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Phytochemical Screening and Cytotoxicity Test of Senduduk (*Melastoma malabathricum* L.) Leaf Ethanol Extract on MCF-7 Cells Using MTT Assay Method

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Abstract:
background: Indonesia is one of the countries with a wealth of flora in the world, has a variety of plants that have the potential for cancer treatment, one of which is the Senduduk Leaf plant (Melastoma malabathricum) or MM. Previous researchers reported that the methanol extract from the leaves showed significant anticancer activity against the MCF-7 cell line with an IC50 of 7.14 g/ml. This research is a follow-up analysis of previous research which aims to examine cytotoxic activity and identify plant chemical compounds used for traditional cancer treatment in Indonesia.
Materials and Methods: The materials used in this study were MM leaf simplicia, 96% ethanol, test tube reagents, MCF-7 cells, RPMI culture media, DMSO, MTT reagents, PBS, SDS. The extraction method used was maceration, phytochemical screening analysis using test tube reagents and TLC profile confirmation along with cytotoxic activity testing using the MTT Assay method.
Results: Results of phytochemical screening of MM leaf ethanol extract contained flavonoids and tannins. The results of the cytotoxic test of the ethanol extract of MM leaves had an average IC_{50} value of 36.056 µg/mL. As well as the IC_{50} value of the positive control doxorubicin of 11.282 µg/mL. The results of statistical analysis carried out on the IC_{50} value found that the ethanol extract of MM leaves and doxorubicin had statistically significant differences with a p-value <0.05.
Conclusion: The ethanol extract of MM leaves positively contained flavonoids, tannins and steroids and was able to inhibit the growth of MCF-7 cells with an average IC50 value of 36.056 µg/mL.
Keyword: Melastoma malabathricum, Cytotoxic, MCF-7 Cells, MTT Assay.

I. Introduction

In 2020, there were 2.3 million women diagnosed with breast cancer and 685,000 deaths globally. As of the end of 2020, there were 7.8 million living women diagnosed with breast cancer in the last 5 years, making it the most common cancer in the world. This condition is predicted to get worse, IARC estimates that by 2040 the incidence of breast cancer will increase by more than a third, to more than 3 million new cases per year, and deaths from breast cancer will increase by more than half, to more than 1 million deaths per year. year [1].

Cancer drug resistance continues to be a major hurdle in medical oncology. Clinically, resistance can arise before or as a result of cancer therapy. Until now, one of the therapies for treating cancer is using chemotherapeutic agents, various mechanisms adapted by cancer cells to resist treatment, including changes in drug transport and metabolism, mutation and target amplification drugs, as well as genetic rewiring that can lead to impaired apoptosis [2].

The use of plants as traditional medicine is related to the content of secondary metabolites and their bioactivity. Indonesia is one of the countries with a wealth of flora in the world, has a variety of plants that have the potential to treat cancer, one of which is the Senduduk Leaf plant (*Melastoma malabathricum*) or MM. Anti-cancer effect MM has significant anticancer activity against the MCF-7 cell line. Previous researchers reported that the methanol extract from the leaves showed significant anticancer activity against the MCF-7 cell line with an IC_{50} of 7.14 g/ml, while the methanol and chloroform extracts from the flowers showed moderate activity against the MCF-7 cell line with an IC50 of 33.63 g/mL. and 45.76 g/mL after 72 hours of treatment respectively (Roslen, et al 2014).

According to Idris 2017 revealed that the MTT Test Senduduk Leaf extract had IC50 > 400 g/ml in both cell lines at 24 hours after treatment. Flow cytometric analysis and fluorescence microscopy of Annexin-V/PI-stained Senduduk Leaf cells revealed that most of the cells had secondary necrosis/late apoptosis. The TUNEL assay showed that little or no DNA nicks were present in the MM-treated cells, indicating that the cells had undergone secondary necrosis, not late apoptosis, at that time point [3].

The bioactive components in Senduduk leaves which are thought to have functional benefits are flavonoids, ursolic acid, 2-hydroxyursolic acid, asiatic acid, gallic acid, p - hydroxy benzoic acid, kaempferol, kaempferol-3-O-(2″,6″ di-O - p - trans -coumaroyl)- -glucoside, -amyrin, uvaol, quercetin, quercitrin, caffeic acid, chlorogenic acid, p - coumaric acid, gallocatechin, epigallocatechin, catechin, quercetin, quercetin-3- O - glucoside, and hesperidin [4]. There are at least four mechanisms of an active substance component to fight cancer: anti-proliferative activity (preventing or slowing the spread of cancer cells, inhibition of angiogenesis (formation of new blood vessels), induction of apoptosis (cancer cells commit suicide), prevention of metastasis [5] .

Broadly speaking, research on the scientific evidence of Senduduk leaves has been published a lot and the benefits of Senduduk leaves have been explored a lot, but no one has identified the chemical compounds contained in MM leaves. Based on this description, it is necessary to conduct a screening of chemical phytochemicals in Senduduk leaves which can kill and suppress the growth of cancer cells, especially in MCF-7 breast cancer cells.

II. Materials And Methods

The equipment used in this study were conical tubes, analytical balance, centrifuge, vortex, incubator 37 ºC 5% CO_2, refrigerator, laminar air flow class II, hemocytometer, object glass, deck glass, inverted microscope, micropipette, Elisa Reader. The tools used for extraction and fractionation include maceration vessels, filter paper, glassware, refrigerators, vacuums, Buchner funnels, rotary evaporators.

The materials used in this study were fresh senduduk leaves (*Melastoma Malabathricum*) obtained from the Pedamaran area of South Sumatra. 96-wellplate, tissue culture flask, 6 cm dish, falcon flask, MCF-7 cells, Culture medium consisting of RPMI 1640 Gibco Life Technologies with phenol red and 2 mM glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin , 1 mM sodium pyruvate, and 10% Fetal Bovine Serum supplement (FBS, Gibco Life Technologies) which had been inactivated by heating at 56°C for 30 minutes. DMSO; MTT (3-(4, 5–dimethylthiazol–2)-2,5- diphenyl-tetrazolium bromide); sodium dodecyl sulphate (SDS, Sigma).

Procedure methodology

Preparation of leaf extract *Melastoma Malabatricum*

The ethanol extract of Melastoma Malabathricum leaves was prepared using the maceration method by weighing 1 kg of dry simplicia powder and then extracting it. Maceration was carried out with 96% ethanol in a container protected from light for 2 days while occasionally stirring every 12 hours. Then the macerate obtained is filtered through a remaceration process with the remaining ethanol solvent until the color of the ethanol solvent is clear which indicates that the solvent is no longer able to attract the compounds contained in the simplicia. The macerate obtained was separated using filter paper and remaceration was carried out 2 times with the same solvent.

Reagent Manufacturing

Mayer's Reagent Solution is 1.36 g of HgCl2 is dissolved in 60 ml of distilled water. 5 gr KI in 10 ml of distilled water. These two solutions were then mixed and diluted with distilled water up to 100 ml. Dregendrof reagent solution is 8 grams of KI dissolved in 20 ml of distilled water and 0.85 grams of bismuth subnitrate. Wagner's Reagent Solution A total of 1.27 grams of iodine and 2 grams of KI dissolved in 5 ml of distilled water. This solution was diluted to 100 ml with distilled water. The precipitate formed was filtered and stored in a brown bottle. Preparation of Lieberman Burchard reagent is 5 mL of anhydrous acetic acid is mixed with concentrated H2SO4 while cooling, the mixture is added to 50 mL of ethanol in a cold state. Preparation of 1% HCL is Pipette 2.7 ml of 1% HCL into a 100 ml volumetric flask, then add distilled water to the mark. Preparation FeCl2 1% is $FeCl_2$ 1% gram is put into a 100 ml volumetric flask and then dissolved with 100 ml of distilled water or up to the mark.

Phytochemical Screening Analysis

Alkaloid Test take 0.5 grams of extract, add 1% HCL then filter. The filtrate was divided into two parts and tested using Mayer's and Dragendorf's reagents. Alkaloid compounds are characterized by the presence of a yellow precipitate with Mayer reagent. An orange precipitate was formed with the addition of dragendorf reagent which showed positive for alkaloids. Triterpenoid Test put 0.5 grams of extract in a test tube, add 1 ml of glacial CH3COOH and 1 ml of concentrated H2SO4 solution. The color change to red indicates the presence of terpenoid compounds.

Flavonoid Test a total of 0.5 grams of extract is put in a test tube, added with one gram of Mg powder, and concentrated HCL solution. A change in the color of the solution to pink/orange indicates the presence of flavonoids. Saponin test a total of 0.5 grams of extract is put in a test tube and 10 ml of hot water is added, cooled and then shaken vigorously for 10 seconds (if the substance being examined is in the form of a liquid preparation, 1 ml of the sample being examined is diluted with 10 ml of water and shaken vigorously - strong for 10 minutes). A positive reaction the foam is formed Test Steroids a total of 0.5 grams of extract was placed on the drip plate and CH3COOH was added until the sample was completely submerged, left for about 15 minutes, put six drops of the solution into a test tube and added 2-3 drops of H_2SO_4 to form a blue color indicating the presence of steroids. Tannin Test put 0.5 gram of extract in a test tube, add 2 ml of 96% ethanol, stir, and add 3 drops of 1% $FeCl_3$. The formation of a characteristic blue, blue-black, green or blue-green color.

Thin Layer Chromatography (TLC) Analysis

The stationary phase used in this screening was silica gel F254 measuring 10 x 10 cm^2 while the mobile phase and stain remover were used as follows: Identification of Flavonoid Compounds, Mobile phase : n-Butanol : acetic acid : water (4:1:5). Identification of Saponin Compounds, Mobile phase : Chloroform : Methanol : Water (1:7:2). Identification of tannin compounds, Mobile phase : n-Butanol : acetic acid : water (4:1:5)[7].

Cytotoxic testing using the MTT Assay

Cytotoxic testing included cytotoxic testing of extracts, fractions, subfractions, and isolates of Senduduk leaves against MCF-7 cells. Cells were grown on a 96-well microplate to obtain a density of 1x104 cells/well and incubated for 24 hours to obtain good growth. After that, the medium was replaced with a new one, then various concentrations of test solutions were added with co-solvent DMSO (Sigma) and incubated at 37°C in a 5% CO^2 incubator for 24 hours. At the end of incubation, the media was discarded and the cells were washed with PBS (Sigma). In each well, 100 µL culture medium and 10 µL MTT (Sigma) 5 mg/mL were added. cells were again incubated for 4-6 hours in a 5% CO^2 incubator at 37°C. The MTT reaction was stopped with stopper reagent (10% SDS in 0.01 N HCL), the wrapped plate was left overnight.[8].

statistical analysis

Data on the results of the MCF-7 cell growth inhibition test in the form of percentages shown at several concentrations were analyzed using the T-Test with the SPSS program, the mean test technique with t distribution at the 95% confidence level.

III. Results

1. Determination Results of *Melastoma Malabathricum* leaf plants

   The results of the determination of the Tembelekan plant show that the sample used is the true *Melastoma Malabathricum* root with the following determination key:

   Determination Key:

   1b-2a (Goal 10. Single leaf plants opposite each other)-239b-243b-244a-245b-246b-247b (Fam 95. Melastomaceae)-1b-4b-5b (Genus Melastoma) species: *Melastoma malabathricum* L.
2. The results of the manufacture of *Melastoma Malabatricum* leaf extract

   The results of extracting *Melastoma malabathricum* leaves can be seen in Table 1.

Table 1. Extraction Results of *Melastoma malabathricum* Leaves

Powder Weight	Extract Weight	yield	Characteristics		
			Form	Color	Smell
1000 grams	260.84 grams	26.08 %	Typical Dark Chocolate Thickness		

Based on the results of the extraction that was carried out, it was obtained that the ethanol extract of *Melastoma malabathricum* leaves was as much as 260.84 grams so the percent yield was 26.08%.

3. Phytochemical Profile Results

   A preliminary test was conducted to determine the chemical content of the ethanol extract of MM (*Melastoma malabathricum* L) leaves. The inspection results can be seen in Table 2.

Table 2. Phytochemical Profile of MM Leaf Ethanol Extract

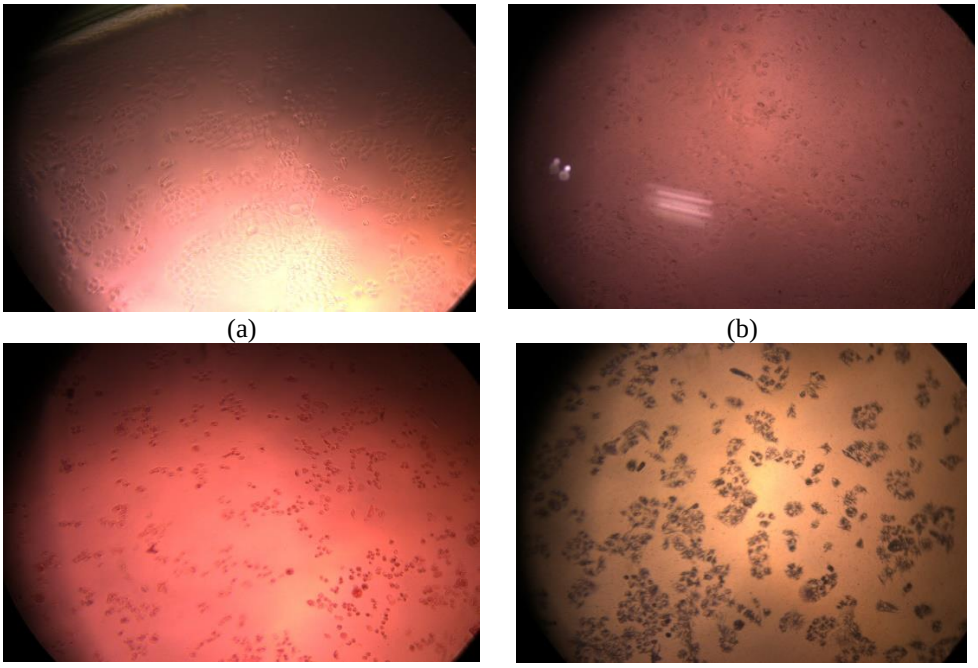
No	Compound	Reactor	Literature	Observation	Result
1	Flavonoids	Mg + concentrated HCL	Red/Orange	Red/Orange	(+)
2	tannins	FeCl3	Blackish blue	Blackish blue	(+)
3	Saponins	H2O Shake vigorously	Formed foam	Formed foam	(+)

4. Thin Layer Chromatography Confirmation Test Results
   The results of the confirmatory test of the ethanol extract of MM leaves (*Melastoma malabathricum* L) using the thin layer chromatography method can be seen in Table 3.

Table 3. Affirmation Test Results

No	Compound	Mobile Phase	Comparison Standard	Solvent mileage	The distance traveled by the stain	sample rf	Rf standard comparator	Result
1	Flavonoids	BAA	Quercetin	10cm	9.5cm	0.95cm	0.93cm	(+)
2	tannins	BAA	Catechins	10cm	9.5cm	0.90cm	0.90cm	(+)
3	Saponins	KMA	Sapogenins	10cm	9.5cm	0.95cm	0.95cm	(+)

5. Cytotoxic Test Results using the MTT Assay method
   Cytotoxicity test is a standard screening procedure to assess the cytotoxicity of a drug substance. One of the cytotoxicity test methods that is often used is the enzymatic test using 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) reagent.
   Observations under an inverted microscope can be seen in Figure 1.



(a) (b) (c) (d)

Figure 1. Observations under an inverted microscope (a) Live Cell Control MCF-7, (b) MM leaf extract concentration of 50 ppm, (c) Doxorubicin concentration of 50 ppm, (d) Formazan Crystals

IC_{50} data calculation results can be seen in Table 2.

Table 4.IC_{50} Data of MM Leaf Extract and Doxorubicin

No.	Sample	IC50 (ppm)	Mean ± SD
1	MM Ethanol Extract	35,285 36,247 36,637	36.056 ± 0.695
2	Doxorubicin	9,613 12,718 11.515	11.282 ± 1.566

Based on Table 2, it can be seen that the ethanol extract sample of MM leaves has cytotoxic activity with an average IC_{50} value of 36.056 µg/ml and for doxorubicin positive control of 11.282 µg/ml. Based on the literature, IC_{50} is said to be potent if the concentration is <50 µg/ml [9]. So it can be said that the ethanol extract of MM leaves can be said to be potent. The results of extract concentrations and mean MCF-7 cell viability can be seen in Figure 2.

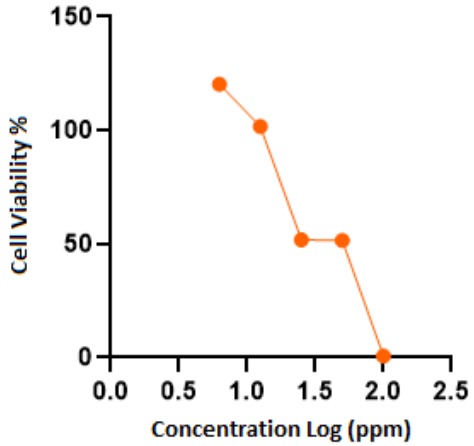


Figure 2. Graph of Relationship of Extract Concentration and Average Viability of MCF-7 Cells from 3 Replications

The results of the comparison of % live cells vs. the concentrations of the ethanol extract of MM leaves and doxorubicin can be seen in Figure 3.

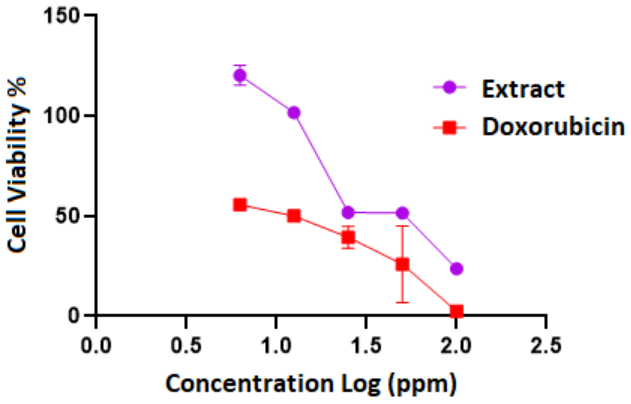


Figure 3.% Live Cells vs. Log Sample concentration MM leaf extract and Doxorubicin

IV. Discussion

1. Plant Determination
   Description:
   Senduduk has the form of a bush or small tree growing up to 5 m high. The stem is reddish, covered with small scales. The foliage is opposite, petiole has leaf blades that are lance-shaped, 2–15 by 0.6–6.5 cm, and bears three prominent veins – one in the center and two on the edges. The leaves are rough on the underside. The flowers are up to 8 cm wide, with petals that are light to dark magenta pink, or occasionally white. The 6–10 mm wide fruit are slightly globose, and open irregularly when ripe to reveal a dark blue flesh with numerous orange seeds. The fruit is edible but somewhat tasteless, with flesh that stains the tongue blue-black [10].
2. MM leaf extract
   Making a viscous extract from senduduk plant using the maceration method used is a fresh sample of senduduk leaf (*Melastoma malabathricum*) which has been dried as much as 1000 grams, the solvent used is 96% ethanol. The extraction process uses the maceration method starting from the wet sorting of *Melastoma malabathricum* leaves which aims to separate the dirt that is still attached, then the drying and chopping process is carried out. The senduduk leaf sample is chopped before maceration aims to expand the surface of the sample so that the contact between the sample and the ethanol solvent is wider so as to speed up the process of dissolving the compounds contained in the sample into the solvent used [11].
3. Phytochemical Profile
   Flavonoid test using magnesium and HCL reagent. The use of concentrated HCL is used to hydrolyze flavonoid compounds into their aglycones, namely by hydrolyzing O-glycosyl. Glycosyl will be replaced by H+ from hydrochloric acid because it is electrolytic. The results of reduction with Mg and concentrated HCL can produce complex compounds that are red or orange in flavonoids, flavonones, flavononols, and xanthone [12]. This shows that the senduduk flower extract (*Melastoma malabathrium* L) shows the presence of flavonoid compounds due to the red/orange color change.
4. Thin Layer Chromatography Assertion Test
   Based on the thin layer chromatography confirmatory test using the mobile phase n-butanol:acetic acid:water (BAA), as well as viewed under UV light with a wavelength of 366 nm and the Rf value of the ethanol extract of MM leaves (*Melastoma malabathrium* L) was 0.95 and using quercetin as a reference standard obtained from research results of Rf of 0.93. So the Rf results obtained between the reference standard and the sample have almost the same value so that the results are said to have the same characteristics and are positive for containing flavonoids.
   The ethanol extract of MM leaves (*melastoma malabathrium* L) produces a greenish-brown or blackish-blue color which indicates a positive tannin content. Then the extract was continued with a thin layer chromatography (TLC) confirmation test using the mobile phase n-butanol:acetic acid:water (BAA), as well as UV light with a wavelength of 366 nm and the results obtained were the ethanol extract of MM leaves (*melastoma malabathrium* L) Rf of 0 .90 while the standard for the comparison of tannins used catechins and it was found from research that the Rf value was 0.90. The high Rf value is influenced by the polarity of the solvent where the polarity of the mobile phase is more polar than the stationary phase so that the tannins that are separated are lifted up following the flow of the eluent, because the tannins are polar.
5. Cytotoxic Testing Using the MTT Assay
   The basis of the MTT enzymatic test is to measure the ability of living cells based on mitochondrial activity from cell culture. This test is widely used to quantitatively measure cellular proliferation or to measure the number of living cells [14].
   Cytotoxic assays were made for extract concentration series by dilution using culture media of 100, 50, 25, 12.5, and 6.25 µg/mL. The in vitro cytotoxic assay was performed with the reagent [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium-bromide] (MTT) assay. MCF-7 cells were distributed into 96 well plates and incubated for 48 hours in DMEM culture medium. Cells were treated with MM leaf extract and doxorubicin positive control with concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL and incubated for 48 hours. At the end of the incubation, 100 µL of DMEM culture medium containing MTT 5 mg/mL was added to each well, and incubated for 3 hours at 37oC. Live cells will react with MTT to form purple formazan crystals. The crystals were dissolved by adding 10% sodium dodecyl sulfate (SDS) stopper reagent in 0.01N HCl, left in a dark place overnight, then read the absorbance with an Enzyme-linked immunosorbent assay (ELISA) reader at a wavelength of 595 nm. The results of the cytotoxicity test are in the form of absorbance data which is then converted to % live cells and then the IC50 value. This IC50 value is an illustration of the cytotoxic effect of the compound given by the test compound, namely the level that can inhibit cells by 50% [15].

MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in the yellow cytotoxic test will be reduced to formazan salt which is purple in color by the enzyme succinate dehydrogenase which is present in the mitochondria of living cells, the reaction allowed to occur for 4 hours. A longer incubation time will result in an increase in color intensity and an increase in sensitivity up to a certain point. However, the incubation time is limited because the nature of the cytotoxic detection reagent requires energy (equivalent to reduction of NADH) from the cell to generate a signal. In cell populations in log phase growth, the amount of formazan product is generally proportional to the number of metabolically viable cells. However, certain conditions can change cell metabolism which will likely affect the rate of reduction of MTT to formazan. For example, when cells are in a confluent state so that cell growth becomes inhibited, thus cell metabolism becomes slow and the reduction in MTT will be low. This situation will cause a loss of linearity between the absorbance and the number of cells [16]. Dead cells will lose the ability to convert MTT into formazan, so the color change that occurs serves as a marker that the cells are still alive [17].

Based on the graphical results of the relationship between the concentration of MM leaf ethanol extract and the average MCF-7 cell viability, the higher the concentration of MM leaf ethanol extract, the lower the % cell viability. It can be assumed that the extract is able to inhibit cell growth as the concentration of the extract increases. In a previous study, the ethanol extract of the herb child had moderate strength cytotoxic activity on MCF-7 cells with an IC50 value of >400 µg/mL [3]. This shows that the sensitivity of growth inhibition of each cancer cell is different. A compound that has a moderate category of cytotoxic activity can be used as a chemoprevention agent, which is able to prevent and inhibit the growth of cancer cells [18].

Based on Figure 3 it can be seen that the % live cells of doxorubicin is lower than the ethanol extract sample of MM leaves. This is also indicated by the IC50 value obtained above. Thus it can be seen that doxorubicin has higher cytotoxic activity compared to the ethanol extract of MM leaves in inhibiting the growth of MCF-7 breast cancer cells. The graph above also shows the number of % living cells depending on the dose of the given compound (dose-dependent phenomenon). The higher the concentration (dose) of the compound, the fewer surviving cancer cells [19].

Based on the data obtained, data analysis was carried out using the T-Test to determine differences between groups in the average activity of the ethanol extract of MM leaves and doxorubicin indicating that the two groups had homogeneous values marked with a significance value of >0.05. For the T-Test test, if the significance value is <0.05, the mean value between groups has a significant difference, so it can be concluded that the two groups have a significant difference.

V. Conclusion

The ethanol extract of MM leaves has cytotoxic activity with an average IC_{50} value of 36.056 µg/mL. As well as the IC_{50} value of the positive control doxorubicin of 11.282 µg/mL. The results of the phytochemical screening of the ethanol extract of MM leaves positively contained flavonoids, tannins and steroids. The results of statistical analysis carried out on the IC_{50} value found that the ethanol extract of MM leaves and doxorubicin had statistically significant differences with a p-value <0.05.

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Tri Oktarina, et. al. "Phytochemical Screening and Cytotoxicity Test of Senduduk (Melastoma malabathricum L.) Leaf Ethanol Extract on MCF-7 Cells Using MTT Assay Method." *IOSR Journal of Pharmacy (IOSRPHR)*, 13(06), 2023, pp. 01-08.