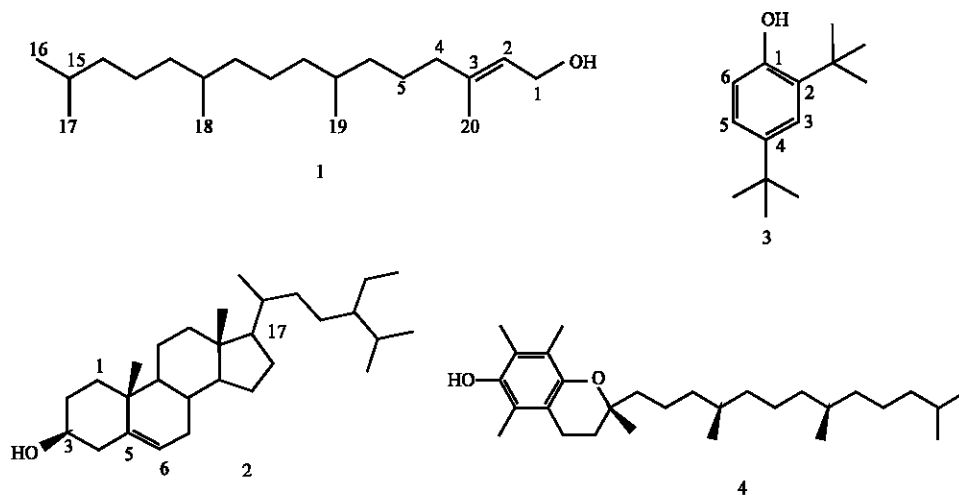


Fig. 2: Chemical compounds isolated from the ethyl acetate fraction of *Pereskia bleo*

## CONCLUSIONS

*Pereskia bleo*, which was selected for the present study based on its traditional medicinal use, showed remarkable inhibitory activity against KB cells. The ethyl acetate fraction showed very good cytotoxic activity against the nasopharyngeal epidermoid carcinoma KB cells but however, a study on the mechanism of signal transduction such as cell cycle and cell death would provide a more convincing evidence. An investigation into this phenomena is now underway. In addition this fraction also showed no cytotoxicity against the normal cell line; if this also occurs *in vivo*, the use of this plant by locals for cancer treatment would have scientific support. Thus, *Pereskia bleo* could be further developed for use in the treatment of the nasopharyngeal epidermoid carcinoma. Studies on the effect of the isolated components (present in the ethyl acetate fraction) on KB cells are now underway. The resulting information will certainly contribute to a better understanding of the anti-carcinogenic activity of the natural constituents in *Pereskia bleo*.

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