Pharmacon: Jurnal Farmasi Indonesia. Vol 21, Special Issue 1, (2024). ISSN 1411-4283
Available online at: http://journals.ums.ac.id/index.php/pharmacon

Cytotoxic of Ethanol Extract of Leaves and Bark Mareme (*Glochidion Arborescens* Blume.) Against 4T1 Cancer Cells

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Abstract

Mareme plant (Glochidion arborescens Blume.) is a plant that has the potential to treat cancer. In a study on the leaves and bark of the Mareme (Glocidion arborescens Blume.) plant, tests for antioxidant, antidiabetic, and anti-inflammatory activities were previously carried out. Secondary metabolites that play a role in cytotoxic activity in Mareme plants are flavonoids, and these compounds can specifically kill the progression of cancer cells. This study aims to determine the content of compounds, examine cytotoxic activity, and determine the percentage of living cells in the leaves and bark of Mareme (Glochidion arborescens Blume.). The leaves and bark of Mareme (Glochidion arborescens Blume.) were extracted by maceration using a 96% ethanol solvent, then identification of the compounds was carried out to determine the content contained in the ethanol extract of the leaves and stem bark of Mareme, and then a cytotoxic test was carried out using the MTT assay method. The compounds contained in Mareme leaves are flavonoids, tannins, steroids, saponins, and polyphenolics, while the chemical compounds in Mareme stem bark are flavonoids, tannins, saponins, and polyphenolics. Tests were carried out at concentrations of 500, 600, and 800 µg/mL. The results of the cytotoxic test showed that the value of live cells in the ethanol extract of Mareme leaves (Glochidion arborescens Blume.) was 114.21, 106.21, and 78.91%, respectively. While the value of live cells in the ethanol extract of Mareme stem bark (Glochidion arborescens Blume.) was 59.56, 56.37, and 42.85%, respectively. The higher the concentration, the smaller the living cells produced.

***Keywords**: % living cells, 4T1 cells, cytotoxic, Glochidion arborescens Blume.*

INTRODUCTION

Cancer is a non-communicable disease that is characterized by abnormalities in cells that develop uncontrollably and have the ability to move between cells and body tissues (Ministry of Health, 2019). Based on data from 2021, 1,898,160 new cases and 608,570 deaths caused by cancer occurred in the United States. The largest number of cancer deaths in men comes from lung, prostate, and colorectal cancer, while in women it is lung, breast, and colorectal cancer (Siegel et al., 2021). Based on the visible characteristics, the prevalence of cancer increases with increasing age, with those aged ⩾ 55 years occupying the highest position among cancer sufferers. The incidence of cancer in women is higher than in men. Cancer prevalence tends to be higher in urban areas than in rural areas (Ministry of Health, 2018).

Breast cancer treatment can be chemotherapy with single or combined drugs (Panigroro et al., 2019). Drugs for chemotherapy can cause side effects (Mallik, 2007). Furthermore, to minimize these side effects, traditional medicines can be used that have anticancer potential (Haryoto & Putri, 2019). Currently, Indonesia is experiencing quite good progress in the use of traditional medicine (Hamzah et al., 2021).

One of the plants that can be used as an anticancer is the mareme plant, whose leaves contain chemicals including flavonoids, phenolics, alkaloids, saponins, tannins, steroids, triterpenoids, and quinones (Anggraeni et al., 2020). The flavonoid content has an effect as a cancer inhibitor by inhibiting the signal transduction pathway from the membrane to the cell nucleus (Anggraini., 2022). Apart from that, the mareme plant also contains antioxidants (Sugihartini, 2019), where antioxidant activity is closely related to anticancer activity. This is because one of the causes of cancer is free radicals, which cause oxidative stress. Meanwhile, antioxidants are compounds that can reduce oxidative stress (Mulia et al., 2016).

Therefore, the leaves and bark of the mareme plant require further research. The ethanol extract from the leaves and stem bark of the mareme plant has not yet undergone cytotoxicity testing against 4T1 breast cancer cells. So in this research, we will carry out a cytotoxic test of the ethanol extract of the leaves and bark of the mareme plant against 4T1 breast cancer cells in vitro. We will test the cytotoxicity of the ethanol extract of the leaves and stem bark of the mareme plant using the MTT assay.

RESEARCH METHODOLOGY

Preparation of a Sample

The leaves and bark of the Mareme plant were cleaned of impurities, then washed thoroughly, and then the samples were dried in an oven at a temperature of 50 °C for 3 days or until completely dry. Next, we use a blender to powder the simplicia.

Extraction of Mareme Leaves and Stem Bark

Extraction was carried out using the maceration method, namely soaking the sample for 3x24 hours using a 96% ethanol solvent. The sample was stirred every day. The results obtained were evaporated at a temperature of 70°C for 2 hours until the solvent flowing through the condenser did not drip again. Next, we evaporate the extract in a water bath until we obtain a thick extract. We calculate the weight of the thick extract from the initial weight of the simplicia using the following formula (Rahmawati et al., 2022).

$$\%\text{Rendement} = \frac{Total\ weight\ of\ thick\ extract}{Total\ weight\ of\ simplicia} \times 100\%$$

Phytochemical Tests

The sample extract was placed in a 3-drop test tube, then added with 2 drops of concentrated sulfuric acid solution, and the changes that occurred were observed. If the solution changes color to dark red or yellow, it indicates that it contains flavonoid compounds (Puspa et al., 2017).

Tannin Test

A total of 1 gram of extract was put in a test tube, then added to 10 mL of hot water and boiled for 5 minutes, adding $FeCl_3$ drops. If it is green and black, then it is positive for tannin compounds (Muthmainnah, 2017).

Steroid Test

Put 2 drops of the extract into a test tube, and then add 1-2 drops of glacial acetic acid and a concentrated sulfuric acid solution. If the color changes to purple or violet, this indicates the presence of steroid compounds (Puspa et al., 2017).

Saponin Test

Put 3–7 drops of extract into a test tube, then add 5 mL of water. After shaking the solution for 30 seconds, we left it for several minutes. If the foam is still stable between 1 and 10 cm, this indicates that it contains saponin compounds (Puspa et al., 2017).

Polyphenolic Test

A total of 0.5 g of sample was dissolved in 10 mL of distilled water and heated, then filtered hot. The filtrate was dripped with $FeCl_3$. If it is blackish blue or dark green, it indicates that it contains polyphenolic compounds. (Haryoto and Niati, 2022)

Cytotoxic Test

The cytotoxic test begins with cell harvesting by taking the cells from the incubator and observing the cells under a microscope. Cells can be used if 80% of the cells are confluent, then the growth medium is discarded and the cells are washed by spraying PBS on the flask wall, then 0.5 mL of 0.05% trypsin is added to release the cells. Next, the flask was closed and incubated in a CO incubator for approximately 10 minutes. After that, it was observed under an inverted microscope, and then the flask was opened and filled with around 3–5 mL of DMEM media. Next, the cells were transferred into a sterile conical tube. 10 µL of the suspension was taken, and the number of cells was counted using a hemocytometer. A certain amount of media was added to the suspension until achieving a cell concentration of 1x104 cells per 1 mL.

This cytotoxic activity test uses an MTT assay. We then place the harvested cells in a 96-well plate, with 104 cells per well. Incubated for 24 hours in a CO2 incubator,

then at the end of incubation, the media was removed from each well and new media and 100 µL of sample were added in each different well until the final sample levels were obtained (800, 600, and 500 µg/mL). Furthermore, more plate incubation was carried out in a CO2 incubator for 24 hours at 37°C. At the end of incubation, the medium in each well was discarded and then washed with 100µL of PBS, and then 100µL of 5 mg/mL MTT in PBS was added. Furthermore, we incubated the plate again for 4 hours at 37 °C (CCRC, 2012). MTT (3-(4,5-dimethylthiazol-2il)-2,5-diphenyltetrazolium bromide) reacts with living cells to make dye that is purple. In the reaction, the formation of formazan was stopped with a 10% SDS solution (Sodium Dodecyl Sulfate) in 0.01N HCl, then incubated overnight at room temperature. We used an ELISA reader at a wavelength of 595 nm to read the absorbance at the end of the incubation.

Data Analysis

Absorbance data was obtained using the MTT assay in the form of absorbance from an ELISA reader and then used to calculate the percentage of living cells. Cell viability can be calculated using the following formula:

$$\% \text{ Cell viability} = \frac{Abs.of\ treatment - control\ media\ abs.}{Cell\ control\ abs. - medium\ control\ abs.} \times 100\%$$

RESULT AND DISCUSSION

The determination of Mareme plants (Glochidion arborescens Blume.) was carried out to determine the correctness of the sample by matching the morphological characteristics of the Mareme plant. Apart from that, determination also has the aim of avoiding errors in the samples used and avoiding mixing samples with other plants during collection. The determination of Mareme plants (Glochidion arborescens Blume.) was carried out at the Jatinangor Herbarium, Plant Taxonomy Laboratory, Biology Department, FMIPA, Padjadjaran University with letter number 50/HB/01/2022.

The extraction of Mareme leaves and stem bark resulted in yields of 2.68% and 1.72%, respectively. Variations in yield results can occur due to several things, namely the method used for extraction, storage conditions, sample particle size, extraction time, storage time, and the ratio and type of solvent used. The results obtained by leaf extract have a higher yield than stem bark extract, this is related to the active compounds in the sample. The greater yield obtained indicates the high level of active compounds contained in the extract (Lamadjido et al., 2019). The extract that was obtained was then tested for the bioactive compounds it contained, and a cytotoxic test was carried out against 4T1 cancer cells.

We conducted phytochemical screening to identify the class of secondary metabolite compounds present in the tested samples. Things that need to be considered in this phytochemical test are the selection of the appropriate solvent and the extraction method used.

Based on Table 1, the phytochemical test of the ethanol extract of Mareme stem bark is positive for containing flavonoids, tannins, saponins, and polyphenolics. Furthermore, Mareme stem bark does not contain secondary steroid metabolite compounds. Based on research, Mareme bark was detected to contain flavonoids, which were characterized by their dark red color and greater quantity. The flavonoids contained in the bark of Mareme stems are what make the test samples have anticancer action.

The ethanol extract of Mareme leaves positively contains flavonoids, tannins, steroids, saponins, and polyphenols. The flavonoid test on the leaves and stem bark of mareme was done with the addition of concentrated sulfuric acid (H_2SO_4) to form flavillium salts (the formation of flavonoid compounds), which can be seen from the color change in the solution (Puspa et al., 2017). The results obtained from testing the ethanol extract of mareme leaves and bark showed positive results, namely a change in color to reddish. We conducted a tannin test on mareme leaves and bark by first adding 10 mL

Table 1. Phytochemical of Mareme (Glochidion arborescens Blume.)

Sample	Compounds	Methods	Results	Intensity
Mareme Stem Bark	Flavonoid	Add H_2SO_4 concetrated	Dark red	++
	Tannin	Add $FeCl_3$	Black, green	+
	Steroid	Add acetic anhydride and H_2SO_4 concentrated	No violet ring is formed.	-
	Saponin	Shake for 30 seconds	Stable foam between 1-10 cm	+
	Polyphenolate	Add $FeCl_3$	Blackish blue	+
Mareme Leaves	Flavonoid	H_2SO_4 concentrated	Dark red	++
	Tannin	Plus $FeCl_3$	Black, green	+
	Steroid	Add acetic anhydride and H_2SO_4 concentrated	No violet ring is formed.	+
	Saponin	Shake for 30 seconds	Stable foam between 1-10 cm	+
	Polyphenolate	Plus $FeCl_3$	Blackish blue	+

of hot water, followed by the addition of $FeCl_3$. The purpose of adding $FeCl_3$ is to find out whether the leaves and bark of mareme contain tannins, which are characterized by the formation of a green-black color due to the content of phenolic groups (Muthmainnah, 2017).

The results obtained from testing the ethanol extract of mareme leaves and bark were positive, this was indicated by the formation of a blackish-green color. We carried out the steroid test by adding a solution of glacial acetic acid and concentrated sulfuric acid. This test is based on the compound's ability to form a concentrated sulfuric acid color in an acetic anhydride solvent (Puspa et al., 2017). The results obtained from the steroid test on Mareme leaves were positive, as indicated by the formation of violet rings, while the results on Mareme stem bark showed negative results. The saponin test on mareme leaves and bark was carried out using the fourth method, namely the formation of stable foam after shaking for a few seconds. For this test, it was positive for saponin, as indicated by stable foam with a height of 1 cm for the leaves and bark of Mareme. The formation and stability of this foam are probably due to the hydrolysis of glycosides into glucose and other compounds in water (Puspa et al., 2017). Test the polyphenols in the leaves and bark of mareme by dissolving heated distilled water and dropping the filtrate formed with $FeCl_3$. The results of this test are positive, indicated by the formation of a blackish blue or dark green color (Ambarsari & Haryoto, 2022).

Previous research stated that the phytochemical screening test of Mareme extract (Glochidion arborescens Blume.) is positive for containing flavonoids, saponins, steroids, terpenoids, tannins, and polyphenolics (Ambarsari & Haryoto, 2022). Compounds that play a role in anticancer effects are flavonoid compounds. This compound modulates the activity of reactive oxygen species enzymes that participate in the cell cycle, induces of apoptosis and autophagy, and suppresses the proliferation and invasion of cancer cells (Kopustinskiene et al., 2020).

The method used to test the cytotoxic activity of the ethanol extract of mareme leaves and bark (Glochidion arborescens) was the MTT method. The yellow MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced by the reductase system. This is how the MTT method works. The reaction between tetrazolium salts found in the mitochondria of

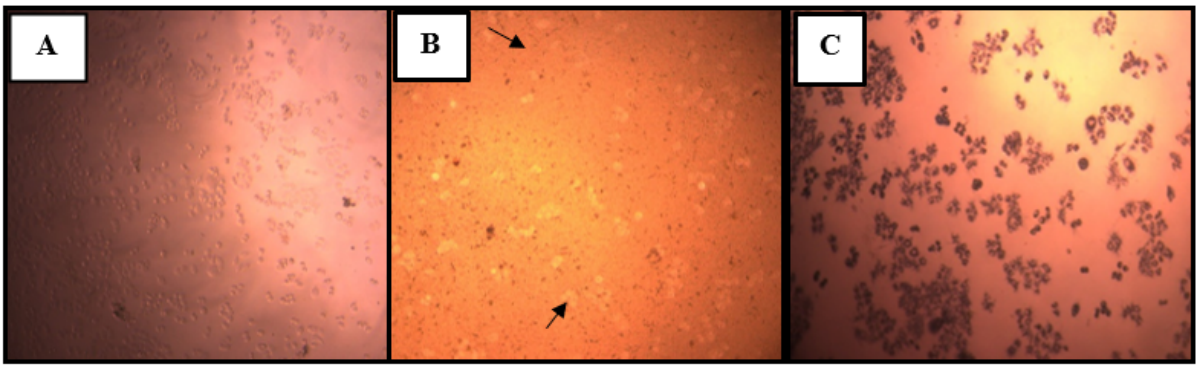

Figure 1. Morphology of 4T1 cells before treatment (A) 4T1 cells after being treated with stem bark extract (B) 4T1 cells after being treated with MTT solution (C)

living cells forms purple formazan crystals (CCRC, 2014). This causes living cancer cells to form more formazan crystals than dead cancer cells (Haryoto et al., 2013).

We add 10% SDS in 0.01N HCl to these formazan crystals, which have alkaline properties and are insoluble in water, to dissolve the salt. HCl has the function of stopping enzymatic reactions and changing the color of phenol in the media to yellow, so that there is no interference during the reading process. Adding the stopper reagent will dissolve the purple crystals, and then measure the absorbance using an ELISA reader. The intensity of the purple color in the MTT test is proportional to the number of living cells. If the intensity of the purple color increases, the number of living cells will increase (CCRC, 2014). The media used is DMEM containing penicillin-streptomycin as an antibiotic to prevent contamination. We can see the results of the cytotoxic test using the MTT assay method on 4T1 cells in Figure 1.

Based on the Figure 1, 4T1 cells have an oval-shaped morphology with clearly visible borders between cells and cluster together to form colonies. Visually, 4T1 cells before being given extract treatment did not experience death as indicated by the formation of formazan salts by the MTT reagent. Meanwhile, 4T1 cells that had been treated with the sample experienced cell death which was characterized by changes in cell size to smaller ones and irregular round shapes with the boundaries between cells not being clearly visible.

Based on the Table 2, percent living cells ethanol extract of Mareme stem bark (Glochidion arborescens Blume.) at concentrations of 500, 600, and 800 µg/mL with average yield of live cells was 59.56, 56.37, and 42.85%. This live cell value shows the percentage of live cells that have been treated. The higher the concentration of mareme bark extract, the lower the live cell yield. Then, to see the distribution of data in the sample, the standard deviation value is

Table 2. Percent of Living Cells Ethanol Extract of Mareme (Glochidion arborescens Blume.)

Sample	Concentration (µg/mL)	Concentration log	Mean of %living cells	SD
Mareme Stem Bark	500	2,90	59,56	14,5
	600	2,84	56,37	10,3
	800	2,69	42,85	18,8
Mareme Leaves	500	2,90	114,21	33,4
	600	2,84	106,21	16,5
	800	2,69	78,91	23,1

used. A good standard deviation value is indicated by results that are smaller than the average of living cells. A large standard deviation value indicates a variety of data variations and describes the data obtained as inaccurate. The percent living cells of the ethanol extract of Mareme leaves (Glochidion arborescens Blume.) at concentrations of 500, 600, and 800 µg/mL obtained an average yield of live cells of 114.21, 106.21, and 78,91% with a standard deviation of 33.4, 16.5. and 23.1. The standard deviation shows results that are smaller than the average percentage of living cells, this shows that the data does not vary and shows the accuracy of the data.

The cause of the differences in data results obtained was sampling so that there were variations in compound content, in addition to the solubility of the ethanol extract in Mareme leaves and stem bark. The Mareme stem bark extract test sample was more difficult to dissolve than the Mareme leaf extract test sample even though vortexing had been carried out for 30 minutes to dissolve the Mareme stem bark sample. The solubility factor of the test sample in the media is important because as one of the requirements for the sample to be tested for cell culture, the solubility of the test sample is assisted by cosolvent DMSO.

The compound content that has cytotoxic activity in the leaves and bark of Mareme is flavonoids. Flavonoids are phenolic compounds that have anticancer activity (Kusumawardani et al., 2020). Examples of flavonoid compounds that have anticancer activity are persicogenin, artemetin, luteolin, penduletin, vitexicarpin, and chrysosplenol-D, where these flavonoid compounds can inhibit the proliferation of cancer cells by inhibiting the cell cycle (Nuraini et al., 2015). Based on previous research on plants Mareme contains flavonoids from the flavone group (Haryoto and Niati, 2022). Previous research also proves that cancer cells can be controlled by flavonoid compounds (Jenie et al, 2019). This is because flavonoid compounds can have a specific effect on cancer cells without affecting normal cells. Previous cytotoxic tests stated that flavonoid compounds have the ability to regulate macrophage function in destroying cancer cells and play a role in inhibiting cell proliferation (Feng et al., 2016) and (Zhou et al., 2019).

CONCLUSIONS

Based on the results and discussion, it is known that the ethanol extract of mareme leaves (Glochidion arborescens Blume.) contains flavonoid compounds, tannins, steroids, saponins, and polyphenols, while the ethanolic extract of mareme stem bark contains flavonoids, tannins, saponins, and polyphenols. Cytotoxic test results of ethanol extract of mareme leaves (Glochidion arborescens Blume.) measured at concentrations of 500, 600, and 800 µg/mL, had an average percent of live cells of 59.56; 56.37, and 42.85%. Meanwhile, the ethanol extract of mareme bark (Glochidion arborescens Blume.) has an average percent of living cells of 114.21, 106.21, and 78.91%.

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