ORIGINAL ARTICLE

Cytotoxic Activity of Ethanolic Extract *Aquilaria malaccensis* Leaves Against MCF-7 Cells

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ABSTRACT

Introduction: *Aquilaria malaccensis*, also known as "Pokok Karas" in Malaysia, is widely used in Southeast Asian countries for the treatment of joint pain, diarrhoea and inflammatory diseases, and has shown beneficial effects as an anticancer agent. The aim of this study was to investigate the effect of ethanol leaf extracts of *A. malaccensis* on MCF-7 cells. **Methods:** MTT-based cytotoxic and antiproliferative assay was used to determine the outcome of ethanolic extract toward MCF-7 cells. The mode of cell death was determined by the AO/PI double staining assay and the depolarisation of the mitochondria membrane potential. **Results:** IC_{50} value of the extract against MCF-7 cells treated for 72 hours was 4.1 ± 2.08 µg/mL, while the IC_{50} value for doxorubicin was 2.92 ± 0.12 µg/mL. The extract showed a lower cytotoxic effect against the NIH/3T3 cells and inhibited the growth of MCF-7 cells in a dose dependent manner. AO/PI double stain showed that the ethanolic extract of *A. malaccensis* leaves induced MCF-7 cells into apoptotic cell death. The present study showed that the ethanolic extract of *A. malaccensis* induced apoptosis through mitochondrial pathway as indicated by its ability to take up JC-1. **Conclusion:** The study found that ethanolic extract obtained from *A. malaccensis* leaves is cytotoxic on MCF-7 cells, resulting to apoptotic cell death of the cells. *Malaysian Journal of Medicine and Health Sciences* (2023) 19(6):215-221. doi:10.47836/mjmhs19.6.29

Keywords: *Aquilaria malaccensis*, breast cancer, MCF-7, cytotoxicity, apoptosis, mitochondria membrane potential

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INTRODUCTION

The World Health Organization (WHO) has identified breast cancer as a major global health concern. The latest GLOBOCAN 2020 data reveals that breast cancer ranks second among the most frequently diagnosed cancers worldwide, representing 11.1% of all new cancer cases, following lung cancer. In Malaysia, female breast cancer accounts for 32.1% of all cancer cases registered from 2012 to 2016, with a reported 21,925 cases according to the latest data from the National Cancer Registry (NCR). Breast cancer is the leading cause of cancer-related deaths for women in the country, underscoring the urgent need for improved prevention, detection, and treatment options. The existing breast cancer treatments are still dominated by chemotherapy and radiotherapy and more recently via hormonal therapy and immunotherapy. Among all currently available medications, tamoxifen is the gold standard and the most commonly prescribed medication for invasive and metastatic breast cancer (1-2), although there is report on resistance toward tamoxifen (3). Such medications pose many negative consequences including improper menstrual cycles, vaginal discomfort, deep-vein thromboembolic events. Despite the side effects stated above, the said treatments reduce the victims' quality of life and do not record high success rate (1). Therefore, there is an obvious need for a new treatment with natural products such as medicinal herbs to minimize the progression of the disease and the side effects of the current invasive breast cancer treatment.

Naturally occurring active compounds from herbs and medicinal plants exert antiproliferative effects and are highly potential alternatives that can be designed and developed into novel treatments for cancer. Many natural products have demonstrated significant anti-cancer properties in laboratory studies and preclinical trials. These natural compounds may inhibit cancer cell

growth, induce apoptosis, and suppress the formation of new blood vessels that support tumor growth. Recent studies suggest that secondary metabolites from *Aquilaria* species, which belong to the Thymelaeaceae family, have the potential to inhibit tumourigenesis by identifying the plant-derived compounds as anticancer agents (4). Various studies have revealed that the leaves of *Aquilaria* species have promising potential for agricultural and pharmaceutical industries due to their diverse biological activities such as antimicrobial (5), antifungal (6), antioxidant (5,7), antinociceptive (8), anti-inflammatory (9-10), antidiabetic (11-12) and anticancer (5,13-14) agents. Although some studies indicated that secondary metabolites of *Aquilaria* species could be used to treat cancer (7,15), there is still no scientific evidence on the effectiveness of *A. malaccensis* leaves as a treatment for breast cancer. The goal of this research was to determine the results of *A. malaccensis* ethanolic extract in terms of cytotoxicity, antiproliferative effect and apoptosis induction on human breast MCF-7 cell line. Results of this study may pave the way for the application of *A. malaccensis* leaves as an anticancer agent especially for treating breast cancer cases.

MATERIALS AND METHODS

Plant materials

Leaves of *A. malaccensis* were collected from a Forest Research Institute Malaysia (FRIM) Forest Reserve in Merchang, Terengganu. Plant autenthication was performed by a competent botanist from the Universiti Sultan Zainal Abidin. The voucher number is UniSZA/A/000000031.

Plant extraction

A. malaccensis extraction process was similar to the suggestion by Wan Mamat et al. (16). Random sampling technique was applied in which young and old leaves from different trees were mixed. The leaves were washed to remove impurities before being dried in a laboratory oven at 40°C for 20 hours. The samples were then ground, stored in glass vials and kept in the freezer at -80°C before extraction. The extraction was carried out with ethanol (EtOH) and the powder sample was macerated in the solvent in the ratio of 1:50 sample to solvent at room temperature (22-24°C). The mixture was soaked for 24 hours before sonication at 40°C for 30 minutes to facilitate the extraction process. Whatman Filter No.1 was then used to refine the mixture after it has been centrifuged at 10,000 rpm for 10 minutes. The extraction steps were repeated three times and the filtrates were combined before being evaporated to dryness using a rotary evaporator (Heidoph, Germany). The extract was stored in a freezer at -20°C until further use.

Cell lines

Normal mouse fibroblast NIH/3T3 and human breast cancer MCF-7 cell lines were purchased from ATCC (Manassas, VA, USA). 10% foetal bovine serum (Gibco, USA) and antibiotics (100.0 units/mL penicillin and 100.0 µg/mL streptomycin) (Gibco, USA) were fed to the cells cultivated in DMEM media (Nacalai Tesque, Japan). Next, the cultivation was placed in a humidified incubator (37°C, 5% CO_2). Subculture of both NIH/3T3 and MCF-7 cells were prepared every 3 days by segregating the cultivation 1:2 in a new flask containing similar growth medium and processed with TrypLE™ Express with phenol red for 7 minutes (Gibco, USA). Before moved to new flask, the cells were re-suspended in the medium with serum. This study utilized cell viability of at least 95%.

MTT cytotoxic assay

Cell viability was determined using the MTT assay as described by Mossmann (1983) with slight modifications (17). Briefly, 100 µL MCF-7 or NIH/3T3 cells were seeded in each well of a 96-well flat-bottomed microtitre plate (Eppendorf, USA) and incubated overnight. One hundred µL of diluted ethanolic extract of *A. malaccensis* at 200 µg/mL or 100 µL of diluted doxorubicin at 20 µg/mL was added into row A and row B. A series of two-fold dilution of extract or doxorubicin was carried out down from row B until row G. The row H was left untouched, and the excess solution (100 µL) was discarded. Then, the cells were incubated for 72 hours in a CO2 incubator before 20 µL MTT solution (5 mg/mL) was added in every well before further kept for 4 hours. Next, 100 µL of 100% DMSO solvent was added to every well to solubilise the formazan blue crystal after the culture medium was taken out. Plate reader (Infinite M200 Pro) at 570 nm with a reference wavelength of 630 nm was utilized to check the plates. A dose-response curve plotted the percentage of cell viability against extract concentration.

Cell proliferation assay

The assay was performed as described by Tajudin et al. (18) with slight modifications. Briefly in this assay, MCF-7 cells at the concentration of 1 x 10^5 cells/mL were incubated with extract at IC_{25}, IC_{50}, and IC_{75} values and doxorubicin as a control for 24-, 48-, and 72-hours in 96-wells plates. Next, 20 µL MTT solution (5 mg/mL) was added in every well and further incubated for 4 hours after consequences incubation period. The next step was removing the culture medium and introducing 100 µL DMSO into every well to dilute the formazan blue crystal. The absorbance of each well was measured using a plate reader (Infinite M200 Pro) at a wavelength of 570 nm with a reference at 630 nm. The graph was plotted against the using OD values. The same procedure was used for 48- and 72-hour treatment periods.

AO/PI double staining assay

The assay was performed as described by Aziz et al. (19) with slight modifications. Plates with 6-well were used to seed the MCF-7 cells before incubated for 24 hours at 37°C. Afterwards, the existing medium was changed with new one with and without treatment, with

the treatment concentration set at IC_{50} of the ethanol extract. On the next day, 15 mL tube was used to gather detached cells in the medium. The flasks were cleaned using PBS before aspirated into the tube. TrypLE™ Express (Gibco, USA) was utilized to release attached cells and also aspirated into the tube. The tube was then centrifuged at 1700 rpm for 5 minutes. The supernatant was discarded. Ten microlitres (10 mg/mL) of acridine orange (AO) and 10 µL of propidium iodide (10 mg/mL) were added to the tube and resuspended. Finally, after 3 minutes of incubation, about 10 µL of the mixture of stained cells was fixed on a slide and mounted with coverslips for further observation under the fluorescence microscope (Nikon, Japan).

Mitochondrial membrane potential (ΔΨm) detection

Polarized and depolarized mitochondrion due to apoptotic activity was detected via the BD™ MitoScreen Kit (Becton Dickinson, USA). Briefly, 100 µL MCF-7 cells at a concentration of 1 x 106 cells/mL were aliquoted into wells of a 96-well microtitre plate and treated with IC_{50} of ethanolic extract of *A. malaccensis* or doxorubicin. After consequences of 1-, 3- and 5-hours, the cell pellet obtained was resuspended in 0.5 mL of JC-1 solvent, stirred for a while, and placed in a CO_2 incubator at 37°C for 15 minutes. Next, 1 x Assay Buffer was used to cleanse the cells for two times and centrifuged at 1700 rpm for 5 minutes. Then, supernatant in every tube was disposed before the pellet was resuspended in 0.5 mL of 1 x Assay Buffer. Finally, data acquisition and analysis of the mitochondrial potential of MCF-7 cells induced by ethanolic extract of *A. malaccensis* was performed in CytoFlex flow cytometer (Beckman Coulter, USA) using CytExpert software.

Statistical analysis

GraphPrism 8 (GraphPad Software, La Jolla, USA) was used to analyse the data in triplicate. Methods used in this study include student's t-test or one-way ANOVA and Tukey's test, with $p \leq 0.05$ deemed significant. Results were taken as mean ± standard error of the mean.

RESULTS

Cytotoxicity of *A. malaccensis* extract against MCF-7 cells

The ethanolic extract of *A. malaccensis* was evaluated for its cytotoxic activity against MCF-7 cells. An over-the-counter medication for breast cancer, doxorubicin, was set as positive control of the experiment. The MTT assay was used to detect the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) by mitochondrial dehydrogenase to formazan blue, which reflects normal mitochondrial function in viable cells. The toxicity of *A. malaccensis* extract and doxorubicin was assessed after 72 hours of treatment compared with untreated cells using a concentration that reduced cell number to 50% (IC_{50}), the same figure employed to represent the cytotoxicity of cell lines. The cytotoxic activity of the extracts of *A. malaccensis* on the growth of MCF-7 and NIH/3T3 cells is shown in Table I. The extract of *A. malaccensis* showed strong inhibition by reducing the viability of MCF-7 cells at an IC_{50} of 4.1 ± 2.08 µg/mL. On the other hand, the IC_{50} of doxorubicin against MCF-7 cells was 2.92 ± 0.12 µg/mL, as shown in Figure 1. Our results indicate that the ethanolic extract from the leaves of *A. malaccensis* has potent cytotoxic feature on MCF-7 cells in a time- and dose-dependent manner. This outstanding effect of *A. malaccensis* extract could possibly reveal its potential usefulness in the treatment of non-communicable diseases such as cancer.

Table I: IC_{50} values of MCF-7 and NIH/3T3 cells after 72 hours of treatment with ethanolic extract of *A. malaccensis* and doxorubicin

Incubation Period (Hours)	Mean Average Values of IC_{50} (µg/mL) * ± S.E.M			
	MCF-7		NIH/3T3	
	A. malaccensis	Doxorubicin	*A. malaccensis*	Doxorubicin
72	4.1 ± 2.08	2.92 ± 0.12	75.1 ± 0.88	12.75 ± 2.60

*IC50 values were determined from the dose response curves that correlate between the cell viability and extract concentration. The IC_{50} values were expressed as Mean ± S.E.M determined from three independent experiment that were performed in three replicates

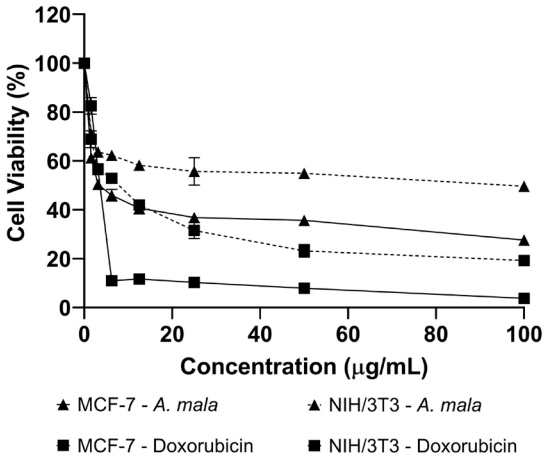

Figure 1: Cytotoxicity of A. malaccensis extract and doxorubicin against MCF-7 and NIH/3T3 cells after 72 hours of treatment. The IC50 for extract and doxorubicin against MCF-7 cells were 4.1 ± 2.08 µg/mL and 2.92 ± 0.12 µg/mL, respectively. Each point represents the mean of the results of three independent experiments. Error bars represent means ± S.E.M. of the three independent experiments.

Anti-proliferative effects of *A. malaccensis* extract against MCF-7 cells

Effects of *A. malaccensis* ethanolic extract at IC_{25}, IC_{50} and IC_{75} concentrations on MCF-7 cells proliferation were studied over a period of 72 hours. Viable cells quantified by MTT assay indirectly reflect the proliferation rate of MCF-7 cells. For IC_{25}, viable cells reduced to 83.04%, 68.02% and 35.75% after 24-, 48- and 72-hours, respectively ($p<0.05$ for controlled cells). Meanwhile, for IC_{50}, viable cells diminished to 82.76% and 21.74% after 24 and 72 hours, respectively. Finally, for IC_{75}, viable cells downed 62.88% and 5.12% after 24

and 72 hours, respectively. On the contrary, according to the OD values, proliferation rate of controlled MCF-7 cells increased to 128.40%, 167.16% and 191.11% after 24-, 48- and 72-hours, respectively. As depicted in Figure 2, decrement in cell proliferation rate is directly related to the dose used, where the rate decreased significantly in the first 24 hours, and further reduced for the next recorded time frames (48 and 72 hours).

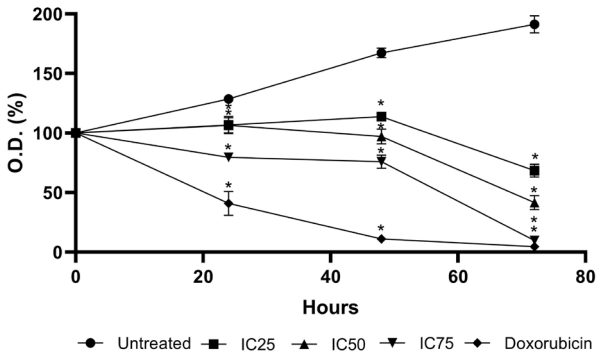

Figure 2: Effects of *A. malaccensis* extract at different concentration on the viability of MCF-7 for 24-, 48- and 72-hours treatment period. Each point represents the mean of the results of three independent experiments. Error bars represent means ± S.E.M. of the three independent experiments. *Statistical significance (P<0.05) between control cells and treatment groups.

Method of cells death

AO/PI double staining assay is considered the appropriate qualitative method for determining the method of cells death induced by any substance. On that note, this study implemented the said strategy to determine viable, apoptotic, and necrotic cells after being treated with ethanolic extract of A. malaccenis. Figure 3 shows the treated and untreated MCF-7 cells after 24-, 48- and 72-hours treatment with IC_{50} of *A. malaccensis* ethanolic extract stained with AO/PI. In general, *A. malaccensis* clearly showed induction of apoptosis as a method of cells death of MCF-7 cells. As early as 24 hours after treatment, an early stage of chromatin condensation and membrane blebbing was observed in the cell's population treated with MCF-7. As expected, additional transformations were evident on MCF-7 cells exposed to the ethanolic extract of *A. malaccensis*. The viable cells population was greatly reduced, with most cells apoptotic and showing severe DNA fragmentation.

Mitochondria membrane potential (ΔΨm) depolarization analysis

One method of detecting early events of apoptosis is to analyse depolarisation of the mitochondrial membrane potential (ΔΨm) of the cells after treatment. Healthy cells have healthy polarised mitochondrial membranes that can take up the JC-1 dye to become brighter red fluorescent colour, which can be distinguished using a flow cytometer. JC-1 is a fluorescent dye commonly used to assess mitochondrial membrane potential (ΔΨm) in living cells. The dye can be taken up by

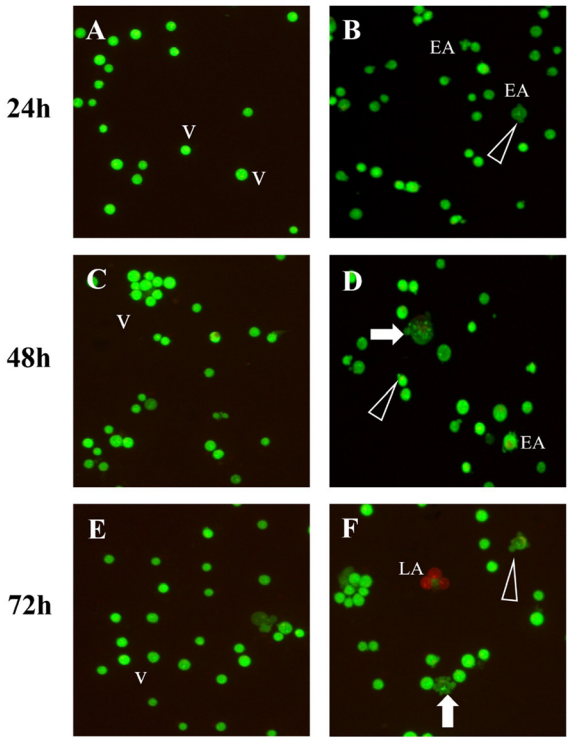

Figure 3: Morphological assessment of MCF-7 cells stained with acridine orange (green) and propidium iodide (red). Cells were incubated (A, C, E) without or with treatment by (B, D, F) 4 µg/mL of *A. malaccensis* for 24, 48 or 72 hours in culture plates. Cells with intact membrane stained green indicate viable cells (V); cell that show membrane blebbing (arrowhead) and chromatin condensation, nuclear genome fragmentation (filled arrow) indicate early apoptosis (EA); cells that are stained orange and contain fragmented DNA represent late apoptosis (LA).

cells and accumulates in the mitochondria, where it undergoes a potential-dependent aggregation that shifts the fluorescence emission from green to red. This shift in fluorescence is indicative of healthy, polarized mitochondria with high membrane potential, while a decrease in red fluorescence and an increase in green fluorescence indicates mitochondrial depolarization and potential loss, which can be indicative of cellular stress, damage, or apoptosis. In the present study, we examined the potential activity of mitochondrial membranes of the MCF-7 cells to detect early signs of apoptosis by incubating the cells for 1, 3, and 5 hours. (Figure 4). Depolarisation in the mitochondria of MCF-7 cells in the treatment groups was normalised for direct comparison with the control group. After treatment with *A. malaccensis* extract, mitochondrial membrane potential (ΔΨm) of the MCF-7 cells started to depolarise at 3.11% after 1 hour. Aggregations of JC-1 dye in the mitochondrial membrane decreased after 3 and 5 hours in the treated groups, resulting in increased depolarisation of mitochondrial membrane in MCF-7 cells. In the group treated with *A. malaccensis* extract, the depolarised mitochondrial membrane potential (ΔΨm) of the MCF-7 cells increased from 3.53% after 3 hours to 4.45% after 5 hours of treatment. Meanwhile, in the doxorubicin-treated group, the

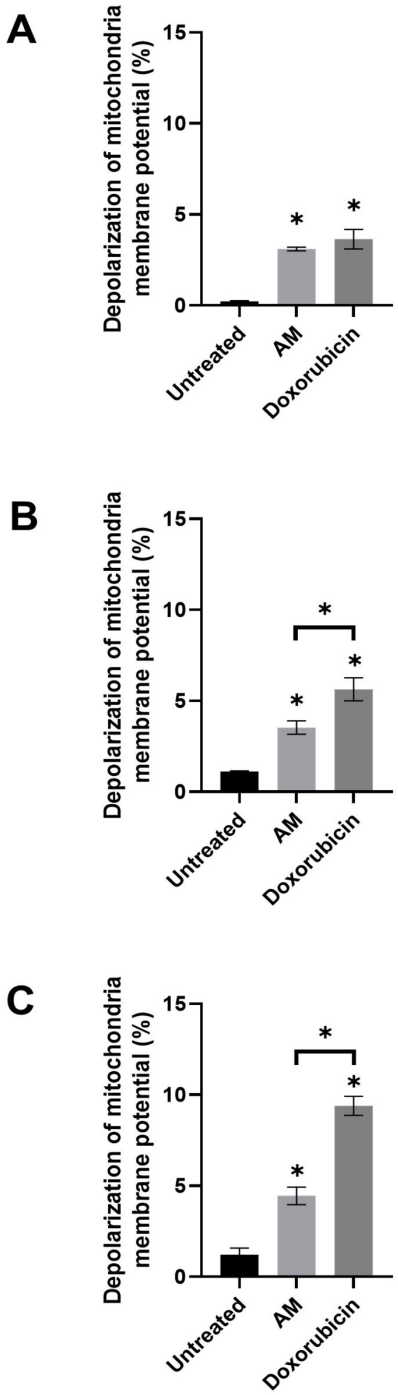

Figure 4: Depolarization of mitochondrial membrane potential (ΔΨm) in the MCF-7 cells after incubation for 1 (A), 3 (B), and 5 (C) hours. The results are expressed as fold change relative to ΔΨm of the untreated control group. Error bars represent means ± S.E.M. of three independent experiments. *Statistical significance (P<0.05) between the control group and the treatment groups

depolarised mitochondrial membrane potential (ΔΨm) of the MCF-7 cells increased from 5.64% after 3 hours to 9.39% after 5 hours of treatment. From the results, it is evident that mitochondrial membrane potential (ΔΨm) depolarisation for treated cells increased with respect to exposure time.

DISCUSSION

In recent years, complementary and alternative medicine (CAM) has been widely practiced especially among cancer patients to alleviate the symptoms and improve the quality of life. Medicinal plants are one of the sources of traditional remedies that have been utilized since antiquities to treat the problem. Application of plant extracts or natural compounds in cancer therapy research has attracted significant attention globally. The literature agreed that phytochemicals derived from medicinal plants are crucial in dealing with various ailments and shown great promise as therapeutic anticancer agents with negligible side effects. Previous studies revealed that *A. malaccensis* oil possesses cytotoxic activity against some cancer cell lines that originated from the colon and breast (20). Due to its promising anticancer properties, the present study aimed further to investigate the potential antitumour activities of the ethanolic extract of *A. malaccensis* against MCF-7 cells. Cytotoxic and antiproliferative outcomes exhibit that this extract is capable of inhibiting the growth of MCF-7 cells. Cytotoxicity of the extract is very strong with regard to MCF-7 cells, recording IC_{50} of 4.1 ± 2.08 µg/mL. According to the American National Cancer Institute (NCI) standards, crude extracts that have IC50 value of less than 20 µg/mL are considered active and potentially cytotoxic against cancer cells. In contrast, IC50 values of 20-100 µg/mL are considered moderately cytotoxic (21-23). Compounds with IC_{50} values of less than 5.0 µg/mL are considered very cytotoxic, those between 5.0-10.0 µg/mL are considered moderately active and compounds with IC_{50} values between 10-25 µg/mL are considered weak (18). However, the ethanolic extract of *A. malaccensis* was only mildly cytotoxic on standard mouse fibroblast cell line NIH/3T3. This finding is similar to that of Dyary et al. (24) who reported IC_{50} of *A. malaccensis* ethanolic extract of more than 100 µg/mL against monkey kidney epithelial Vero cells. Nevertheless, these results clearly show that *A. malaccensis* exerts selective anticancer effects on cervical cancer cells.

The occurrence of apoptosis is described as a cascade of programmed processes involving a series of characteristic morphological and biochemical criteria (25). Among the main reasons for cell death, apoptosis relies on mitochondria or death receptor and causes a shift in the cell membrane integrity (26). Further experiments were conducted to investigate the type of cell death caused by ethanolic extract of *A. malaccensis* on MCF-7 cells using AO/PI double staining assay and to determine polarisation of mitochondrial membrane potential. Fluorescent dyes, for example AO and PI, can be used to colour apoptotic cells to determine how a medicine causes cell death (27). Having nucleic acid-selective and cationic properties, AO produces protonated positive charge after it enters the plasma membrane of normal or

pre-apoptotic cells, resulting to green fluorescence with it fuses with DNA and RNA (28). On the other hand, nucleic acids in cells with lysed membranes responds to PI, resulting to orange-coloured cells (29). Using this hybrid method, the study successfully tells apart various subgroups of apoptotic cells from normal, membrane-intact pre-apoptotic and necrotic cells groups in terms of nuclear morphology, e.g., perinuclear chromatin condensation, membrane vesicles, cell shrinkage, nuclear collapse, and eventual DNA fragmentation of MCF-7 treated cells. As a note, normal MCF-7 cells have vivid green nuclei with undamaged structures. As shown in Figure 3, apoptosis rather than necrosis occurred in MCF-7 cells treated with ethanolic extract of *A. malaccensis*.

Another way to study apoptosis is to determine polarisation of the mitochondrial membrane potential of the cells after treatment (29,30). The ethanolic extract of *A. malaccensis* mediates apoptosis via depolarisation of the mitochondrial membrane ($\Delta\Psi m$). This is consistent with the previous statement that apoptosis can be induced by mitochondria dependent or a death receptor pathway. From the MTT assay experiment, it is evident that mitochondrial activity in MCF-7 cells was inhibited by *A. malaccensis*, although there was no proof of apoptosis happening. Determination of J-aggregates formation in the cells thus provided additional evidence that *A. malaccensis* triggered polarisation of the mitochondrial membrane potential in MCF-7 cells.

CONCLUSION

The present in vitro study showed that the ethanolic extract of *A. malaccensis* possesses cytotoxic properties and potential pharmacological activity against human breast cancer MCF-7 cells. The results found that majority of MCF-7 cells died due to apoptosis—making it vital for future researchers to separate cytotoxic matters in the extract. Further work should focus on evaluating the efficacy of the extract in an in vivo model.

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