

## **CYTOTOXICITY TEST OF N-HEXANE, ETHYL ACETATE, AND WATER FRACTIONS FROM THE ETHANOL EXTRACT OF SENDUDUK LEAVES (*Melastoma malabathricum* L.) ON MCF-7 CELLS USING THE MTT ASSAY METHOD**

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### **ABSTRACT**

*The world's richest flora may be found in Indonesia, where a variety of plants, such as the sesame plant (*Melastoma malabathricum* L) or MM, may be used to treat cancer. According to earlier studies, the methanol extract of the leaves had a substantial anticancer effect on the MCF-7 cell line, with an  $IC_{50}$  of 7.14  $\mu\text{g/mL}$ . The purpose of this study was to evaluate the cytotoxic potential of the water, ethyl acetate, and n-hexane fractions of senduduk leaf ethanol extracts. Maceration was the extraction technique, partitioning (liquid-liquid extraction) was the fractionation technique, and the MTT method was employed to assess cytotoxic activity. The n-hexane, ethyl acetate, and water fractions showed cytotoxic effects with  $IC_{50}$  values of 137.200  $\mu\text{g/mL}$ , 17.108  $\mu\text{g/mL}$ , and 610.863  $\mu\text{g/mL}$ , respectively. The water and n-hexane fractions were classified as fragile, based on the results of the cytotoxicity test. The ethyl acetate fraction, on the other hand, was added to the active category for suppressing MCF-7 cells.*

**Keywords:** *Melastoma malabathricum L, Cytotoxic, Cells, MCF-7, MTT assay*

### **INTRODUCTION**

Globally, there were 685,000 breast cancer-related deaths and 2.3 million new cases of breast cancer in women in 2020. Breast cancer is the most common disease worldwide, with 7.8 million people living with a diagnosis as of the end of 2020. According to the International Agency for Research on Cancer (IARC), things will worsen by 2040. According to the organization's predictions, there will be a more than 25% increase in the incidence of breast cancer, amounting to over 3 million new cases annually, and a more than 50% increase in breast cancer mortality, amounting to over 1 million deaths annually. (WHO, 2021).

Drug resistance in cancer remains a major challenge in medical oncology. Clinically, resistance may develop in response to cancer treatment. Chemotherapy drugs have been used for cancer treatment. Cancer cells have developed several resistance mechanisms to chemotherapy, such as altered drug metabolism and transport, mutations, and drug target amplification (Zahreddine, 2013).

The bioactivity and concentration of the secondary metabolites found in plants are linked to their traditional use in medicine. The senduduk plant (*Melastoma malabathricum* L), also known as MM, is one of the many plants that may be used to treat cancer. Indonesia has the richest flora worldwide. MM leaves significantly inhibited the ability of MCF-7 cells to spread malignancy. The leaf methanol extract demonstrated significant anticancer activity against the MCF-7 cell line, according to previous research, with an  $IC_{50}$  of 7.14  $\mu\text{g/mL}$ . On the other hand, after being exposed to the MCF-7 cell line for 72 hours, the methanol and

chloroform extracts of the flowers demonstrated moderate activity, with IC<sub>50</sub> values of 33.63 µg/mL and 45.76 µg/mL, respectively (Roslen *et al.*, 2014).

According to Idris (2017), 24 hours after exposure, senduduk leaf extract had the IC<sub>50</sub> of the cell lines MCF-7 and A549 that was greater than 400 µg/mL in the MTT assay. When Annexin V/PI-stained MCF-7 cells were analyzed using cytometric and fluorescence microscopy, the majority of the cells exhibited secondary necrosis or late apoptosis. The TUNEL assay revealed that cells treated with the MM extract exhibited little to no DNA nicks, suggesting that the cells had experienced secondary necrosis rather than late apoptosis.

Senduduk leaves contain bioactive substances such as flavonoids, ursolic acid, 2-hydroxy ursolic acid, aspartic acid, gallic acid, p-hydroxy benzoic acid, kaempferol, and kaempferol-3-O-(2,6-di-O-p-transcoumaroyl). These substances are considered to exert functional effects. Among these compounds are gallocatechin, epigallocatechin, catechin, quercetin, quercetin-3-O-glucoside, uvaol, α-amyrin, caffeic acid, chlorogenic acid, p-coumaric acid, and hesperidin. The constituents of the material proactively combat cancer via four methods:

- Anti-proliferation: a strategy to stop or limit the growth of cancer cells
- Inhibition of angiogenesis, which stops new blood vessels from growing
- Apoptosis induction (keeping cancer cells from taking their own lives)
- Avoiding metastasis (Salleh *et al.*, 2017)

Scientific evidence for senduduk leaves has been extensively studied, and the advantages of senduduk leaves have been investigated. Even then, the majority are still unrefined extracts that have not yet been separated. This indicates that cytotoxicity tests and fractionation of the senduduk leaf extract are required to kill and inhibit the growth of cancer cells, particularly MCF-7 breast cancer cells.

## RESEARCH METHODS

### Equipment and Materials

The apparatus used in this study included a vacuum, Buchner funnel, rotary evaporator, hemocytometer, laminar air flow class II, 37 °C 5% CO<sub>2</sub> incubator, and an inverted microscope.

Fresh senduduk leaves (*Melastoma malabathricum* L) from the Pedamaran area of South Sumatra, a 96-well plate, tissue culture flask, 6 cm dish, falcon flask, MCF-7 cells, and culture media made of RPMI 1640 (Gibco Life Technologies) with phenol red and two mM glutamine, one mM sodium pyruvate, as well as 10% Fetal Bovine Serum/FBS supplement (Gibco Life Technologies), were heated at 56°C for 30 minutes to inactivate it, DMSO, MTT (3-(4,5-dimethylthiazol-2)-2,5-diphenyl tetrazolium bromide), and sodium dodecyl sulfate/SDS (Sigma).

### Research Procedure

#### 1. Extraction

Maceration was used as an extraction method. The powdered leaves weighed 1 kg. Next, the leaves were extracted with 3,250 mL of 96% ethanol solvent. The mixture was kept under direct light for two days, with stirring every 12 hours. After filtering the resulting macerate, 1,750 mL of ethanol was used to perform the remaceration procedure. A Buchner funnel with filter paper lining was used to separate the resulting mass, and a vacuum procedure was then applied. To create a thick ethanol extract, the resulting group was collected, placed in a water bath below 65°C, and evaporated from the filtrate of the senduduk leaf extract using a rotary evaporator set at 78°C.

#### 2. Fractionation

The liquid-liquid method was used in the fractionation process. One hundred thirty-five grams of thick extract from senduduk leaves was suspended at a ratio of 1:9 v/v (20 mL:180 mL) of water to methanol for each fractionation (four fractionations totaling 135 grams of thick extract were conducted in this study). Three partitioning processes using

200 mL of n-hexane were then performed, with each separation yielding the n-hexane fraction and methanol-water phase. Evaporation was performed in the methanol-water step to remove the methanol. To obtain the water and ethyl acetate fractions, the filtrate was suspended in water, partitioned again using the same volume of ethyl acetate (200 mL), separated, and repeated three times.

### 3. Cytotoxic Test

The MTT assay was used for cytotoxic testing, and dilutions of RPMI culture media at concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL were used to create a sample concentration series. The n-hexane, ethyl acetate, and water fractions of the senduduk leaves were tested for cytotoxicity against MCF-7 cells. For optimal growth, the cells ( $1 \times 10^4$  cells/well) were seeded in 96-well microplates and incubated for 24 hours. Subsequently, fresh RPMI medium was added and the test solutions at different concentrations were mixed with a DMSO co-solvent. The mixture was then incubated for the whole day at 37 °C in an incubator with 5% CO<sub>2</sub>. Following the incubation period, the medium was disposed of and the cells were washed with PBS. 100 µL of culture media and 10 µL and 5 mg/mL MTT were applied to each well. The cells were then re-incubated at 37 °C for 4–6 hours in an incubator with 5% CO<sub>2</sub>. The plate was covered and left overnight using a stopper reagent (10% SDS in 0.01 N HCl) to halt the MTT reaction. The absorbance was measured using an ELISA reader at a wavelength of 595 nm.

### Data Analysis

Data analysis was performed using a one-way analysis ANOVA. Differences in results between test groups for each IC<sub>50</sub> measurement can be determined through data analysis using MANOVA-LSD with a confidence level of 95% using the SPSS version 25 application.

## RESULTS AND DISCUSSION

### Results

#### 1. Result of Extract and Fraction

*Melastoma malabathricum* leaf thick ethanol extract yielded 260.84 grams of the product, or 26.08% of the total (**Table I**). The extract results met the requirements for a satisfactory yield, which included a value greater than 10% of the weight of the extract. The maximum yield was obtained from the water fraction, followed by the n-hexane and ethyl acetate fractions (**Table II**). The vast number of compounds drawn to the sample may be the reason for the variation in yield results; the more compounds attracted, the higher the yield (Nugroho *et al.*, 2018).

**Table I.** Extraction Result

Powder Weight	Extract Weight	Yield	Characteristics		
			Shape	Color	Smell
1000 grams	260,84 grams	26,08%	Thick	Brown	Specific

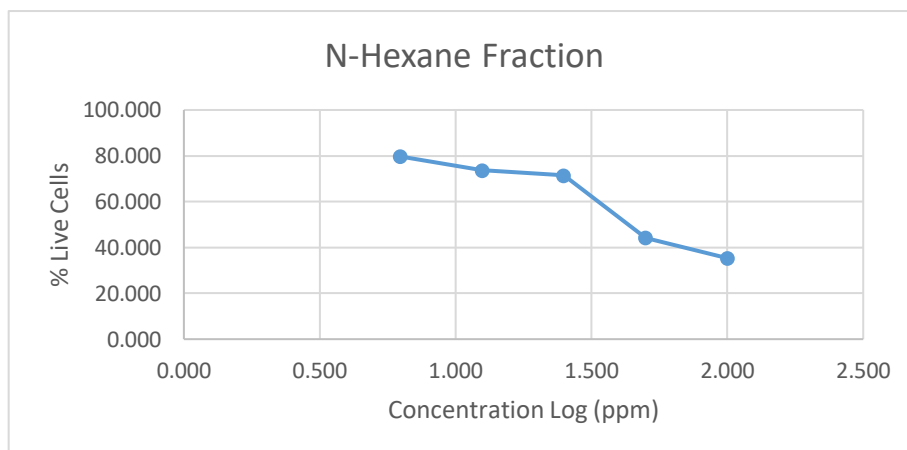
**Table II.** Fractionation Result

Totals Extract Weight	Fraction	Fraction Weight (grams)	Yield (%)
135 gram	Hexane	41,02	30,38
	Ethyl acetate	45,65	33,81
	Water	47,14	34,91

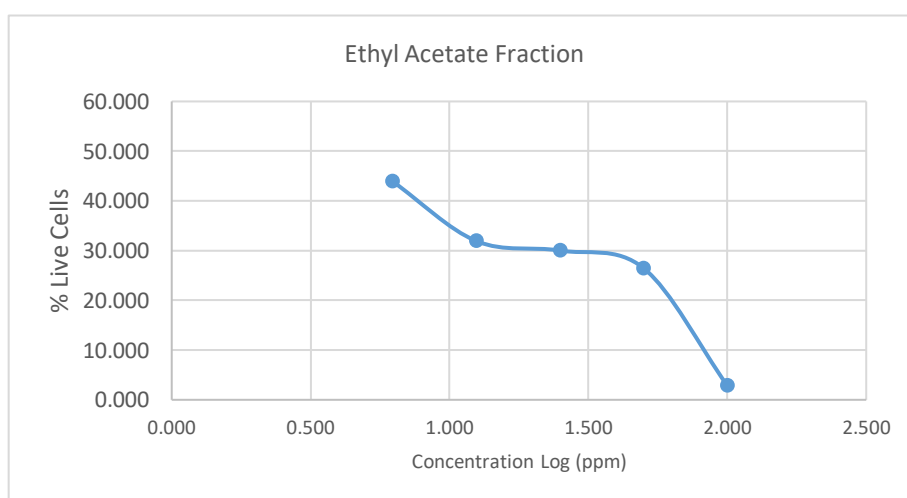
## 2. Cytotoxic Test Result

Samples of the n-hexane fraction, ethyl acetate fraction, water fraction, and positive control doxorubicin are shown on the log concentration vs. % living cell graph (Figure 1, Figure 2,

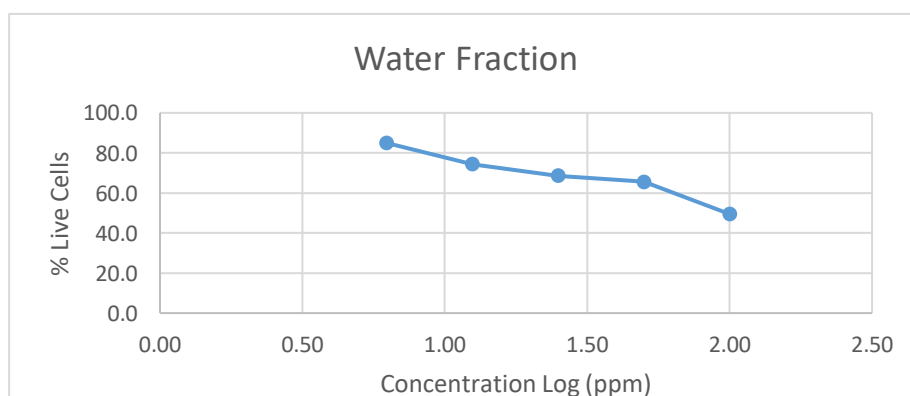
Figure 3, and Figure 4), where the greater the sample concentration, the lower is the % of live cells. The results of the  $IC_{50}$  value calculations support this hypothesis.

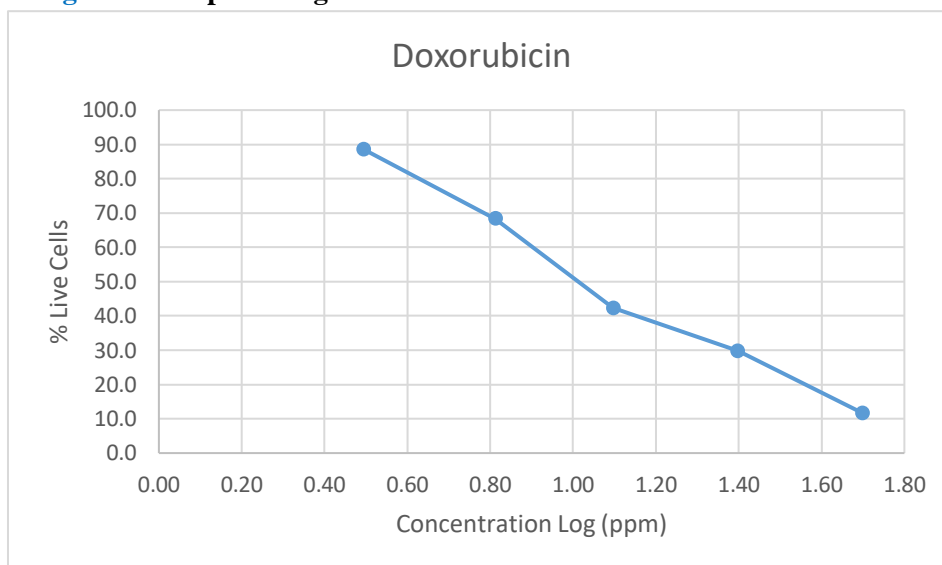


**Figure 1.** Graph of Log Concentration vs % Live Cells n-Hexane Fraction



**Figure 2.** Graph of Log Concentration vs % Live Cells Ethyl Acetate Fraction



**Figure 3. Graph of Log Concentration vs % Live Cells Water Fraction****Figure 4. Graph of Log Concentration vs % Live Cells Doxorubicin**

**Table III** displays the cytotoxic activity of the MM leaf n-hexane fraction ( $IC_{50}$  value: 137.200  $\mu\text{g/mL}$ ), ethyl acetate fraction (17.108  $\mu\text{g/mL}$ ), and water fraction 610.863  $\mu\text{g/mL}$ . The positive control doxorubicin had an  $IC_{50}$  value of 11.282  $\mu\text{g/mL}$ . The results indicated that the cytotoxic activity increased with decreasing  $IC_{50}$  values. The cytotoxic activity of an agent can be classified based on its  $IC_{50}$  value. The highly active category's  $IC_{50}$  value is  $< 20 \mu\text{g/mL}$ , the functional category's is 20-100  $\mu\text{g/mL}$ , the fragile category's is 100-1000  $\mu\text{g/mL}$ , and  $\geq 1000 \mu\text{g/mL}$ , respectively (Suhendi *et al.*, 2017). Thus, it can be concluded that while the ethyl acetate fraction is highly active in suppressing MCF-7 cells, the water fraction and n portion of MM leaf hexane were classified as very weak.

**Table III. IC<sub>50</sub> Results of Fractions from Senduduk Leaf Ethanol Extract**

No.	Sample	IC <sub>50</sub> (ppm) $\pm$ SD
1	n-Hexane Fraction	137,20 $\pm$ 0,07
2	Ethyl Acetate Fraction	17,108 $\pm$ 0,045
3	Water Fraction	610,86 $\pm$ 0,54
4	Doxorubicin	11,28 $\pm$ 0,04

## Discussion

The maceration method of extraction begins with the making of a simplicia. The first step involves wet sorting the leaves of *Melastoma malabathricum* L in order to separate organic material from other plant parts, such as grass or other parts of the plant that are not used. Washing the plant material is intended to remove dirt and reduce the number of microbes or contaminants. Finally, chopping was performed to facilitate drying and pollination to accelerate the process of dissolving the compounds within the sample into the solvent used. Samples of dried senduduk leaves were ground into a powder using a blender and sieved before maceration (Sari *et al.*, 2016). This increased the surface area of the sample and the contact between it and 96% ethanol solvent.

The most straightforward maceration approach involves soaking and stirring senduduk leaves to produce a thick extract. This process causes structural damage to the leaf

cells and exposes the chemical components of the cells to the solvent. According to [Purwaniati and Aliku \(2020\)](#), 1000 g of powdered senduduk leaves was utilized, and 96% ethanol was used as the solvent. Liquid-liquid partition is a crude method for separating extracts with highly complex contents. Therefore, dividing the sections according to their degree of polarity is imperative, starting with nonpolar extracts using n-hexane, semi-polar extracts using ethyl acetate, and polar extracts using water. Fractionation was performed by dissolving the sections in a solution of water and methanol. The water fraction yielded the most significant yield, followed by the ethyl acetate and hexane fractions, as seen from the fraction yield statistics. The vast number of compounds drawn to the sample may be the reason for the variation in yield results; the more compounds attracted, the higher the yield ([Noviyanty et al., 2021](#)).

Cytotoxicity testing is a standard screening procedure for assessing the cytotoxicity of medicinal substances. One of the cytotoxicity test methods is an enzymatic test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT enzymatic test is used to measure the ability of living cells based on mitochondrial activity in cell cultures. This test is widely used to quantitatively measure cellular proliferation and number of living cells ([Haryanti et al., 2017](#)).

The IC<sub>50</sub> (Median Inhibitory Concentration) value is a measure of potential toxicity that is used to express cytotoxic potential. The 50% of the population's ability to inhibit cell growth is known as the IC<sub>50</sub> price. Using the linear regression method, the IC<sub>50</sub> value was determined by finding a linear equation relating the log of concentration to the percentage of life produced.

The log of levels that inhibit 50% of the cell population was then obtained by plugging the probit value of 5 into the straight-line equation. The data indicate a linear association between the concentration and percentage of surviving cells (Figure 1-4). These results showed that, when tested on MCF-7 cells, the ethyl acetate fraction of the ethanol extract of senduduk leaves had a lesser IC<sub>50</sub> value ( $17.108 \pm 0.045 \mu\text{g/mL}$ ) than the n-hexane fraction ( $137.20 \pm 0.07 \mu\text{g/mL}$ ) and the water fraction ( $610.86 \pm 0.54 \mu\text{g/mL}$ ). Compounds with lower IC<sub>50</sub> values are more likely to be developed as anticancer agents.  $11.28 \pm 0.04 \mu\text{g/mL}$  was the IC<sub>50</sub> value determined from the positive control sample. Statistical analysis showed that the ethyl acetate component of senduduk leaves had a significance value of less than 0.05 when compared to doxorubicin. The results for the n-hexane and water fractions indicated a significance of  $>0.05$ , when compared with doxorubicin; however, the ethyl acetate fraction had the same impact as doxorubicin.

The following describes the mechanism that inhibits MCF-7 cells: In the cytotoxic test, the MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) is yellow and is converted to purple formazan salt by the enzyme succinate dehydrogenase. The process is permitted to occur for four hours and is found in the mitochondria of living cells. Longer incubation periods produced more vibrant colors and heightened sensitivity. However, owing to the nature of cytotoxic detection reagents, which require energy from the cell (equal to NADH reduction) to produce a signal, the incubation time is limited.

The amount of formazan product in cell populations undergoing log-phase growth is typically correlated with the number of metabolically viable cells. It is possible that specific circumstances alter the metabolism of cells, which will probably have an impact on the rate at which MTT is reduced to formazan. For example, confluent cells exhibit reduced MTT reduction, slow metabolism, and decreased cell development. As a result, absorbance and cell number are no longer linear ([Riss et al., 2013](#)). A color shift indicated that the cells were still alive because dead cells were unable to convert MTT into formazan ([Riss et al., 2013](#)).

The findings of phytochemical screening of MM leaf ethanol extracts were reported by [Jofry \(2017\)](#). These results showed the presence of secondary metabolites, such as flavonoids, phenolics, and terpenoids, which are molecules that coagulate proteins. The ethyl acetate extract of MM leaves also contained flavonoid naringin, kaempferol, and kaempferol-3-OD-glucoside, whereas the methanol extract contained kaempferol-3-O-(200,600-di-Op-trans-coumaroyl)-glucopyranose and kaempferol-3-OD-glucoside



(Septisetyani *et al.*, 2014). However, the ethyl acetate fraction of the MM methanol extract proved hazardous ( $IC_{50} > 400 \mu\text{g/ml}$ ) to MCF-7 and A549 cells after 24 hours of exposure and induced cell death, according to research conducted in 2017 by Idris from Tutong Brunei Darussalam. By MM, secondary necrosis is the primary cause.

The collection of flavonoid and phenolic compounds extracted from the ethyl acetate fraction is believed to possess cytotoxic properties against HepG2 liver cancer cells by impeding their growth and triggering apoptosis by activating caspase-3 while maintaining Bax protein expression. The ethyl acetate fraction of MM leaves contains a group of flavonoid compounds that inhibit DNA topoisomerase I/II activity, help reduce reactive oxygen species (ROS), regulate heat shock protein expression, modulate the apoptotic pathway, activate caspase-9 and caspase-3, and decrease nuclear transcription factor expression, reducing Mcl-1 protein, endonuclease activity, and factor kappa B (Chear *et al.* 2019).

One-way analysis of variance was used for data analysis based on the obtained  $IC_{50}$  data. Normality and homogeneity tests were performed before one-way ANOVA. A significant value of  $>0.05$  in the normality test findings indicates that the data distribution is expected. A significance value of  $>0.05$  shows a homogenous distribution of data in the homogeneity test results. Subsequently, one-way ANOVA yielded a significance value of less than 0.05, indicating a significant difference in the average value between the sample and positive control groups. Post-hoc test results indicated a significant difference between the two groups. The sample group (water and hexane fractions) was compared to the positive control, and the post-hoc test revealed a significant difference of  $>0.05$ , indicating that the ethyl acetate fraction sample group and the control group did not differ significantly from one another.

## CONCLUSION

Based on the cytotoxic testing results, the ethyl acetate fraction had an  $IC_{50}$  value of  $17.108 \mu\text{g/mL}$ , including the active category. In contrast, the water and n-hexane fractions from the ethanol extract of senduduk leaves had  $IC_{50}$  values of  $610.863 \mu\text{g/mL}$  and  $137.200 \mu\text{g/mL}$ , including in the fragile category. The ethyl acetate fraction was the active fraction that suppressed MCF-7 cells in the senduduk leaf ethanol extract.

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## REFERENCES

- Ali, D.M.H., Keng, C.W. and Boey, P.L., (2020). Triterpenoids, glycolipids and flavonoids of *Melastoma malabathricum*: Isolation, spectrometric characterization and antibacterial activity. VDM Publishing.
- Chear NJY, Fauzi AN, Khaw KY, Choi SB, Yaacob NS, Lai CS. (2019) Free radical scavenging and cytotoxic properties of acylated and non-acylated kaempferol glycosides from *Stenochlaena palustris*: A perspective on their structure – activity relationships. *Pharm Chem J* 53: 188–193.
- Fitria, M., Septhea, D.B., Ikawati, A.H.M. and Meiyanto, E., (2011). Ekstrak etanolik herba ciplukan (*Physalis angulata* L.) berefek sitotoksik dan menginduksi apoptosis pada sel kanker payudara MCF-7. *Bionatura*, 13(2).
- Haryanti, S., Murwanti, R., Putri, H., Ilmawati, G.P.N., Pramono, S. and Meiyanto, E., (2017). Different 4T1 cells migration under *Caesalpinia sappan* L. and *Ficus septica* Burm. f ethanolic extracts. *Indonesian Journal of Cancer Chemoprevention*, 8(1), pp.21-26.
- Idris, A., Zulkipli, I.N., Zulhilmi, N.R., Lee, H.F., Rajabalaya, R., Chee, L.Y., Majid, M. and David, S.R., (2017). *Melastoma malabathricum* ethyl acetate fraction induces secondary necrosis in human breast and lung cancer cell lines. *Pharmacognosy*

- Magazine, 13(Suppl 3), p.S688.
- Joffry, S.M., Yob, N.J., Rofiee, M.S., Affandi, M.M.R., Suhaili, Z., Othman, F., Akim, A.M., Desa, M.M. and Zakaria, Z.A., (2017). *Melastoma malabathricum* (L.) Smith ethnomedicinal uses, chemical constituents, and pharmacological properties: a review. *Evidence-Based Complementary and Alternative Medicine*.
- Noviyanty, Y. and Linda, A.M., (2020). Profil fitokimia senyawa metabolit sekunder ekstrak etanol bunga senduduk (*Melastoma malabathricum* L). *Journal of Pharmaceutical and Sciences*, 3(1), pp.1-6.
- Nugroho, P., Dwiloka, B., & Rizqianti, H. (2018). Rendemen, Nilai pH, Tekstur, dan Aktivitas Antioksidan Keju Segar dengan Bahan Pengasam Ekstrak Bunga Rosella Ungu (*Hibiscus sabdariffa* L.). *Jurnal Teknologi Pangan*, 2(1), 33–39.
- Riss, S., Mohamed, F., Dayal, S., Cecil, T., Stift, A., Bachleitner-Hofmann, T. and Moran, B., (2013). Peritoneal metastases from colorectal cancer: patient selection for cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. *European Journal of Surgical Oncology* (EJSO), 39(9), pp.931-937.
- Roslen NA, Alewi NA, Ahamada H, Rasad MS. (2014). Cytotoxicity screening of *Melastoma malabathricum* extracts on human breast cancer cell lines in vitro. *Asian Pac J Trop Biomed*. Jul;4(7):545-8.
- Salleh, M.M., Eshak, Z. and Ismail, W.W., (2017). Acacia honey induces apoptosis in human breast adenocarcinoma cell lines (MCF-7). *J. Teknol*, 79, pp.9-16.
- Sari, E.R., Nova, A. and Sahitri, L., (2016). Skrining Senyawa Sitotoksik dari Ekstrak Daun, Bunga, Buah, Batang dan Akar pada Tumbuhan Senduduk (*Melastoma malabathricum*. L) Terhadap Larva *Artemia salina* Leach dengan Metode Brine Shrimp Lethality Bioassay. *Scientia*, 6(1), pp.66-72.
- Septisetyani, E.P., Ningrum, R.A., Romadhani, Y., Wisnuwardhani, P.H. and Santoso, A., (2014). Optimization of sodium dodecyl sulphate as a formazan solvent and comparison of 3-(4,-5-dimethylthiazo-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay with wst-1 assay in mcf-7 cells. *Indonesian Journal of Pharmacy*, 25(4), p.245.
- Suhendi, A., Wikantyasning, E.R., Setyadi, G., Wahyuni, A.S. and Da'i, M., (2017). Acetoxy Chavicol Acetate (ACA) concentration and Cytotoxic Activity of *Alpinia galanga* Extract on HeLa, MCF7 and T47D cancer cell lines. *Indonesian Journal of Cancer Chemoprevention*, 8(2), pp.81-84.
- World Health Organization, (2021). WHO handbook for reporting results of cancer treatment. World Health Organization.
- Zahreddine, H. and Borden, K.L., (2013). Mechanisms and insights into drug resistance in cancer. *Frontiers in pharmacology*, 4, p.28