

# Cytotoxic Activity of Ethanol Extract Legundi Leaf (*Vitex trifolia* L.) and N-Hexan, Ethyl Acetate and Ethanol-Water Fraction against MCF-7 Breast Cancer Cells

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## ABSTRACT

Indonesia's biodiversity provides many potential anticancer agent. One of the plants that has anticancer properties is legundi (*Vitex trifolia* L.). This study was conducted to determine the cytotoxic effects of ethanol extract, ethanol, ethyl acetate, and n-hexane fractions of legundi leaves in MCF-7 cells and to identify compounds contained in the legundi ethanol extract and their fractions. The extraction process was carried out using the soxhlet method with 96% ethanol. Fractionation was done using a liquid-liquid partition. Cytotoxic tests were performed using the MTT assay method. Phytochemical screening was carried out by TLC test using silica GF<sub>254</sub> stationary phase, mobile phase n-hexane:ethyl acetate (7:3) and chloroform:methanol:acetic acid (9:3:3). Observation of spot results was done using spray reagents and visualized using UV 366. The cytotoxic test results showed that the ethanol extract and the n-hexane fraction of legundi leaves had cytotoxic effects with IC<sub>50</sub> values of 98.69 µg/mL and 41.06 µg/mL, respectively. While ethyl acetate and ethanol-air fractions did not have cytotoxic effects. The results of phytochemical screening from ethanol extract and n-hexane fraction of legundi leaves showed flavonoid, phenol, tannin, alkaloid, and terpenoid compounds. The ethyl acetate fraction contains a mixture of steroid, saponin, phenol, tannin, and terpenoid. The ethanol-water fraction contains compounds containing steroid, saponin, phenol, tannin, and alkaloid.

**Keywords:** MCF-7, MTT assay, Cytotoxic, *Vitex trifolia* L

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## INTRODUCTION

Breast cancer is a malignant growth of breast tissue. This disease is clinically or radiologically can be detected and called with metastatic breast cancer. This condition generally incurable (DiPiro, 2015). In 2016, in America, breast cancer was the second most common cancer after lung cancer as a cause of death in women. As many as 249,260 people are estimated to be diagnosed with breast cancer and 40,890 people will die of breast cancer (DiPiro, 2017). In Indonesia in 2013, the prevalence of breast cancer was ranked 2nd after cervical cancer with an estimated number of 61,682 people (Ministry of Health, 2015).

Legundi plants (*Vitex trifolia* L.) are known to produce a variety of diterpenoids that show antioxidant, cytotoxic, and trypanoside activities (Saklani et al., 2017). This plant is also rich in flavonoids which are traditionally used to treat rheumatic pain and swelling. Pharmacologically this plant is also known as an antibacterial, and has hepatoprotective activity (Mathankumar et al., 2015).

Other studies suggest that methanol extracts and petroleum ether legundi leaves show strong inhibition of MCF-7 cell proliferation. Petroleum ether extract of legundi leaves has an IC<sub>50</sub> value of 0.41 µg/ml and methanol extract of legundi leaves has an IC<sub>50</sub> value of 6.71 µg/ml (Garbi et al., 2015). Extraction of active compounds from a plant tissue with various types of solvents at different polarity levels aims to obtain optimum results, both the amount of extracts and active compounds contained in the sample. Phytochemical examination of legundi leaf ethanol extract in Jose et al (2017) research showed that there were sterols, terpenoids, flavonoids, alkaloids, glycosides, saponins, and in Saklani et al (2017) there were carbohydrate, phenol and tannin compounds. While the petroleum ether extract showed the presence of sterols, flavonoids, alkaloids, glycosides (Jose et al., 2017), and saponins (Saklani et al., 2017). Petroleum ether has a low polarity characteristic so that it cannot

attract complete compounds, whereas ethanol has a very high polarity so that it can extract more material. Methanol has a high solubility like ethanol but its toxicity is higher than ethanol. According to Saifudin (2014) extracts from materials obtained from the withdrawal of compounds of simplicia whose contents are very complex by organic solvents or water, still require separation. One of the compounds contained in the extract can be separated by fractionation. Therefore, it is necessary to further investigate the effect of ethanol extract of legundi leaves and their fractions on MCF-7 cells to develop their potential activity, and also determine the class of compounds contained in legundi leaves.

## METHOD

### Materials

The tools used are blenders, drying cupboards, Soxhlet equipment (Soxhlet tubes, distillation flasks, coolers, and heaters), glassware (beaker cups, measuring flasks, test tubes, funnels, stirring rods, etc.), Buchner funnel, rotary evaporator (Heidolph), water bath, laminar air flow (ESCO), analytical balance (Ohaus), micropipette (Socorex), dropper, microscope (Olympus), camera documentation, CO<sub>2</sub> incubator (Binder), ELISA reader (Bio-tek ELX800), refrigerator, waterbath (Mettler), hemocytometer (Neubauer), pasteur pipette, vortex (Maxi Mix II), microscope (Olympus), centrifugator (PLC Series).

The material used is legundi leaves (*Vitex trifolia* L.) obtained from Ngrambe District, Ngawi, Central Java, dimethyl sulfoxide DMSO, 96-well plate (Iwaki), 6-well plate, conical tube, yellow type, blue type, white tip, Phosphate Buffer Saline (PBS), filter paper, Dulbecco's Modified Eagle's Medium (DMEM) culture media, 10 mM methotrexate, streptomycin-penicillin, 96% ethanol, Fetal Bovine Serum (FBS), Sodium Dodecyl Sulfate (SDS), MTT solution, MTT, streptomycin-penicillin, 96% ethanol, Fetal Bovine Serum

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(FBS), Sodium Dodecyl Sulfate (SDS), MTT solution, MTT, streptomycin-penicillin, ethanol 96%, Fetal Bovine Serum (FBS), Sodium Dodecyl Sulfate (SDS) trypsin 0.025%, MCF-7 cells from the Laboratory of Cell Culture Mammalia, Faculty of Pharmacy UMS.

### Extraction

Extraction was carried out by soxhletation method for 9 hours, by means of 30 g of legundi leaf powder put in a sample bag placed on the soxhletation tube. The solvent used is 96% ethanol as much as 200 mL. The ethanol extract of legundi leaves that have been obtained is then collected and the liquid solution is evaporated in a rotary evaporator and water bath until a thick ethanol extract of the legundi leaves is obtained.

### Fractionation

Fractionation is done using the liquid-liquid partition method. The fractionation was carried out using a thick extract of legundi leaves as much as 5 grams which was dissolved with a mixture of 96% ethanol solvent and distilled water (1:1) each of 50 mL. Then the legundi leaf extract is fractionated based on its polarity in the order of n-hexane, ethyl acetate with each volume of 50 mL by shaking out using a separating funnel. The fractionation results obtained were thickened with a rotary evaporator which was then placed in a porcelain cup and placed on a water bath at 60°C.

### Cytotoxicity Test

The cytotoxic activity of legundi leaf extract and its fractions was tested using the MTT assay method (CCRC, 2013). The test begins with cell culture and cell harvesting. Cells are counted under a microscope with the help of a counter. Ethanol extract of legundi leaves and their fractions weighed approximately 10 mg, plus DMEM culture media up to 1000 µL. Subsequently, the ethanol extract concentration of legundi leaves (250, 125, 62.5, and 31.25 µg/mL), and the fraction of legundi leaves (16, 8, 4, 2, 1 µg/mL) by diluting the stock in DMSO using DMEM culture media.

MCF-7 cells with a density of 10,000 cells per mL were transferred into wells in a volume of 100 µL and 3 blanks were left for media control. Then the cell condition was observed using an inverted microscope. Cells were incubated at 37°C in a CO<sub>2</sub> incubator for 48 hours. After that, the series of extract concentrations and fractions of legundi leaves, solvent control (DMSO), and positive control (methotrexate) were put into the well and incubated at 37°C in a CO<sub>2</sub> incubator for 24 hours. Cell media was discarded, 0.5 mg/mL MTT reagent of 100 µL was put into each well (including control media), cells were incubated for 2 hours in a CO<sub>2</sub> incubator then a stopper reagent was added (10% SDS in 0.01 N HCl) as much 100 µL. The plate is wrapped in aluminium foil and incubated in a dark place at room temperature overnight. Then the absorbance of each well was read with an ELISA reader with a wavelength of 550 nm. Then the percentage of viable cells is calculated and IC<sub>50</sub> is calculated.

### Data Analysis Technique

The percentage of viable cells can be calculated with a certain formula from the absorbance obtained then looking for a linear regression relationship between log concentration with % of viable cells resulting in the equation  $y = bx + a$ . IC<sub>50</sub> calculation is calculated by substituting the value of 50 on Y so that the value of x is obtained and the value of IC<sub>50</sub> is the antilog of x. The formula for calculating % of viable cells is as follows:

If the absorbance of the solvent control is smaller than the absorbance of the control cell, then calculate the percentage of viable cells using the following formula:

$$\% \text{ viable cell} = \frac{(\text{Absorbance test} - \text{Absorbance media control})}{\text{Absorbance of solution control} - \text{Absorbance media control}} \times 100\%$$

### Phytochemical screening with TLC

Phytochemical screening by TLC was carried out using the mobile phase n-hexane: ethyl acetate (7: 3) for ethanol extract and n-hexane fraction of legundi leaves, for the ethanol-water fraction and ethyl acetate of legundi leaves using the mobile phase of chloroform: methanol: acetic acid (acetate acid) 9: 3: 3). The compound content in the extract and fraction of legundi leaves can be detected using several spray reagents. Spray reagents used are:

- Citroborate reagents are used to detect flavonoid compounds. The color formed is yellow-greenish color on UV366 (Markham, 1998).
- Lieberman-Burchard reagent for detecting steroid saponin compounds. The color formed is blue or green UV366 (Farnsworth, 1966).
- FeCl<sub>3</sub> reagent for detecting phenolic compounds and tannins. The resulting color is black (Pascual, *et al*, 2002).
- Dragendorff reagents for detecting alkaloid compounds. The resulting color is brownish orange on visible light (Saifudin, 2014).
- Vanillin-sulfuric acid or anisaldehyde-sulfuric acid to detect terpenoid compounds. The resulting color is purple (Saifudin, 2014).

## RESULT AND DISCUSSION

### Extraction and Fractionation

The extraction process is carried out by the soxhletation method. The search was carried out repeatedly so that the results obtained were perfect and the solvents used were relatively few. When the filtering has been completed, the solvent is evaporated again and the remainder is the contaminated substance. The yield of legundi leaf ethanol extract was 34.43%. The yield value shows how much the amount of content that can be extracted by the solvent in percent (%). The solvent used in this extraction process is 70% ethanol. The choice of 70% ethanol solvent as a solvent is based on optimal search so it is expected that many active compounds are contained therein.

The obtained thick extract was then fractionated with 5 grams of liquid-liquid partition using n-hexane, ethyl acetate and ethanol-water. Fractionation has the aim to classify the compound content contained in the extract based on its polarity, the yield obtained from the fraction of n-hexane, ethyl acetate and ethanol-water is 13.4%, 24.2%, 21.4%. The amount of extract which is diffracted by n-hexane solvent has the smallest amount of yield than ethylacetate and ethanol-water solvent. This is made possible by the small amount of non-polar content that can be absorbed by n-hexane from legundi leaf ethanol extract. The content of semi-polar compounds is more concentrated by ethyl acetate than legundi leaf extract.

### Cytotoxic Test

Cytotoxic test was carried out to determine the toxicity of an extract and fraction of legundi leaves against MCF-7 cells by the MTT assay method. The solvent used is DMSO because it is a solvent capable of dissolving polar and non-polar compounds (Liu, 2008). The DMSO level contained in the sample is 1%. DMSO with levels less than 3% can be used as a solvent for this cytotoxic test, because it is not toxic or kills cancer cells (Purwaningsih *et al*, 2014). The addition of MTT reagents serves to determine cancer cells that are still viable, through the reaction of the enzyme reductase to form purple formazan crystals.

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Figure 1. The morphology of MCF-7 cells showed the activity of hexane fraction of *Vitex trifolia* L. The control of MCF-7 cells without any treatment (left), MCF-7 cells was dying after treatment with hexane fraction with concentration of 16 µg/mL (centre), formation of formazan crystals in MCF-7 cells (right)

Figure 1 showed the morphology of live MCF-7 cells (before treatment), after treatment, and formazan crystals formed during cytotoxic tests. The morphology of MCF-7 viable cells is round and irregularly surrounded by clear colored membranes. After being treated with a sample (n-hexane fraction) the morphology of the cancer cells changes, the cell nucleus turns black and the cell shrinks. This shows that the cancer cells have died due to sample treatment. Formazan crystal forming comes from the enzymatic reaction between MTT reagents and enzymes found in viable cells so that they form purple and not water-soluble formazan crystals. The addition of the stopper reagent will dissolve the colored crystals which will then be measured

for absorbance using an Enzyme-linked Immunosorbent Assay (ELISA) reader. Purple intensity formed linearly with the number of viable cells. So that if the intensity of the purple color is greater, then that means the number of viable cells more and more.

The MTT assay test method obtained absorbance data used to obtain the percentage value of viable cells. The percentage of viable cells was used to determine cell death due to the administration of sample treatments (ethanol extract and ethanol fraction, ethyl acetate and n-hexane legundi leaves) from the concentration series. The greater the concentration of the sample, the smaller the percentage of viable cells produced.

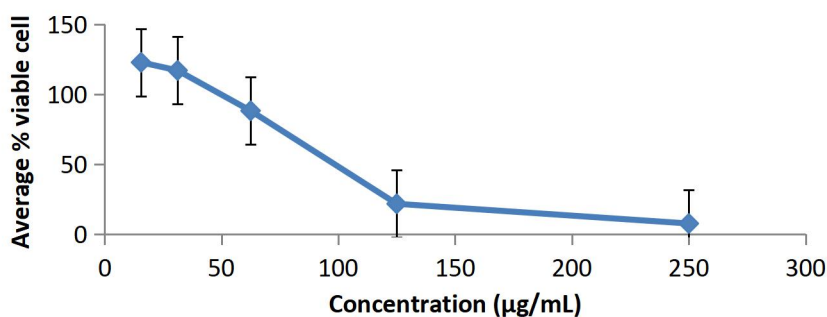


Figure 2. Graph of ethanol extract vs% concentration of MCF-7 viable cells

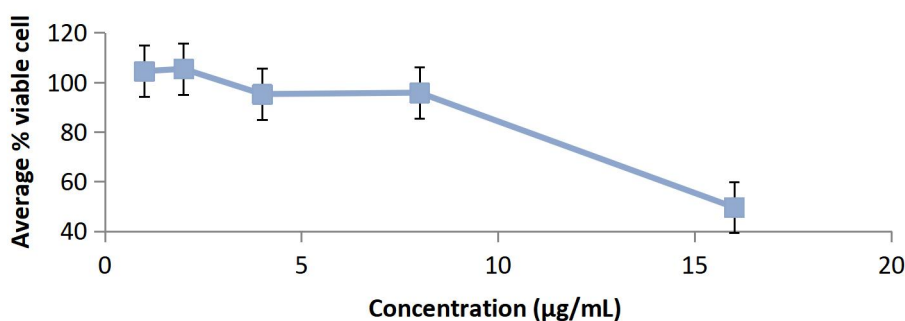


Figure 3. Graph of n-hexane fraction concentration vs % MCF-7 viable cell

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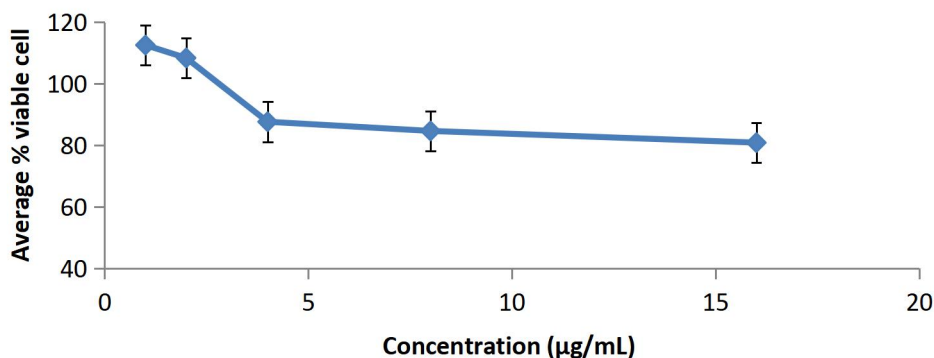


Figure 4. Graph of concentration of ethyl acetate fraction vs % viable cells of MCF-7

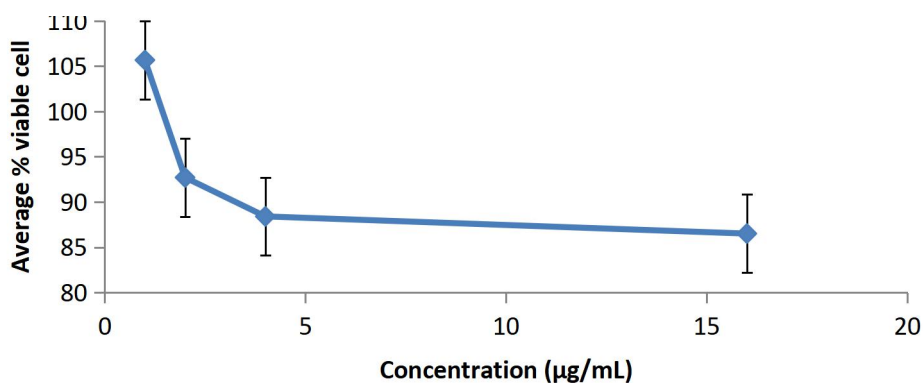


Figure 5. Graph of concentration of etanolic-water fraction vs % viable cells of MCF-7

IC<sub>50</sub> value calculation was obtained from the log plot of concentration vs% of viable cells (Suhendi et al., 2014) from each sample tested by taking several concentrations. The results of data processing can be seen in Figure 2-5. The data obtained showed that ethanol extract at concentrations of 125 and 250 µg/mL, as well as the n-hexane fraction at concentrations of 16 µg/mL could produce a 50% reduction in cell count. But in the ethyl acetate and ethanol fraction has a value of % of viable cells more than 50%, so the IC<sub>50</sub> value cannot be calculated. The experimental results also showed the number of % of viable cells depends on the

concentration of the compound given. The higher the concentration of the compound, the less the number of cancer cells that live.

In the study by Fodor et al (2016) the use of 10 µM methotrexate combination therapy with 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) 100 µM in MCF-7 breast cancer cells resulted in a 50 % reduction in cell count. In this study, 10 µM methotrexate was used but it produced a viable cell % of 115.13% so that the IC<sub>50</sub> value could not be calculated. This is likely due to the relatively small concentration used.

**Table 1.** Cytotoxic test results of ethanol extract of legundi leaves and n-hexane fraction against MCF-7 breast cancer cells

Sample	Linear Regression	R <sup>2</sup>	IC <sub>50</sub> (µg/mL)
Ethanolic extract	Y = -108,16x + 265,7	0,92	98,69
n-hexane fraction	Y = -39,651x + 113,97	0,67	41,06

According to the National Cancer Institute (NCI) a compound is classified as a potential anticancer if the IC<sub>50</sub> value is < 20 µg/mL (NCI, 2015). Compounds can be used as anticancer if they have potential cytotoxic activity (Tussanti et al, 2014). The cytotoxic test results showed that ethanol extract and hexane fraction had no potential cytotoxic effects with IC<sub>50</sub> values 98.69 and 41.06 µg/mL (Table 1). The ethyl acetate and ethanol-water fractions cannot be calculated for IC<sub>50</sub> values because the percentage of viable cells is more than 50%. In previous studies, methanol

extracts and petroleum ether of legundi leaves had potent activity against MCF-7 cells, each of which had IC<sub>50</sub> values of 6.71 µg/mL and 0.41 µg/mL (Garbi et al., 2015). In this study, ethanol extract of legundi leaves had IC<sub>50</sub> 14 times greater than methanol extract and 2 times petroleum ether extract. While the results of fractionation are n-hexane fractionation which shows potential cytotoxic activity against MCF-7 cells having IC<sub>50</sub> values 6 times greater than methanol extract and 100 times that of petroleum ether extract. This is due to differences in

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polarity used, so that it can affect the amount and type of content of the compound of interest that can affect inhibition of MCF-7 cells.

**Fitochemical Screening**

Phytochemical screening using Thin Layer Chromatography (TLC) is used to detect the presence of flavonoid compounds, saponins, steroids, phenolics, tannins, alkaloids,

and terpenoids. The stationary phase used in this test is silica GF254, and the mobile phase used is n-hexane: ethyl acetate (7: 3) for ethanol extract and n-hexane fraction of legundi leaves, for ethanol-water fraction and ethyl acetate of legundi leaves using chloroform mobile phase: methanol: acetic acid (9: 3: 3). Spotting results were observed using spray reagents and visualized using visible light and 366 nm.

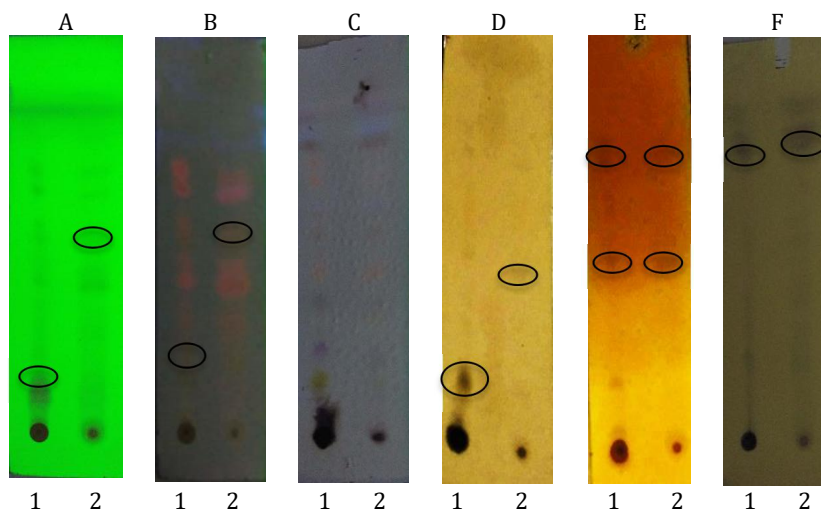


Figure 6. TLC results for ethanol extract (1) and n-hexane fraction (2). Plate before spraying was observed at UV 254 nm (A), after spraying with citroborate reagents in UV light 366 nm (B), Liebermann-Burchard in UV light 366 nm (C), FeCl<sub>3</sub> in visible light (D), Dragendorff in visible light (E), vanillin-sulfuric acid in UV366 nm (F) light.

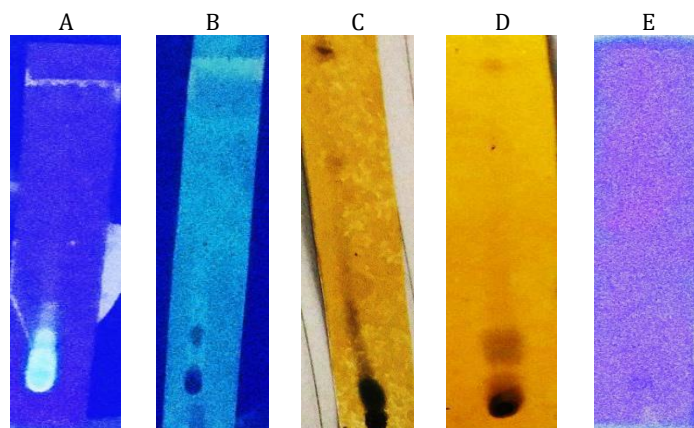
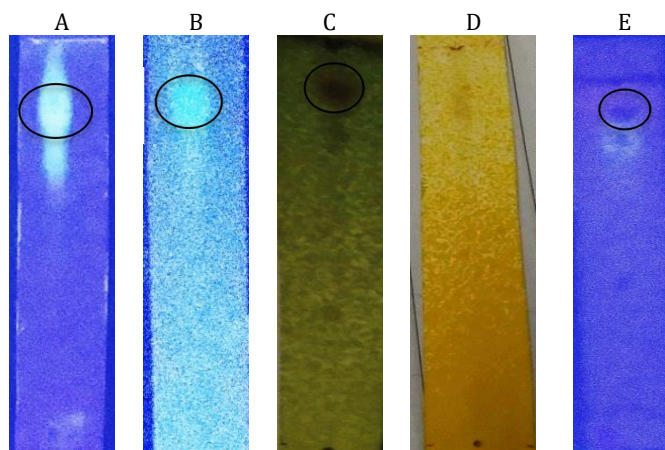


Figure 7. Results of ethanol-water TLC (1) after spraying with citroborate reagents in UV light 366 nm (A), Liebermann-Burchard in UV light 366 nm (B), FeCl<sub>3</sub> in visible light (C), Dragendorff in visible light (D), vanillin-sulfuric acid in UV light 366 nm (E).



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Figure 8. TLC results of ethyl acetate fraction (1) after spraying with citroborate reagents in UV light 366 nm (A), Liebermann-Burchard in UV light 366 nm (B), FeCl<sub>3</sub> in visible light (C), Dragendorff in visible light (UV) D), vanillin-sulfuric acid in UV light 366 nm (E).

Table 2. Detection results of legundi leaf extract compounds

	UV 254	Spray reagent					Compound Prediction
		Citroborate UV 366	Lieberman-Burchard UV366	FeCl <sub>3</sub> Visible	Dragen- dorff Visible	Anisaldehyde-H <sub>2</sub> SO <sub>4</sub> UV <sub>366</sub>	
Ethanolic extract	Dark	Yellow	-	Black	Brown	Purple	Flavonoid, Phenol, tannin, alkaloid, and terpenoid
Fraction of N-Hexane	Dark	Yellow	-	Black	Brown	Purple	Flavonoid, Phenol, tannin, alkaloid, and terpenoid
Fraction of Ethyl Acetate	NA	Green	Green	Black	-	Purple	Steroid, saponin, Phenol, tannin, and terpenoid
Fraction of Ethanol-Water	NA	Green	Blue	Black	Brown	-	Steroid, saponin, Phenol, tannin, and alkaloid

Detection of compound content was carried out using spray reagents namely citroborate reagent, Lieberman-Burchard, and anhydrous dehyde-H<sub>2</sub>SO<sub>4</sub>, which was observed at UV 366 nm. FeCl<sub>3</sub> reagent, and Dragendorff and the results are observed in visible light. Ethanol extract and n-hexane fraction were thought to contain flavonoid compounds, phenols, tannins, alkaloids, and terpenoids (Figure 6, Table 2). The ethyl acetate fraction of legundi leaves is thought to contain steroids, saponins, phenols, tannins, and terpenoids (Figure 7, Table 2). The ethanol-water fraction of legundi leaves is thought to contain steroids, saponins, phenols, tannins, and alkaloids (Figure 8, Table 2). The TLC results of the ethyl acetate and ethanol-water fractions of the legundi leaves have yet to be ascertained for their compound content due to method limitations.

The results of the compound content in the ethanol extract are in line with previous research, that the chemical tests qualitatively on the ethanol extract of legundi leaves showed the presence of sterols, terpenoids, saponins, carbohydrates, tannins, alkaloids, glycosides, and flavonoids (Jose et al., 2017). The difference in the content of compounds in the n-hexane, ethyl acetate, and ethanol-water fractions is due to differences in the polarity of the compounds that can be drawn in the fraction solvent. Based on the test results of flavonoid compounds in *Vitex trifolia* plants have anticancer activity. Li et al (2005) isolated six flavonoids isolated from *Vitex trifolia*, namely persicogenin, artemetin, luteolin, penduletin, viteksikarpin and krisosplenol-D. The six flavonoids inhibit the polyperation of K562 cancer cells and are thought to have a mechanism of action to induce apoptosis in these cancer cells through the apoptotic pathway regulated by mitochondria. Differences in cytotoxic effects on extracts and fractions of legundi leaves are possible because they have different content of flavonoid compounds. The test results of the compound content in ethanol extract and n-hexane fraction of legundi leaves showed the presence of flavonoid compounds and did not indicate the presence of saponin and steroid compounds. The cytotoxic test results of ethanol extract and n-hexane fraction have IC<sub>50</sub> values

and% of viable cells which are smaller than the results of cytotoxic tests of ethyl acetate and ethanol-water fractions which contain saponin and steroid compounds and cannot detect flavonoid compounds.

#### CONCLUSION

Ethanol extract and n-hexane fraction of legundi leaves have the same compound contents, namely flavonoids, phenols, tannins, alkaloids, terpenoids and have cytotoxic effects with IC<sub>50</sub> values 98.69 µg/mL and 41.06 µg/mL against MCF- cells 7. Ethyl acetate and ethanol-water fractions do not have cytotoxic activity.

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#### CONFLIC OF INTEREST

The authors declare no conflict of interest.

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