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Cytotoxic Activity of *Eugenia polyantha* Wight Young Leaves Purification Extract and Fraction on T47D

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ABSTRACT

Breast cancer is a cancer that ranks the highest incidence in Indonesia. One of the plants that can be used for this treatment is a bay leaf (*Eugenia polyantha* Wight). Purification techniques can optimize the extraction of flavonoid compounds to create larger concentrations of these substances. Old bay leaves' ethyl acetate fraction has lethal effects on T47D cancer cells. In this work, the amounts of flavonoids and the cytotoxic activity of young bay leaves' pure extract and ethyl acetate fraction against T47D cells are to be determined. By using the maceration process, two phases of methanol extraction from young bay leaves were produced. In the first stage, ethyl acetate was used to partially purify the material, and in the second stage, n-hexane, ethyl acetate, and water. Cytotoxic tests of purified extract and ethyl acetate fraction of young bay leaves were carried out at 200; 350; 500; 750; and 1000 µg/mL. Cytotoxicity test using the MTT assay method. IC_{50} value analysis using linear regression. Determination of total flavonoid content using a UV-Visible spectrophotometer with a comparator compound, quercetin. The results showed that the purified extract of young bay leaves and the ethyl acetate fraction of methanol extract of young bay leaves had cytotoxic activity with IC_{50} values of 570.57 ± 30.72 µg/mL and 588.45 ± 6.90 µg/mL, respectively. Total flavonoid levels were 9.80 ± 0.05 mgQE/g and 5.99 ± 0.06 mgQE/g. The purified extract and ethyl acetate fraction on young bay leaf extract (*Eugenia polyantha* Wight) had cytotoxic activity on T47D cells and the highest levels of flavonoids in the purified extract.

Keywords: *Eugenia polyantha* Linn; Purified extract; Ethyl acetate fraction; T47D; Cytotoxic

INTRODUCTION

Cancer is a disease caused by abnormal genes characterized by continuous proliferation signals, damage to growth suppressor genes, absence of cell death processes, uncontrolled cell replication, and stimulation of angiogenesis which causes cell metastasis and invasion of surrounding tissues[1]. According to Globocan[2] that 396,914 new cases of cancer and 234,511 deaths were reported in Indonesia, with breast cancer (16.6%), cervical cancer (9.2%), and lung cancer (8.8%) having the highest prevalence rates.

The high prevalence encourages the development of herbal medicines that have the potential as anticancer[3]. Natural ingredients can support breast cancer treatment because they can prevent and reduce side effects and increase the effectiveness of breast cancer treatment[4]. In order to improve the quality of life for breast cancer patients, research on natural substances must be conducted as a supporting therapy. Bay leaves are one of

the organic components that may be used as a supportive therapy for cancer treatment[5].

Salam plants (*Eugenia polyantha* Wight) contain flavonoids, tannins, alkaloids, and essential oils[6]. Flavonoids, which have been demonstrated to have a variety of anticancer effects including modulating reactive oxygen species (ROS) training, taking part in stopping the cell cycle, inducing apoptosis and autophagy, and suppressing cancer cell proliferation and invasion, are substances thought to have anticancer activity[7]. According to Prahastuti et al.[8], the flavonoid compound contained in bay leaves is a type of quercetin. Quercetin has potential as an anticancer by inhibiting the proliferation of breast cancer cells[9]. According to Fauzizah's research[10], With IC_{50} values of 436.713 µg/mL, 798.808 µg/mL, and 171.946 µg/mL, respectively, young and old bay leaf methanol extract (*Eugenia polyantha* Wight) and the ethyl acetate fraction of aged bay leaf methanol extract all displayed cytotoxic activity against T47D breast cancer cells.

According to Rivai et al.[11], The amount of total flavonoids in the ethanol extract of bay leaves was higher than the amounts of alkaloids, phenols, and tannins combined. The amount of quercetin in the condensed bay leaf extract, which is the measure of total flavonoid concentration, is not less than 1.14%[12]. The total flavonoid contents in the methanol extracts of young and old bay leaves (*Eugenia polyantha* Wight) and the ethyl acetate fraction of aged bay leaf methanol extract were 3.54 mgQE/g, 1.60 mgQE/g, and 5.27 mgQE/g, respectively[10].

Purification methods can increase the pharmacological activity of herbal medicines. The purification method is one way to obtain pure natural materials free from other unwanted chemicals, and the purity level of the material must be 95-100%[13]. Therefore, it is hoped that the purification method can extract purer chemical compounds, so their pharmacological activity was expected to increase. The purification process using hot water and ethyl acetate solvents can attract flavonoid compounds[14]. Based on research by Hidayati et al.[14], the total flavonoid content of the purified ethyl acetate extract of old bay leaves was 9.3 0.83 mg QE/gram, while the ethyl acetate fraction of the methanol extract of old bay leaves was 5.27 0.13 mg QE/gram. Young bay leaves were chosen as the test material for this investigation because, according to the literature review, they have a cytotoxic impact and contain more flavonoids. They were purified and fractionated to produce bigger secondary metabolites. Based on the aforementioned details, young bay leaves were used as the starting material for the purification and fractionation procedure. After that, the IC_{50} value and the measurement of the amounts of flavonoids were used to assess the young bay leaves' cytotoxic activity against T47D cells.

METHODS

Materials and Methods

The materials for this study were young bay leaves (Eugenia polyantha Wight) taken from Ngadiwarno Village, Sukorejo District, Kendal, Central Java, methanol (Bratacem), ethyl acetate (Bratacem), n-hexane (Bratacem), distilled water (Bratacem), Mg powder (Merck), concentrated HCl (Merck), amyl alcohol (Merck), Mayer's reagent (Merck), Dragendorf's reagent (Merck), Wagner's reagent (Merck), NaCl (Merck), gelatin (Merck), $FeCl_3$ (Merck), Liebermann-Burchard's reagent (Merck), ethanol (Merck), quercetin (Merck), $AlCl_3$ (Merck), 1 M potassium acetate (Merck), breast cancer cells T47D Collection of Cell Culture Laboratory, Faculty of Medicine and Science Muhammadiyah Yogyakarta the University of Health, RPMI 1640 medium (Gibco), Penstrep (Rmbio), 10% FBS (Rmbio), 0.5% fungizone (Rmbio), PBS (Rmbio), 5 mg/mL MTT solution, stopper and trypsin-EDTA (Gibco).

The research tools were drying cupboard (Memmet), a set of maceration

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tools, rotary vacuum evaporator (Heidolph), electric balance (Ohaus), UV-Vis 1800 spectrophotometer (Shimadzu), CO_2 incubator (Thermoscience), Biosafety Cabinet-level 2 (Esco Airstream), inverted microscope (Magnus), centrifuge (Hettick Ebba), vortex (Cleaver), 96-well plate, and ELISA reader (Biorad).

Determination

Plant determination was carried out at the Ecology and Biosystematics Laboratory, Departement of Biology, Faculty of Mathematics and Natural Sciences, diponegoro University, Semarang. The results of the determination are1b-2b-3b-4b-12b-13b-14b-17b-18b-19b-19b-21b-22b-23b-24b-25b-26b-27a-28b-29b-30b-31a-32a-33b-35b-37b-38b-39b-41b-42b-44b-45b-46e-50b-51b-53b-54b-56b-57b-58b-59b-70b-72b-73b-74a-75b-76a-77a-78b-103c-104b-106b-107b-186b-287b-288b-289a-290b-291a-292b-293b-294b-285b-296a (Famili 84. Myrtaceae) 1a-2b-3b-7b-8b-9b-10b (Genus 9. Syzygium) 1b-7b-8b-11b-12b (Species. Syzygium polyanthum (Wight) Walp. Sinonim: Eugenia polyantha Wight

Preparation of young bay leaf purified extract

Ten liters of methanol were used to macerate one thousand grams of bay leaf powder. Five days were spent on the maceration process, which was broken up into three days of maceration and two days of re-maceration. The filtrate was then heated to 60°C in a Rotary Evaporator and evaporated. 20 grams of the condensed extract were added to 400 mL of boiling water before being followed by 1:1 ethyl acetate. To create a thick pure extract, the ethyl acetate phase was extracted and thickened[14].

Preparation of young bay leaf ethyl acetate fraction of methanol extract

In a separatory funnel, 200 ml of water and 200 ml of n-hexane solvent (1:1) were added after 20 grams of the condensed extract were added. To create the two phases of n-hexane and water, shaking was used. The water and n-hexane phases were divided, then repeated until the n-hexane was clear and fit into an Erlenmeyer. 200 ml of ethyl acetate solvent were added to the water phase in a 1:1 ratio, agitated, and split into the two phases. The two phases ethyl acetate and water were separated via replication until the ethyl acetate solvent was clear, at which point they were accommodated in an Erlenmeyer. At 50°C, the rotary vacuum evaporator was used to condense the ethyl acetate fraction[14].

Identification of compounds

Flavonoids

Each sample was dissolved in 10 mL of methanol for 50 mg before being filtered. Mg metal powder and 3 mL of acetone were added to the solution. Then, through the tube wall, a few drops of a strong HCl solution were introduced. The appearance of red, orange, or yellow in the upper phase (amyl alcohol layer) is a sign of success[15].

Alkaloids

Each sample was split into three test tubes after being dissolved in 10 mL of 2N HCl. Dragendorf's, Mayer's, and Wagner's reagents were introduced to tubes 1, 2, and 3, respectively. A white precipitate in tube 1, a brownish-orange precipitation in tube 2, and a brownish precipitate in tube 3 were signs of successful results[15].

Tanin

Each sample was divided into two test tubes after being dissolved in 10 mL of hot distilled water. A 1% NaCl solution and 5% gelatin solution were added to tube 1, and a 5% FeCl3 solution was added to tube 2. A yellowish-white precipitate forming in tube 1 and a dark blue or greenish-black color change in tube 2 are signs of a successful experiment[15].

Fenolic

10 mL of methanol were used to dissolve 50 mg of each sample before filtering it. 5% $FeCl_3$ was added in a few drops to the solution. If the extract solution yields a dark blue, blackish blue, or black color, this indicates a good result to the phenolic test[15].

Cytotoxic test

Confluent T47D cells were harvested for the cytotoxicity test using the MTT tech-

nique, and 5000 cells were added to each well of a 96-well microplate. For adaptation, the cells were cultured for 24 hours at 37˚C in a 5% CO2 incubator. This allowed the cells to adhere to the bottom of the well and become treatment-ready. The media was then removed, cleaned with PBS (Phosphate Buffer Saline), and added to the pure extract sample test solution and the ethyl acetate fraction of the methanol extract at concentrations of 1000, 750, 500, 350, and 200 µg/mL for three replications, respectively. This time, the incubation period was extended to 24 hours. We employed T47D cells, DMEM medium (Dulbecco's Modified Eagle's Medium), and solvent control (DMSO absolut). After the incubation period, the culture medium in the plate was removed, and each well was then cleaned with PBS. Then 100 l of MTT (0.5 mg/ml) was put to each well. Formazan was generated after 4 hours of additional 37°C incubation. MTT will be changed into the dark blue substance formazan by living cells. After dissolving the formazan crystals with a 10% SDS stopper reagent in 0.1 N HCl, the cells underwent an overnight incubation at room temperature with protection from light. The plate was shaken with a horizontal shaker for 10 minutes at the conclusion of incubation before being read with an ELISA reader at a wavelength of 595 nm. The results of the readable absorbance are converted to a percentage of life.

Determination of flavonoid levels

Determination of maximum wavelength of operating time using a UV-VIS spectrophotometer with quercetin as a comparison was obtained. The results obtained are a wavelength of 431 nm and an operating time of 30 minutes.

Then determine the standard curve was series of quercetin concentrations of 1000 µL at each concentration (2, 4, 6, 8, 10, and 12 ppm) added 200µL of 10% $AlCl_3$ and 200 µL of 1 M potassium acetate into the flask measure 5 mL and fill up to the mark with ethanol p.a. The absorbance was read with a UV-VIS spectrophotometer at 431 nm during 30 minutes.

Using a pipette, 1000 µL of the purified extract sample and the ethyl acetate fraction of the methanol extract of young bay leaves were combined. 200 µL of 10% $AlCl_3$ and 200 µL of 1 M potassium acetate were then added, along with 5 mL of ethanol p.a., to make the required volume. Absorbance is read using spectrophotometry at 431 nm during 30 minutes. The treatment was replicated three times [16].

Analysis

Percent cell life is calculated using the equation:

“% viable cell=(“sample absorbance- control medium absorbance”/”control cells absorbance- control medium absorbance”)x100%”

The obtained cell viability was then examined using linear or probit regression between the log concentration and the cell viability percentage, which was then utilized to get the IC_{50} value.

Quercetin standard solution concentration series data were created, and a linear regression equation with a standard curve y = bx+a with y = absorbance, x = quercetin content in g/mL was then used to analyze the data. The total flavonoid content of each sample from the purified extract and the ethyl acetate fraction of the methanol extract of young bay leaves was calculated in µgQE/gram, where QE stands for equivalent to quercetin as a comparison compound. The absorbance of the purified extract solution and the ethyl acetate fraction of the methanol extract of young bay leaves was obtained. Total flavonoid levels are calculated using the formula:

$$\mathrm{KFT} = \frac{\mathrm{c\ x\ V\ x\ fp}}{\mathrm{m}}$$

Note:

KFT = Total flavonoids content (mg QE/gram)
c = Concentration of total flavonoid from quercetin (mg/L)
V = volume (L)
fp = dilution factor
m = mass (g)

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RESULTS AND DISCUSSION

Fresh young bay leaves as much as 3.355 grams were dried to obtain 1,140 grams of dried young bay leaves with a drying shrinkage of 66.02% and a moisture content of 5%. As much as 1000 grams of bay leaf powder was extracted, and a viscous extract of 186 grams was obtained with a yield of 18.6%. The characteristics of the methanol extract of young bay leaves are dark green with a thick texture and a distinctive odor of bay leaves.

The methanol extract of young bay leaves was then purified and fractionated. The purpose of purification is to obtain pure natural ingredients free from other unwanted chemicals[13]. The results of the purified ethyl acetate extract of young bay leaves obtained were 21.2 grams from 60 grams of young bay leaf methanol extract with a yield of 35.33%. Fractionation of methanol extract of young bay leaves of as much as 80 grams produces 21.20 grams of ethyl acetate fraction with a gain of 26.5%.

Young bay leaf methanol extract (EMDSM), young bay leaf purified extract (ETEADSM), and young bay leaf ethyl acetate fraction (FEAEMDSM) were all subjected to phytochemical screening in order to identify flavonoids, alkaloids, tannins, phenolics, saponins, and steroids. Table 1 lists the outcomes of the EMDSM screening.

The next step was to determine the total flavonoid content in ETEADSM and FEAEMDSM using the colorimetric method using a standard quercetin solution. According to the Indonesian Ministry of Health[17], bay leaves contain total levels of flavonoids which are counted as quercetin. The results of determining total flavonoid content ETEADSM contained a total flavonoid content of 9.8 ± 0.05 mgQE/g, and FEAEMDSM contained a total flavonoid content of 5.99 ± 0.06 mgQE/gram.

The results of the purified cytotoxic test of young bay leaf extract (ETEADSM) obtained an IC_{50} value of 570.57 ± 30.72 µg/mL, and the ethyl acetate fraction of methanol extract of young bay leaf (FEAEMDSM) IC_{50} value of 588.45 ± 6.90 µg/mL. The results of the ETDSM and FEAEMDS cytotoxic tests on T47D cells are shown in Table 2. The forms of live and dead T47D cells after being treated with ETDSM and FEAEMDS samples are shown in Figure 1. The method used is the MTT Assay, where the living cells are characterized by the formation of colored formazan crystals purple (figures 1C and F).

ETEADSM has a weak category of cytotoxic activity in the range of 200 µg/mL to 1000 µg/mL[17]. This is presumably because the content of flavonoid compounds in FEAEMDSM can cause a biphasic effect. Research by Xi et al.[18] showed that flavonoids were shown to have a biphasic effect on breast cancer cells at a low dose flavonoids. Flavonoids at low concentrations stimulated the growth of breast cancer cells, while at high concentrations, they inhibited cell proliferation.

ETEADSM has an activity of 588.477 µg/mL. This is in line with the research by Hidayati et al.[14], Results revealed that T47D cells were sensitive to the pure ethyl acetate extract of old bay leaves, with an IC_{50} value of 593.826 µg/mL. Compared with research by Fauzizah[10], Young bay leaves' methanol extract had cytotoxic activity against T47D cells with an IC_{50} value of 436.713 µg/mL. Based on this, young bay leaves' methanol extract has more cytotoxic activity than ETEADSM. The chemical composition of EMDSM contains alkaloids, flavonoids, steroids, and phenolic compounds.

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Table 1. Phytochemical screening of methanol extract, purified extract and ethyl acetate fraction of methanol extract from young bay leaves

Compound screening	Chemical reagent	Phytochemicals			Result		
		EMDSM	**ETEADSM**	**FEAEMDSM**	**EMDSM**	**ETEADSM**	**FEAEMDSM**
Flavonoids	Mg + HCl + amyl alcohol	Orange on the top layer	Orange on the top layer	Orange on the top layer	+	+	+
Alkaloids	Mayer	Green with an orange precipitate	Green	Green	+	-	-
	Dragendorf	Orange with an orange precipitate	Orange	Orange			
	Wagner	Brown with a brown precipitate	Orange	Orange			
Tanins	NaCl 1% + gelatin 5%	Green	Green	Green	-	-	-
	$FeCl_3$	Blackish blue	Blackish blue	Blackish blue			
Fenolic	$FeCl_3$ 5%	Blackish blue	Blackish blue	Blackish blue	+	+	+
Saponin	Aquadest	No foam	No foam	No foam	-	-	-
Steroids	Liebermann-Burchard	Greenish blue	Green	Green	+	-	-

Table 2. Cytotoxic test results of purified extract and ethyl acetate fraction of methanol extract of young bay leaves against T47D cells

Concentration (μg/mL)	Average cell viability (%) ± SD		Average IC_{50} Value (μg/mL)	
	ETEADSM	**FEAEMDSM**	**ETEADSM**	**FEAEMDSM**
1000	3.30 ± 0.19	3.7 ± 0.59	570.57 ± 30.72	588.45 ± 6.90
750	6.30 ± 4.16	10.9 ± 0.21		
500	81.20 ± 0.20	70.9 ± 3.77		
350	144.10 ± 0.85	124.9 ± 3.78		
200	149.50 ± 2.42	173.4 ± 2.33		

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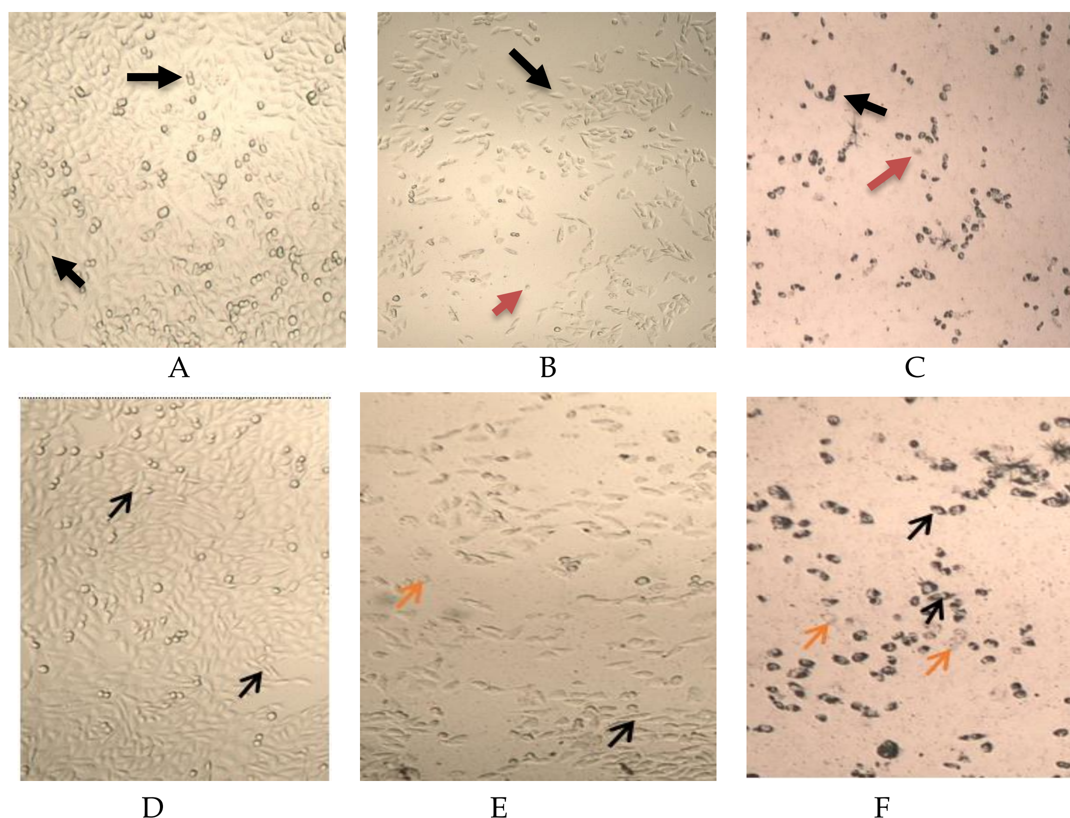

Figure 1. Effect of ETEADSM (A-C) and FEAEMDSM (D-F) treatment on T47D cell morphology
A dan D =Control cells without treatment
B = ETEADSM 500 µg/mL
C = ETEADSM 500 µg/mL after being given MTT
E = FEAEMDSM 500 µg/mL
F = FEAEMDSM 500 µg/mL after being given MTT
= live cells
= dead cells

Meanwhile, ETEADSM and FEAEMDSM contain flavonoids and phenolic compounds. The age difference of the leaves can affect the bioactive compounds and secondary metabolites produced, affecting the IC_{50} value. The ethyl acetate fraction of the old bay leaf methanol extract exhibits superior cytotoxic activity to FEAEMDSM, which is indicated by the fact that FEAEMDSM has a larger IC_{50} value than the old bay leaf methanol extract. According to research by Mar'atussolikhah[19], Old bay leaf methanol extract contains flavonoids and steroid chemicals in the ethyl acetate fraction. Based on this, it is known that the ethyl acetate fraction of the methanol extract of young bay leaves differs from the ethyl acetate fraction of the extract of old bay leaves in that the latter does not contain steroids. The methanol extract of bay leaves, on the other hand, contains steroids in the ethyl acetate fraction. Therefore, it is believed that steroid molecules with anticancer action are involved.

Numerous anticancer actions of flavonoid compounds have been demonstrated, including modulation of ROS-scavenging enzyme activities, cell cycle arrest, induction of apoptosis and autophagy, and suppression of cancer cell proliferation and invasiveness[7]. According to Meiyanto et al.[20], phenolic compounds are efficacious in the antiproliferation and apoptosis of MCF-7 breast cancer cells.

It can be assumed that the high cytotoxic activity in the methanol extract of young bay leaves is due to the presence of alkaloid and steroid compounds. Alkaloid compounds are generally semi-polar, and steroid compounds are non-polar[21], so it is necessary to carry out an extraction process with a solvent that can attract alkaloid compounds and steroid compounds in young bay leaves and then test their cytotoxic activity on T47D cells[21].

CONCLUSION

The purified extract and ethyl acetate fraction on young bay leaf extract (*Eugenia polyantha* Wight) had cytotoxic activity on T47D cells and the highest levels of flavonoids in the purified 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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