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In vitro Cytotoxic Potential of *Sterculia quadrifida* Leaf Extract Against Human Breast Cancer Cell Lines

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ABSTRACT

Breast cancer is the most common type of cancer among women worldwide. The main treatments for breast cancer are surgery and chemotherapy, but the medications used are associated with several side effects. *Sterculia quadrifida* R. Br is traditionally used by the people of East Nusa Tenggara as a medicinal plant to treat various diseases. This study was conducted to determine the potential of the ethanol fraction of *S. quadrifida* leaf extract as an anti-breast cancer agent. Human breast cancer cell lines (MCF-7, T47D, 4T1, MDA-MB-231, and Vero) were obtained for the study. An ethanol solvent (80%) was employed to extract the leaves of *S. quadrifida* using the maceration method. The *S. quadrifida* leaf extract was then fractionated with n-hexane, ethyl acetate and n-butanol with a liquid-liquid partition. An *in vitro* cytotoxicity assay was conducted on the MCF-7, T47D, 4T1, MDA-MB-231, and Vero cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method. The results showed that the ethyl acetate fraction of the *S. quadrifida* leaf extract exhibited considerable cytotoxic activity in T47D and 4T1 breast cancer cells, with an IC_{50} value of 19.86 ± 3.48 and 16.70 ± 3.33 µg/mL, respectively. Furthermore, the n-hexane fraction had the highest activity in MCF-7 cells with an IC_{50} value of 33.78 ± 7.82 µg/mL. The findings of this study revealed that the leaves of *S. quadrifida* have the potential to act as a cytotoxic agent on breast cancer cells.

Keywords: Breast cancer, Cytotoxic, Leaf extract, MTT assay, *Sterculia quadrifida*

Introduction

Cancer in recent decades has become a major concern in the health sector.[1] This is attributable to the increasing prevalence of cancer in several developing and developed countries.[2] Breast cancer ranks first in cancer cases in women worldwide, with an incidence rate of 1.6 million.[3] It is the leading cause of cancer death in women.[4] Breast cancer is classified into four major molecular subtypes: luminal A (HR+/HER2−), luminal B (HR+/HER2+), HER2+, and triple-negative. Each of them has its own set of risk factors for incidence, disease progression, preferred organ locations of metastases, and therapeutic response.[5] Although surgery and chemotherapy are the primary treatments for breast cancer, selective medications are still required to improve efficacy, and selectivity, and reduce side effects.[6]
The use of natural products in cancer therapy is increasing rapidly at the moment, owing to the belief that conventional medications have frequent negative effects.[7] Many researchers have been inspired to investigate plants and secondary metabolites with anti-breast cancer activities. Taxol compounds, for example, are terpenoid compounds identified from *Taxus brevifolia* as the first microtubule stabilizing agents that interact molecularly with the hydrophobic pocket in the β-tubule.[8] Scientific evidence shows that *Catharanthus roseus* produces vinblastine and vincristine chemicals via a mechanism involving α, and β-tubulins.[9] Furthermore, luteolin, a flavone found in *Salvia tomentosa* Mill. has been shown to delay or block the development of cancer cells, protect DNA from a carcinogenic stimulus, and induce apoptosis even in multidrug-resistant cancer cells.

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This is achieved through reactive oxygen species (ROS) generation, DNA damage, activation of ataxia telangiectasia and rad3-related/checkpoint kinase 2/p53 (ATR/Chk2/p53) signaling, depletion of antiapoptotic proteins and inhibition of NF-κB signaling and p38.[10] Furthermore, the natural product is thought to have significant cytotoxic potential.[11]
This study was aimed at investigating the potential of *S. quadrifida* leaf extract as a cytotoxic agent in breast cancer cells.

Materials and Methods

Source of cell lines
The MCF-7, T47D, 4T1, MDA-MB-231, and Vero cells were obtained from the American Type Culture Collection (Manassas, USA). Briefly, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco, USA), 150 IU/mL penicillin, 150 µg/mL streptomycin (Gibco, USA) and 12,5 µg/mL amphotericin B (Gibco, USA).

Source and identification of plant material
The leaves of *S. quadrifida* were collected in January 2022 at the Research and Development Center for Environment and Forestry, Kupang, East Nusa Tenggara, Indonesia. The collection locations are S 10° 07' 40"–10°17' 39" and E 123° 31' 35"–123° 41' 00". Kupang City has a low average annual rainfall (1,290 mm/year). The average humidity is 77%, and the average temperature is 27.5°C. The plant was identified at the Laboratory of Herbal Materia Medica Batu, Indonesia. The root, stem, and leaf parts of the plant were authenticated using the Flora of Java book. The remaining plant sample was labeled as *Sterculia quadrifida* and deposited in the Pharmacognosy-Phytochemical Laboratory of Ma Chung University, Indonesia with a voucher number: FA:023-MACHUNG-2022.

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Preparation and fractionation of plant extract

The leaves of *S. quadrifida* were washed and dried. They were blended and sieved through a 100 mesh sieve after being reduced in size with a blender. A total of 3 kg of leaf powder was macerated in 9 L of ethanol (80%) for 24 hours. The dry extract was fractionated with n-hexane, ethyl acetate, and n-butanol with a liquid-liquid partition in a ratio of 1:1. Each dry fraction was used as a test material.

Preparation of cancer cell lines.

The *in vitro* test was performed using the method described by Hariono et al.[6] The breast cancer cells in the cryo tube were first removed from a liquid nitrogen tank. Then, they were placed in an alcohol-sprayed laminar airflow (LAF) and waited for them to melt. Following that, 4T1 cells were placed in conical tubes with high glucose complete media from Dulbecco's modified Eagle Medium (DMEM). The cell culture was centrifuged in a conical tube at 600 rpm for 5 minutes, and the supernatant was discarded. The new medium was then added to the conical tube with the pellet and suspended until homogenous. Cell suspensions were grown in a tissue culture dish (TCD) and incubated in a CO_2 incubator at 37°C. The cell conditions were then examined under a microscope before being cultured in a 5% CO_2 incubator. After the confluent cells reached 80%, they were harvested by removing the culture first and washing them with 3 mL PBS twice. Then, 0.25% trypsin-EDTA was added to separate the cells from the TCD. After 30 seconds, the 0.25% trypsin-EDTA solution was discarded, and the cells were incubated for 1 minute in a CO_2 incubator. The cells were then re-suspended in 2-3 mL of medium using a micropipette on the walls and bottom of the TCD so that they would not clump together. The cell suspension was then transferred into a new sterile conical tube. A haemocytometer and a cell counter were used to count the number of cells, and a cell suspension with the requisite concentration was prepared. A cell density of $8x10^4$ cells/well was utilized in a single cytotoxic test. All instruments used for cell maintenance were used under sterile conditions and sprayed with 70% alcohol when placed in the LAF. During the study, the LAF environment remained sterile by sprinkling 70% alcohol on it.

Cytotoxicity assay

The *in vitro* cytotoxicity test was performed according to the method described by Rollando et al.[12] Cells were harvested at a concentration of $8x10^3$ cells/well and diluted with culture media, then planted into a 96-well microplate of 100 µL/well and incubated for 24 hours in a 5% CO_2 incubator. Before being used for treatment, the media in the plate was discarded and then washed once with PBS in the amount of 14 µL/well. Then, the PBS was discarded, and 100 µL of a fraction test solution (15.62; 31.25; 62.5; 125; 250; 500; 1000 µg/mL) was added to each well. The cells were then incubated again for 24 hours. After incubation, they were washed with PBS and 100 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reagent was added to each well. Finally, the cells were incubated for 4 hours at 37°C. Following that, 100 µL of a stopper solution (SDS 10% in 0.01 N HCl) was added to each well and incubated overnight at room temperature in the dark before being read with an ELISA reader at 595 nm. The absorbance values were used to estimate the surviving cells. The absorbance data of a single treatment was converted into percent viability and used to calculate the IC_{50}. The absorbance data from each well was transformed into a percentage of cell viability using the following calculations:[11]

$$\%\,\text{cell viability} = \frac{(\text{Absorbance of treatment} - \text{Absorbance of control media})}{(\text{Absorbance of control of cells} - \text{Absorbance of control media})} \times 100\%$$

The selectivity index was calculated using the formula:[12]

$$\textit{Selectivity Index} = \frac{\textit{IC50 No Cancer Cell}}{\textit{IC50 Cancer Cell}}$$

Statistical analysis

The percentage of cell viability was calculated using Microsoft Excel 2010 software to derive a linear correlation value (r) from the curve description between the concentration log and the percentage of cell viability, which was then used to determine the IC_{50} value. The linear correlation value from the selectivity index calculation was tested for significance using 5% alpha. The efficiency of the test substance was assessed by the percentage decrease in cell viability. The results were expressed as mean ± standard deviation of the mean (SD), and statistical comparisons were made using one-way analysis of variance (ANOVA) to test the null hypothesis. A Tukey's test was done to compare the sample means. The data were considered significantly different from other fractions when the significance level was $p<0.05$.

Results and Discussion

Cytotoxicity test was conducted on several breast cancer cell lines, namely T47D, MCF-7, 4T1, and MDA-MB-231 cells. The MTT technique was utilized for testing. The assay is a colorimetric method where the MTT reagent is a tetrazolium salt that can be broken down into formazan crystals by the succinate tetrazolium reductase system found in the cellular respiratory pathway in the mitochondria, which is active in living cells.[13] Formazan crystals form a purple colour that can be used to measure absorbance using an ELISA reader. In this study, a cytotoxicity test was performed to identify the potential toxicity of compounds to breast cancer cells. The cytotoxicity test parameter is the IC_{50} value. The IC_{50} is a concentration that can cause up to 50% of the cancer cell population to live, while the remainder is predicted to die.[14] The lower the IC_{50} value, the higher the potential of the test compound as a cytotoxic agent. As observed in Figure 1, cell viability decreased in a dose-dependent manner after 24 hours of treatment.

The results of the cytotoxicity test on T47D cells showed that the ethyl acetate fraction was the most active fraction with an IC_{50} value of 19.86 ± 3.48 g/mL, followed by the n-butanol and n-hexane fractions with IC_{50} values of 127.05 ± 6.93 and 131.50 ± 2.31 µg/mL, respectively. It was observed that the cytotoxic activity of the n-hexane fraction on MCF-7 cells was the highest, with an IC_{50} value of 33.78 ± 7.82 µg/mL. Furthermore, the cytotoxicity testing on 4T1 cells revealed that the ethyl acetate and n-butanol fractions were the most active, with IC_{50} values of 16.70 ± 3.33 and 47.82 ± 2.31 µg/mL, respectively. The cytotoxicity testing on cancer cell type MDA-MB-231 revealed the most active fraction of n-butanol with an IC_{50} value of 108.25 ± 21.33 g/mL (Table 1). The lower the IC_{50} value of a compound, the greater its cytotoxic effect. According to the NCI (National Cancer Institute), the IC_{50} value for a potent compound that can be further developed as an anticancer agent is <20 µg/mL.[15] From this result, the fractions that have the potential to be further developed are the ethyl acetate and n-hexane fractions.

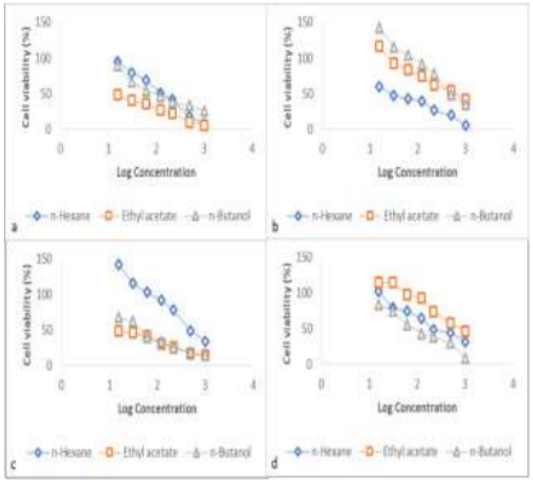

Figure 1: Effect of *Sterculia quadrifida* leaf extract fractions on breast cancer cell viability. A: T47D cells; B: MCF-7 cells; C: 4T1 cells; D: MDA-MB-231 cells.

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Table 1: The IC_{50} values of *Sterculia quadrifida* leaf extract fractions on human breast cancer cells.

Fraction	IC_{50} ± SD (µg/mL)				
	T47D	MCF-7	4T1	MDA-MB-231	Vero
n-Hexane	131.50 ± 2.31	33.78 ± 7.82*	549.66 ± 13.12	286.94 ± 23.47	554.30 ± 23.82
Ethyl acetate	19.86 ± 3.48*	533.67 ± 3.12	16.70 ± 3.33*	904.17 ± 33.12	83.66 ± 1.82
n-Butanol	127.05 ± 6.93	549.66 ± 13.23	47.82 ± 2.31*	108.25 ± 21.33	257.82 ± 2.32

Values are presented as mean ± SD; n = 5 replicates; p-value < 0.05 indicates statistically significant differences in comparison to other fractions.

Moreover, the morphology of cancer cells indicates the potential for cytotoxic activity. Figure 2a-f depicts the morphology of cells following treatment. Fraction treatment reduced cell viability when compared to control cells. In addition, fraction treatment caused the cells to undergo morphological changes. The cell nucleus appeared shriveled leading to cell death, and the number of cells was reduced, whereas untreated cells had normal morphology (Figure 2 a-f). Cells displayed morphological changes that were conceivable because the cytoskeleton was severed and the proteins involved in cell attachment did not polymerize, causing cell bonds to be released and lipid membranes to round. Cell shrinkage is a marker leading to cell death.[5]

The selectivity index (SI) can be defined as the ratio of the toxic concentration of a sample against its effective bioactive concentration. An ideal drug should have a relatively high toxic concentration but a low active concentration. Evaluation of the SI value for any research on herbal drugs and/or isolated compounds is crucial for determining whether further research should be carried out.[18] Jantamat *et al*.,[19] proposed a lower SI value (>3) for classifying prospective anti-cancer samples. The test results revealed that the n-hexane and ethyl acetate fractions had SI values of 4.21 in T47D cancer cells, followed by the n-hexane fraction with SI values of 16.41 in MCF-7 cells. The fractions of ethyl acetate and n-butanol with SI values of 5.01 and 5.39, respectively, were determined in 4T1 cells as presented in Table 2.[20] Ideally, the medicine should be able to destroy cancer cells while not affecting normal cells.

Sterculia quadrifida is widely used in traditional medicine. For example, Siswadi *et al*.,[21] reported that the bark of *S. quadrifida* was used to treat hepatitis. In addition, several studies have shown that the bark of *S. quadrifida* has cytotoxic activity. Rollando and Siswadi,[22] discovered that the ethanol fraction of *S. quadrifida* stem bark has a cytotoxic effect on T47D cells with an IC_{50} value of 21.89 g/mL. In another investigation, the ethyl acetate fraction was found to have the maximum cytotoxic activity in T47D cells, with an IC_{50} value of 24.88 µg/mL, and able to induce apoptosis in the S phase (27.43%).[23] Other research revealed that the ethyl acetate fraction had cytotoxic activity in MCF-7 with an IC_{50} value of 7.62 µg/mL and the selectivity index was 2.52.[24]

Isolation of cytotoxic compounds from *S. quadrifida* revealed naphthoquinone derivatives, specifically 2,3-dihydro-6-hydroxy-2-methylenenaphtho [1,2-b] furan-4,5-dione, which has anticancer activity with an IC_{50} value of 9.88 g/mL and a selectivity index value of 30.23 in cancer T47D cells.[25] The isolated compound, 2-iminoethyl 2-(2-(1-hydroxybutan-2-yl)phenyl) acetate, demonstrated cytotoxic activity against T47D breast cancer cells with an IC_{50} value of 7.12 µg/mL and selectivity index value of 47.53.[26] Furthermore, an auron compound known as (2E)-2-[(3,4-dihydroxy phenyl) (hydroxy) methylidene] -4,6-dihydroxy-2,3-dihydro-1-benzofuran-3-one isolated from n-butanol fraction was reported to have a cytotoxic effect on 4T1 cells with an IC_{50} value of 4.05 µg/mL in 4T1 cells.[27]

Naphthoquinone and auron compounds have been isolated from *S. quadrifida*. Several investigations have revealed that naphthoquinone compounds have cytotoxic activity on breast cancer cells. Carcass *et al*.,[28] characterized the compound, 1,4-naphthoquinone, which had high cytotoxic activity on MCF-7 and MDA-MB-231 cancer cells with IC_{50} values of 2.3 and 3.9 µM, respectively. The compound, 2-methoxy-1,4-naphthoquinone was found to be active against human breast adenocarcinoma cells, namely MDA-MB-231 cells with an IC_{50} value of 1.3 µM. Auron compounds have also been shown to exhibit cytotoxic activity against breast cancer cells. Szilárd et al.,[29] synthesized azaaurone compounds with an IC_{50} 0.3 µM that were active in 4T1 breast cancer cells. In addition, aurone derivatives, including simple unsubstituted coumaranone, exhibited cytotoxic action against BT20 breast cancer cells with an IC_{50} value of 0.98 µM.[30]

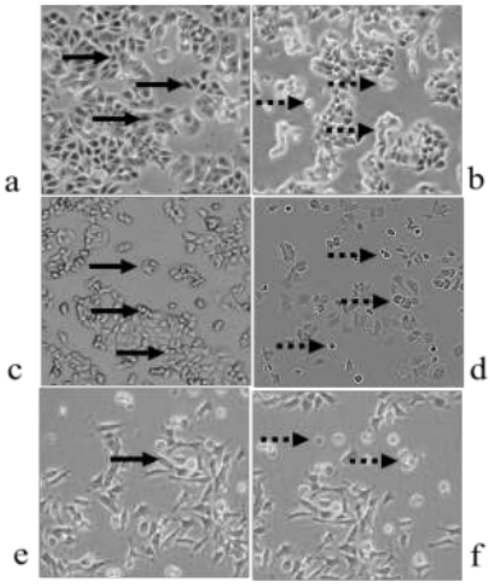

Figure 2: The effect of *Sterculia quadrifida* leaf extract fractions on human breast cancer cell morphology. A: Untreated T47D; b: IC_{50} concentration of ethyl acetate fraction on T47D; c: Untreated MCF-7; d: IC_{50} concentration of n-hexane fraction on MCF-7; e: Untreated 4T1; f: IC_{50} concentration of ethyl acetate fraction on 4T1; Cells ($2x10^4$) were seeded in 96 well plate and incubated for 24 h. The morphology of living cells is shown by arrows (➔) and cells undergoing morphological changes are indicated by dashed arrows (▪▪▪▶).

Table 2: The selectivity index of *Sterculia quadrifida* leaf extract fractions on human breast cancer cells.

Fraction	Selectivity Index (SI)			
	T47D	MCF-7	4T1	MDA-MB-231
n-Hexane	4.21	16.41	1.01	1.93
Ethyl acetate	4.21	0.16	5.01	0.61
n-Butanol	2.03	0.47	5.39	2.38

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Conclusion

The findings of this study revealed that the ethyl acetate fraction of *S. quadrifida* leaf extract exhibited considerable cytotoxic activity in T47D and 4T1 breast cancer cells, with IC_{50} values of 19.86 ± 3.48 and 16.70 ± 3.33 µg/mL, respectively. More so, the n-hexane fraction had the highest activity in MCF-7 cells with an IC_{50} value of 33.78 ± 7.82 µg/mL. Future research could be done to determine the active ingredients in the fraction of ethanolic leaf extract.

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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