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Cytotoxic, Antiproliferation, and Necrosis Effects of the n-Hexane Fraction Extract of Gendola Leaf (*Basella rubra Linn.*)

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Abstract

Breast cancer is the second most common cancer in women. Several studies have been conducted on natural materials to see the ability of its activity as a new anticancer compound. The purpose of this study was to determine the cytotoxic activity of Gendola leaf (*Basella rubra Linn.*) extracts and fractions, in inhibiting proliferation and inducing apoptosis of T47D breast cancer cells. The design of this study was experimental laboratory research using the in-vitro method. Cytotoxic and anti-proliferation tests on T47D cancer were using the MTT method, and the apoptosis test was using the flow cytometry method. Cytotoxic of extracts and fractions of n-hexane Gendola leaves were tested with a concentration series of 1000; 500; 250; 125; 62.5 µg/mL and doxorubicin was studied a series concentration of 0.5; 0.25; 0.125; 0.0625; 0.03125 µg/mL for 24 hours. Antiproliferation test used n-hexane fraction with a concentration series of $1IC_{50}$, $½IC_{50}$, $¼IC_{50}$, and $⅛IC_{50}$ with an incubation time of 24 and 48 hours. Apoptosis test utilized n-hexane fraction with a concentration series of $1IC_{50}$, $½IC_{50}$ with an incubation time of 24 hours. The results showed that the ethanol extract had IC_{50} 424 µg/mL, n-hexane fraction 292 µg/mL. The n-hexane fraction had an anti-proliferation effect at a concentration of $1IC_{50}$ within 32 hours but was unable to induce T47D cell apoptosis. These results indicate that the n-hexane fraction of Gendola leaf has a cytotoxic effect, which can inhibit proliferation, that is unable to induce apoptosis but induce necrosis of T47D breast cancer cells.

1. Introduction

Cancer is one of the leading causes of death worldwide. In 2015, there were 17.5 million cases of cancer worldwide and 8.7 million deaths. Between 2005 and 2015, cancer cases increased by 33%. For men, the most common cancer is prostate cancer (1.6 million cases). For women, the most common cancer is breast cancer (2.4 million cases) [1]. Cancer can be treated with surgery, chemotherapy, or radiation. Treating cancer with surgery is not effective for cancers that have metastasized, and treatment with chemotherapy and radiation is often less selective. The use of chemotherapy has toxic side effects in healthy tissue and causes resistance to cancer cells. One strategy for developing anti-breast cancer drugs is through the discovery of new compounds. Several studies have been conducted on natural materials to see the ability of its activity as a new anticancer compound. One of these natural potentials is Gendola leaf (*Basella rubra Linn.*), which is used by the community as inflammation and anticancer medication. Gendola leaf is a plant from the family *Basellaceae*, which is a natural medicinal plant in Indonesia [2, 3].

The research conducted with *in-vivo* showed that Gendola plant water extracts activated apoptosis (p53 and caspase-3) and inhibited proliferation in rat colon cancer cells [4]. In *in-vitro* research shows that methanol extract

from Gendola fruit has intense cytotoxic activity against cervical cancer cells [2]. Much research has supported this study, but no one has yet examined the cytotoxic, anti-proliferation, and apoptotic effects of extracts and fractions of n-hexane from Gendola leaves (*Basella rubra Linn.*) on T47D breast cancer cells.

2. Methodology

2.1. Sample Preparation

The leaves were picked from plants that were approximately 1 (one) year old, harvested in the district of Kalidoni, Palembang Regency, South Sumatra Province, in March 2018. Gendola (*Basella rubra Linn.*) leaves have a red stem; they can grow up to 10 cm long, creeping, and wrapped around the stake. The leaves are single, ovoid, pinnate, green leaves with red leaf bones. Blackish red fruit with whitish red seeds. Gendola leaves have been identified by the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Sriwijaya University. T47D breast cancer cells were obtained from the Parasitology Laboratory, Faculty of Medicine, Gajah Mada University (FK-UGM) Yogyakarta.

2.2. Extraction and Fraction Making

Gendola (*Basella rubra Linn.*) leaves were dried in the open air and not exposed to any direct sunlight. Then Gendola leaves were pulverized with a blender to get simplicia from Gendola leaves. A precise amount of 250g Gendola leaves simplicia was weighed and then extracted by the maceration method for 2x24 hours in 96% ethanol. The extraction results were evaporated with a rotary evaporator until a viscous extract was obtained. The viscous extract was dried by a hairdryer to obtain a dry extract. The fractionation process was carried out using the FCC (Liquid-Liquid Fractionation) method, where the dry extract was dissolved into 200 mL ethanol and distilled water with a ratio of 1: 1. Subsequently, the extract was put into a separating funnel, and 200 mL of n-hexane was added and shaken slowly until it was mixed and allowed to appear separate layers. Then, the fraction solution into the bottle and the process was repeated several times until the solution becomes clear. The fractionation was continued using ethyl acetate solvent in the same manner and volume with the n-hexane fraction. The n-hexane fraction was evaporated with a rotary evaporator until a thick fraction was obtained and dried with a hairdryer to obtain a dry fraction. [5].

2.3. Phytochemical Screening

Phytochemical screening tests were carried out qualitatively; phytochemical screening aims to determine the content of alkaloids, steroids, terpenoids, tannins, saponins, and flavonoids, in the n-hexane fraction of Gendola leaves (*Basella rubra Linn.*). Phytochemical screening was conducted at the Integrated Testing Laboratory of the Department of Chemistry, Faculty of Mathematics and Natural Sciences, Sriwijaya University.

Alkaloid identification was conducted by adding 1 mL of HCN 2N and 6 mL of distilled water into 3 mL of the tested material solution, then heated for 2 minutes, then cooled and filtered. The filtrate obtained was reacted with Mayer's reagents; the formation of white deposits marked the presence of alkaloids.

Identification of terpenoids and steroids was carried out using the test material dissolved with chloroform, and 0.5 mL of acetic acid anhydride was added, 2 mL of concentrated sulfuric acid added through the tube wall. The presence of a triterpenoid was characterized by the formation of a brownish or violet ring, while the formation of a greenish-blue ring characterizes the presence of steroids.

Tannin identification was conducted by adding 10% $FeCl_3$ into the solution of the test material; the presence of tannin was characterized by the formation of dark blue or greenish-black. Saponin identification was performed by adding distilled water into the test material, then shaken vertically for 10 seconds. The presence of saponins was identified by a stable foam for several minutes. Flavonoid identification was made by adding Mg reagents and 2 mL of 2N HCL into 2 mL of test material solution. A positive result of flavonoids was marked by the formation of red or yellow [6].

2.4. Cytotoxic Test Using the Cytotoxic MTT Method

Cells were diluted with culture media (RPMI) with a concentration of $1x10^4$ cells/mL. Cells were transferred into 96 well-plates, 100 µL each, leaving three blanks (media control). Cells were incubated in a CO_2 incubator for 24 hours. Ethanol extract and n-hexane fraction of Gendola leaves were tested for their anticancer activity against T47D cells with five variations in the concentration of the test compounds, i.e., 1000; 500; 250; 125; 62.5 µg/mL. Doxorubicin as a positive control was tested in 5 concentrations which were 0.5; 0.25; 0.125; 0.0625; 0.03125 µg/mL. Media control (DMSO 1%) as a negative control and cell control used was T47D breast cancer cell control.

The sample concentration series was put into a well (triplo), as much as 100 µL of the test extract was added to the test cell well and well blank (MK), then incubated in a CO_2 incubator for 24 hours. The medium in all wells was discarded, and 100 µL MTT reagent was added and then incubated for 4 hours in a CO_2 incubator. Cell condition was examined under an inverted microscope by adding 100 µL 10% SDS. The plate was wrapped in paper or aluminum foil and incubated in a dark place at room temperature overnight. The absorbance of each well was read with a microplate reader with λ = 550-600 nm [7].

2.5. Anti-proliferation Test with Doubling Time Technique

The number of cells needed for the apoptosis test was $5x10^3$ cells/wells, which were then planted on a six-well microplate, then incubated for 24 hours. The next day the cells were added by n-hexane Gendola fraction with concentration values of $1IC_{50}$ and $½IC_{50}$, 292 µg/mL, and 146 µg/mL. The negative control used was the T47D cell control and then re-incubated for 24 hours. The media in each well was put in a 15 mL conical tube then washed with PBS and collected in the same conical. 250 µL trypsin was added to each well and then incubated for 3 minutes at 37°C. 1 mL of culture media was added and put in a 15

mL conical tube, centrifugated at 6000 rpm for 5 minutes, the supernatant removed, finally added by 1 mL PBS. Next, the media was transferred to the conical tube and centrifuged again at a speed of 2000 rpm for 3 minutes, after that the supernatant was thrown away. Continue with the addition of Annexin V/Propidium Iodide (Annexin V/PI) and measured with a flow cytometer [8].

2.6. Data Analysis

2.6.1. Cytotoxic

$$\% \text{ Living cell: } \frac{(\text{Absorbance treatment} - \text{Absorbance media control})}{(\text{Absorbance cell control} - \text{Absorbance media control})} \times 100\%$$

The value of IC_{50} was determined by linear equation and cell viability and converted using *Microsoft excel*.

2.6.2. Anti-proliferation

Data on the number of living cells at hours 0, 24, 48 were graphed between the number of living cells and the length of incubation time (hours), then it was determined the difference between time to reach the number of two initial cells (knowing doubling time) by making a regression equation between the log number of living cells and the time of each treatment Then from the equation, the log value is entered two times the initial value as the value of y so that the value of x is obtained with a doubling time that is longer than the negative control. If this is obtained, then the test material has antiproliferative power.

2.6.3. Apoptosis

A flowmeter in the percentage of apoptosis measures apoptosis.

3. Result and Discussion

3.1. N-hexane Extract and Fraction Result

The Gendola leaf from the 250 g dried simplicia that has been macerated and evaporated produced 28.69 g of Gendola leaf extract. An amount of 6.31 g of the extract was separated to be dried for the use of ethanol extract test material. The rest of the Gendola leaf extract of 22.38 g was used in the fractionation process with the FCC method to obtain the n-hexane fraction. The weight of the n-hexane fraction obtained was 3.66 g.

3.2. Phytochemical Screening Result

The results obtained from the phytochemical screening of ethanol extract and n-hexane fraction of Gendola leaves can be seen in Table 1. The phytochemical screening on ethanol extracts shows that Gendola leaves contain steroid compounds (stigmasterol), terpenoids, tannins, and saponins. The fraction of n-hexane contains steroid compounds (stigmasterol), terpenoids, and saponins. From several studies, Singh *et al.* [3] reported that the chemical compounds found in the leaves of Gendola were saponins and phenols. Priya *et al.* [9] found steroids and triterpenoids compounds, and also found flavonoids, β-cyanine, and 7,4-di-ortho methyl kaempferol compounds.

Table 1. Phytochemical screening result of Ethanol extract and n-hexane fraction of Gendola leaves

Chemical compound	Ethanol Extract	n-hexane fraction
Alkaloid	-	-
Steroid	+	+
Terpenoid	+	+
Tannin	+	-
Saponin	+	+
Flavonoid	-	-

3.3. Cytotoxic Test Result Using the MTT Method

Based on the results of the ethanol extract cytotoxic test, n-hexane fraction of Gendola leaves, and doxorubicin (Table 2), the IC_{50} value of doxorubicin was smaller than that of ethanol extract and n-hexane fraction. Cytotoxic activity of ethanol extract at a concentration of 424 µg/mL, n-hexane fraction at a concentration of 292 µg/mL, and doxorubicin at a concentration of 0.1 µg/mL was able to inhibit 50% of the T47D cancer cell growth. The IC_{50} value of doxorubicin includes a very active cytotoxic group, ethanol extract, and n-hexane fraction classified in a moderately active cytotoxic based on Weerapreeyakul *et al.* [10]. Based on IC50 values, cytotoxicity can be grouped into: (i) cytotoxic is very active if IC50 value <10µg/mL, (ii) cytotoxic is active if IC50 value is between 10/-100µg/mL, (iii) cytotoxic is quite active if IC50 value is around 100 - 500µg/mL.

Table 2. The cytotoxic test result of ethanol extract and n-hexane fraction of Gendola leaves and doxorubicin

Material	Concentration (µg/mL)	% living cell	Value of IC_{50} (µg/mL)
Ethanol extract	1000	2.375	424
	500	51.388	
	250	71.161	
	125	63.801	
	62.5	74.875	
n-hexane fraction	1000	0.468	292
	500	8.096	
	250	65.841	
	125	71.328	
	62.5	64.269	
Doxorubicin	0.5	10.000	0.1
	0.25	10.392	
	0.125	41.451	
	0.0625	78.549	

From the results of the phytochemical examination of the n-hexane fraction, it is found that Gandola leaves contain terpenoids, steroids, and saponins. These three metabolites likely possess the n-hexane fractions, which have the potential as anticancer agents [3, 10]. Manik *et al.* [11] explained that the results of n-hexane extract isolate from the leaves of *Anredera cordifolia* (Tenore) Steenis by using FT-IR and LC-MS spectrophotometers, allegedly the active compound was stigmasterol compound (steroid alcohol), which had a biological activity as an antitumor and showed powerful cytotoxic activity against tumor cells in humans. Ardji *et al.* [12], on the

characterization of steroid compounds from the dichloromethane fraction of the stem of the Andong plant (*Cordyline fruticosa*) and their cytotoxic activity against cells, reported that steroids, especially steroid glycosides, were known to have biological activity as antitumor and showed powerful cytotoxic activity against tumor cells in humans.

Nurcahyanti *et al.* [13] also support that the compound isolated from n-hexane extract from *Dysoxylum alliaceum* bark was a steroid compound, which was stigmatist-5-en 3β-ol and stigmasterol and showed potent cytotoxic activity with an IC_{50} value of 8.65 µg/mL against MCF-7 breast cancer cell. According to Kim *et al.* [14], the steroid compound, which was stigmasterol, was isolated from the marine microalgae *Navicula incerta* had cytotoxic activity, anti-proliferation, and apoptosis in human hepatoma HepG2 cells. Astuti *et al.* [15] stated that the Binahong plant (*Anredera cordifolia* (Tenore) Steenis) contained triterpenoid saponins and steroid saponins that had anticancer properties. Podolak *et al.* [16] stated that the saponin compound had toxic properties; in *Cucumariafrondosa*, it contained saponin, which had a cytotoxic potential with IC_{50} concentration of <10 µg/mL against MCF-7 cells [16].

3.4. Anti-proliferation Test Result Using *Doubling Time* Method

The anti-proliferation test was performed by *doubling time* technique. Anti-proliferation test used n-hexane fraction with four different concentrations of 1 IC_{50}, ½IC_{50}, ¼IC_{50}, and ⅛IC_{50} and was observed and incubated at 0 hours, 24 hours, and 48 hours. Anti-proliferation test results of the n-hexane Gendola fraction in figure 1 showed that the control cells had the fastest *doubling time* values. That indicates that the rate of cell proliferation can be slowed by introducing Gendola n-hexane fraction, where Gendola n-hexane fraction shows the inhibition of T47D breast cancer cell proliferation, with a longer *doubling time* value of 32 hours at a concentration of one IC_{50}. The n-hexane fraction of Gendola leaves able to extend the *doubling time*. This shows the ability of the extract to inhibit the proliferation of cancer cells through the mechanism of cell *cycle arrest* [8]. The n-hexane fraction at IC_{50} concentration can inhibit cell growth through *cell cycle arrest*, thereby causing the ability of cell proliferation to decrease. The ability to inhibit cell proliferation is attributed to the mechanism of *cell cycle arrest* that causes DNA or RNA damage and triggers the activation of the p53 gene hence the *cell cycle* is temporarily stopped for the process of DNA or RNA repair. If the damage is severe enough and cannot be repaired, the cell will undergo apoptosis or suicide. However, in this study, only the number of living cells could be determined, but the molecular mechanism could not be known to inhibit cancer cell proliferation [17].

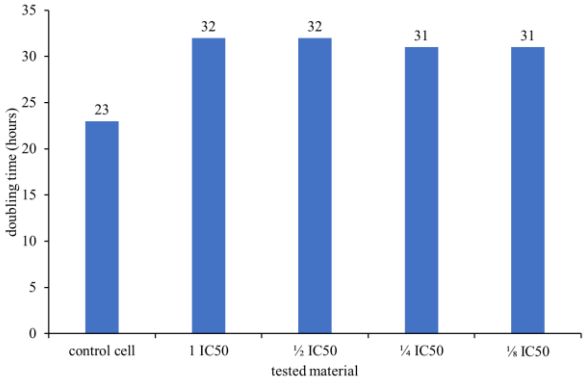


Figure 1. Antiproliferation test result of n-hexane fraction of Gendola leaves (*Basella rubra Linn.*)

The content of secondary metabolites in the n-hexane fraction of Gendola leaves is possibly the leading cause of the inhibition of T47D cell proliferation; secondary metabolites include steroids (stigmasterol), terpenoids, and saponins. Several previous studies have explained that these compounds have the potential to inhibit cancer cell proliferation [11, 15].

Nirwana [18] argued that the possibility of steroid compounds and terpenoids in their function as an anticancer agent due to its ability to able to block the cell cycle in the G2/M phase by stabilizing the *spindle* threads in the mitotic phase so that the mitosis process was inhibited. In the next stage, there was an inhibition of cell proliferation and apoptotic tracking. Steroid and terpenoids compounds were also able to inhibit the enzyme topoisomerase in mammalian cells. There are two classes of topoisomerase enzymes in mammalian cells, type I, which cuts and breaks up a single strand of DNA, and type II, which cuts and breaks down a double-stranded DNA. The topoisomerase enzyme inhibitor stabilizes the topoisomerase complex, and the DNA is cut off; therefore, it can cause DNA damage. Damaged DNA can induce the expression of proapoptotic proteins so that they can stimulate apoptosis.

Nirwana [18] also argued that saponin compounds had been known to inhibit the formation of Bcl-2, which is overexpressed, induce caspase-3 proteins that are expressed too low, increase p53 expression, and also trigger G1 cell cycle arrest. Antiapoptotic members of the Bcl-2 family (Bcl-2, Bel-xL) inhibit the release of cytochrome C, Smac/Diablo, and mitochondrial apaf-1 via the formation of apoptosomes can activate caspase nine which ultimately activates downstream caspase 3. Domination of antiapoptotic family members such as Bcl-1. 2 and Bcl-xL can promote cell survival because apoptosis depends on the balance of Bcl-2 family members. Xu et al. [19] claimed that the active compound of triterpenoids and saponins were ginsenoside Rg3 had anti-proliferation activity in *hepatocellular carcinoma* HepG2 at IC_{50} concentration of 64 µM in 24 hours [19].

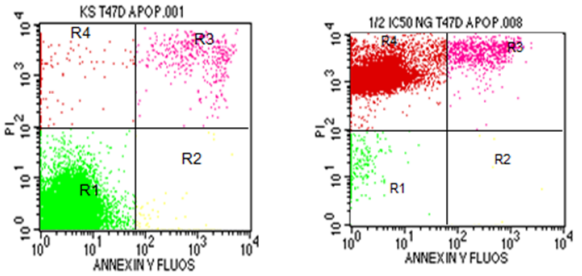
3.5. Cell Death Induction Test Using *flowcytometry* Method

The principle of flow cytometry was to use the reagent of annexin v to bind phosphatidylserine to the

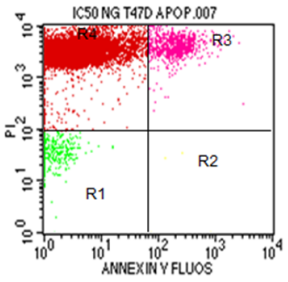
surface of cells undergoing apoptosis and propidium iodide (PI) to differentiate living cells, apoptosis, and necrosis. Induction of cell death by the n-hexane fraction of Gendola leaves with concentrations of 1 IC_{50}, and ½ IC_{50} was 292 µg/mL and 146 µg/mL, respectively. The positive control used was T47D cell control, with a concentration of 0.1 µg/mL, as shown in Table 3 and Figure 2.

Table 3. The induction percentage of *n*-hexane fraction from Gendola leaves towards breast cancer cell T47D using the *Flowcytometry* method.

Test material	Living cell (%)	Early Apoptosis cell (%)	Final Apoptosis cell (%)	Total apoptosis (%)	Necrosis (%)
Control cell	96.16	0.78	2.05	2.83	1.04
1/2 IC50	1.67	0.06	4.21	4.27	94.08
1 IC50	2.52	0.02	4.94	4.96	92.54



cell control ½ IC_{50} of n-hexane fraction



1 IC_{50} of n-hexane fraction

Figure 2. The result of the induction of cell death using Flowcytometry (a) control cell, (b) cell with the treatment of ½IC_{50}, (c) cell with the treatment of 1IC_{50}. explanation: R1= phase of life, R2 = early apoptosis, R3 =final apoptosis, R4 = necrosis

Based on the cell death induction test of Gendola n-hexane fraction, there was no increase in the percentage of cells undergoing apoptosis in cells with Gendola leaf n-hexane fraction treatment, ½IC_{50} concentration was 4.27%, and 1IC_{50} was 4.94%, compared to control cells. An increase in the percentage of necrosis at a concentration of ½IC_{50} was 94.08%, and 1IC_{50} was 92.54%, so it can be concluded that the n-hexane fraction of the Gendola leaf was unable to induce cell death through the apoptotic pathway but through the necrosis pathway.

It is important to understand various ways of cell death by cytotoxic agents to describe the mechanism and action of the drugs and identify any significant side effects. The direct result of compound treatment on cell death is often temporary and difficult to capture using standard endpoint tests. For example, apoptosis only occurs in a short window of time, usually within a few hours, and often followed by secondary necrotic events. It is, therefore, essential to conducting relevant tests, measuring either cell viability or cell death at an optimal experimental time point. Considering that the ability of compounds to kill cells may be different, it is essential to monitor survival and toxicity continuously, to determine the optimal time points by conducting endpoint tests to obtain precise information.

This study is not in line with the study of Kilari *et al.* [4], which showed the results that Gendola leaf extract could activate apoptosis (p53 and caspase-3) and inhibit proliferation in rat colon cancer cells [2]. Many factors influence the different results provided by each study besides the content of various metabolites and the characteristics of the cancer cells used. T47D breast cancer cells undergo *missense mutation* in residue 194 (in the zinc-binding domain L2) p53 gene. This L2 loop plays an essential role in DNA binding and protein stabilization. If p53 cannot bind to the response element in DNA, its ability to regulate cell cycles can be reduced or lost. In tumor cells with p53 mutations, there is a known reduction in response to agents that induce apoptosis, and the tumors are likely to become resistant to antineoplastic drugs that target DNA destruction [20]. Mutations in the p53 gene can cause loss of tumor suppressor function with the mechanism of alteration of the function of p53 protein as an apoptotic induction factor and as a transcription factor. Most p53 gene mutations are localized to the DNA binding region, and about 90% of mutations that occur are missense point mutations [21]. With the characteristics of T47D cancer cells, where the mutated p53 protein is thought to result in secondary metabolite content in the n-hexane fraction of the Gendola leaf from a steroid compound namely stigmasterol not able to spur the expression of p53 protein so that apoptosis does not occur, then what happens is the cell experiences necrosis. The danger of necrosis is to create inflammation around cells, where dead cells kill surrounding cells by releasing digestive enzymes.

4. Conclusion

The n-hexane fraction of Gendola leaves (*Basella rubra Linn*) has a cytotoxic effect with an IC_{50} value of 292 µg/mL, which can inhibit proliferation for 32 hours by doubling time technique but is unable to induce T47D cell death through apoptotic pathways.

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