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Original Research Article

Effect of African Leaves (*Vernonia amygdalina* Delile) on the Development of T47D Breast Cancer Cells

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ABSTRACT

Cancer is still a health problem in the world. The number of sufferers and deaths from cancer continues to increase from year to year, especially breast cancer. Cancer treatment is often associated with high toxicity and low selectivity, and the emergence of resistance to chemotherapeutic agents is a serious problem. Therefore, the development of new anticancer agents is a priority. This research provides information regarding the potential of the *Vernonia amygdaline* Delile plant for the development of breast cancer. Several studies show that this plant has the potential to be used as an anticancer agent. The research was conducted by extracting and fractionating this plant's leaves and then assessing the cytotoxic effect on T47D cell viability using Microtetrazolium. Next, cell cycle inhibition, apoptosis, p53 and Akt protein expression were analyzed using flow cytometry techniques. The results showed that the dichloromethane fraction had the highest yield. The dichloromethane fraction had the best IC_{50} value, 76.72 ± 1.79 µg/mL. Able to inhibit the cell cycle in the G_2/M phase, stimulate apoptosis, increase p53 protein expression and inhibit Akt protein expression in T47D cells. The dichloromethane fraction can potentially be developed in treating breast cancer development.

Keywords: African leaves, fraction, *Vernonia amygdalina* Delile., breast cancer, in vitro.

Introduction

Cancer is a major contributor to global death, causing approximately 1 in every six deaths.[1] Globally, it is estimated that there will be 20 million new cases of cancer and 9.7 million deaths from cancer in 2022. The burden of cancer will increase by around 77% by 2050, placing an increasing burden on health systems, society and communities.[2] In general, some of the most frequent cancers are breast, lung, colon and rectal cancer, and prostate cancer. In 2020, breast cancer was the cancer with the highest prevalence in the world.[3,4]Approximately 7% of breast cancer patients are found to have distant metastases at the time of diagnosis. 20 – 30% of early breast cancer patients at the time of diagnosis will develop distant metastases with a five-year relative survival rate of only 27%.

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Currently, due to metastasis, breast cancer is still considered an incurable disease with a relatively low survival rate. New approaches to breast cancer treatment to increase survival times are needed.[5,6,7,8]The increasing incidence of drug resistance in cancer patients, especially breast cancer, is a major problem in conventional chemotherapy treatment and targeted therapy.[9]Approximately 90% of patients experience chemotherapy failure due to drug resistance, even in initial treatment. The main mechanisms of drug resistance in cancer cells are diverse and complex processes, including activating DNA repair, reducing drug entry, sequestering drugs in intracellular organelles, increasing drug elimination, inactivating apoptotic pathways, and triggering immune responses.[10,11]In addition, conventional first-line chemotherapy agents have severe side effects in patients, including nausea and vomiting, alopecia, signs of erythropoietic affection and decreased immune function. Chemotherapy agents are expensive and not readily available in developing countries.[12,13]Therefore, new anticancer agents are urgently needed to overcome these problems.
African leaves are one plant with the potential to be an anticancer agent (*Vernonia amygdaline* Delile.). Several studies have been conducted on African leaves in experimental animals, including antioxidant, antimutagenic, anticancer, antidiabetic, analgesic, cardiotonic, antimalarial, cardioprotective, anti-inflammatory, antimicrobial, and antiobesity.[14,15,16,17,18,19,20,21,22] *Vernonia amygdalina* leaves contain phytochemical compounds such as sesquiterpenes, triterpenes, saponins, flavonoids, tannins, and steroid glycosides such

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as vernonioside B1 and vernoniol. Polyphenols, vernonioside, vernodaline, and vernomygdine are also found in this plant.[23,24,25] *Vernonia amygdalina* contains sesquiterpene lactone compounds proven to possess anticancer properties against various forms of cancer, including lung, colorectal, cervical, and breast cancer.[26] This compound shows anticancer activity against cancer cell lines. Additionally, the leaves of this plant have been shown to increase the efficacy of gemcitabine.[27,28,29]

This research aims to test African leaves for the development of breast cancer so that it can be developed into a therapeutic model to overcome anticancer resistance. The content of flavonoid compounds in these leaves has the potential to inhibit the development of cancer by inhibiting cell proliferation, causing inhibition of the cell cycle, stimulating apoptosis by activating caspase 9 and 8 and activating proteins that cause apoptosis.[30,31,32,33,34,35] Meanwhile, several studies have revealed that glycosides have both in vitro and in vivo anticancer activity by inhibiting cancer cell proliferation in small concentrations.[36,37,38,39] The research began with extraction and fractionation, then testing for cytotoxic activity on T47D cell viability, followed by analysis of cell cycle inhibition, apoptosis promotion, and p53 (protein 53) and Akt (protein kinase B) expression.

Materials and Methods

Extraction and fractionation

African leaves were obtained from the Faculty of Pharmacy, Universitas Sumatera Utara, on May 15, 2017, and identified at the Medanense Herbarium, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, with identification number 1712/MEDA/2017. Extract preparation was carried out by reflux (800 g powder) using n-hexane (8 L) (SmartLab) solvent followed by methanol (8 L) (Merck). The methanol extract (50 g) obtained was then fractionated using dichloromethane (1 L) (SmartLab), ethyl acetate (1 L) (SmartLab) and n-butanol (1 L) (SmartLab) solvents using the liquid-liquid extraction method[40,41.]

Cytotoxic test on T47D cell viability

Cells were planted in 96-well microplates to obtain a density of 3 x 10^3 cells/well and incubated for 24 hours for good growth. After that, the RPMI (Gibco) medium was replaced with a new one, and then the test solution of various concentrations was added with the co-solvent DMSO (Sigma) and incubated at 37ºC in a 5% CO_2 incubator (HeraCell) for 48 hours. At the end of incubation, the medium was discarded, and the cells were washed with PBS (Sigma). Each well added 100 µL of culture medium and 10 µL of 5 mg/mL MTT (Sigma). Cells were incubated again for 4-6 hours in a 5% CO_2 incubator, 37ºC. The MTT reaction was stopped with stopper reagent (10% SDS (Sigma) in 0.01 N HCl (Merck), and the wrapped plate was left for one night. The absorbance was read with an ELISA reader (Benchmark Bio-Rad) at a wavelength of 595 nm.[42,43,44,45]

Analysis of inhibition of cell cycle, apoptosis stimulation, p53 and Akt protein expression by flow cytometry

Cells (3x10^5 cells/well) were placed in 6 wells and incubated for 24 hours. After that, the cells were exposed to the test solution and incubated for 48 hours. The floating and attached cells were harvested by treating them with 0.025% trypsin (Gibco) and then placed into a conical tube. Cells were washed twice with 1 ml PBS and centrifuged at 2500 rpm for 5 minutes; the top layer was removed, the precipitate was collected and resuspended in PBS and centrifuged (Eppendorf) at 3000 rpm for 3 minutes, the supernatant was removed, and the precipitate was added with the RNase/PI kit dye solution (BioLegend) (cycle cells). Annexin V + PI (apoptosis) (BioLegend), FITC p53 (BioLegend) and PE Akt (BioLegend) left for 10 minutes in a dark place at 37ºC.[26, 46]

Statistical analysis

Data are presented as mean values and standard deviation. The normality of the data was determined and analyzed using the ANOVA test to determine the differences observed between the two groups; each test was biologically replicated a minimum of three times.

Results and Discussion

Results of extraction and fractionation

African simplicia leaves powder was extracted by reflux with n-hexane solvent and then by methanol. The methanol extract was then fractionated using the solvents dichloromethane, ethyl acetate, and n-butanol from 800 g of African simplicia leaves powder to obtain extracts and fractions, as seen in Table 1.

African leaves extracts and fractions have different percentage yields. Fractionation is carried out to separate polar, semi-polar and non-polar extracts. The high yield size is influenced by the active ingredients contained.[47,8] Based on these results, the dichloromethane fraction was chosen to continue testing because it had the highest yield (33.62%).

Table 1. Data on the yield of African leaves extracts and fractions

Sample	Weight (grams)	Percentage (%)
n-hexane extract	14.62	1.83
Methanol extract	92.11	11.51
Dichloromethane fraction	16.81	33.62
Ethylacetate fraction	5.58	11.16
N-butanol fraction	11.14	22.28
Residual fraction	9.20	18.40

Table 2. Cytotoxic activity against T47D cells

Sample	IC_{50} (µg/mL)
Dichloromethane fraction	76.72 ± 1.79
Doxorubicin	0.113 ± 0.002

Note: The data is presented as an average and standard error. Repetition was carried out three times.

Cytotoxic activity testing against T47D cells

Cytotoxic test results can be seen in Table 2. In the dichloromethane and doxorubicin fraction treatment, the values were 76.72 ± 1.79 µg/mL and 0.113 ± 0.002 µg/mL. Doxorubicin is an anticancer drug that has emerged as one of the most potent broad-spectrum antitumor anthracyclines. It has cytotoxic effects on malignant cells but has the potential for undesirable cardiotoxic effects.[49] The dichloromethane fraction has a value that is included in the active group because the value ranges between 10 – 100 µg/mL.[50] The results were quite good in the dichloromethane fraction because the contents contained in the dichloromethane and ethylacetate fractions were cytotoxic. The dichloromethane and ethylacetate fractions contain cardiac glycosides and flavonoids. Based on research reports, African leaves contain compounds that have been isolated, including vernolide, vernolepin, vernodalin, hydroxyvernolide, vernodalol, vernomygdin, vernomenin, 11, 13 dihydrovernorodeline, 4,15 dihydrovernodalin, 1, 2, 11, 12', 3' hexahydrovernodalin, 1, 2, 4, 15, 11, 13, 2′,3′-octahydrovernodalin, epivernodalol, and vernonioside A1, A2, A3, A4, B1, B2, B3, and B4.[51]

The cytotoxic test aims to determine the cytotoxic effect of the test solution on cells. This test is the first step to determine the concentration of the test compound used in subsequent tests.[52,53] The cytotoxic activity testing method used is the micro tetrazolium (MTT) (3-[4,5-dimethylthiazol-2yl]2, 5-diphenyltetrazolium bromide) technique, which uses a colourimetric measurement principle based on the formation of insoluble purple formazan salts from the tetrazolium reduction reaction. The MTT dissolves in water to produce a yellow

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solution. Living cells will have high absorbance due to the formation of purple formazan crystals.[54,55]

Analysis of cell cycle inhibition of T47D cells

The cell cycle test results can be seen in Table 3 and Figure 1. The cell cycle inhibitory activity test was carried out using the dichloromethane fraction of African leaves with concentrations of 16 μg/mL (1/5 IC_{50}) and 40 μg/mL (1/2 IC_{50}). The control used was T47D cells without cytotoxic substance treatment. The results above show that the most considerable cell accumulation in the dichloromethane fraction treatment occurred in the G_2-M phase compared to control cells. Many

studies show that flavonoids and glycosides increase cell accumulation in the G_2-M phase of the cell cycle.[56]Anticancer compounds can inhibit the cell cycle and stimulate cell apoptosis. The activity of a compound in inhibiting the cell cycle can be tested using flow cytometry. This method can detect each phase in the cell cycle based on the number of chromosomes in each phase.[57,58,59]

Table 3. Cell cycle inhibitory activity on T47D Cells

Sample	Concentration (μg/mL)	Phase		
		G_0-G_1	S	G_2-M
Cell Control	-	45.9	25.5	23.1
Dichloromethane fraction	40	34.0	23.8	32.5
Dichloromethane fraction	16	38.9	25.2	30.6
Doxorubicin	0.05	30.7	23.1	38.2

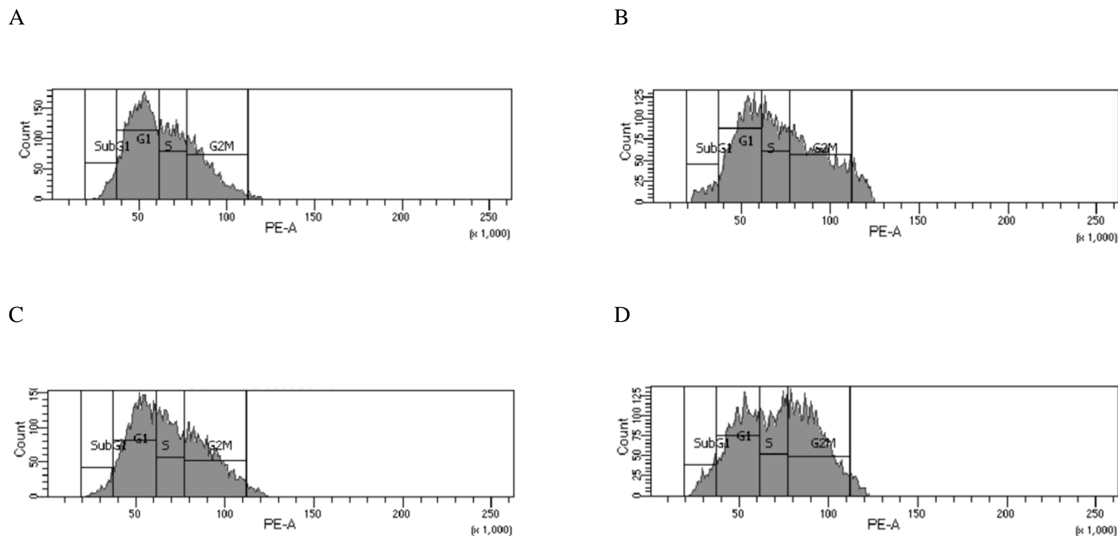

Figure 1. Cell Cycle Inhibitory Activity on T47D Cells, A: Cell Control, B: Dichloromethane fraction 40 μg/mL, C: Dichloromethane fraction 16 μg/mL, D: Doxorubicin 0.05 μg/mL

Analysis of apoptosis stimulation of T47D cells

The results of the apoptosis-inducing test on T47D cells can be seen in Table 4 and Figure 2. Apoptosis induction was observed to determine the cause of cell death, both apoptosis and necrosis. The flow cytometry method can differentiate live cells, early apoptosis, late apoptosis and necrosis by staining with Annexin V and Propidium Iodide. The mechanism of cell death can be seen from the total percentage in each quadrant. Quadrants are divided into four, namely Quadrant I (Lower Left), which indicates the number of living cells; Quadrant II (Lower Right), indicating initial apoptosis; Quadrant III (Upper Right), indicating cells that have undergone apoptosis; and Quadrant IV (Upper Left) shows the total cells that experienced necrosis.[60,61,62] The results showed that treatment with dichloromethane fraction increased early apoptosis, late apoptosis/early necrosis and even late necrosis of T47D cells.

Table 4. Analysis of Apoptosis Stimulation of T47D Cells

Sample	Concentration (μg/mL)	LL	LR	UR	UL
Cell Control	-	97.7	0.4	0.5	1.5
Dichloromethane fraction	40	83.0	7.8	4.7	5.3
Dichloromethane fraction	16	85.6	2.1	2.4	6.6
Doxorubicin	0.05	63.7	0.5	6.6	32.5

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Note: LL= Lower Left, LR= Lower Right, UL= Upper Right, UR= Upper Left

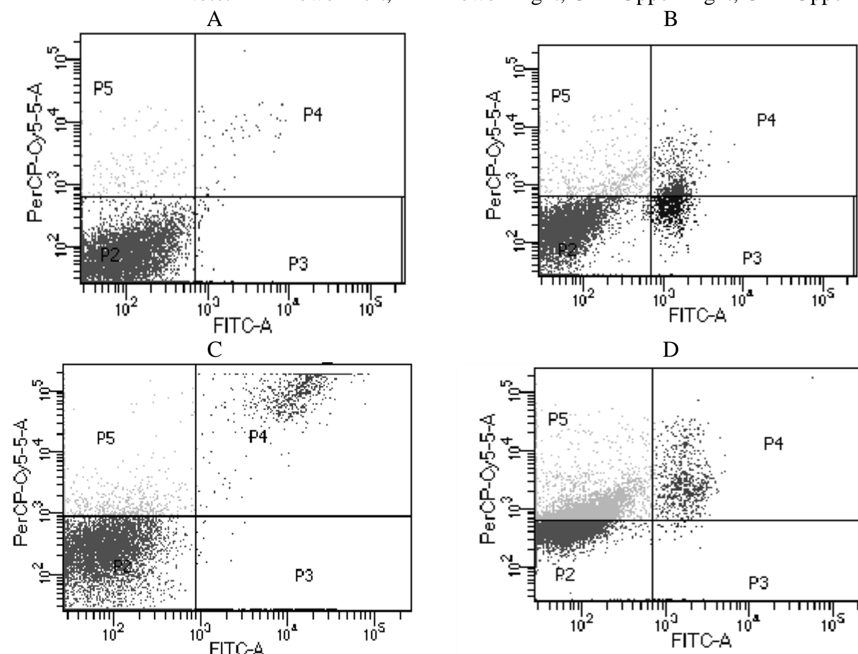

Figure 2. Apoptosis stimulating activity on T47D Cells, A: Cell Control, B: Dichloromethane fraction 40 µg/mL, C: Dichloromethane fraction 16 µg/mL, D: Doxorubicin 0.05 µg/mL

Analysis of p53 and Akt protein expression

The results of p53 and Akt protein expression testing on T47D cells can be seen in Table 5 and Figure 3. Expression analysis was carried out to determine the ability of the dichloromethane fraction to inhibit the expression of p53 and Akt. p53 and Akt are proteins that play an essential role in the cell cycle process.[63] p53 is activated in response to cellular stress and DNA damage. Through genotoxic stress, p53 is activated, stimulating DNA repair, cell cycle arrest, and apoptosis. In breast cancer, p53 activity is often inactivated by overexpression of the negative regulator MDM_2, which occurs in 30-35% of all breast cancer cases.[64] Observations showed that administration of the dichloromethane fraction increased p53 protein expression and decreased Akt in T47D cells. Akt activation promotes tumour progression and drug resistance.[65,66] Increased Akt activity has been demonstrated in many types of cancer, sending a solid survival/anti-apoptotic signal.[67] Akt activation is initiated by phosphorylation of threonine 308, which regulates various target proteins involved in critical cellular processes such as cell growth, proliferation, cell cycle progression, transcription, protein synthesis, cell survival, and glucose metabolism. This activation is essential in tumour progression and inhibits apoptosis by inactivating pro-apoptotic proteins. Many research reports indicate that Akt can be activated in response to DNA damage.[68]

Table 5. Analysis of p53 and Akt Protein Expression in T47D Cells

Sample	Concentration (µg/mL)	p53	Akt
Cell Control	-	3.5	2.0
Dichloromethane fraction	40	18.1	7.1
Dichloromethane fraction	16	6.5	3.4
Doxorubicin	0.05	9.1	3.4

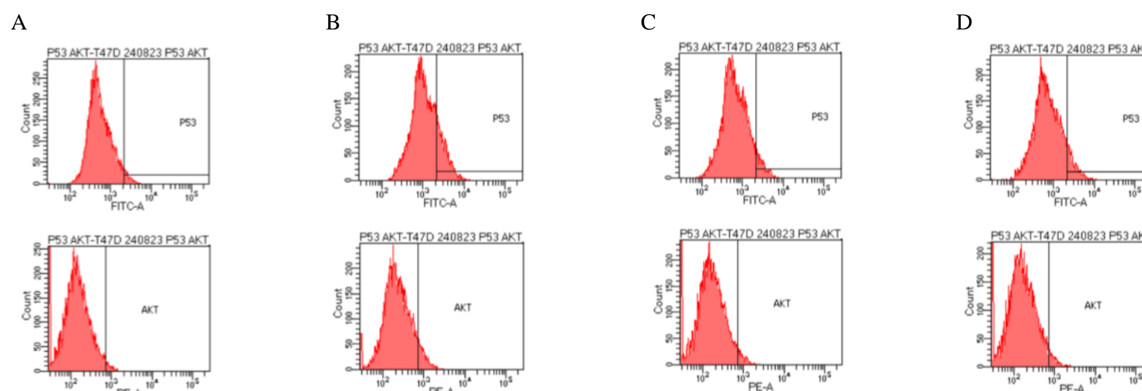

Figure 3. The activity of promoting p53 expression and inhibiting Akt expression on T47D cells, A: Cell Control, B: Dichloromethane fraction 40 µg/mL, C: Dichloromethane fraction 16 µg/mL, D: Doxorubicin 0.05 µg/mL

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Conclusion

This study investigated the anticancer characteristics of dichloromethane fractions derived from African leaves. The findings show that this plant has cytotoxicity against T47D breast cancer cells, with an IC_{50} of 76.72 ± 1.79 µg/mL. It can inhibit the cell cycle, induce apoptosis, increase p53 protein expression, and decrease Akt protein expression in T47D cells. This study shows the capacity of this plant to function as an anticancer agent. Additional investigations can be carried out to confirm the bioactive components present in the dichloromethane fraction of African leaves.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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