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Cytotoxic Activity and Apoptosis by Extract and Ethyl Acetate Fraction of *Hibiscus tiliaceus* Linn in 4T1 Cell Line

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ABSTRACT

Waru leaves are proven to have cytotoxic activity. The activity of waru leaves needs to be developed in a molecular direction to see its apoptotic activity. This study aims to determine the cytotoxic activity of methanol extract (EWL) and ethyl acetate fraction fraction (EAFWL) of *Hibiscus tiliaceus* Linn as well as the induction of apoptosis in 4T1 cells. The methanol extract of hibiscus leaves was obtained by the maceration method, followed by a fractionation process with n-hexane, ethyl acetate, and water as solvents. Cytotoxic test of EWL was carried out at a concentration of 31.25; 62.5; 125; 250; 500 and 1000 µg/mL while EAFWL was at a concentration of 100; 200; 400; 600 and 100 µg/mL, using MTT assay. Analysis of IC_{50} values using linear regression. The apoptosis induction test at IC_{50} and ½ IC50 concentrations using flowcytometry. Data on the percentage of apoptosis were analyzed using One Way Anova statistical analysis. The results were EWL and EAFWL cytotoxic tests against 4T1 had IC50 values of 649 µg/mL and 746 µg/mL. EWL obtained a percentage of total apoptosis at a concentration of 324.5 µg/mL of 48.76%; 649 µg/mL was 67.06% and control cells were 36.16%. EAFWL percentage of total apoptosis at a concentration of 746 µg/mL of 30.8%; 373 µg/mL was 42.13% and control cells were 9.6%. So EWL and EAFWL can induce apoptosis of 4T1 cells.

***Keywords*:** *Hibiscus tiliaceus* leaves; 4T1; Cytotoxic; Apoptotic

INTRODUCTION

Breast cancer is an important public health problem, and its mortality and morbidity increase worldwide every year. Breast cancer has the highest incidence of 16.7%, with a mortality rate of 11.0%.[1] Breast cancer cases in Indonesia rank first, with a prevalence of 16.6% of all cancer cases in Indonesia. In addition, the death rate due to breast cancer is 9.6%, ranking second. The incidence of breast cancer is estimated at 148.1 per 100,000 population.[2] Hibiscus leaves (*Hibiscus tiliaceus* Linn.) are a natural ingredient that can be used as a chemopreventive agent. Based on the phytochemical screening, the methanolic extract of waru leaves contains saponins, flavonoids, terpenoids, glycosides, steroids, proteins, carbohydrates, and polyphenols. Methanol extract from hibiscus leaves has cytotoxic activity on MDA-MB-435S breast cancer cells with an IC_{50} of 1.14 mg/mL because it falls within the range (IC_{50} 1.1–1.6 mg/mL).[3] The methanol extract of waru leaves is known to contain a flavonoid compound, namely quercetin.[4] The flavonoid derivative quercetin compound has anticancer activity, which is thought to induce apoptosis and inhibit the cell cycle.[5]

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Research into apoptosis induction of methanol extract and ethyl acetate fraction of hibiscus leaves on 4T1 cancer cells has so far never been carried out. Quercetin is able to the loss of cell viability, apoptosis, and autophagy though the modulation of PI3K/Akt/mTOR, Wnt/β-catenin, and MAPK/ERK1/2 pathways.[6] Induction of apoptosis in 4T1 cancer cells was demonstrated in the ethyl acetate fraction from the methanol extract of the stem bark of *Pterygota alata* (Roxb.) R. expressed Bcl-2.[7] In addition, flavonoid compounds are reported to be able to induce apoptosis through down-regulation of downstream molecules in the Bcl-2 and Blc-xL proteins.[8] In addition, quercetin has apoptotic activity in human malignancies.[9]

Flavonoids induces apoptosis by modulating Bcl-2, Bax and Caspase activity of Triple Negative Breast Cancer (TNBC).[10] The cytotoxic test of the ethanol extract of waru leaves was found to have an IC_{50} value of 892 µg/mL.[11] The activity of waru leaves needs to be developed with different solvens to extraction and fractionation. So their activity a molecular direction to see its apoptotic activity. Based on the description above, this study aims to determine the cytotoxic activity and apoptosis induction from methanol extract and the ethyl acetate fraction of waru leaves against 4T1 cancer cells.

METHODS

Materials and Methods

Source of plant material

Hibiscus leaves from Kunduran Village, Kunduran District, Blora Regency, Central Java Province. Waru leaves were determined at the Laboratorium Ekologi dan Biosistematika Jurusan Biologi Matematika dan Ilmu Pengetahuan Alam Universitas Diponegoro Semarang and the material was proven to be correct.

Materials

The research materials used methanol (technical), ethyl acetate (technical), distilled water, and n-hexane (specialized) as solvents in fractionation. 4T1 cells were obtained from the collection of the In Vitro Cell Culture Laboratory, Faculty of Medicine and Health Sciences, Muhammadiyah University of Yogyakarta, DMEM media (Gibco), Fetal Bovine Serum (FBS) 10% (Sigma), Penicillin-Streptomycin 2% (Sigma), Fungizon 0.5 % v/v (Gibco), DMSO (Merck), MTT (Sigma), Stopper containing SDS 10% (Sigma), Trypsin EDTA 0.1% (Gibco). FITC Annexin V Apoptosis Kit with PI reagent (BioLegend) was used in the apoptosis assay.

Equipment

Preparing maceration test samples, scales (Hanherr), and oven (IRASTAR). Tools for cytotoxic and apoptotic tests are CO_2 incubator (Heraeus), vortex (Gemmy), centrifuge (Biosan), centrifuge tube, LAF (Laminar Air Flow) (Labconco), micropipette (Gilson), yellow and blue tip (Brand), autoclave (Hirayama), hemocytometer (Neubauer), cell counter (Kenko), tread tissue culture disk (Iwaki), test tube, conical tube (Falcon), inverted microscope (Zeiss), ELISA reader (Bio-Red), 96-well plate (Nunc), 6 well-plates (Nunc), sterile Eppendorf (Plasti Brand), electric scales (Sartorius), flowcyometry (BD Accuri).

Extraction of waru leaves

1000 mg of hibiscus leaf powder was macerated using 10 L of methanol solvent. The maceration process was carried out for 5 days, divided into maceration (3 days) and remaceration (2 days). Next, the filtrate was evaporated using a Rotary Evaporator at a temperature of 60°C.[11]

Preparation of waru leaf ethyl acetate fraction

Forty grams of EWL was dissolved in 400 ml distilled water with a ratio of 1:10. The extract solution was put into a separating funnel and mixed with 400 ml n-hexane (non-polar) solvent in a 1:1 balance. The n-hexane fraction was separated. The water fraction was added with ethyl acetate solvent in the same ratio of 1:1 and carried out as in the previous step until the ethyl acetate solvent was

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clear in color. The ethyl acetate fraction was collected in an Erlenmeyer flask so that three fractions were obtained, namely the n-hexane fraction, the ethyl acetate fraction, and the water fraction. However, in this study, only the ethyl acetate fraction of the methanol extract of waru leaves was used. The ethyl acetate fraction of the methanol extract of waru leaves was thickened using a rotary vacuum evaporator at a temperature of 50°C.

Cytotoxic test

Cytotoxic tests were carried out on the methanol extract of waru leaves (EWL) and the ethyl acetate fraction of the methanol extract of waru leaves (EAFWL) against 4T1 cancer cells. The harvested 4T1 cell cultures were distributed into 96 well plates $1x10^4$ cells/well, and 3 well cells were left for media control. Cells were incubated in a CO_2 incubator at 37°C and observed under a microscope. The cell medium is removed by turning the plate 180o above the waste site; then, the scale is gently pressed on the tissue to drain the liquid. Cells were washed with 100 PBS per well. The EWL and EAFWL concentration series that had been made were put into the wells and incubated for 24 hours in a CO_2 incubator at 37°C. After incubation, the culture medium was removed and washed using 100 PBS per well. Then, all wells were added with MTT 100 reagent and incubated for 2-4 hours in a CO_2 incubator at 37°C. In the next step, a 10% SDS reagent was added in 0.01 N HCl. The plate was wrapped in white paper, incubated overnight at room temperature, and protected from light. After incubation, the absorbance was read using an ELISA reader at a wavelength of 595 nm[12]. The IC_{50} value is used as the basis for the concentration of the apoptosis induction test.

Apoptosis test with flowcytometry

The cells that have been prepared are then transferred into a conical tube and washed in each well using PBS ad 500; PBS is taken using a micropipette and moved to the same conical tube. Harvesting was carried out by adding 200% trypsin-EDTA into the wells and incubating for 3 minutes. Culture media was added at a rate of 1000 per well and suspended until the cells were released one by one. The cell suspension was transferred into a conical tube, and the wells were rinsed using 500 PBS, then put into the same conical box and centrifuged at 2000 rpm for 5 minutes. The precipitate formed was arranged in a conical tube and wrapped in aluminum foil. The residue (pellet) was dissolved with 100 of the Annexin V-FLOUS kit buffer, and 2 FITC Annexin V and 2 Propidium Iodide (PI) were added. The cell suspension was homogenized and then incubated for 15 minutes at room temperature, then analyzed using flowing (FACS Calibur) to determine the apoptosis profile.

Analysis

The cell viability obtained was analyzed by linear or probit regression between the log concentration and the percentage of cell viability, which was then used to get the IC_{50} value. The data apoptosis produced by flowcytometry was analyzed using the flow program to see the percentage of cells in 4 quadrants, namely Q1, Q2, Q3 and Q4. Q2 and Q3 were percentage total apoptosis, was compared with control cells. The data was analyzed statistically using SPSS software with one way Anova.

RESULTS AND DISCUSSION

Methanol extract and ethyl acetate fraction of hibiscus leaves

Fresh hibiscus leaves were processed into simplicia, resulting in a yield of 33.02%. The next stage is to make it in the form of a thick extract with a gain of 12.98%. This yield showed greater results than using 96% ethanol solvent, namely 10.2%.[11]

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Macroscopically, the methanol extract of waru leaves has a blackish-green color, is thick, and has a distinctive odor. The methanol extract of waru leaves (EWL) was then fractionated using ethyl acetate solvent. The selection of the ethyl acetate fraction was based on the previous research that the ethyl acetate solvent was able to extract the content of flavonoid nonpolar compounds.[13] The results of the ethyl acetate fraction of methanol extract of waru leaves (EAFWL) were obtained as much as 3.7 grams with a yield of 9.25% with a characteristic brown color, with a rather thick texture.

Cytotoxic test results

The EWL cytotoxic test results obtained an IC_{50} value of 649.053 µg/mL. These results were obtained from log concentration vs. % cell viability, obtained by the equation Y = -18.469x + 101.94. The data are presented in Table 1. These results are smaller than previous research, which extracted hibiscus leaves using 96% ethanol solvent with an IC_{50} value of 892.4 µg/mL.[11] Other studies categorize the cytotoxic effects of extracts based on the IC_{50} value, namely very active (IC_{50}≤ 20 µg/mL), moderate (IC_{50} > 20-100 µg/mL), weak (IC_{50} ≥ 100-1000 µg/mL), and inactive (IC_{50} > 1,000 µg/mL).[14] The results of the FEAMDW cytotoxic test on 4T1 cells are presented in Table 2, which was then analyzed using linear regression; the regression equation obtained Y= - 0.0657x + 99.067 so that the IC_{50} value was 746.8 µg/mL.

Apoptosis Test

One of the mechanisms for cancer to develop is inhibiting the process of apoptosis (cell death) so that tracking of therapeutic targets can be seen for its activity in increasing apoptosis. Apoptotic induction test on 4T1 cancer cells, treated with EWL with a concentration of 649 µg/mL (IC_{50}) and 324.5 µg/mL (½ IC_{50}) and EAFWL with a concentration of 746 µg/mL (IC_{50}) and 373 µg/mL (½ IC_{50}). In this apoptosis induction test using two concentrations based on previous research, the apoptosis induction test can be carried out using 2 concentrations, namely IC_{50} and ½ IC_{50}.[15] Display of EWL flow cytometry results on 4T1 cancer cells at two concentrations, namely ½ IC50 (324.5 µg/mL) and IC_{50} (649 µg/mL) and EAFWL at concentrations of ½ IC_{50} (373.4 µg/mL)

Table 1. EWL cytotoxicity test results on 4T1 cells

Concentration EWL (µg/mL)	% cell viability ± SD
1000	48.52 ± 0.75
500	50.11± 0.64
250	56.43± 0.73
125	63.08± 0.77
62.5	70.67± 0.87
31.25	73.77± 0.78

Table 2. EAFWL cytotoxicity test results on 4T1 cells

Concentration EAFWL (µg/mL)	Cell viability ± SD
1000	30.70 ± 0.87
600	61.74 ± 2.89
400	77.61 ± 0.87
200	84.95 ± 0.89
100	89.22 ± 1.73

and IC_{50} (746 µg/ mL) is presented in figure 1. EWL obtained the percentage of total apoptosis at a concentration of 324.5 µg/mL of 48.76% while at a concentration of 649 µg/mL of 67.06%. It is known that the percentage of total apoptosis was greater in the treatment group than in the control group, which was 36.16%. 4T1 cells treated with EAFWL at a concentration of 373.4 µg/mL resulted in a total apoptosis percentage of 42.13%, while at a concentration of 746 µg/mL, it was 30.8%. These results were greater than those in the control group of cells, which only produced a total apoptosis percentage of 9.6%. The results of the rate of total apoptosis are presented in Table 3.

These results show that EWL and EAFWL have significant apoptosis induction ability ($p<0.05$) compared to control cells. Methanol extract contains flavonoid, phenolic, terpenoid, alkaloid, and steroid compounds.[16]

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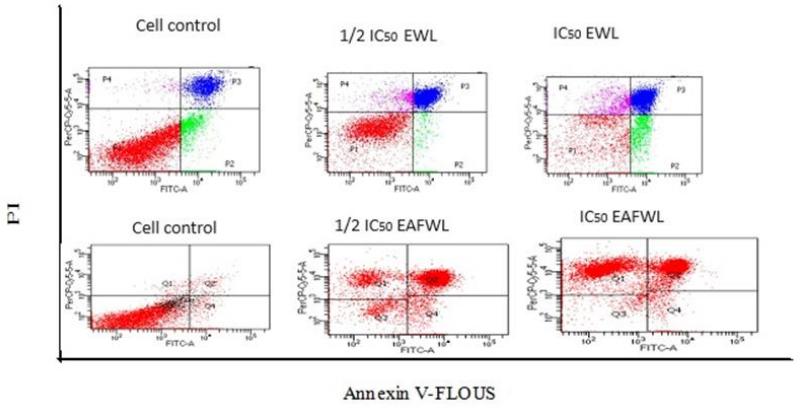

Figure 1. Results of apoptotic 4T1 cancer cell apoptotic flow cytometry. Treatment of methanol extract of waru leaves (EWL) and ethyl acetate fraction of methanol extract of waru leaves (FEAEDMW) at concentrations of ½ IC_{50} and IC_{50}. Control cells: no treatment

Table 3. The results of the percentage of total apoptosis after being treated with EWL and EAFWL on 4T1 cancer cells. *p<0.05 there are significant differences

Percentage Cell	EWL			EAFWL		
	Cell control ± SD (n=3)	**½ IC_{50} (324,5 µg/mL) ± SD (n=3)**	**IC_{50} (649 µg/mL) ± SD (n=3)**	**Cell control ± SD (n=3)**	**½ IC_{50} (373,4 µg/mL) ± SD (n=3)**	**IC_{50} (746 µg/mL) ± SD (n=3)**
% cell viability	61.96 % ± 7.27	42.53 % ± 1.31	20.6 % ± 2.89	88.9 % ± 5.50	9.4 % ± 0.29	2.7 % ± 1.14
% early apoptosis	9.53 % ± 6.33	4.8 % ± 0.22	13.03 % ± 2.41	5.3 % ± 2.62	5.03 % ± 4.02	1.2 % ± 1.63
% late apoptosis	26.53 % ± 7.30	43.96 % ± 1.81	54.03 % ± 4.53	4.36 % ± 2.89	37.1 % ± 21.48	29.6 % ± 6.98
% total apoptosis*	36.16 % ± 6.62*	48.76 % ± 1.84*	67.06 % ± 2.36*	9.6 % ± 5.49*	42.13 % ± 5.48*	30.8 % ± 8.21*
% necrosis	2.43 % ± 0.29	10 % ± 0.67	14.16 % ± 1.31	1.4 % ± 0.29	48.53 % ± 25.33	66.33 % ± 8.91

Some of these compounds have anticancer potential. Flavonoid compounds the callus of *Ampelopsis grossedentata* have anti-cancer potential against 4T1 cancer cells.[14] Purification extract and fraction of young leaves *Eugenia polyantha* Wight compounds flavonoids and had cytotoxc on T47D cells.[17]

The methanol extract of waru leaves contains the flavonoid derivative quercetin.[4] Ethyl acetate extract from waru leaves contains flavonoids, saponins, phenolics, and fumaric acid.[13] Quercetin is able to induce apoptosis by activating the caspase cascade (mitochondrial pathway) in MDA-MB-231 cancer cells.[9] Quercetin is able to induce apoptosis in MCF-7 breast cancer cells because there is an increase in the regulation of the Bax protein and a decrease in the law of the Bcl-2 protein, so it is known that a reduction in the expression of the antiapoptotic protein (Bcl-2) and an increase in the face of the pro-apoptotic protein (Bax) results in the level of apoptosis higher after treatment with quercetin.[10]

CONCLUSION

Methanol extract of waru leaves and ethyl acetate fraction of methanol extract of waru leaves had cytotoxic activity against 4T1 cells with IC_{50} values of 649.053 µg/mL and 746.8 µg/mL, respectively. Methanol extract from waru leaves and ethyl acetate fraction of methanol extract from waru

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leaves were able to induce apoptosis in 4T1 cells.

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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REFERENCES

1. Cancer Country Profiles. WHO Report on Cancer: Country Profile. https://www.who.int/cancer/country-profiles/IDN_2020.pdf.
2. Globocan. International Agency for Research on Cancer. The Global Cancer Observatory. 2021;1–2.
3. Uddin J, Grice ID, Evelin T. Cytotoxic Effects of Bangladeshi Medicinal Plant Extracts. Evidence-Based Complementary and Alternative Medicine. 2009;1–7.
4. Mustapa MA, Taupik M, Kadir NI. Liquid Chromatography-Tandem Mass Spectroscopy Assay for Quercetin and Conjugated Quercetin in Skin Rods (Hibiscus tiliaceus). Journal of Pharmaceutical Sciences and Research. 2019;11:2598–603.
5. Kopustinskiene DM, Jakstas V, Savickas A, and Bernatoniene J. Flavonoids as Anticancer Agents.PubMed Central. 2020;Feb 12 (2):457.doi: 10.3390/nu12020457
6. Farias MR, and Pozo CC. The Anti-Cancer Effect of Quercetin: Molecular Implications in Cancer Metabolism. International Journal of Molecular Sciences. 2019. 20(13): 3177. https://doi.org/10.3390/ijms20133177
7. Rollando R, Warsito W, Masruri M, Widodo W. Pterygota alata (Roxb.) R.Br. Bark Fraction Induced Intrinsic Apoptotic Pathway in 4T1 Cells by Decreasing Bcl-2 and Inducing Bax Expression. Pakistan Journal of Biological Sciences. 2021;24:172–81: DOI: 10.3923/pjbs.2021.172.181
8. Zhang, Hong W, Hu JJ, Ruo-Qiu F, Xin L, Yan-Hao Z, et al. Flavonoids Inhibit Cell Proliferation and Induce Apoptosis and Autophagy Through Downregulation of PI3Kγ Mediated PI3K/AKT/mTOR/p70S6K/ ULK Signaling Pathway in Human Breast Cancer Cells. Scientific Reports. 2018;8:1–13: doi: 10.1038/s41598-018-29308-7.
9. Shahbaz M, Naeem H, Momal U, Imran M, Alsagaby SA, Abdulmonem WA et al. Anticancer and apoptosis inducing potential of quercetin against a wide range of human malignancies. International Journal of Food Properties. 2023:26 (1): 2590-2626: https://doi.org/10.1080/10942912.2023.2252619
10. Chinnkrishnan P, Ibrahim IAA, Alzahrani AR, Shahzad N, Sivaprakasam P, Pandurangan AK. The Role of Selective Flavonoids on Triple-Negative Breast Cancer. Separations. 2023: 10(3): 207: https://doi.org/10.3390/separations10030207
11. Hidayati DN, Safitri EI, Alviani DL, Putri MNA. Aktivitas Sitotoksik dan Penghambatan Migrasi Sel kanker 4T1 Dari Ekstrak Daun Waru (Hibiscus tiliaceus Linn.). Jurnal Tumbuhan Obat Indonesia. 2022;15(1): DOI: https://doi.org/10.22435/jtoi.v15i1.5710
12. CCRC. Sel T47D [Internet]. 2021. Available from: http://ccrc.farmasi.ugm.ac.id/?page_id=1239
13. Luna SLRD, Garza RER, and Saldivar SOS. Environmentally Friendly Methods for Flavonoid Extraction from Plant Material: Impact of Their

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ISSN: 2354-8770 (Electronic)

Operating Conditions on Yield and Antioxidant Properties. 2020: 28: doi 10.1155/2020/6792069

14. Li Y, Kumar PS, Tan S, Huang C, Xiang Z, Qiau J, et al. Anticancer and antibacterial flavonoids from the callus of Ampelopsis grossedentata; a new weapon to mitigate the proliferation of cancer cells and bacteria. Royal society of Chemistry. 2022;12.24130–24138: doi 10.1039/D2RA03437A
15. Setiawan SI, Safitri EI, Nailul ML, Hidayati DN. Aktivitas Sitotoksik dan Induksi Apoptosis dari Ekstrak Etanol Kulit Apel Hijau (Pyrus malus L.) terhadap Sel MCF-7. Jurnal Pharmascience. 2021;8:69–78: DOI: http://dx.doi.org/10.20527/jps.v8i2.10729
16. Samsudin MS, Andriani Y, Purbowatiningrum RS, Desy FS. Study on Hibiscus tiliaceus Leaves as Antibacterial and Antioxidant Agent. Jurnal Pendidikan Ilmu Kimia. 2019;3:123–31: https://doi.org/10.33369/atp.v3i2.9874
17. Hidayati DN, Safitri EI, Yumni GG, Salsabiila I, Rahayu P. Cytotoxic Activity of Eugenia polyanthaWight Young Leaves Purification Extract and Fraction on T47D. Jurnal Kefarmasian Indonesia. 2023; 13 (2); 131-139; DOI: https://doi.org/10.22435/jki.v13i2.6607