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The Phytochemical Components of Kelantan Grown *Annona muricata* Leaves and Its Anti-Proliferative Properties on MCF-7 Breast Cancer Cells

(Komponen Fitokimia Daun *Annona muricata* Ditanam di Kelantan dan Sifat Anti-Proliferatifnya terhadap Sel Kanser Payudara MCF-7)

Norzila Ismail[1], Harishini Rajaratinam[2], Khairunnisa Che Ghazali[3], Siti Farhanah Mohd Salleh[1], Wan Suriyani Faliq Adeeba Wan Ibrahim[4], Maya Mazuwin Yahya[3,]& Wan Zainira Wan Zain[3]

[1]*Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[2]*School of Health Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[3]*Department of Surgery, Hospital Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[4]*Faculty of Bioengineering & Technology, Universiti Malaysia Kelantan, 17600 Jeli, Kelantan, Malaysia*

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ABSTRACT

Annona muricata leaves exhibit anti-cancer properties against breast cancer. In this study, the phytochemical components, anti-proliferative activity, and cytotoxicity of Kelantan (Malaysia) grown *Annona muricata* leaves were evaluated using MCF-7 breast cancer cell line. The preparation of *Annona muricata* leaves extracts was performed with various organic solvents (ethyl acetate, *n*-hexane, methanol, and aqueous) using Soxhlet method. Each extract was analysed using gas chromatography-mass spectrometry (GCMS) for phytochemical analysis and characterised with Wiley and NIST library searchers. The effects of all extracts on cell proliferation were analysed using MTT assay. The anti-proliferative activity was determined by evaluating the IC_{50} value. Based on the lowest IC_{50} value, further experiments were done to observe the apoptosis effect using the Annexin V-FITC technique. While for cell cycle analysis, Cycletest Plus DNA Reagent Kit was used. Both were further determined using flow cytometry analysis. The ethyl acetate-based *Annona muricata* extract contained terpenoids, phenolic compounds, and alkaloids. Ethyl acetate extract of *Annona muricata* exhibited a significant cytotoxic effect on MCF-7 breast cancer cells. The most potent IC_{50} value obtained was 21.8 ± 3.85 µg/mL. The changes in morphology were more profound after 72 h of treatment with ethyl acetate extract of *Annona muricata,* including cell shrinkage. Flow cytometry analysis showed that it was induced in early and late apoptosis *in vitro* and arrest at G_0/G_1 cell cycle phase in time-dependant manners. In short, ethyl acetate extract of *Annona muricata* exhibited a range of phytochemical components, anti-proliferative activity and cytotoxic effect on MCF-7 breast cancer cells.

Keywords: *Annona muricata*; apoptosis; breast cancer; cytotoxicity; MCF-7

ABSTRAK

Daun *Annona muricata* menunjukkan sifat anti-kanser terhadap kanser payudara. Dalam kajian ini, komponen fitokimia, aktiviti anti-proliferatif dan kesitotoksikan daun *Annona muricata* yang ditanam di Kelantan (Malaysia) dinilai menggunakan titisan sel kanser payudara MCF-7. Penyediaan ekstrak daun *Annona muricata* dilakukan dengan pelbagai pelarut organik (etil asetat, *n*-heksana, metanol dan akueus) dengan menggunakan kaedah Soxhlet. Setiap ekstrak dianalisis menggunakan spektrometri jisim kromatografi gas (GCMS) untuk analisis fitokimia dan dicirikan dengan pencari perpustakaan Wiley dan NIST. Kesan semua ekstrak pada pertumbuhan sel telah dianalisis menggunakan asai MTT. Aktiviti anti-proliferatif ditentukan melalui nilai IC_{50}. Berdasarkan nilai IC_{50} yang paling rendah, uji kaji lanjutan telah dilakukan untuk memerhatikan kesan apoptosis menggunakan teknik Annexin V-FITC. Manakala untuk analisis kitaran sel, Cycletest Plus DNA Reagent Kit digunakan. Keputusan daripada kedua-dua uji

kaji dikukuhkan dengan kaedah analisis sitometri aliran. Ekstrak *Annona muricata* berasaskan etil asetat mengandungi terpenoid, sebatian fenol dan alkaloid. Ekstrak etil asetat *Annona muricata* menunjukkan kesan sitotoksik yang ketara pada sel kanser payudara MCF-7. Nilai IC_{50} yang paling berkesan diperoleh ialah 21.8 ± 3.85 µg/mL. Perubahan morfologi sel lebih ketara selepas 72 jam rawatan dengan ekstrak etil asetat *Annona muricata* termasuk pengecutan sel. Analisis sitometri aliran menunjukkan bahawa ia telah diinduksi pada apoptosis awal dan lewat secara *in vitro* dan perencatan pada fasa kitaran sel G_0/G_1 secara berkadar langsung dengan masa. Ringkasnya, ekstrak etil asetat *Annona muricata* menunjukkan pelbagai komponen fitokimia, aktiviti anti-proliferatif dan kesan sitotoksik terhadap sel kanser payudara MCF-7.

Kata kunci: *Annona muricata*; apoptosis; kanser payudara; kesitotoksian; MCF-7

Introduction

The complementary use of herbal medicines is well established to be safe and effective. It has been used long in history for disease prevention and the ailment of chronic diseases. Thus, they are accepted by national authorities worldwide. World Health Organization (WHO) plays an important role in making policies and stimulating strategic research into traditional medication by evaluating its safety and efficacy (WHO Traditional Medicine Strategy 2013). Breast cancer is one of the common forms of cancer that occurs among females globally. According to the latest GLOBOCAN report released by International Agency for Research on Cancer (IARC) in 2020, breast cancer has the highest percentage of incidence (24.5%), followed by colorectal cancer (9.4%), lung cancer (8.4%), and cervical cancer (6.5%). The report also highlighted that breast cancer holds the record for the highest number of prevalent cases in 5 years (33.7%) and the number of death cases (15.6%). In Malaysia, breast cancer exhibited the highest age-standardised incidence rate (ASR), accounting for up to 34.1, followed by colorectal (11.1) and cervix uteri (6.2) cancer. These figures were included in the Malaysian National Cancer Registry report (MNCR) 2012-2016 that was published in 2019 (MNCR 2019).

Malaysian women who suffer from breast cancer are mostly presented with larger tumours, hence a poorer prognosis. Few factors influence the late stage of presentations, including lack of education or awareness, poverty, complementary and alternative medicine usage, lack of autonomy in making decisions, and avoidance of evidence-based medicine. Five-year survival varies among different institutions in Malaysia. Surgery and chemotherapy have been shown to improve survival, however, because of disfigurement and compromising one's immune system, these complications remain significant barriers for patients to accept these treatment options (Yip, Pathy & Teo 2014). To date, few complementary medicines have been tested through sturdy clinical trials especially those medicinal plants, where their metabolites or active compounds are discovered to have anti-cancer potential (Syed Najmuddin et al. 2016).

The tropical tree *Annona muricata* belongs to the family of Annonaceae. It is a widespread small tree and has its native to central America. The plant of interest is widely distributed in most tropical countries. It is also known as *Graviola* or soursop. All parts of this tree can be used as natural medicine including the twigs, leaves, roots, fruits, and seeds (Gajalakshmi, Vijayalakshmi & Devi 2012). Various research was done on the chemical composition of the leaves and seeds to explain their therapeutic effects. *Annona muricata* has been identified to display promising compounds that could potentially be utilised for cancer treatment (Ilango et al. 2022). The most prevalent phytochemical components detected and isolated from this plant are alkaloids, phenols, and acetogenins (Ilango et al. 2022). The anti-cancer effect of *Annona muricata* leaves in retarding breast cancer has been sufficiently addressed by various studies (Hadisaputri et al. 2021; Ilango et al. 2022; Sulistyoningrum et al. 2017).

A particular study by Endrini, Suherman and Widowati (2014) cultivated our interest in selecting *Annona muricata* as our plant of interest. The study addressed that *Annona muricata* Linn leaves have stronger cytotoxic activity against MCF-7 breast cancer cell line as compared to *Hedyotis corymbosa* (pearl grass). Considering the similar cell line used, we have decided to further explore the anti-cancer properties of *Annona muricata* leaves. However, the previous study by Endrini, Suherman and Widowati (2014) utilised *Annona muricata* leaves that were obtained in Indonesia.

According to Syed Najmuddin et al. (2016), the *Annona muricata* leaves grown in various states (Johor,

Melaka, Negeri Sembilan, Selangor, Perak, and Perlis) in Malaysia showed varied IC_{50} values, showing the influence of the secondary metabolite constituents composed in them. This circumstance could be explained by the geographical difference of the sample cultivation area. The geographical difference of the cultivated plant means that each plant is introduced to different climate and environmental stress factors such as humidity, temperature, and soil composition (Gull et al. 2012). To the best of our knowledge, there is no study till date that tested the anti-cancer properties and studied the phytochemical components of *Annona muricata* leaves grown in Kelantan, using MCF-7 breast cancer cells.

Therefore, the main objective of the present study was to determine the phytochemical components, anti-proliferative activity, and cytotoxicity of Kelantan (Malaysia) grown *Annona muricata* leaves on MCF-7, a hormone-receptor positive breast cancer cell line. It is a prospective study to investigate the effect of *Annona muricata* extract on breast cancer cell proliferation morphologically. The *Annona muricata* extracts were also tested on MCF-10A, a normal breast cell line, to determine whether it has potential toxicity towards normal cells or otherwise. In addition, the induction of apoptosis and its effect on cell cycle arrest in MCF-7 breast cancer cells via the introduction of *Annona muricata* extract were also investigated.

Materials and Methods

Plants Material

The leaves of *Annona muricata* were collected from Kota Bharu, Kelantan, Malaysia. The leaves, twigs, and flowers were authenticated in Universiti Sains Malaysia Herbarium, by our botanist Dr. Rahmad Bin Zakaria. The voucher specimens are under reference number 11765. The leaves were plucked fresh from the tree, cleaned, and ground. They were dried using an oven till 40 °C and ground to become powder.

Preparation of the Extracts

The preparations of *Annona muricata* leaves extracts were carried out using the Soxhlet method. The *Annona muricata* leaves were dried in an oven at 40 °C and then ground into powder. Approximately, 20 g of powdered leaves were then extracted using ethyl acetate until the colour cleared. The extract was then filtered and dried under reduced pressure by a rotary evaporator. The same procedure was repeated to prepare extracts using different solvents (*n*-hexane, methanol, and aqueous). All crude extracts were measured in grams.

Cell Culture

The MCF-7 and MCF-10A breast cell lines were utilised to examine the anti-cancer effect of *Annona muricata*. The MCF-7 and MCF-10A cell lines were obtained from the American Type Culture Collection (ATCC, USA). The cells were stable as they were not mixed with other tissues, hence enabling us to produce more consistent results for comparison. MCF-7 is a human breast cancer cell line with oestrogen, progesterone, and glucocorticoid receptors (Horwitz, Costlow & McGuire 1975). MCF-10A, on the other hand, is a human breast epithelial cell line that is non-tumorigenic (Amaro et al. 2020) (Figure 1).

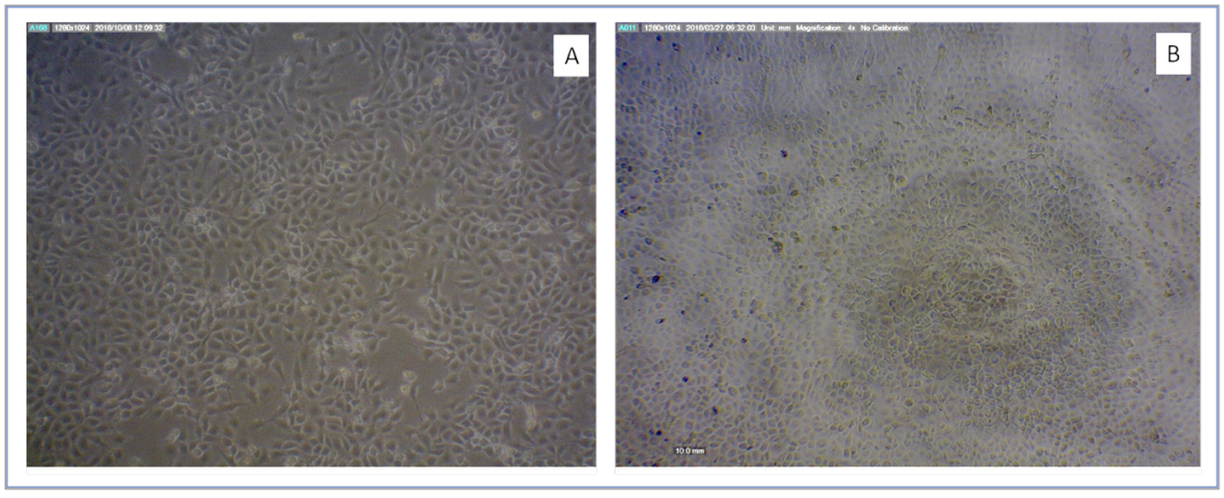

FIGURE 1. (A) MCF-7 breast cancer cell and (B) MCF-10A breast cell under microscope view (4X magnification), which have been cultured

The MCF-7 and MCF-10A cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% of fetal bovine serum (FBS). Antibiotics were added to the cell media to inhibit the growth of bacteria (100 µg/mL penicillin and 100 µg/mL streptomycin). The cells were maintained at 37 °C and 5% carbon dioxide (CO_2) in a humidified atmosphere in the incubator.

CELL VIABILITY (CYTOTOXIC) ASSAY

About 10 mg/mL of crude extracts (ethyl acetate, *n*-hexane, methanol, and aqueous) were used to carry out the cytotoxicity assay. Tamoxifen, a well-known hormonal modulator that blocks the oestrogen receptors was prepared in the form of a stock solution in dimethyl sulfoxide (DMSO) and used as a positive control. Eight serial dilutions were made with concentrations starting from 0.99 mg/mL to 0.039 mg/mL. Negative control for all the assays was represented by untreated medium containing DMSO (0.1%) whereas Tamoxifen was considered as the positive control.

Once the MCF-7 and MCF-10A achieved 80% confluence, they were trypsinised. A 100 µl medium that contained 5×10^4 cells/mL was seeded into a 96-well plate. Only central 60 wells of micro-titre filled in with cells. The four edges of the well were filled with distilled water to prevent the plate from drying during incubation. The plate was incubated overnight. Then after 24 hours, the old media was removed and 200 µL of new media was filled in. The MCF-7 and MCF-10A cells (5×10^4 cells/mL) were treated with the presence of 4 types of leaf extracts; *n*-hexane, acetyl acetate, methanol, and aqueous, at different concentrations. Cells in the first column of the seeded well were treated with 2 µL of stock solution extract (2 µL of 10 mg/mL *Annona muricata* extract). For the subsequent column of cells from second to 9th, cells were treated with serial dilutions of extract starting from 2 µL of 5 mg/mL of extract until 0.0391 mg/mL. In the 10^{th} column, cells were treated with 2 µL of DMSO 0.1% as negative control (DMSO is supposedly non-toxic to cells). Another fresh set of 96-well plate was prepared as discussed earlier for treatment with different concentration of Tamoxifen as a positive control.

After 72 h, the culture media was replaced with fresh media containing thiazolyl blue tetrazolium bromide (MTT) reagent. After 4 h of incubation at 37 °C in 5% CO_2 in the humidified atmosphere, the media was replaced with 200 µL of DMSO. The plates were placed on a shaker at room temperature for 5 min and then covered with aluminium foil. The absorbance of cell viability was measured using an ELISA plate reader at the wavelength of 570 nm. The corresponding cytotoxic values (IC_{50}) were calculated (IC_{50} is a value that produces inhibitory concentrations of cancer cells by 50%) (Gavamukulya et al. 2014; He et al. 2016). The IC_{50} value was determined from a graph of the percentage of cell viability versus $\log_{10}$ concentration (mg/mL) of extract. The percentage of cell viability was determined from the formula as follows:

$$\text{Percentage of cell viability} = \frac{\text{mean absorbance of treatment}}{\text{absorbance of DMSO}} \times 100\%$$

The best extract was then selected to proceed for further analysis i.e., morphological effect, apoptosis, and cell cycle. The best extract is defined as the one with the lowest IC_{50}. From this cytotoxic assay, ethyl acetate extract of *Annona muricata* leaves showed the most potent IC_{50} (the lowest IC_{50}). Hence, we used only ethyl acetate extract to continue the study.

MORPHOLOGICAL EFFECTS

The best crude extract was used to observe the morphology of the treated MCF-7 breast cancer cell line. The cells were incubated in a 5% CO_2 incubator at 37 °C for 0, 24, 48, and 72 h. The morphology of the cells was observed using a light microscope and recorded by Dino eye software.

APOPTOTIC EFFECT

The apoptotic effects of *Annona muricata* extract were done by using Annexin V-FITC, BD Pharmingen (BD 556420) kit. The MCF-7 breast cancer cell line (5×10^4 cells/mL) was seeded into 96-well plates and followed by treatment with the best concentrations of extracts according to IC_{50} obtained from the previous study. The treated MCF-7 cells were incubated for 0, 24, 48, and 72 h.

The cells were washed using PBS and then re-suspended in a binding buffer at a concentration of 1×10^6 cells/mL. 100 µL of the cell suspension was transferred to a 5 mL culture tube. 5 µL of Annexin V-FITC and 5 µL of PI were added to the cell suspension. Then incubated for 15 min at room temperature (25 °C) in the dark. Another 400 µL of binding buffer Annexin V- FITC solution was added into the test tube containing treated cells. The apoptotic effects were then analysed under a flow cytometer and 10,000 events were acquired

using the green channel for Annexin V-FITC and the red channel for PI. The test was done triplicate at different time points (0, 24, 48, and 72 h) and the result was analysed.

The data was obtained in the form of a dot plot that represents the cell population. From the dot plot, the characteristics of the cells within the population were determined. The Annexin V-FITC was utilised to identify the apoptotic effect where the apoptotic cell would take up the markers and represent the population of interest. The percentage of the cell population that displayed the characteristic of apoptosis and the population that did not display such characteristic were counted via flow cytometry.

CELL CYCLE STUDY

The MCF-7 breast cancer cell lines were treated with the best concentration of extract and Tamoxifen according to IC_{50} obtained from previous study and incubated overnight. The cells are washed with phosphate-buffered saline (PBS) and then collected and put in the test tube. The cell cycle study was conducted using Cycletest Plus DNA Reagent Kit. The cells were then analysed using a flow cytometer (Moghadamtousi et al. 2014). The data was presented in the form of a dot plot. The data represented the cell population. Using Cycletest Plus DNA reagent kit, the dot plot described the characteristic of cell cycle differentiation. The cell cycle phase at which affected by *Annona muricata* extract was observed via this technique.

RESULTS

PHYTOCHEMICAL ANALYSIS (GCMS)

The outcomes of the GCMS analysis of different solvent extracts of *Annona muricata* leaves were displayed in Tables 1-4. The chemical compounds of each solvent and aqueous extracts were different due to the different polarity of the solvents and water (Ab Rahman et al. 2018). Terpenoids and phenolic compounds were amongst the main phytocompound groups identified from ethyl acetate and hexane-based *Annona muricata* leaves extracts.

Phytochemical compounds identified from the ethyl acetate extract of *Annona muricata* were terpenoids (20.27%), phenolic compounds (10.14%), alkaloids (0.17%), and others (24.17%) (Table 1). Among the listed terpenoids within the extract, Neophytadiene stood out the most or had the highest expression, with approximately, 9.39% of the area (Table 1). Interestingly, none of the other extracts showed any presence of alkaloids (Table 1).

Similarly, the hexane-based extract also expressed high amounts of terpenoids (25.46%) and phenolic compounds (18.25%), followed by other compounds (21.62%) (Table 2). However, alkaloids were not identified in the hexane-based extract of *Annona muricata*. In this extract, gamma-Sitosterol had the highest percentage of the area (Table 2) as compared to other terpenoids.

In contrast to ethyl acetate and hexane-based extracts, the aqueous-based extract did not portray any presence of terpenoids, phenolic compounds or alkaloids (Table 3). With not much difference, the percentage of terpenoids in the methanol-based extract of *Annona muricata* was very small (0.12%) (Table 4).

The ethyl acetate and hexane-based extracts of *Annona muricata* leaves were found to exhibit better efficiency in extracting various polar chemical compounds such as terpenoids, phenolic compounds, and alkaloids, as compared to aqueous and methanol-based extracts.

CELL VIABILITY (CYTOTOXIC) ASSAY

The extracts of *Annona muricata* exhibited different suppressive effects on MCF-7 and MCF-10A normal breast cells. The cytotoxic effect is displayed below in Table 5, with the IC_{50} values listed. The ethyl acetate extract was more active and superior as it portrays an IC_{50} value of 21.8 ± 3.85 µg/mL against MCF-7, whereas *n*-hexane extract yields an IC_{50} of 38.6 ± 3.29 µg/mL. Tamoxifen is used as standard hormonal therapy for receptor-positive breast cancer, undeniably, it gives an ideal IC_{50} of 3.4 ± 0.64 µg/mL. On the other hand, methanol and aqueous extract did not affect cell viability in MCF-7 cells, hence no IC_{50} value was obtained.

For the MCF-10A cells, *n*-hexane and ethyl acetate extract were unable to give reasonable results as most cells were not viable after 72 h of treatment, despite multiple experiment repetitions. However, the aqueous extract did not exhibit any cytotoxic effect as the IC_{50} value was beyond 100. In contrast, the methanol extract exhibited a cytotoxic effect with an IC_{50} of 8.9 ± 3.15 µg/mL, and Tamoxifen showed an IC_{50} of 9.2 ± 1.59 µg/mL (Table 5) in MCF-10A cells.

TABLE 1. Compounds identified in ethyl acetate extract of *Annona muricata* leaves via GCMS analysis

Compounds	Percentage of total ion chromatogram
TERPENOIDS	
beta.-elemene	0.21
Caryophyllene	1.92
Ledene	0.53
alpha.-Muurolene	0.37
delta.-Cadinene	1.02
(-)-Loliolide	1.28
Neophytadiene	9.39
Isophytol	3.80
Campesterol	0.24
Stigmasterol	0.19
gamma.-Sitosterol	1.32
PHENOLIC	
Pyrocatechol	0.26
4-vinylphenol	1.25
2-Methoxy-4-vinylphenol	1.03
Phenol, 2,6-dimethoxy-	0.13
Benzeneethanol, 4-hydroxy	0.96
4-(1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	3.99
beta.-Tocopherol	1.61
Vitamin E	0.91
ALKALOIDS	
1H-Indole	0.17
OTHERS	24.17

TABLE 2. Compounds identified in hexane extract of *Annona muricata* leaves via GCMS analysis

Compounds	Percentage of total ion chromatogram
TERPENOIDS	
I-Limonene	0.29
beta.-elemene	0.06
trans-Caryophyllene	0.03
delta.-Cadinene	0.28
Spathulenol	0.06
Caryophyllene oxide	0.10
trans-Carveol	0.16
.tau.-Cadinol	0.17
alpha.-Cadinol	0.24
(-)-Loliolide	0.50
Neophytadiene	2.47
Farnesyl acetone B	0.21
Isophytol	0.07
Phytol	6.91
4,8,12,16-Tetramethylheptadecan-4-olide	0.29
Farnesyl acetone B	0.51
Geranylgeraniol	0.21
Farnesol	0.18
Squalene	1.1
cis-Farnesol	0.44
Campesterol	1.54
gamma.-Sitosterol	9.35
Lanosterol	0.29
PHENOLICS	
3-Buten-2-one,4-(4-hydroxy-2,2,6-trimethyl-7-oxabicyclo	0.13
beta.-Tocopherol	0.43
gamma.-Tocopherol	2.32
Vitamin E	15.37
OTHERS	21.62

TABLE 3. Compounds identified in aqueous extract of *Annona muricata* leaves via GCMS analysis

Compounds	Percentage of total ion chromatogram
OTHERS	
Benzoic acid,4-(trimethylsilyl)oxy)-tms	0.07
Raffinose TMS	7.33
Hexadecanoic acid, tms	0.76
Xylobiose	0.97
Xylobiose MEOX1 TMS	4.62

TABLE 4. Compounds identified in methanol extract of *Annona muricata* leaves via GCMS analysis

Compounds	Percentage of total ion chromatogram
TERPENOIDS	
β-Sitosterol tms	0.12
OTHERS Pyrrolidine,1-(trimethylsilyl)- Diethyl disulfide	28.49

TABLE 5. IC_{50} values of *Annona muricata* leaves extract on MCF-7 and MCF-10A breast cell line after 72 hours of treatment with different extracts

Cells	IC_{50} (µg/mL)				
	Tamoxifen	*n*-Hexane	Ethyl Acetate	Methanol	Aqueous
MCF-7	3.4 ± 0.64	38.6 ± 3.29	21.8 ± 3.85	>100	>100
MCF-10A	9.2 ± 1.59	-	-	8.9 ± 3.15	>100

Each data was expressed as mean ± standard error mean (SEM) of triplicate determination

MORPHOLOGICAL EFFECT

The visible changes in cell morphology, such as cell shrinkage, were noted after treatment with ethyl acetate extract of *Annona muricata*. The changes in morphology were more profound after 72 h of treatment. Cell shrinkage increased in a time-dependent manner (similar appearance to those treated with the positive control, Tamoxifen). Whereas in DMSO, cells appeared comparable between the initiation of treatment and after 72 h (Figure 2).

APOPTOSIS EFFECT

Apoptotic cells were examined by counting the percentage of early and late apoptotic cells. Early apoptotic was defined by Annexin V positive and PI-negative (at right lower quadrant) while late apoptotic was defined by both Annexin V and PI-positive (at right upper quadrant) (Figure 3). To examine the effect of ethyl acetate *Annona muricata* extract on MCF-7 breast cancer cells, an Annexin V-FITC kit was used to determine the percentage of cells undergoing apoptosis. After treatment with ethyl acetate extract, both the percentages of early and late apoptotic cells were elevated in a time-dependent manner (Figure 3).

After 72 h of treatment with ethyl acetate extract, the early apoptotic cell population increased gradually from 1.40 ± 0.06% in the control group to 4.30 ± 0.40% in the treatment group. There was also an increment in the population of late apoptotic cells from 2.07 ± 0.24% in the control group to 44.97 ± 2.54% in the treatment group. After 72 h of treatment, the percentage of cell viability reduced remarkably. As in this experiment, live cells decreased from 95.17 ± 0.52% to 36.47 ± 0.52% at the end of 72 h (Table 6). This implies that ethyl acetate extract of *Annona muricata* leaves can effectively induce apoptosis in MCF-7 breast cancer cells.

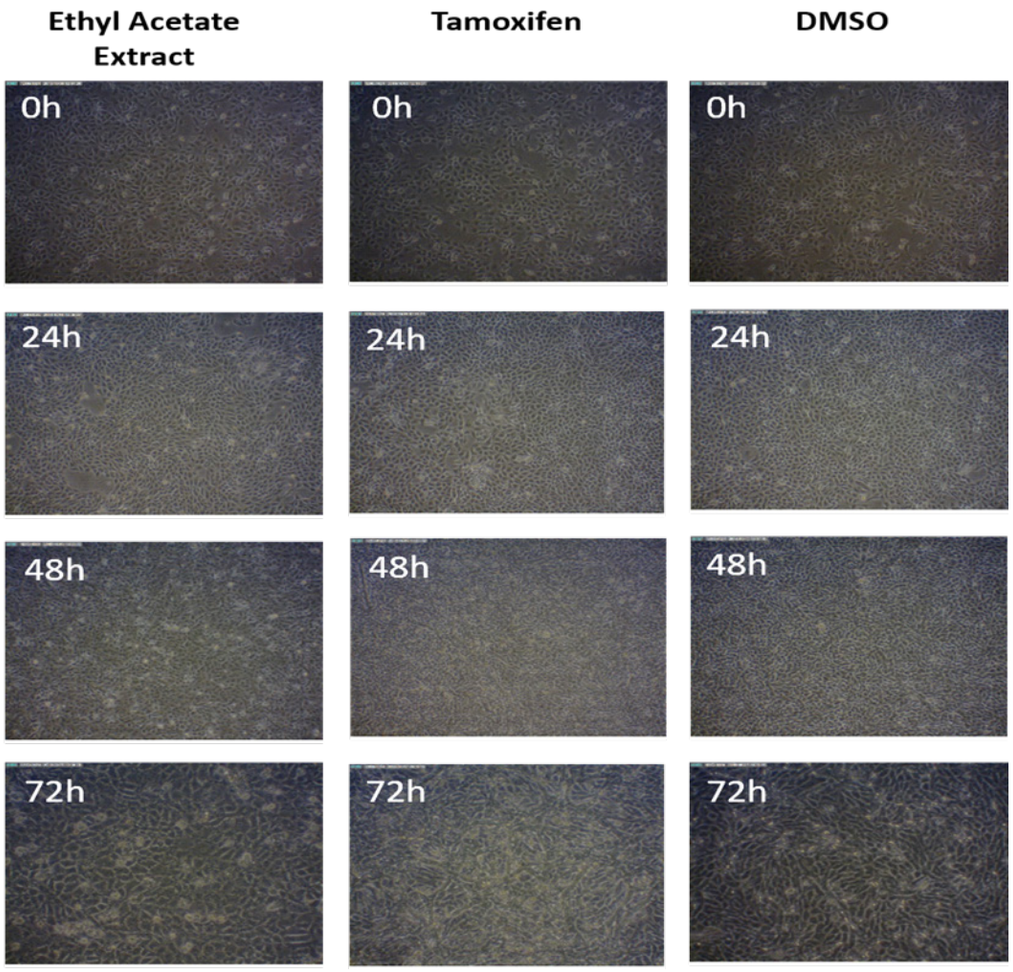

FIGURE 2. Morphological changes of MCF-7 breast cancer cell line after treated with ethyl acetate extract *Annona muricata* at different time frames (4X magnification under microscope view)

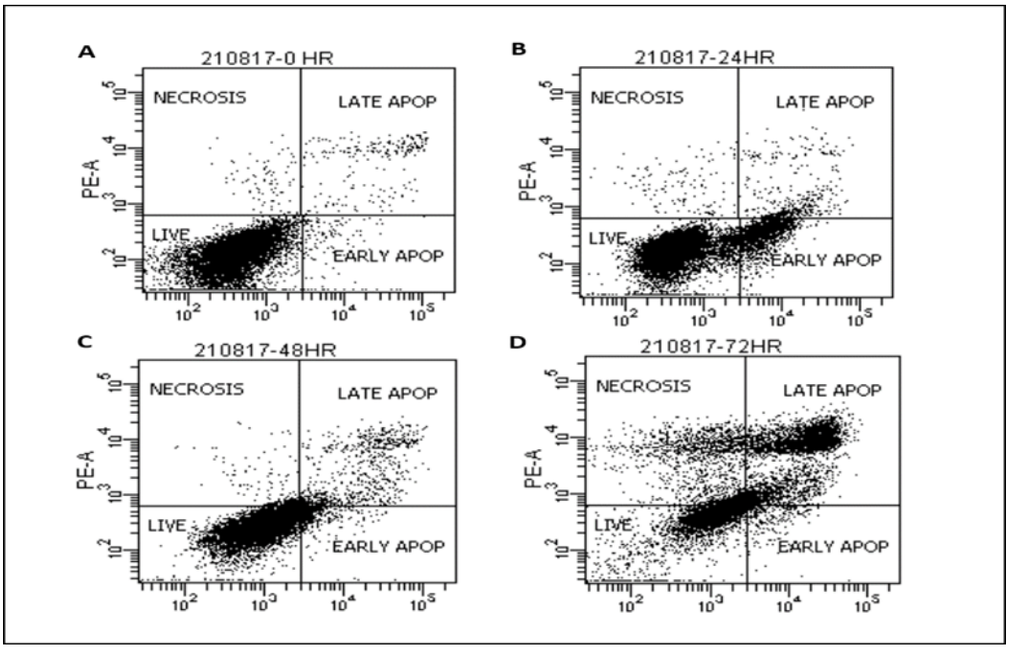

FIGURE 3. Time-dependant apoptosis rate of MCF-7 breast cancer cells treated with ethyl acetate extract of *Annona muricata* detected by flow cytometry after (A) control, (B) 24 h, (C) 48 h and (D) 72 h

TABLE 6. Percentage of cells undergoing early and late apoptosis after treatment with ethyl acetate extract of *Annona muricata* based on the duration of time

Treatment group	Percentage of the cells (%)			
	Live	Early apoptosis	Late apoptosis	Necrosis
Control	95.17 ± 0.52	1.40 ± 0.06	2.07 ± 0.24	1.30 ± 0.35
24 h	77.53 ± 0.37	16.07 ± 0.52	4.10 ± 0.17	2.23 ± 0.41
48 h	83.90 ± 0.36	6.53 ± 0.07	7.90 ± 0.45	1.73 ± 0.03
72 h	36.47 ± 0.52	4.30 ± 0.40	44.97 ± 2.54	14.33 ± 2.18

The data represent the mean ± SEM of three independent experiments

CELL CYCLE ARREST

As shown in Figure 4, there was a significant G_0/G_1 phase arrest in treated MCF-7 breast cancer cells in a time-dependent manner. After 72 h of treatment, the percentage of cells in G_0/G_1 phase markedly rose from 46.81 ± 0.28% in the control group to 82.49 ± 0.44%. On the other hand, the percentage of cells in the S phase also showed a significant decrement in promoting cell cycle arrest in G_0/G_1 phase (from 48.11 ± 0.56% to 8.39 ± 0.33% after 72 h) (Table 7). This result suggested that ethyl acetate extract of *Annona muricata* leaves arrested cell cycle at G_0/G_1 phase and induced apoptosis *in vitro*.

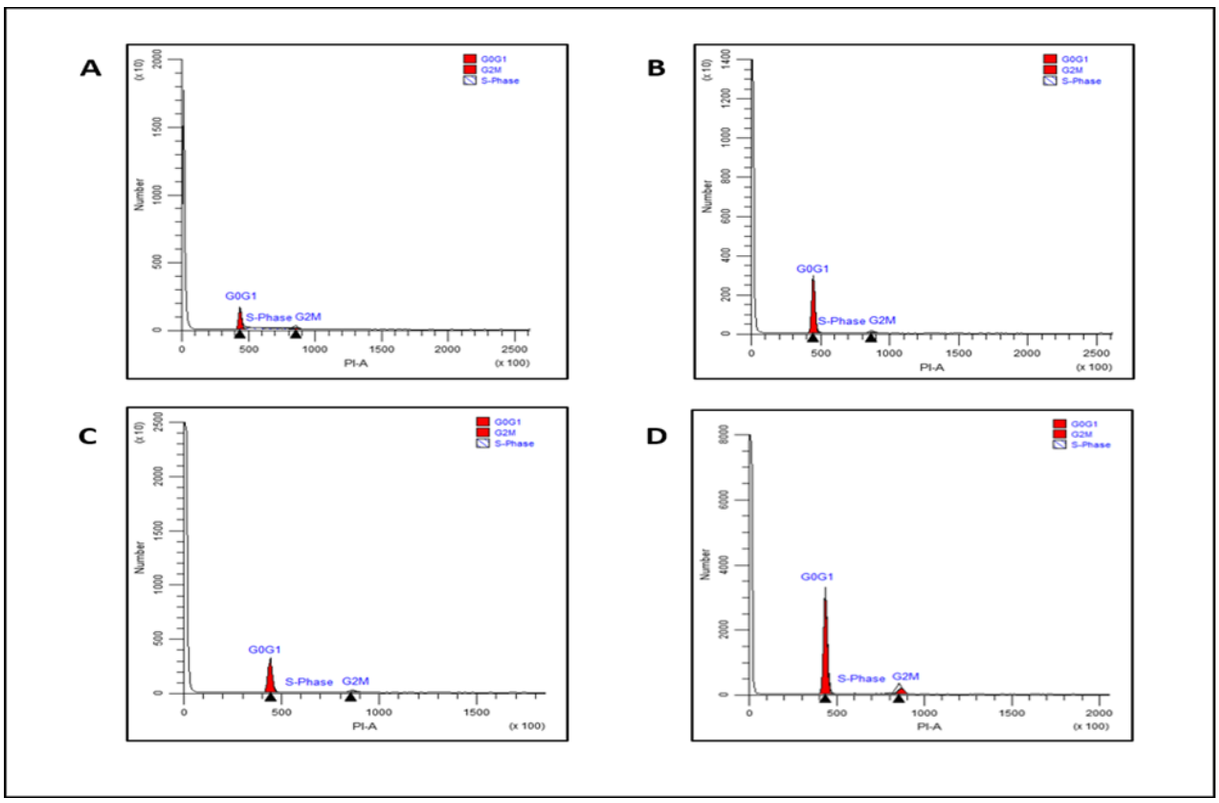

FIGURE 4. Flow cytometry analysis of cell cycle arrest of breast cancer cells treated with IC_{50} of ethyl acetate extract of *Annona muricata* in a time-dependant manner; (A) control, (B) 24 h, (C) 48 h and (D) 72 h

TABLE 7. Percentage of cells in each cell cycle phase after treatment with ethyl acetate extract of *Annona muricata* for 72 h

	Percentage of the cells (%)		
	G_0/G_1	G_2M	S
Control	46.81 ± 0.28	5.08 ± 0.31	48.11 ± 0.56
24 h	83.94 ± 0.27	2.43 ± 0.81	13.63 ± 0.33
48 h	85.56 ± 0.11	6.47 ± 0.26	6.97 ± 0.34
72 h	82.49 ± 0.44	9.12 ± 0.26	8.39 ± 0.33

The data represent means ± SEM of three independent triplicates

DISCUSSIONS

Medicinal plants have been used to treat ailments since before recorded history (Gajalakshmi, Vijayalakshmi & Devi 2012). According to WHO, people still seek herbal medicines and traditional treatment as part of healthcare, as they are natural treatment sources for chronic illnesses. Particularly in cancer, the usage of complementary and alternative medicines (CAM) is common among Malaysian cancer patients (Farooqui et al. 2016; Salleh et al. 2021). It may help cope with the quality of life and emotional distress; however, CAM may impose risk if there is interaction with conventional therapies (Farooqui et al. 2016). Therefore, it is important to explore the various natural resources, particularly in the field of ethnomedical, and investigate its diverse pharmacological aspects (Abdul Wahab et al. 2018). According to a study by Mohd Mujar et al. (2017), the highest CAM users are among Kelantanese compared to other states in Malaysia. The study added that the prevalence of CAM use among breast cancer patients was high, and women of Malay ethnicity was significantly associated with the use of CAM. Considering the majority of Kelantanese are of the Malay ethnicity (Department of Statistics 2022), it is ideal to conduct research on the pharmacological activities of the medicinal plants grown in Kelantan to verify the safety of using medicinal plants as part of CAM.

The present study was conducted to investigate whether *Annona muricata* leaves originating from Kelantan exhibit anticancer properties on breast cancer cells. Many studies have been done to look for the potential of medicinal plants as chemotherapeutic-escort agents. Studies have shown that different parts of *Annona muricata* trees have their own unique therapeutic effects. These parts either have the potential as anticancer (Abdul Wahab et al. 2018; Moghadamtousi et al. 2014; Pieme et al. 2014; Rachmani et al. 2012; Syed Najmuddin et al. 2016), antiviral (Astirin et al. 2013) and antimicrobial agents (Solomon-Wisdom, Ugoh & Mohammed 2014).

The phytochemical analysis is essential to identify the chemical compound of the active ingredients. Quantitative analysis of the ethyl acetate and hexane extracts of *Annona muricata* leaves showed that there were rich in secondary metabolites such as terpenoids (20-26%), phenolics (10-19%), alkaloids, fatty acids, and hydrocarbons. These secondary metabolites play a major role in influencing the anti-cancer effects of the plant (Abdul Wahab et al. 2018; Moghadamtousi et al. 2014). Comparatively, the aqueous extract showed no presence of terpenoids, phenolics or alkaloids. In contrast, the methanol extract displayed detectable terpenoids, however the percentage was lower (0.12%) compared to the percentage of terpenoids detected in the ethyl acetate (20.27%) and hexane (25.46%) extracts. The main compounds identified in the methanol and aqueous extracts were fatty acids, carbohydrates, and others. Due to such variation in the secondary metabolites in these extracts, it makes sense that ethyl acetate and hexane-based extracts of *Annona muricata* portrayed a better cytotoxic effect on MCF-7 breast cancer cells.

Plant-based natural products such as phenolic compounds (Anantharaju et al. 2016; Nayyab et al. 2020), alkaloids (Isah 2016), and terpenoids have recently received an astounding amount of interest due to the growing request for the discovery of novel anti-cancer drug candidates (Farooqi et al. 2020; Tomko et al. 2020).

According to a recent study by Hemalatha et al. (2020), terpenoids are not detected in the phytochemical extracts of *Annona muricata* fruit using solvents such as ethyl acetate and hexane. Such contrast indicates the importance of distinguishing the correct part of the plant to imply the concept of using terpenoids against breast cancer. As reported by Ateba et al. (2018), natural terpenoids such as thymoquinone, costunolide, tanshinone IIA, triptolide, cucurbitacin B, celastrol, and lycopene have shown potential therapeutic spectrum to overcome treatment failure in breast cancer. Furthermore, terpenoids have shown effects on the progression of breast cancer cells. Such as reported by Yang and Dou (2010), terpenoids could prevent tumour cell proliferation and induce tumour cell death or apoptosis by inhibiting various cancer-specific targets including the proteasome, NF-κB, and anti-apoptotic protein BCL-2. In the current study, amongst all the other terpenoids, neophytadiene (from ethyl acetate extract) consists of the highest percentage area. Recently, a study conducted by Bhardwaj et al. (2020) stated that neophytadiene could suppress lipopolysaccharide-induced inflammatory responses in *in vivo* model. Such valuable data may justify the ability of neophytadiene extracted from *Annona muricata* to combat breast cancer, as inflammation acts as one of the most important hallmarks in cancer progression (Taniguchi & Karin 2018).

As noted in the results of the phytochemical analysis, alkaloids were only noted in the ethyl acetate-

based extract of *Annona muricata*. Various studies have acknowledged the anti-cancer properties of alkaloids (Xu & Xu 2020). According to a study by Sallam et al. (2013), indole diterpene alkaloids may act as novel inhibitors of the Wnt/β-catenin pathway in breast cancer cells. Aside from that, the indole alkaloid meleagrin extracted from the olive tree endophytic fungus *Penicillium chrysogenum*, has shown potential as a possible regulator of c-Met-dependent breast cancer proliferation (Mady et al. 2016). Since alkaloids were only detected in ethyl acetate extract, it gives the solvent an upper hand in tackling the proliferation of breast cancer cells. Pertaining to the hormonal receptor profile of MCF-7, it is clear that oestrogen plays a part in the proliferation of the MCF-7 breast cancer cells. Interestingly, Martínez-Campa et al. (2006) demonstrated that alkaloids influence the oestrogen receptor-alpha levels and estradiol-induced responses in MCF-7 cells.

In analysing the hexane-based *Annona muricata* extract, it was reported that it also contained high amounts of terpenoids and the most dominant was gamma-sitosterol. According to a study by Musini, Rao and Giri (2015), gamma-sitosterol extracted from *Salacia oblonga* was one of the potential candidates that are responsible for exerting anti-cancer biological activities against breast cancer. Bearing that in mind, it was relevant to anticipate that the hexane-based extract may produce a cytotoxic effect against MCF-7. In such discussion, the aqueous and methanol-based *Annona muricata* extracts contained a very small amount of terpenoids or none of the main phytochemical compounds, thus reducing their ability to exert anti-cancer effects.

However, it was unexpected that the methanol-based *Annona muricata* extract exhibited a cytotoxic effect in MCF-10A. The phytochemical profile of the extract was reassessed, and it was observed that it contained a minute expression of terpenoids particularly, β-Sitosterol. However, a study by Xu et al. (2018) noted that β-Sitosterol-D-glucoside depicted relatively milder cytotoxicity in MCF-10A cells as compared to MCF-7 and MDA-MB-231 cells, which implies that the low amount of β-Sitosterol should not have caused serious toxicity in MCF-10A.

In a study by Syed Najmuddin et al. (2016), the anti-cancer effect of crude extract *Annona muricata* on breast cancer cell lines was tested on MCF-10A. The study discovered that crude extract treatment was less toxic to normal cells as it required a higher concentration to kill the cells (four times higher) (Syed Najmuddin et al. 2016). Based on the findings reported by Syed Najmuddin et al. (2016), it is clear that the *Annona muricata* plant composition should not have affected the viability of MCF-10A cells. Rather, such occurrence may have been caused by external factors such as instability of cells (passage number) or effects of the methanol composition. In addition, it is also plausible that such occurrence could be due to difference in the characteristics of these two cell lines, where cancer cells tend to divide at a higher rate compared to slow-dividing cells like MCF-10A. The comparison on the effect of methanol-based extract of *Annona muricata* using MCF-10A and MCF-7, may not be reliable since the methanol-based extract was not active against MCF-7 cancer cells. Therefore, it is relevant to repeat the experiment using methanol-based extract on different cell line passages.

Our study had shown that ethyl acetate extract of *Annona muricata* leaves has a potent cytotoxic effect on the MCF-7 breast cancer cell line. Such potency was displayed by the lowest IC_{50} value that has been achieved i.e., 21.8 ± 3.85 µg/mL. On the other hand, the hexane-based extract also has a potent cytotoxic effect, however, it was lower compared to that of the ethyl acetate extract. In a comparison study by Endrini, Suherman and Widowati (2014), it was reported that the ethanolic extract of soursop leaves has the strongest cytotoxic activity against MCF-7 breast cancer cell line after 48-hour of incubation (IC_{50} 14.678 µg/mL). We considered plant extract with IC_{50} values ≤30 µg/mL as pharmaceutically active (Suffness & Pezzuto 1990). Therefore, the consecutive tests were conducted using ethyl acetate-based extract of *Annona muricata*.

Anti-proliferative activities of *Annona muricata* have been studied on several types of cancer cells with acceptable results. For example, a study by Torres et al. (2012) on pancreatic cancer cells showed that *Graviola* inhibits tumorigenicity and metastasis of pancreatic cancer cells as a novel promising natural-derived drug by altering cellular metabolism (Torres et al. 2012).

In the current study, it was concluded that ethyl acetate extract of *Annona muricata* leaves arrested cell cycle at G_0/G_1 phases and induced apoptosis *in vitro*. In agreement with the present study, Moghadamtousi et al. (2014) demonstrated that *Annona muricata* leaves affected colon cancer cells HCT-116 and HT-29, where it induced cell cycle arrest at the G_1 phase and apoptosis. Similar to the present study, Moghadamtousi et al. (2014) reported that ethyl acetate extract exerted a

significant cytotoxic effect with an IC_{50} value of 11.43 ± 1.87 µg/mL in HCT-116 and 8.98 ± 1.24 µg/mL in HT-29 colon cancer cells. Furthermore, Prasad et al. (2020) showed that the phytochemical fractions of *Annona muricata* fruit pulp also managed to induce cell arrest at G_0/G_1 phases in breast cancer cell lines, MCF-7 and MDA-MB-231.

Basically, several mechanisms may shed some light on cancer dormancy. These include the interference of crosstalk between growth factors and adhesion signalling, which prevents the tumour cells from interpreting their microenvironment, leading to cellular tumour dormancy via a G_0/G_1 arrest (Aguirre-Ghiso 2007). Quiescence, also known as the G_0 phase, is an out-of-cycle condition that cells can reach at any point during G_1 prior to restriction. Different cyclin–cyclin-dependent kinase (CDK) complexes are active at different stages of the cell cycle (Coller 2007). Cell cycle re-entry from quiescence is linked to cyclin D–CDK4 and cyclin D–CDK6 complexes. In the late G_1 phase, cyclin E–CDK2 complexes are dominantly active whereas in the S phase, cyclin A–CDK2 complexes are perceived as active (Coller 2007).

One interesting point that caught out attention was that both the methanol and aqueous extract did not affect cell viability in MCF-7 cells in the present study, therefore, no IC_{50} value was obtained. The results reported by Syed Najmudin et al. (2016) were different as compared to the outcomes of the present study. The previous study by Syed Najmudin et al. (2016) reported that the aqueous leaf extract of soursop samples was more selective towards MCF-7, followed by 4T1, murine mammary cell line and MDA-MB-231 cell line.

Evading apoptosis is a concept that is often highlighted under resisting cell death of cancer hallmark (Hanahan & Weinberg 2011). In the current study, the effect of ethyl acetate was used to induce apoptosis in MCF-7 cells. Since the ethyl acetate-based *Annona muricata* contains terpenoids, it is anticipated that the extract could induce apoptosis in breast cancer cells. According to previous literature by Telang (2018) and Yang and Dou (2010), terpenoids have the potential to cause apoptosis in cancer cells, including breast tumour cells. The morphology of MCF-7 cells changed after treatment with ethyl acetate extract in a time-dependent manner, as compared to control cells in DMSO. Similar morphology changes were seen in cells treated with Tamoxifen. It was suggested that the cells were undergoing apoptosis. The morphology of MCF-7 cells undergoing apoptosis was indicated via several observations, including fragmentation of the nucleus, constriction of the cytoplasm, and formation of apoptotic bodies (Muhamad et al. 2011). Recently, Naik and Sellappan (2020) discovered that the leaf extract of *Annona muricata* induced apoptosis via the caspase-3 independent pathway, which involved ROS accumulation.

Flow cytometric analysis of Annexin V-FITC conducted in a time-dependent manner separates cell population of live, early apoptosis, late apoptosis, and necrosis (Agrawal, Bhaskar & Rao 2015). Our result demonstrated that ethyl acetate extract of *Annona muricata* caused early and late apoptosis in breast cancer cells. This result is comparable to the study conducted by Syed Najmuddin et al. (2016) on breast cancer cell lines, and in accordance with the results reported by Moghadamtousi et al. (2014) on the apoptosis effect of *Annona muricata* on colon cancer cells.

Recently, a study by Hadisaputri et al. (2021) portrayed that the apoptotic mechanism could not only be derived from the changes in cell morphology but also via the expressions of BCL-2, caspase-9, and caspase-3 mRNA. These molecular markers could mediate the cytotoxic activity of the ethyl acetate fraction of *Annona muricata* leaves on MCF-7 cells (Hadisaputri et al. 2021). The study by Hadisaputri et al. (2021) was presumably conducted in Indonesia. In 2018, our previous team studied the phytocompounds of *Anonna muricata* leaves extract and cytotoxic effects on breast cancer cells, where the leaves were imported from Indonesia (Ab Rahman et al. 2018). There was a large difference in the phytochemical analysis of the ethyl acetate extract of the current study (leaves originating from Malaysia) and the ethyl acetate extract of our previous study (Ab Rahman et al. 2018) (leaves originating from Indonesia). Among the distinct differences observed was the absence of terpenoids in the ethyl acetate extract of the previous study conducted using Indonesian *Annona muricata* leaves. This raises a question as to whether the geographical difference may affect the downstream signalling pathways in MCF-7 breast cancer cells due to the changes in the phytochemical profiles. In a review by Abdul Wahab et al. (2018), the leaves of *Annona muricata* were reported to be rich in annonaceous acetogenins in which most of the studies were done in China. In contrast, *Annona muricata*-derived alkaloids and phenols were reported by studies from France, Japan, India, and Cairo. The differences in active secondary metabolites isolated showed that variations in climate, soil pH, and temperature might alter the outcomes.

Therefore, the future direction of the project would focus more on the characterisation of specific phytochemical compounds in the ethyl acetate-based *Annona muricata* leaves (originating from Kelantan, Malaysia) extracts that might contribute to the inhibition of the breast cancer cells. Our further analysis may also include several oncology-based markers such as BCL-2, caspases, NF-κB, CDK complexes and oestrogen-receptor level.

Conclusions

In conclusion, the Kelantan (Malaysia) grown *Annona muricata* leaves exhibited anti-proliferative properties on the MCF-7 breast cancer cell line. The ethyl-acetate based *Annona muricata* extract displayed a range of phytochemical constituents including terpenoids, phenolics, and alkaloids. Among all the four extracts of *Annona muricata*, the ethyl acetate-based *Annona muricata* extract showed the lowest IC_{50} result and was able to reduce cell viability and induce apoptosis in the MCF-7 cell line. In addition, the ethyl acetate-based *Annona muricata* also caused G_0/G_1 cell cycle arrest.

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*Corresponding author; email: zainira@usm.my