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Methanolic extract of *Abrus precatorius* promotes breast cancer MDA-MB-231 cell death by inducing cell cycle arrest at G_0/G_1 and upregulating Bax

Wan Suriyani Wan-Ibrahim[1], Norzila Ismail[1]✉, Siti Farhanah Mohd-Salleh[1], Aidy Irman Yajid[2], Michael Pak-Kai Wong[3], Mohd Nizam Md Hashim[3]

[1]*Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia,16150 Kubang Kerian, Kelantan, Malaysia*

[2]*Department of Pathology, School of Medical Sciences, Universiti Sains Malaysia,16150 Kubang Kerian, Kelantan, Malaysia*

[3]*Department of Surgery, School of Medical Sciences, Universiti Sains Malaysia,16150 Kubang Kerian, Kelantan, Malaysia*

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ABSTRACT

Objective: To determine the anti-proliferative activity of *Abrus precatorius* (*A. precatorius*) leaf extracts and their effect on cell death.

Methods: *A. precatorius* leaves were extracted successively with hexane, ethyl acetate and methanol by Soxhlet extraction. Aqueous extract was prepared by decoction at 50 ℃. Extracts of *A. precatorius* leaves were used to treat selected cancer and normal cell lines for 72 h. Furthermore, 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide assay was performed to determine cell viability. Analysis of cell cycle arrest, apoptosis assay and apoptosis protein expressions were determined by flow cytometry.

Results: Methanolic extract of *A. precatorius* leaves showed the lowest IC_{50} on MDA-MB-231 cells at (26.40±5.40) μg/mL. Flow cytometry analysis revealed that cell arrest occurred at G_0/G_1 phase and the apoptosis assay showed the occurrence of early apoptosis at 48 h in MDA-MB-231 cells treated with methanolic extract of *A. precatorius* leaves. Methanolic extract of *A. precatorius* leaves induced apoptosis by upregulation of Bax, p53 and caspase-3 and downregulation of Bcl-2.

Conclusions: Methanolic extract of *A. precatorius* leaves promotes MDA-MB-231 cell death by inducing cell cycle arrest and apoptosis possibly *via* the mitochondrial-related pathway.

1. Introduction

Apoptosis is a characterized form of cell death and is mostly studied. Known commonly as programmed cell death, apoptosis is the packaging of dying cells into fragments that are easily consumed and eliminated by phagocytes without disturbing the normal function of surrounding tissues[1]. Equilibrium between cell death and cell proliferation is important to avoid disruption of the cellular balance. Excessive apoptosis or deficient apoptosis is the cause of many clinical diseases including cancer[2]. Apoptosis can be initiated through two separate pathways, the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. In cancer management, apoptosis has become an important tool as a target by potent apoptosis-inducing agents, including both chemical and biological[3].

✉Corresponding author: Dr. Norzila Ismail, Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
Tel: +6097676135
Fax: +6097653370
E-mail: norzila_ismail@usm.my

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Cancer prevalence is increasing and has been one of the main causes of death. World Health Organization[4] reported that cancer is the second leading cause of death worldwide with an estimated 9.6 million of deaths in 2018, in which 1 in 6 deaths is caused by cancer. The leading cancers causing deaths include lung, colorectal, stomach and breast cancer. About 627 000 death cases were reported due to breast cancer globally. In Malaysia, 43 837 new cases were reported in 2018 with breast cancer amounting to 7 593 cases[5]. Advances in the medical field have proven to be the preferable choice to combat cancer. However, these advances are used with concern due to their side effects and limitations. This scenario has generated increasing demand of traditional medicines with medicinal plants either as complementary to the allopathy treatments or as a complete alternative. Many issues were raised with this development especially concerning the toxicity and efficacy of the medicinal plants extract. Therefore, it is noted that more studies are needed in order to provide better understanding of the biological activities and underlying mechanism of the medicinal plants.

Medicinal plants are widely sought as an alternative in various treatments including cancer. These plants are extensively studied to screen for potential phytochemical as anticancer agents. *Abrus precatorius* (*A. precatorius*) is a flowering plant that belongs to the legume family, Fabaceae. This plant is native to India, however, it is also found in other parts of tropical areas, like Malaysia[6]. This plant is phenotypically recognized by its slender, perennial, climbing twigs with pinnate leaves arranged in pairs. *A. precatorius* seeds are red with black dot. In Malaysia, the leaves of *A. precatorius* are traditionally used to treat simple illness such as mouth ulcer and fever. Moreover, *A. precatorius* is also reported to be used as an anticancer agent[7-13].

Gul *et al*[7,12] reported anti-proliferative activities of *A. precatorius* against human acute monocytic leukemia cell line (THP-1), while Sofi *et al*[8,13] reported its anti-proliferative activities against MDA-MB-231 by using aqueous extract and fractions from gradient elution of ethyl acetate extract. Our previous study[6] firstly reported the phytochemical analysis of this plant in Malaysia. Therefore, this study aimed to further elucidate the anticancer properties of *A. precatorius* leaves extracts on cancer cell lines and investigate their effect on cancer cell death.

2. Materials and methods

2.1. Plants collection and extract preparation

A. precatorius leaves were collected from Kampung Sabak, Pengkalan Chepa Kelantan and given a specimen voucher USM 11730 by the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia for future references. About 22 g of ground *A. precatorius* were subjected to successive Soxhlet extraction with hexane, ethyl acetate and methanol. Aqueous extract was prepared by decoction of the ground leaves at 50 ℃ in 500 mL of distilled water until the water was reduced to a third of the initial volume. All extracts were kept at -20 ℃ until use.

2.2. Cell culture

Human breast cancer cell lines, MDA-MB-231 and MCF-7; human liver cancer cell lines, HepG2; human colon cancer cell lines, SW480; human cervical cancer cell line, SiHa and HeLa; human normal breast cell, MCF-10a and mouse normal fibroblast cell, NIH were obtained from American Type Cell Culture Collection, Maryland, USA. Cells were seeded in 25 cm^2 tissue culture and grown at 37 ℃ under humidified 5% CO_2 in Dulbecco's Modified Eagle's medium supplemented with 5% fetal bovine serum and 1% penicillin-streptomycin. Confluent cells were harvested by trypsinization (0.25%).

2.3. Anti-proliferative activity by 3-(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) assay

To determine the anti-proliferative activity of *A. precatorius*, human breast cancer cell lines, MDA-MB-231 and MCF-7; human liver cancer cell lines, HepG2; human colon cancer cell lines, SW480; human cervical cancer cell line, SiHa and HeLa; human normal breast cell, MCF-10a and mouse normal fibroblast cell, NIH were treated with all extracts. The anti-proliferative activities were investigated by MTT assay. IC_{50} values were determined, with lower IC_{50} values indicating higher anti-proliferative activity. Cytotoxicity of plant extracts against cancer cell line was categorized based on US National Cancer Institute and Geran Protocol as follows: highly cytotoxic ($IC_{50} \leq 20$ μg/mL), moderately cytotoxic (21 μg/mL$\leq IC_{50} \leq 200$ μg/mL), weakly cytotoxic (201 μg/mL $\leq IC_{50} \leq 500$ μg/mL), and, no cytotoxicity ($IC_{50} \geq 501$ μg/mL)[14].

Cells were seeded into 60 wells at the centre of a 96-wells plate with the concentration of 5×10^4 cells/mL per well. Extracts of *A. precatorius* leaves were added following a serial dilution starting from 99 μg/mL until 0.39 μg/mL in each well. Anti-proliferative activity of *A. precatorius* extracts was measured by the MTT assay, which was performed after a 72-h incubation post treatment with the extracts, tamoxifen (positive control) and dimethyl sulfoxide (negative control). Absorbance was read at OD of 570 nm. The absorption value at this wavelength directly represents the relative cell numbers in comparison with the control group[15]. The percentage of cell viability was determined according to the following equation:

Percentage of cell viability (%) = Absorbance of treated cells (extracts or tamoxifen)/Absorbance of treated cells (dimethyl sulfoxide) ×100

Anti-proliferative activity of *A. precatorius* leaves extract was screened on selected cancer and normal cells. Extract that exhibited the lowest IC_{50} value with its corresponding cell was used for subsequent analysis in this study.

Table 1. IC_{50} values of *A. precatorius* leaf extracts against selected normal and cancer cell lines (μg/mL).

Types of cancer cell lines	Hexane extract	Ethyl acetate extract	Methanol extract	Aqueous extract	Tamoxifen
HeLa (cervix)	>99	>99	73.60±6.17	>99	4.32±0.40
SiHa (cervix)	>99	>99	>99	>99	3.27±0.93
MCF7 (breast)	52.65±7.14	99.00±11.86	59.03±9.40	>99	1.81±1.78
MDA-MB-231 (breast)	45.60±11.60	54.50±9.05	26.40±5.40	>99	2.27±0.38
SW 480 (colon)	>99	>99	77.23±6.39	>99	2.31±0.59
HepG2 (liver)	>99	99.00±10.32	67.72±6.21	>99	4.10±0.88
MCF-10a (normal breast)	>99	>99	>99	>99	3.78±7.79
NIH (normal fibroblast)	>99	>99	>99	>99	3.78±1.78

2.4. Cell cycle assays

MDA-MB-231 cells were treated with IC_{50} of the methanolic extract of *A. precatorius* leaves (APME) and incubated for 24, 48 and 72 h. Cells were harvested by trypsinization and the cell cycle assay was performed according to the manufacturer protocol, BD Cycletest™. All samples readings were acquired with FACSCANTO Ⅱ (BD Bioscience). Data obtained was analysed with ModFit LT 5.0 software.

2.5. Apoptosis assays

2.5.1. Annexin-Ⅴ and propidium iodide (PI) staining

MDA-MB-231 cells were treated with IC_{50} of the APME and incubated for 24, 48 and 72 h. Cells were harvested by trypsinization and the apoptosis assay was performed according to the manufacturer protocol, Annexin Ⅴ-FITC detection kit Ⅰ (BD Bioscience). All samples readings were acquired with FACSCANTO Ⅱ (BD Bioscience). Data was analysed with FlowJo software.

2.5.2. Bax, Bcl-2, caspase-3 and p53 activity

MDA-MB-231 cells were treated with IC_{50} of the APME and incubated for 24, 48 and 72 h. Cells were harvested by trypsinisation following each incubation time and washed twice with phosphate buffer saline (PBS). Ethanol (70%) was used to fix the cells at 4 ℃ for 1 h. Cells were washed twice with PBS and then blocked with 2% bovine serum albumin for 10 min at room temperature. Another cell wash was performed, and cells were resuspended in PBS.

About 100 μL of the cell suspension (1×10^6 cells) were mixed independently in different tubes, with antibodies (SantaCruz); Bax-PE (sc-7480), Bcl-2 – Alexa Fluor 647 (sc-7382), p53 – Alexa Fluor 488 (sc-126) and Caspase-3 – Alexa Fluor 488 (sc-7272). These cells-antibodies mixtures were incubated for 20 min at room temperature, then washed once and resuspended in 500 μL PBS. All samples readings were acquired with FACSCANTO Ⅱ (BD Bioscience). Data was analysed with FlowJo software.

2.6. Statistical analysis

The data were expressed as mean ± SD of three repeated experiments. The level of statistical significance was tested using repeated measure one-way ANOVA, followed by Dunnett's multiple comparison test. The difference was considered significant if $P<0.05$. Analyses were all done using GraphPad Prism7.

3. Results

3.1. Screening of A. precatorius extracts on various cancer and normal cells

The IC_{50} values are summarized in Table 1. Methanol extract had the lowest IC_{50} value on MDA-MB-231 cells at (26.40±5.40) μg/mL, which could be categorized to moderate toxicity. IC_{50} values of all extracts were determined by plotting the graph of concentration of the extract or tamoxifen *versus* percentage of cell viability as depicted by Figure 1 which represents the data for MDA-MB-231 cells.

3.2. Effects of APME on cell cycle progression in MDA-MB-231 cells

The ability of the APME to reduce cell viability could be due to cell death mediated by cell cycle arrest. Therefore, cell cycle arrest was investigated using flow cytometric analysis of PI stained DNA. Representative profiles of the cell cycle progression are presented in Figure 2(a) and percentage of each phase of cell cycle is presented in Figure 2(b). Significant increment of the arrest was found at G_0/G_1 phase of the cell cycle, suggestive of cell death consistent with the reduction of cell growth as the percentage of cell population decreased in both S-phase and G_2/M phase.

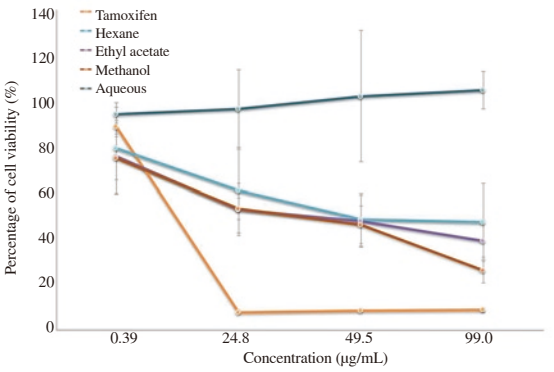

Figure 1. Antiproliferative effects of all extracts on MDA-MB-231 cells. Data were expressed as mean±SD of three repeated experiments with three replicates.

3.3. Effects of APME on apoptosis in MDA-MB-231 cells

To determine the ability of the APME to induce apoptosis, an apoptosis assay was performed using the Annexin Ⅴ-FITC detection kit Ⅰ (BD Bioscience). Representative profiles of the apoptosis assays are presented in Figure 3(a) and percentage of each phase of cell death progression is presented in Figure 3(b). Live cells did not uptake any stain and represented at Q4 (Figure 3a). Annexin Ⅴ bound to the phosphatidylserine of the plasma membrane which was exposed in early apoptosis (Q3; annexin Ⅴ positive, PI negative). Late apoptotic cells lost their cell integrity thus allowing the penetration of PI (Q2; annexin Ⅴ positive, PI positive), while necrotic cells were stained with PI only (Q1 PI positive). APME induced early apoptosis in MDA-MB-231 cells at 48 h and late apoptosis at 72 h after treatment.

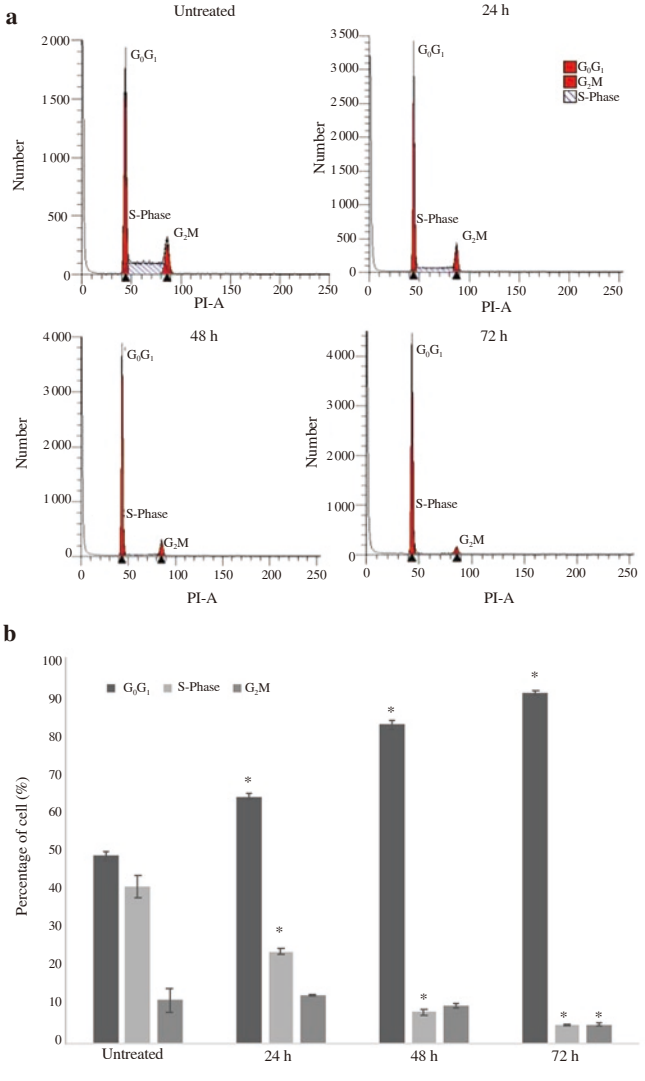

Figure 2. Effects of methanolic extract of *A. precatorius* leaves on cell cycle progression in MDA-MB-231 cells. (a) Cell cycle arrest analysis of MDA-MB-231 cells; (b) percentage of each phase of cell cycle. Data are expressed as mean±SD of three repeated experiments. *P<0.05 is considered significant when comparing treated cells *vs* untreated cells.

3.4. Bax, Bcl-2, caspase-3 and p53 protein expression in MDA-MB-231 cells treated with APME

To determine whether Bax, Bcl-2, caspase-3 and p53 proteins are involved in APME-induced apoptosis in MDA-MB-231 cells, the proteins expressions were measured by flow cytometry at 24, 48, and 72 h (Figure 4a). The MDA-MB-231 cells treated with the APME increased pro-apoptotic protein, Bax and reduced anti-apoptotic protein, Bcl-2 expressions in a time dependent manner (Figure 4b). Significant increase was shown in the p53 protein expressions in the treated cells compared with untreated ones. Caspase-3 protein expressions were also increased. These findings indicated that APME induced apoptosis in MDA-MB-231 cells by upregulating Bax, p53 and caspase-3 protein and downregulating Bcl-2 protein. These findings suggest that the apoptosis in MDA-MB-231 cells was possibly triggered in the intrinsic pathway.

4. Discussion

Medicinal plants have been actively studied over the years in research labs and used traditionally worldwide. *A. precatorius* is one of the medicinal plants that belongs to the legume family Fabaceae. The legume family has many plants that exhibit excellent anticancer properties[16-19].

Anti-proliferative study gives an insight on the possibility of an occurrence of cell death of selected cancer cells induced by the extract. Our study demonstrated that the methanol leaves extract had the best anti-proliferative activity at 26.4 μg/mL against the human breast cancer cells, MDA-MB-231, an androgen-independent human breast cancer cells that express wild-type p53. However, these values are not comparable to the control, tamoxifen, which demonstrated the IC_{50} values of 2.27 μg/mL in MDA-MB-231. Based on the National Cancer Institute criteria, these results indicated that APME has a moderate cytotoxicity on MDA-MB-231 cell lines. Sofi *et al*[13] demonstrated that the IC_{50} of the aqueous extract on MDA-MB-231 cells was 98 μg/mL. Our results showed better inhibitory activity on MDA-MB-231 cells by treatment with the APME. The aqueous leaves extract of our experiment did not show any significant activity at maximum concentration of 99 μg/mL. Another study by Gul *et al*[12] claimed the hexanoic and ethanolic extracts of *A. precatorius* leaves had stronger anti-proliferative activity both in human colon adenocarcinoma cells (Colo-205) and human retinoblastoma cancer cells (Y79), while milder anti-proliferative activities were observed in human hepatocellular carcinoma cells (HepG2) and leukemia cells (SupT1).

Cell growth and proliferation of mammalian cells occurs through cell cycle; thus, the inhibition of the cell cycle progression is the ideal target for anticancer agents[20,21]. APME exhibited growth inhibitory effects on the MDA-MB-231 cells, inducing cell cycle

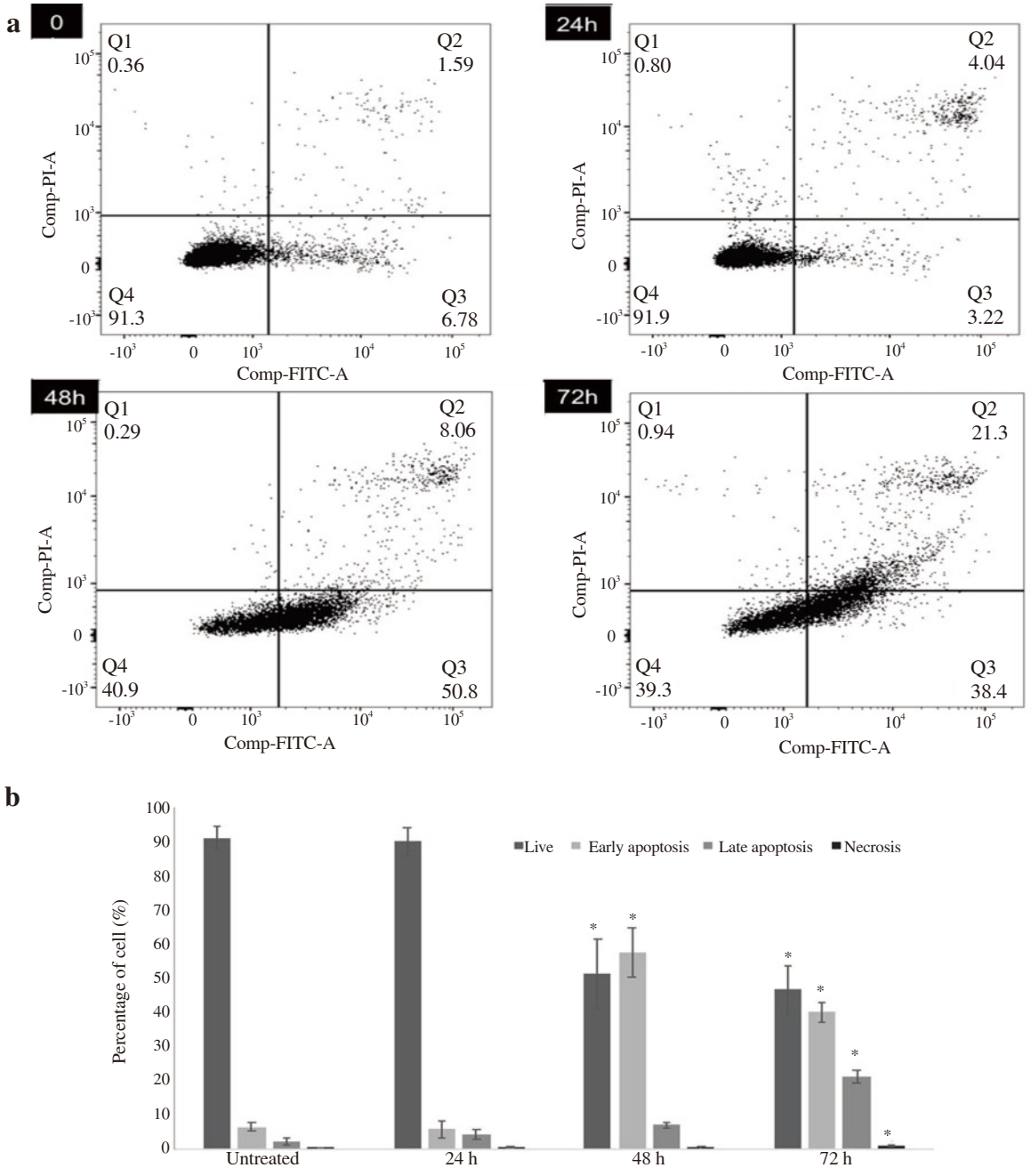

Figure 3. Effects of methanolic extract of *A. precatorius* leaves on apoptosis in MDA-MB-231 cells. (a) Apoptosis assay in a time-dependent manner. Quadrants represent the percentage of cell populations; Q1- Necrosis, Q2- Late apoptosis, Q3-Early apoptosis, Q4-Live cells; (b) Percentage of each phase of cell death. Data are expressed as mean±SD of three repeated experiments. **P*<0.05 is considered significant when comparing treated cells *vs* untreated cells.

arrest at G_0/G_1 phase. Increased percentage of cell population in G_0/G_1 phase and reduction of the population in S-phase proved this claim. In S-phase, genetic information is transferred from one cell generation to another. Genome replication in S-phase is important to segregate two daughter cells during mitosis or the M-phase. Mitosis only occurs when S-phase is completed. Two gaps separate between M- and S-phase. Between M- and S-phase, there is the G_1, and between S- and M-phase, there is G_2. DNA damage activates these checkpoints. When growth arrest occurs at any checkpoints, cells will repair the damage. If the damage is repaired, cell progression will successfully resume, otherwise the cell will be eliminated through apoptosis[21]. DNA arrest occurred during G_0/G_1 phase in this current study, which indicated that the cell proliferation was inhibited, thus showing reduction of the cell percentage in S-phase and G_2/M phase. At this point, it is clear that cell proliferation was halted by DNA arrest at G_0/G_1 phase. Furthermore, it is important to find out if the cell inhibition was caused by apoptosis.

Apoptosis induction is regarded as the best strategy in cancer treatment. It is an important programmed cell death to eliminate unnecessary cells and thus became the common mode of action for most chemotherapeutic agents[2]. Induction of apoptosis signifies the success of plant products as anticancer agents and it is the

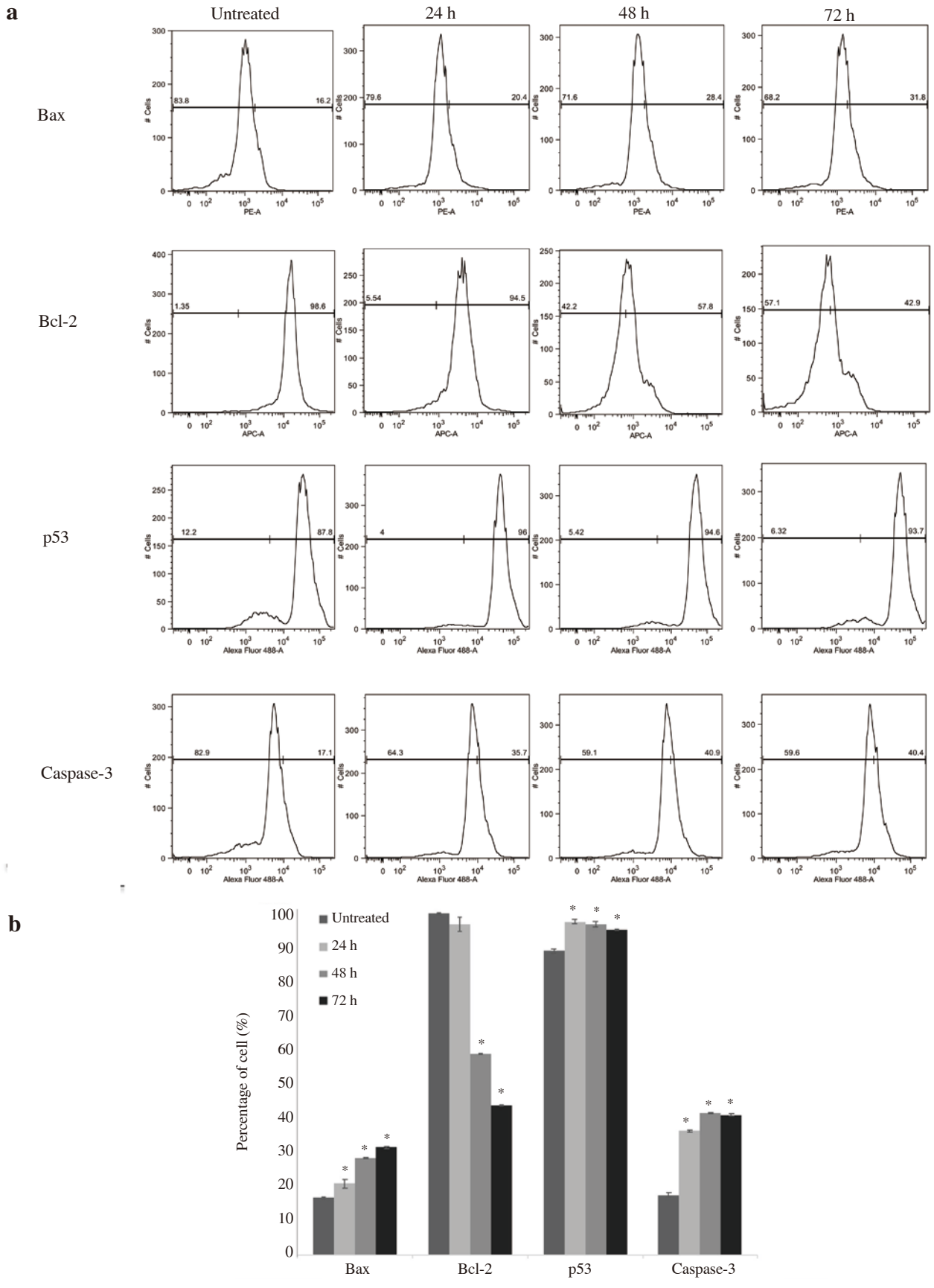

Figure 4. Bax, Bcl-2, caspase-3 and p53 protein expression in MDA-MB-231 cells treated with methanolic extract of *A. precatorius* leaves. (a) Apoptosis protein expression by flow cytometry (Bax, Bcl-2, p53, caspase-3). (b) Proteins expressions of Bax, Bcl-2, p53, and caspase-3. Data are expressed as mean±SD of three repeated experiments. *P<0.05 is considered significant when comparing treated cells *vs* untreated cells.

optimal way in cancer treatment. In order to confirm whether the inhibition of cell proliferation induced by APME is due to apoptosis, rather than necrosis, apoptosis assay using Annexin Ⅴ-FITC and PI staining was performed following the treatment with the extract. Annexin Ⅴ stains the phosphatidylserine of the inner cell membrane which is exposed during the early stage of apoptosis. Our results demonstrated that the APME promotes cell death *via* apoptosis. Early apoptosis occurred after 48 h and eventually led to late apoptosis following 72 h of treatment.

Decrease of mitochondrial outer membrane permeability (MOMP) indicated the irreversible events of early apoptosis. MOMP is highly regulated by anti-apoptotic and pro-apoptotic proteins[22]. Bax is a pro-apoptotic protein and Bcl-2 is an anti-apoptotic protein[23]. Upon stimuli in the intrinsic pathway, Bax is activated which induces MOMP. In this pathway, Bcl-2 prevented MOMP by inhibiting the activity of the BH3-only proteins that are responsible to activate Bax. MOMP allows the release of intermembrane space proteins, such as cytochrome c and SMAC. Cytochrome c forms an apoptosome complex after binding to the APAF-1 protein. This complex is responsible to activate caspase-3 and caspase-7 which eventually lead to apoptosis. Our results indicated that the expression of Bax proteins increased while Bcl-2 proteins decreased. Chien *et al*[24] demonstrated that quercetin induced apoptosis in MDA-MB-231 cells also by reducing Bcl-2 and increasing Bax protein levels. Overexpression of Bcl-2 like proteins such as Bcl-2, Bcl-xL and MCL-1 was detected in various cancers including lymphoma[25], lung cancer[26], neuroblastoma[27] and breast cancer[28]. MOMP is an important event of apoptosis because once it is activated, cell will face death sentence regardless of caspase activation[29].

Increased expression of caspase-3 protein signified the apoptosis events in the MDA-MB-231 cells treated with the APME. Caspase-3 activation can occur both in the extrinsic or intrinsic pathway. Upon activated, caspase-3 will cleave hundreds of other proteins, which subsequently leads to the biochemical and morphological signals of apoptosis. These hallmark events include DNA fragmentation, plasma membrane blebbing and phosphatidylserine exposure[30]. As shown in the apoptosis assay, exposure of phosphatidylserine was detected by Annexin Ⅴ-FITC and this indicates the early apoptosis event.

The results of this current study exhibited that APME inhibited MDA-MB-231 cell proliferation by DNA arrest at G_0/G_1 and induced apoptosis through Bax/Bcl-2 and caspase-3 proteins regulation. These findings suggest that APME successfully promotes cell death in breast cancer cell, MDA-MB-231 *via* apoptosis.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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