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## *Pereskia bleo* Leaves Extract Induces Cell Death via Cell Cycle Arrest and Apoptosis in Cervical Cancer Cells HeLa

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### ABSTRACT

**Introduction:** *Pereskia bleo* is a leafy and edible plant, locally known as “Pokok Jarum Tujuh Bilah” which has anticancer properties. This study purposed to determine the cytotoxic effects of *P. bleo* leaves extracts on several well-known cancer cells and elucidate its underlying mechanism in inducing cell death.

**Methods:** Cytotoxic activity on selected cell lines was determined using MTT assay. Mechanism of cell death was investigated through cell cycle and Annexin V assay. Expression of apoptotic proteins was measured by flow cytometry method.

**Results:** Ethyl acetate extract of *P. bleo* leaves (PBEA) appeared to have the strongest IC<sub>50</sub> value (14.37 ± 8.40 µg/ml) and most active against HeLa cells was further studied for apoptosis. The cell cycle investigation by flow cytometry evidenced the increment of PBEA treated HeLa cells in G<sub>0</sub>/G<sub>1</sub> phase and apoptotic event was detected in Annexin V assay. Analysis of apoptotic protein showed pro-apoptotic proteins (Bax, p53 and caspase 3) were triggered where as anti-apoptotic protein Bcl-2 was suppressed in treated HeLa cells.

**Conclusions:** Our findings demonstrated that PBEA treatment induced cell death in HeLa cells by p53-mediated mechanism through arresting cell cycle at G<sub>0</sub>/G<sub>1</sub> phase and mitochondrial-mediated pathway with involvement of pro-apoptotic proteins, anti-apoptotic protein, and caspase 3.

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### Introduction

Cancer is a condition that occurs when abnormal cells in the body proliferate uncontrollably causing normal cells to die (1). To date, cancer remains the primary cause of death in humans worldwide in spite of the advancement in tools for its diagnosis, treatment, and prevention (2). It remains a global public health issue as its mortality and morbidity rate are predicted to increase every year (3). Conclusive cancer treatment is important and urgently needed for future management of the disease. Conventional cancer treatments such as surgery, chemotherapy, and radiotherapy, even though proven to be effective, remains unsatisfactory in some types of cancer such as detrimental side effects, cancer prevalence, and decline of general health in cancer patients (4). Thus, the discovery of novel potent anticancer agents for cancer treatment will always be in demand.

Apoptosis, or programmed cell death, is critical in eliminating cancer cells via the stimulation by certain chemicals (5,6). Therefore, apoptosis activation has

been pointed out as the main strategy in inhibiting the proliferation of cancer cells (7). Recently, the application of natural products including medicinal plants has caught the attention of oncologists for alternative treatment of cancer. Both plants and their products including crude extracts and pure compounds are recognized as pro-apoptotic agents due to their various mechanism of actions and their side effects are often insignificant (8,9). Nevertheless, in traditional practice, plants are often consumed raw or in the form of crude extracts for medication purposes. Studies have reported the efficacy of plant crude extracts compared to single compounds in cancer research (10,11). Besides that, drugs that are derived from plants are often accessible, inexpensive, and safe with minimal side effects (12). Furthermore, synergistic actions from various phytochemicals in the crude extracts may be the reason behind the healing potential of the plants (13).

*Pereskia bleo* belongs to the family Cactaceae (14), a plant well known for its medicinal properties. In the Malay language, *P. bleo* is called “Pokok Jarum Tujuh

Bilah” while the Chinese calls it “Cak Sing Cam” (15,16). It is a deciduous, undergrowth, tree-like plant with a height ranging from 0.6 m to 8 m. Its leafy trunk often exists with 5–7 black spines (14). Its flowers are orange-red in color while its leaves are thin, glossy, and succulent with oblong to oblanceolate in shape (14,17,18). This plant is popular in Malaysia as well as China and the leaves are edible usually consumed raw or concocted and brewed to be taken as tea acting as a remedy for hemorrhoid, hypertension, diabetes, infections, headaches, rheumatism, asthma, and for other dietary benefits (19–21). In addition, the locals believe that it can also be used for cancer prevention (20,22,23). Earlier studies have reported that *P. bleo* biological activities include anticancer, antitumor, antirheumatic, anti-ulcer, anti-inflammatory, anti-oxidant, and antimicrobial (16,22,24).

Several in vitro studies have shown that the leaves extracts from *P. bleo* leaves demonstrated cytotoxic effects in various cancer cell lines such as human hormone dependent breast carcinoma cell line (MCF7), human lung carcinoma cell line (A549), human colon carcinoma cell line (HCT 116), human nasopharyngeal epidermoid carcinoma cell line (KB), and human cervical carcinoma cell line (CasKi) (20,22). However, there is a lack of information regarding its effects on other common cancer cell lines. Therefore, the current study aims to explore the cytotoxic response of *P. bleo* leaves extracts on HeLa, MDA-MB-231, HepG2, and SW480 cell lines. To understand the mechanism of cell death in this study, further investigation on apoptosis induction was conducted to identify the most significant cytotoxic activity among the cell lines.

## Materials and Methods

### Sample Collection

The leaves of *P. bleo* were collected from Kota Bharu, Kelantan and verified by Dr. Rahmad Zakaria. A voucher specimen (11575) was submitted to the herbarium at the School of Biology, Universiti Sains Malaysia (USM), Penang.

### Preparation of the Extracts

The leaves of *P. bleo* were rinsed thoroughly with water before being subjected to oven-drying at 50 °C and then processed into powder form. Twenty grams of powdered leaves were extracted by using n-hexane, ethyl acetate, and methanol by using Soxhlet apparatus. The extracts were then concentrated via the rotary evaporator. Aqueous extract was prepared by

decoction method where 10 g of the sample was boiled in 450 ml water at 50 °C until the water was reduced to one-third of its initial volume then proceed filtration with Whatman filter paper no. 1. After that, the extract was freeze-dried. All the extracts were then dissolved in dimethylsulfoxide (DMSO; Merck) and later diluted to several working concentrations.

### Cell Lines and Cell Culture

Cancer cell lines namely cervical (HeLa), breast (MDA-MB-231), colon (SW480), liver (HepG2), and normal mouse fibroblast cell line (NIH 3T3) were purchased from ATCC. A complete medium containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco), 10% of fetal bovine serum (FBS; Gibco), 1% of penicillin-streptomycin (Gibco) under a humidified air atmosphere containing 5% CO<sub>2</sub> at 37 °C were used in the culture of the cell lines.

### Cytotoxic Activity Assay

An assay using 3-[4,5-dimethyl thiazol-2-yl] 2,5-diphenyl tetra-zolium bromide (MTT; Merck) were carried out to measure the cytotoxicity activity of *P. bleo* extracts. The cells were seeded in 96-well flat bottomed plates (Eppendorf) at a density of  $5 \times 10^4$  cells per well in a final volume of 100 µl/well. After 24 h incubation at 37 °C with 5% CO<sub>2</sub>, the cells were treated with n-hexane, ethyl acetate, methanol, and aqueous extracts at different concentrations (0.3–99 µg/ml). After 72 h, 50 µl of MTT solution (2 mg/ml) were added to each well and further incubated for 4 h at 37 °C. Then, 200 µl of DMSO was added to each well to dissolve the MTT crystals and absorbance was taken at 570 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. Each experiment was performed in triplicates. IC<sub>50</sub> values of the treated cancer and normal cells were determined as formula below to determine the cytotoxicity effect of all extracts.

Percentage of cell viability (%) = (Absorbance of treated cells/Absorbance of control) × 100%

Since ethyl acetate extract of *P. bleo* leaves (PBEA) showed the strongest IC<sub>50</sub> value against most active cancer cells HeLa, only PBEA and HeLa cells were used further in the study.

### Cell Cycle Assay

Flow cytometry was used to evaluate the changes in cell cycle distribution of HeLa cells induced by PBEA.

**Table 1.** IC<sub>50</sub> values of *P. bleo* leaves extracts on selected cancer and normal cell lines after 72 h incubation period.

Types of cell lines	IC <sub>50</sub> values (μg/ml)				
	Hexane extract	Ethyl acetate extract	Methanol extract	Aqueous extract	Tamoxifen
HeLa (cervical cancer)	20.24 ± 11.69	14.37 ± 8.40	>99	>99	2.71 ± 0.88
MDA-MB-231 (breast cancer)	43.76 ± 36.86	41.60 ± 35.68	>99	>99	2.24 ± 0.95
HepG2 (liver cancer)	>99	>99	>99	>99	3.80 ± 1.07
SW480 (colon cancer)	>99	>99	>99	>99	2.66 ± 0.22
NIH/3T3 (normal mouse fibroblast)	>99	>99	>99	>99	3.78 ± 1.46

The IC<sub>50</sub> data are the average value of three independent experiments and represented as mean ± SD.

The cells were treated with PBEA at a concentration of IC<sub>50</sub> and incubated at 37 °C with 5% CO<sub>2</sub> for certain periods (24 h, 48 h, and 72 h). After that, the samples were processed using BD Cycletest™ Plus DNA Kit (BD Bioscience) as per kit's instruction. The cells were resuspended in buffer solution at concentration of 5 × 10<sup>5</sup> cells per sample. Then, the cells were incubated with trypsin buffer (10 min, room temperature), trypsin inhibitor and RNase buffer (10 min, room temperature) and lastly cold PI stain solution (10 min, 2 °C–8 °C) in the dark on ice. After incubation, the samples were analyzed using FACSCANTO II (BD Bioscience). A minimum of 10,000 events was acquired per sample and the data was analyzed by using ModFit LT 5.0 software.

### Annexin V-FITC Assay

Annexin V-FITC Detection Kit I (BD Bioscience) was used to identify the distribution of early or late apoptosis induced by PBEA towards HeLa cells. The assay was conducted as instructed in manufacture's manual. The cells were later incubated with the IC<sub>50</sub> concentration of PBEA for 24 h, 48 h, and 72 h at 37 °C with 5% CO<sub>2</sub>. After that, the cells were washed two times with cold PBS and stained with 5 μl of FITC Annexin V and 5 μl of propidium iodide (PI) for 15 min at room temperature under dark condition. Results for the stained cells were obtained (10,000 events per sample) through FACSCANTO II (BD Bioscience). The data acquired were analyzed by using FlowJo\_V10 software. The test was repeated thrice independently.

### Apoptosis Proteins Assay

Expression level of apoptosis proteins Bax, Bcl-2, p53 and caspase-3 in HeLa cells following treatment with PBEA were determined by the means of flow cytometry. Cells were incubated with the IC<sub>50</sub> concentration of PBEA for 24 h, 48 h, and 72 h at 37 °C with 5% CO<sub>2</sub>. The proteins were stained with antibody conjugate according to the manufacturer's protocol. The cells were later harvested and washed with PBS. After that, the cells (1 × 10<sup>6</sup> cells) were fixed with ice cold

70% ethanol for 1 h at 4 °C. The cells were washed twice after fixation and blocked in 2% bovine serum albumin for 10 min at room temperature. After the blocking process, 100 μl of cells (1 × 10<sup>5</sup> cells) were transferred into flow tubes and stained with antibodies: Bax-PE (Santa Cruz), Bcl-2-Alexa Fluor 647 (Santa Cruz), p53-Alexa Fluor 480 (Santa Cruz), and caspase 3-Alexa Fluor 480 (Santa Cruz). Results for 10,000 events per sample were recorded with FACSCANTO II (BD Bioscience). The data were then analyzed using FlowJo\_V10 software. The experiment was carried out thrice independently.

### Statistical Analysis

Each data was presented as mean ± SD. The results were analyzed using repeated measure one-way ANOVA ( $P < 0.05$ ). All statistical analysis were performed with GraphPad Prism 7 software.

## Results

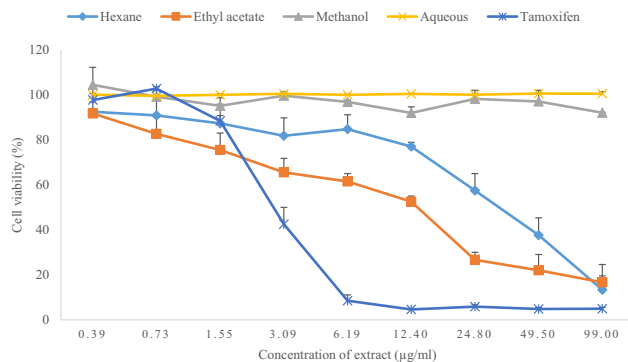
### Cytotoxicity of *P. bleo* Leaves Extracts on Selected Cancer Cell Lines

The cytotoxicity of *P. bleo* leaves extracts on selected cancer and normal cell lines were determined using MTT assay. The control treatment in this study was Tamoxifen. *P. bleo* that were extracted using ethyl acetate (PBEA) showed higher cytotoxic effects on HeLa (14.37 ± 8.40 μg/ml) and MDA-MB-231 cells compared to hexane (Table 1). The cell viability was reduced significantly in a dose and time-dependent manner (Fig. 1). On the other hand, *P. bleo* extracted with methanol and aqueous leaves extracts showed no cytotoxic effects towards all cell lines, with the maximum cut off concentration at 99 μg/ml. In addition, all extracts do not exert cytotoxic effects against normal cell line (NIH/3T3).

### Cell Cycle

The effects of PBEA on cell cycle distribution in HeLa cells were evaluated by flow cytometry method. Cells

treated with  $IC_{50}$  value of PBEA for 72 h were analyzed for the distribution of  $G_0/G_1$ , S and  $G_2/M$  phases of cell cycle (Fig. 2). The results demonstrated that PBEA treatment resulted in a significant accumulation of cells in the  $G_0/G_1$  phase but decreased in S and  $G_2/M$  phases ( $P < 0.05$ ) compared to control. This showed that PBEA induced changes in the cell cycle progression of HeLa cells by causing the arrest of  $G_0/G_1$  phase.



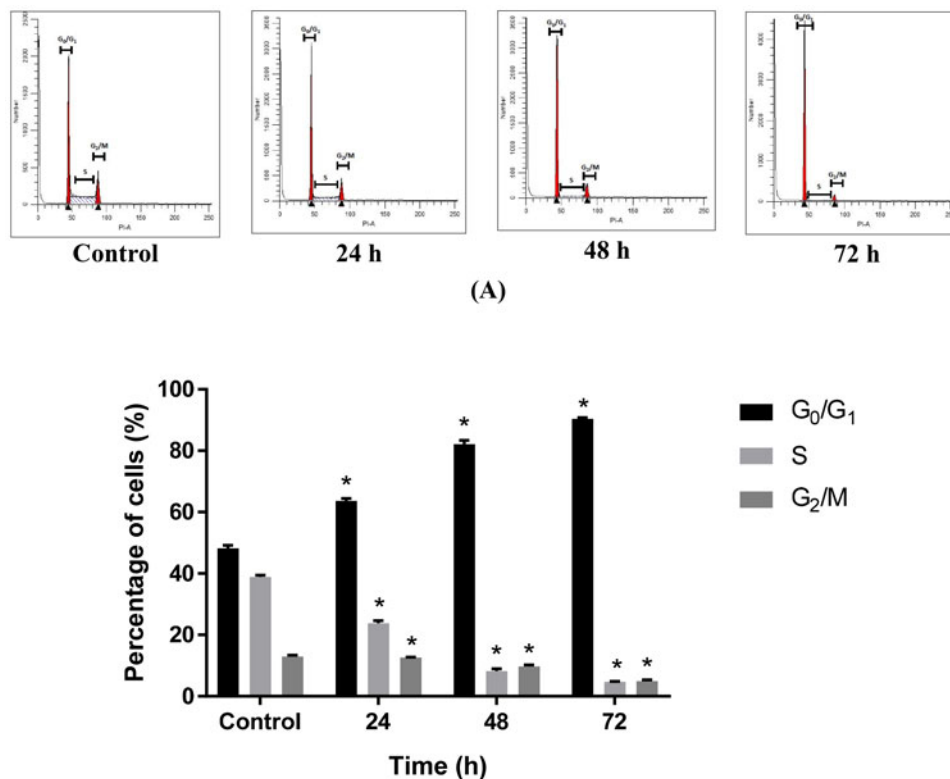
**Figure 1.** Cytotoxicity of *P. bleo* leaves extracts (hexane, ethyl acetate, methanol, and aqueous) and tamoxifen on HeLa cells treated with different concentrations for 72 h. The data of three independent experiments are represented as mean  $\pm$  SD.

## Apoptosis

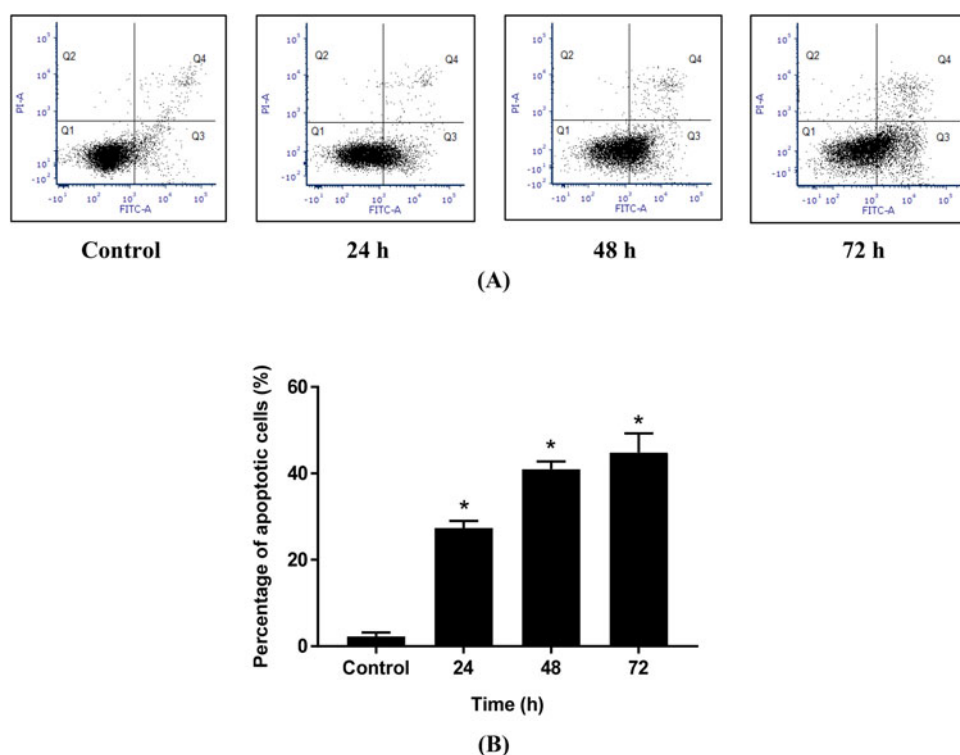
To identify whether or not the cells underwent apoptosis, control and treated HeLa cells with PBEA were stained with Annexin V and PI to measure the presence of apoptotic cells. The event of apoptosis has occurred in HeLa cells within 72 h upon treatment with PBEA as per flow cytometric analysis result shown in Fig. 3. The apoptosis rate was significantly different for every incubation period in treated groups compared to control group ( $P < 0.05$ ).

## Flow Cytometry of Apoptosis Proteins Expression Levels

Apoptotic proteins (Bax, Bcl-2, p53, and caspase-3) expression levels in HeLa cells following treatment with  $14.37 \pm 8.40 \mu\text{g/ml}$  of PBEA were assessed by flow cytometry analysis (Fig. 4). PBEA upregulated protein levels of Bax and p53, whereas Bcl-2 was downregulated in a time-dependent manner following the 72 h treatment period. There were significant differences observed for the expression levels of Bax, p53 and Bcl-2 treated and control cells. Even though there was only a slight upregulation in the expression of



**Figure 2.** The effects of PBEA on cell cycle of HeLa cells. (A) The flow cytometry histogram shows the DNA content and the corresponding percentage of cell distribution in control and treated HeLa cell for 24 h, 48 h, and 72 h. Cell cycle distribution showing accumulation of treated cells in  $G_0/G_1$  phase. (B) The bar columns are represented as mean  $\pm$  SD from three independent experiments. \* $P < 0.05$  when compared with the control group.



**Figure 3.** (A) Distribution of Annexin V-FITC staining dot plots in control and PBEA treated HeLa cells detected by flow cytometry after 24 h, 48 h, and 72 h incubation. Four quadrants represent viable cells (Q1), necrotic cells (Q2), early apoptotic cells (Q3), and late apoptotic cells (Q4). (B) The bar column illustrated the percentage of HeLa cells undergoing apoptosis. The data represent the mean  $\pm$  SD of three independent experiments. \* $P < 0.05$  when compared with the control group.

caspase-3, the results remain significantly different between treated and control cells.

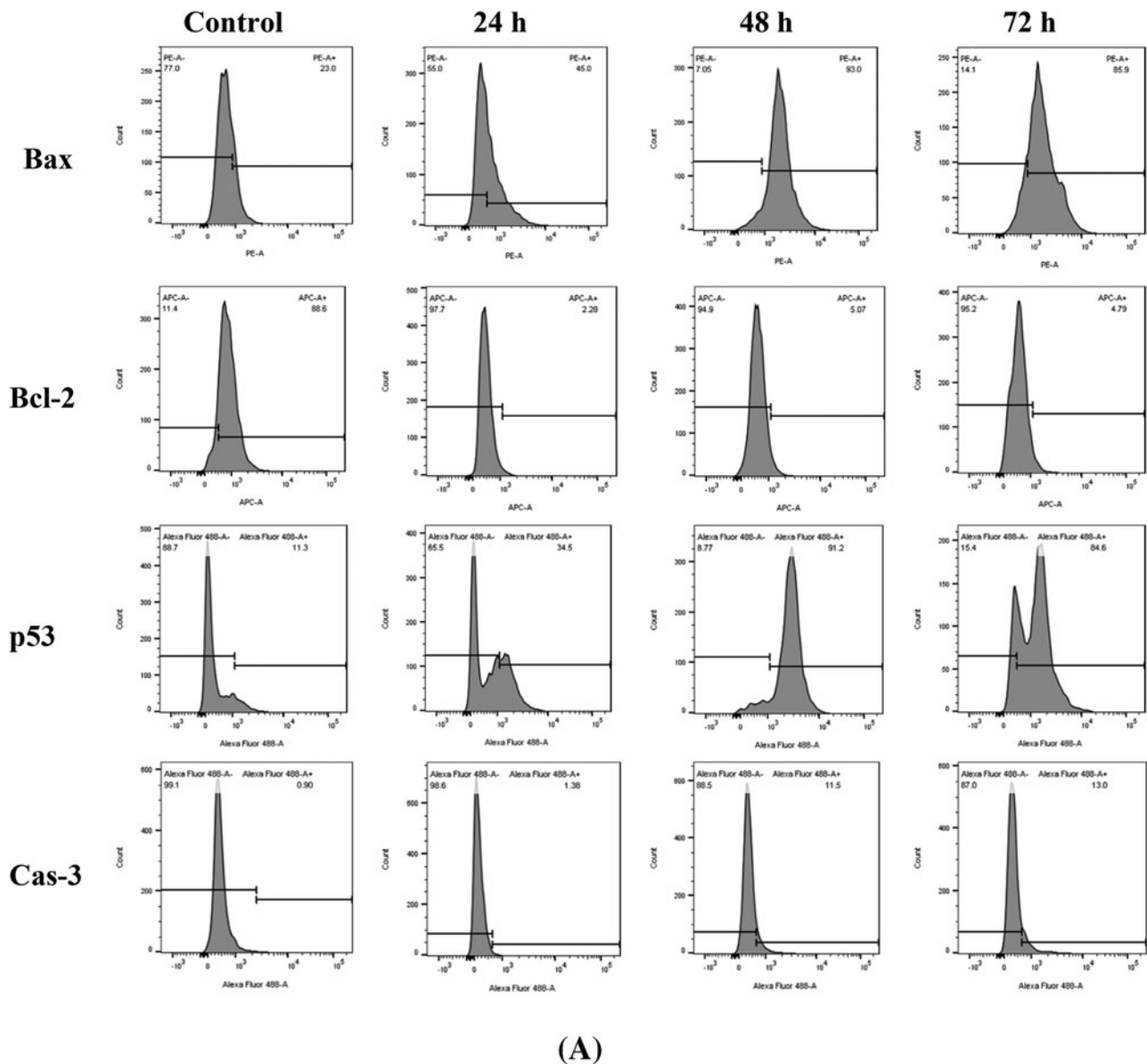
## Discussion

The search for anticancer agents from botanical sources has been gaining popularity worldwide due to their potential in arresting cancer cell proliferation with negligible side effects besides being domestically accessible. At present, a large number of active compounds from natural sources such as herbs and medicinal plants have been isolated and developed as new drugs for cancer treatment (25). *P. bleo* leaves is one of the important sources of medication and traditionally used to treat various ailments such as hemorrhoid, hypertension, cancer, diabetes, infections, headaches, rheumatism, asthma as well as being consumed for dietary benefits (19–22).

Pharmacology screening of plants is essential in the search for novel and effective drugs with minimal side effects (26). According to the National Cancer Institute (NCI), in vitro cytotoxic activity of crude extract with  $IC_{50}$  value  $\leq 20 \mu\text{g/mL}$  is considered highly cytotoxic (27). The  $IC_{50}$  value of a good anti-cancer agents should be low to avoid undesirable effects (28). In the present study, PBEA exhibited the

highest cytotoxic activity with  $IC_{50}$  value of  $14.37 \pm 8.40 \mu\text{g/mL}$  and selectively against HeLa cells while no cytotoxicity was observed in normal cells. The ability of the extract to distinguish between normal and malignant cells is a crucial aspect in the development of an anticancer agent (29). The characteristic of PBEA is consistent with this concept hence possess high potential to become an anticancer agent for cervical cancer. PBEA is known to contain several useful phytochemicals such as terpenoids, and phenolic compounds (20,22,30). Various studies have reported that terpenoids and phenols exhibited cytotoxic activity against several cancer cells like colon and liver (31,32). Thus, the presence of these phytochemicals might contribute to the cytotoxic activity of PBEA.

Cell cycle dispersal and induction of apoptosis were tested to determine the mechanism involved in the inhibition of cell growth by PBEA leading to cytotoxicity in HeLa cells. Cell cycle analysis demonstrated that PBEA arrested HeLa cells at  $G_0/G_1$  phase in a time-dependent manner as the accumulation of the cells were noticeable at this stage after 72 h. This finding indicates that PBEA may inhibit the progression of proliferation in HeLa cells at  $G_0/G_1$  phase. During cell division, checkpoints that exist at each phase of



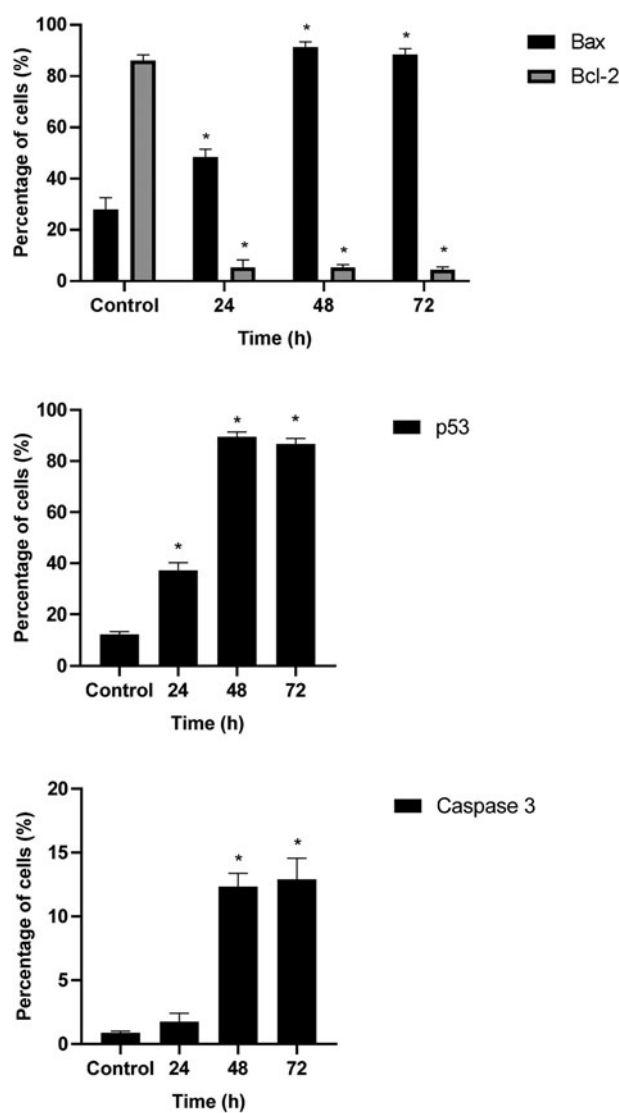
**Figure 4.** Flow cytometry analysis of apoptosis proteins expression in HeLa cells induced by PBEA. (A) Histograms of apoptosis protein Bax, Bcl-2, p53, and caspase-3 (cas-3) expression level measured in HeLa cells for control and treated with PBEA after 24 h, 48 h, and 72 h incubation. (B) The bar columns showed the percentage of apoptosis proteins expression in control and HeLa cells treated with PBEA. PBEA, in a time-dependent manner has significantly upregulated expression of Bax, p53 and caspase-3, whereas Bcl-2 was downregulated. The data were shown as mean  $\pm$  SD which represent for three independent experiments. \* $P < 0.05$  when compared with control group.

the cell cycle will identify the potential DNA impairment which allows for cell repair to take place (33). Arresting DNA replication at  $G_0/G_1$  phase can direct the cells either to be repaired or to undergo apoptosis (34–36). This is the synergistic mechanisms for the cytotoxic effects of cytotoxic agents in cancer cells (37).

To confirm the onset of apoptosis following cell cycle arrest, HeLa cells treated with PBEA were stained with Annexin V-FITC and analyzed using flow cytometry. Apoptosis activation in a time-dependent manner was observed due to the

accumulation of apoptotic cells in both early and late stage of apoptosis. This finding confirmed that HeLa cells had undergone apoptosis as a result of the cytotoxic effects exerted by PBEA.

Apoptosis can be activated via different pathways and controlled by pro-apoptotic and anti-apoptotic proteins. Thus, protein analysis was performed via flow cytometry to further explain the role of apoptotic proteins in apoptosis of HeLa cells induced by PBEA. The findings of this study showed that PBEA stimulated the expression of pro-apoptotic protein Bax and suppressed the anti-apoptotic protein Bcl-2 which



(B)

Figure 4. Continued

eventually leads to apoptosis. PBEA acted as an apoptosis inducer, repressing Bcl-2 expression through the increment of p53 level in HeLa cells. This observation is in line with a study by Wang et al. (38) who reported that barberin hydrochloride acted as an efficient apoptotic inducer in HeLa cells. In healthy cells, the tumor suppressor p53 remained at low concentration. p53 protein level will elevate due to various stimuli and regulate apoptotic gene expression (39). It induces apoptosis by suppressing anti-apoptotic proteins such as survivin and Bcl-2. At the same time, pro-apoptotic protein such as Bax is activated thus initiating the caspase cascade (40,41). Activation of pro-apoptotic proteins promoted mitochondrial membrane permeability which stimulates the occurrence of apoptotic cell death (42).

The caspase cascade signaling system is an essential component in the process of apoptosis as it is controlled by various proteins that either promote or inhibit apoptosis (43). In this study, caspase-3 expression level was slightly increased in HeLa cells upon treatment with PBEA. Similar finding was reported in breast carcinoma cell line (T47-D) treated with methanol extract of *P. bleo* where apoptosis was induced naturally via caspase-3 and c-myc pathway (15). Thus, activation of caspase-3 through extrinsic or intrinsic pathway initiate the action of other proteins which leads to apoptosis.

## Conclusions

The results of this study clearly demonstrated that PBEA induced apoptosis in cervical cancer HeLa cells



through Bax/Bcl-2 signaling pathway with the involvement of caspase-3, while inducing G<sub>0</sub>/G<sub>1</sub> phase cycle arrest via p53-mediated mechanism. Therefore, it can be concluded that *P. bleo* has the potential to become a cancer prevention agent. Further research, particularly in vivo study, should be carried out to provide further evidence of the anticancer properties of this plant.

## Disclosure Statement

Authors have no conflict of interest to declare.

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