



Cytotoxic Test of Fraction of Gaharu Leaves (*Aquilaria Malaccensis*) on Cervic Cancer Cells (Hela Cells) Using MTT Assay Method

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ABSTRACT

Cervical cancer is one type of cancer that attacks women in both developed and developing countries. Cancer can arise from cell division errors or DNA damage from certain environmental exposures that cause free radicals as a factor of cancer cells. The agarwood plant (*Aquilaria malaccensis* Lam) has been known by society as a health tea ingredient. The content of compounds contained in tea is believed to have anti-cancer and antioxidant activity. In previous studies found some of the active compound ingredients contained in the leaves of agarwood, such as alkaloids, flavonoids, triterpenoid, saponins and tannins. Tests that have been done previously that agarwood plants have good cytotoxic activity against cancer cells MCF-7 from one of the fractions, the n-hexane fraction. In this study, another cytotoxic potential test was carried out from aloe plants on other cancer cells, namely HeLa cells. Extraction was carried out by the Soxhlet method using 96% ethanol solvent, then fractionated with n-hexane solvent, ethyl acetate, and methanol: water. The MTT assay method is used to test the cytotoxic activity of sample research on cell cultures used. Measurements were performed using an ELISA reader at 550 nm wavelength. The results showed that the IC₅₀ values of the four samples tested were ethanol extract (299,506 µg / ml), n-hexane fraction (120,913 µg / ml), ethyl acetate fraction (3490,476 µg / ml) and methanol fraction: water (294,060 µg) / ml). from extracts and fractions it is known that the highest value is shown by the n-hexane fraction in the moderately active/moderate category.

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INTRODUCTION

Breast cancer and cervical cancer are the 2 most common types of cancer in women in both devel-

oped and developing countries. (NCI, 2019). In Indonesia, the incidence of cancer is at number 8 in Southeast Asia, while in Asia, it is at number 23. Cervical cancer is the growth of abnormal cells in the cervix or cervix. In 2018 the incidence of breast cancer in Indonesia was 42.1, followed by cervical cancer of 23.4 with an average death rate of 13.9 out of 100,000 population (Guidelines for Breast Cancer Management, 2015). Gaharu plant is a medicinal plant that has antioxidant activity and contains secondary metabolites such as flavonoids, alkaloids, terpenoids and phenolic compounds (Khalil *et al.*, 2013). The purpose of this study was to determine the activity of agarwood leaves against HeLa cells. Gaharu leaves were extracted by the Soxhlet method using 96% ethanol as a solvent. The extract

obtained was then fractionated with the liquid-liquid extraction method using solvents with different polarity, namely n-hexane, ethyl acetate and methanol-water. Extracts and fractions of n-hexane, ethyl acetate and methanol-water were tested for activity against HeLa cells using the MTT Assay method.

MATERIALS AND METHODS

The tools used in this study were a blender, Erlenmeyer (*Duran*) glassware, measuring cups (*Duran*), Beaker glass (*Pyrex*), silica crucibles, porcelain plates, ovens, rotary evaporators (*Ika*), 100-1000 μ L micropipettes. 96-Well Plate, pH meter, Vortex mixer, CO2 incubator, Inverted Microscope, Petri dish, and *ELISA reader*.

The materials used are gaharu leaves (*Aquilaria malaccensis*). Obtained from PT.Mitra Dulur Sejahtera. Traditional Medicine Factory (HERBAL), Palembang, South Sumatra. Ethanol 96% (technical), aquadest, ammonia 25% (technical), chloroform (technical), hydrochloric acid 10%, methanol (technical), ethyl acetate (technical), n-hexane (technical), Dragendorff reagent, Mayer reagent, ether (technical), Liberman-Bauchard reagent, Steasny reagent, magnesium powder, amyl alcohol (technical), formaldehyde (technical), hydrochloric acid 2 N, ferric chloride (pa), sulfuric acid (pa). 1 N sodium hydroxide, gelatin, dimethyl sulfoxide (DMSO), MTT reagent, cervical cancer cells (HeLa cells).

Determination of Agarwood Leaves

The determination process was carried out at the Herbarium Jatinangor Plant Taxonomy Laboratory of the Department of Biology, FMIPA UNPAD, which was held on December 17, 2019, number 53/HB/12/2019

Simplicia Characterization

The Simplicia characterization includes:

Determination of total ash content

A total of 2-3 grams of gaharu leaf simplicia were carefully weighed and put into the silicate crucible, which had been incandescent and tared, then flattened the crucible containing the sample and then slowly incanded it until charcoal was formed. Then it is incubated in the furnace to form ash. The crucible containing ash is weighed, and its exact weight is determined ([Indonesian Herbal Pharmacopoeia edition II, 2017](#)).

Determination of acid-insoluble ash content

The ash obtained on the determination of total ash is boiled with 25 mL dilute hydrochloric acid for 5 min-

utes. The insoluble part of the acid was collected, then filtered using an ash-free filter paper, then washed with hot water and annealed at a temperature of 450°C until a fixed weight was then weighed. The acid-insoluble ash content was calculated on the sample ([Indonesian Herbal Pharmacopoeia edition II, 2017](#)).

Determination of water-soluble extract

A total of 5 grams of gaharu leaf simplicia was extracted for 5 hours with 100 mL of ethanol using a clogged flask while shaking it occasionally in the first 6 hours. Then left for 18 hours and then filtered. The filtrate obtained is then taken 20 mL and then evaporated to dry in a cup that has been tared beforehand. The rest is heated at a temperature of 105 °C until a fixed weight is obtained, then weighed. The water-soluble content of the juice was calculated against the air-dried material ([Indonesian Herbal Pharmacopoeia edition II, 2017](#)).

Determination of ethanol-soluble extract

A total of 5 grams of dried agarwood leaf simplicia powder, extracted for 5 hours with 100 mL 95% ethanol using a clogged flask while occasionally shaking for the first 6 hours, then left for 18 hours. The sample was filtered quickly to avoid 95% ethanol evaporation, and as much as 20 mL of the filtrate was evaporated to dryness in a pre-tared plate. The rest is heated at 105 °C until a fixed weight is obtained, then weighed. The ethanol-soluble content of 95% was calculated for the air-dried material ([Indonesian Herbal Pharmacopoeia edition II, 2017](#)).

Determination of drying shrinkage

The drying shrinkage of the *Aquilaria malaccensis* Lam simplicia were determined using the *Moisture Balance* tool. A total of 2 grams of simplicia powder was put into an aluminium foil-coated container that has been weighed, then the level of drying shrinkage was measured at 105°C until the tool shows a constant number ([Harborne, 1998](#))

Phytochemical Screening

Phytochemical screening was done to determine the class of compounds found in Agarwood leaves. Phytochemical screening carried out includes.

Quinone Test

A total of 5 ml of C solution was added with a few drops of 1N sodium hydroxide solution. The formation of red indicates the presence of quinone. However, false-positive reactions can occur with tannin. Then the examination was continued with the addition of gelatin, then the precipitate was filtered, and the filtrate was added with 1 N sodium hydrox-

ide. When it remains yellow means the presence of quinones (Farnsworth, 1966).

Tannin test

A total of 2 grams of the extract was dissolved with water and then reacted with 10% iron (III) chloride solution, dark blue, or greenish-black colour indicates the presence of tannin (Farnsworth, 1966).

Saponin Test

A total of 2 grams of the extract was dissolved with hot water and added one drop of 2N HCl, then shaken vigorously. Saponins will produce a stable foam that is visible for 5 minutes and does not disappear will show positive saponins (Farnsworth, 1966).

Flavonoid Test

A number of samples were added 0.1 mg magnesium powder and 4 mL amyl alcohol (30% hydrochloric acid mixture and 90% Ethanol with the same volume) and 4 mL alcohol, then the mixture was shaken. Positive reactions are shown in red, yellow, or orange in the amyl alcohol layer (Farnsworth, 1966).

Alkaloid Test

A total of 10 mg extract was added with 5 mL of 25% ammonia, then added 20 mL of chloroform. The mixture was filtered to obtain a layer of water and an organic layer. The layer of water was added to two drops of *Dargendorff* reagent or *Mayer* reagent. If orange is formed with *Dragendorff* reagent or white precipitate formed with the addition of *Mayer* reagents means the extract contains alkaloids (Farnsworth, 1966).

Steroid/triterpenoid test

A total of 500 mg of extract was added with 20 ml of ether, macerated for 2 hours, then filtered, eight drops of the filtrate were transferred into the watch glass and given *Liebermann-Bouchard* reagent. Then it was observed if a red-purple colour was formed indicating triterpenoids and the green-blue colour was established indicating the presence of steroids (Farnsworth, 1966).

Extraction

Extraction was performed using the Soxhlet method with ethanol as a solvent. *Simplicia*, 40 grams of agarwood leaves wrapped in filter paper, tied with threads, put into the soxhlet device, input 400 mL of 96% ethanol solvent into the shock flask. Carry out the soxhletation with a temperature of 70°C until the droplets cycle is colorless or approximately 5 hours. The resulting extract is then filtered and evaporated using a rotary evaporator to obtain

a concentrated extract (Indonesian Herbal Pharmacopoeia edition II, 2017).

Fractionation

The ethanol extract of concentrated gaharu leaves was dissolved in 20% methanol and fractionated using the liquid-liquid extraction method with water, n-hexane, then ethyl acetate, respectively 3 times. The fraction obtained was then monitored by Thin Layer Chromatography (TLC).

Extraction and Fractionation Monitoring

Extracts and fractionations obtained were evaluated qualitatively using thin-layer chromatography methods by silica gel as stationary phase and polar solvent (ethyl acetate:methanol:water (8:1:1, v/v)), semi-polar solvent (chloroform:methanol (9:1, v/v)), and non-polar solvent (n-hexane: ethyl acetate (9:1, v/v)) as mobile phase. The spot on TLC surface was sprayed with 5% AlCl₃, 10% FeCl₃, and 10% H₂SO₄ (Indonesian Herbal Pharmacopoeia edition II, 2017).

Test of HeLa cell breast cancer cell cytotoxic activity using MTT Assay method

Cell culture media was made, growing cells, cell sub-culture, and cell counts. 100 µL of cells were put into the well, and three blank wells were left for media control. The cells were incubated in an incubator for 1x24 hours so that the cells recovered after being harvest. The series concentration of the sample was inserted into the well, incubated in CO₂ incubators. The length of incubation depends on the effect of the treatment on cells. If within 24 hours, no cytotoxic effects have been seen, re-incubate for 24 hours. Towards the end of the incubation period, MTT reagents were prepared for treatment (0.5 mg/ml) by taking 1 ml of MTT stock in *Phosphate Buffer Saline* (5 mg/ml), the culture media was diluted by adding up to 10 ml (for one unit of 96 wells) plate). Cell conditions were examined using an *inverted microscope*; when formazan was formed, 100 µL of 10% DMSO stopper was added in 0.1 N HCl. Then the *ELISA reader* was turned on, waiting for the progressing process to finish. The absorbance of each well was read using an *ELISA reader* at a wavelength of 550 nm. Furthermore, absorbance graphs were made after subtracting media control. Calculate live cell percentage and IC₅₀ value analysis (Cancer Chemoprevention Research, 2013).

RESULTS AND DISCUSSION

Simplicia characterization

Simplicia characterization is a standard parameter that is carried out to ensure the quality and qual-

Table 1: Simplicia characterization results and extract density

Test	Result (%) w/w
Total ash content	1,345
Acid insoluble ash content	0,22
Ethanol-soluble extract	12
Water-soluble extract	9
Drying shrinkage	9.857
Density	0.7971 w/v

Table 2: Phytochemical screening results

Compound	Test	Results	Description
Alkaloid	Filter Paper	Orange Precipitate	+
	Mayer	White Precipitate	+
	Deggendorf	Brick-red Precipitate	+
Flavonoids	+ Amyl Alcohol	Yellow-orange in the amyl alcohol layer	+
Tannin	FeCl ₃	Blackish green	+
	Gelatin	White precipitate	+
	Steasny	Pink after heating	+
Quinones	NaOH	Red	+
Saponin	2 N HCl	Stable foam	+
Steroid / Triterpenoid	LiebermanBuchard	Bluish-green	+

Note: + (detected)

Table 3: IC₅₀ values of Cerviks cancer cells for extract and its fraction

Samples	IC ₅₀ Value (μg/mL)
Extract	299,506
Methanol-water fraction	294,060
Ethyl acetate fraction	3490,476
n -hexane fraction	120,913

Note: IC₅₀ value = sample concentration needed to inhibit 50% of Cerviks cancer cells

Table 4: Cytotoxicity Categories According to the United State National Cancer Institute

IC ₅₀ (μg/mL)	IC ₅₀ value (μg/mL)
≤ 20	Very toxic
21 < IC ₅₀ < 200	Moderate / quite active
201 < IC ₅₀ < 500	Weak
>500	Not toxic

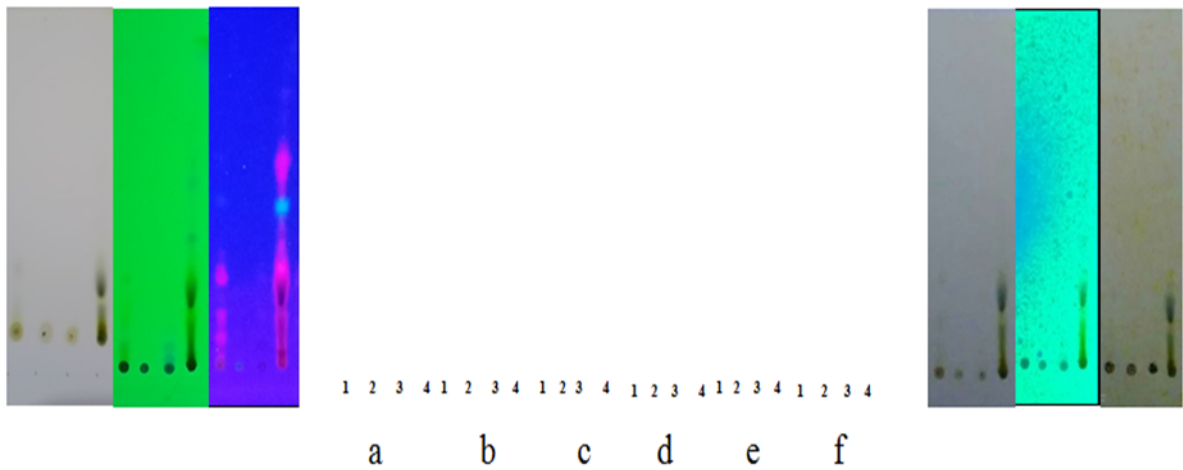


Figure 1: TLC of extracts and fractions by using n-hexane: ethyl acetate (9:1, v/v) as mobile phase

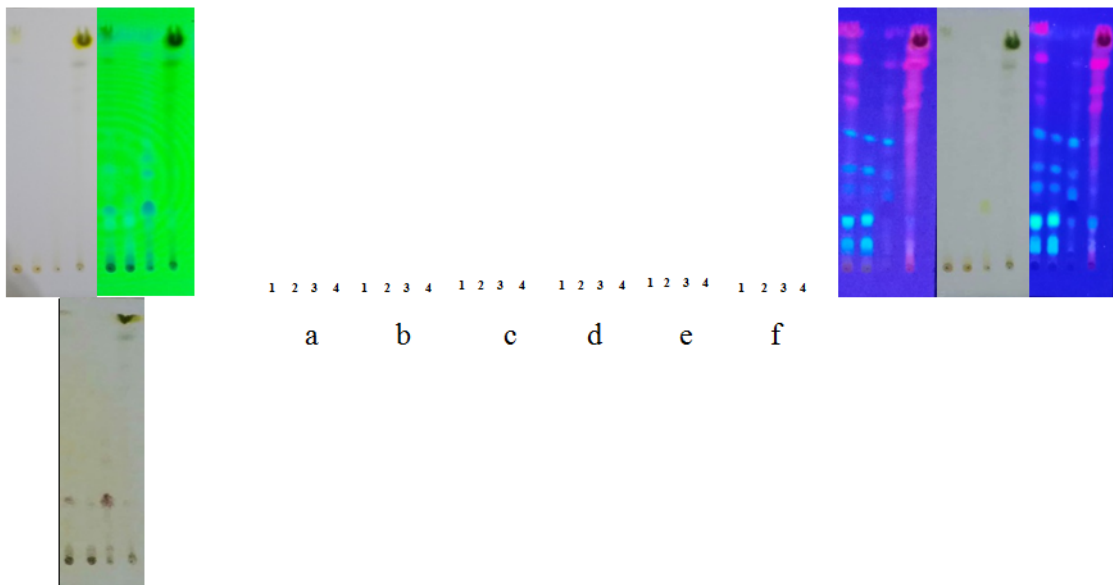


Figure 2: TLC of extracts and fractions by using chloroform: methanol (9:1, v/v) as mobile phase

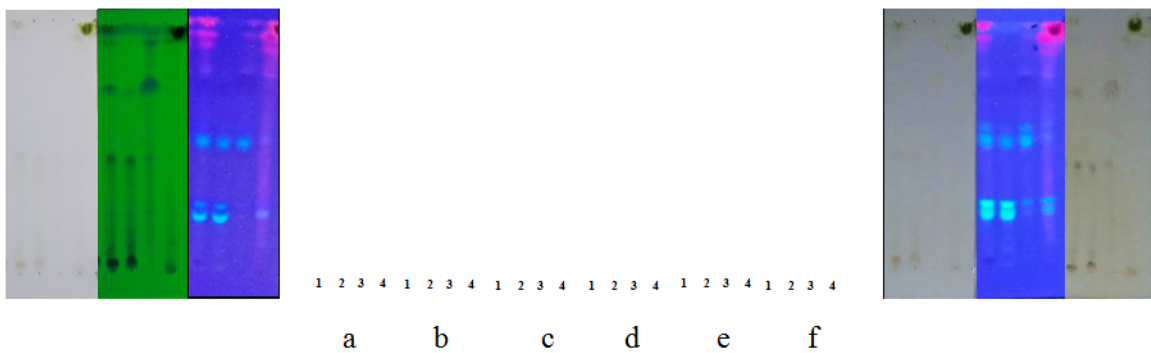


Figure 3: TLC of extracts and fractions by using ethyl acetate: methanol:water (8:1:1, v/v) as mobile phase

ity of simplicia. Simplicia characterization included determination of total ash content, acid insoluble ash content, ethanol-soluble extract, water-soluble extract, and drying shrinkage and extract density. The results of simplicia characterization and extract standardization can be seen in Table 1.

The results of total ash content showed a description of the mineral content from the initial process to the extract formation. Determination of acid-insoluble ash content aims to determine the amount of ash obtained from external factors sourced from impurities originating from sand and soil. Determination of water-soluble extracts and ethanol soluble extracts aims to determine the number of compounds in Simplicia dissolved in water and ethanol solvents. Compounds in Agarwood leaves are more soluble in ethanol solvents. Determination of drying shrinkage of Simplicia aims to provide maximum limits on the amount of water and compounds lost in the drying process of Agarwood leaves (*Aquilaria malaccensis* Lam). The evaluation of extract density was carried out to determine the quality of Agarwood leaf extract (*Aquilaria malaccensis* Lam) because it gives an overview of the chemical content dissolved in the extract (Shoebm *et al.*, 2010).

Phytochemical Screening

Phytochemical screening is the initial stage in identifying secondary metabolites compounds contained in plants. The results of phytochemical screening can be seen in Table 2. Phytochemical screening results show that Agarwood leaves (*Aquilaria malaccensis* Lam) contain alkaloids, flavonoids, tannins, quinones, saponins, steroids, and triterpenoids (Ashraf *et al.*, 2014). These results indicate that Agarwood leaves have antioxidant activity because they contain flavonoid compounds that act as antioxidants.

Extraction

The extraction results obtained in the simplicia used as much as 650 grams produced concentrated extract as much as 96.3768 grams. The yield calculation of the extract obtained was 14.8272%

Fractionation

The yield fraction obtained from the three solvents, namely n-hexane, ethyl acetate, and water-methanol. Amounted to 9.8505% in N-hexane solvent, the yield of ethyl acetate solvent fraction was 10.112%, and the yield of methanol: water solvent fraction was 26.8865% obtained from the viscous extract weighing 20 grams.

Evaluation of Extraction and Fractionation

In the process of extracts and fractions monitoring, a qualitative analysis was performed using the Thin

Layer Chromatography (TLC) method, which aims to ensure the presence of the desired compound. The TLC system was carried out by using silica gel as stationary phase and polar solvent as mobile phase (ethylacetate: methanol: water (8:1:1, v/v)), semi-polar solvent as mobile phase (chloroform: methanol (9:1, v/v)), non-polar solvent as mobile phase (n-hexane: ethyl acetate (9: 1, v/v)). The results of monitoring can be seen in Figure 1.

In Figure 1, 1. Extract, 2. Methanol: water fraction, 3. Ethyl acetate fraction, and 4. -Hexane fraction. (a) Under regular sunlight, (b) Under UV light at 254 nm, (c) Under UV light at 365 nm, (d) Using H₂SO₄ reagent under regular sunlight, (e) Using H₂SO₄ reagent under UV light at 365 nm, (f) Using AlCl₃ reagent under UV light at 365 nm, and (g) FeCl₃ reagent.

TLC spot evaluation of extracts, n-hexane fraction, ethyl acetate fraction and methanol: water fraction of Agarwood leaves (*Aquilaria malaccensis*) by using silica gel as stationary phase and non-polar n-hexane: ethyl acetate (9: 1, v/v) as mobile phase and then sprayed using AlCl₃ showed the presence of active flavonoids in extract and n-hexane fraction under UV light at λ 365 nm in the form of yellow spots. However, detection by using reagent FeCl₃ did not show black spots when observed under visible light (Figure 2). In Figure 2, 1. Extract, 2. Methanol: water fraction, 3. Ethyl acetate fraction, and 4. n-Hexane fraction. (a) Under regular sunlight, (b) Under UV light at 254 nm, (c) Under UV light at 365 nm, (d) Using H₂SO₄ reagent under regular sunlight, (e) Using H₂SO₄ reagent under UV light at 365 nm, (f) Using AlCl₃ reagent under UV light at 365 nm, and (g) FeCl₃ reagent.

TLC spot evaluation of extracts, n-hexane fraction, ethyl acetate fraction and methanol: water fraction of Agarwood leaf (*Aquilaria malaccensis*) by using silica gel as stationary phase and a semi-polar [chloroform: methanol (9:1, v/v)] as mobile phase and sprayed using AlCl₃ showed the presence of active flavanoids in the extract and the ethyl acetate fraction under UV light at λ 365 nm in the form of yellow spots. Detection by using a reagent of FeCl₃ showed black spots when observed under visible light, which shows the presence of active phenolics (Figure 3).

In Figure 3, 1. Extract, 2. Methanol: water fraction, 3. Ethyl acetate fraction, and 4. -Hexane fraction. (a) Under regular sunlight, (b) Under UV light at 254 nm, (c) Under UV light at 365 nm, (d) Using H₂SO₄ reagent under regular sunlight, (e) Using H₂SO₄ reagent under UV light at 365 nm, (f) Using AlCl₃ reagent under UV light at 365 nm, and

(g) FeCl₃ reagent.

TLC spot evaluation of extract, *n*-hexane fraction, ethyl acetate fraction, and methanol: water fraction of Agarwood leaves (*Aquilaria malaccensis*) by using silica gel as stationary phase and a polar solvent [Ethyl acetate: methanol: water (8:1:1, v/v) as mobile phase. And then sprayed using AlCl₃ showed the presence of active flavanoids in the extract and methanol: water fraction, the ethyl acetate fraction under UV light at λ 365 nm in the form of yellow spots. Detection by using a reagent of FeCl₃ and observed under visible light showed green spots, which means the presence of active phenolics.

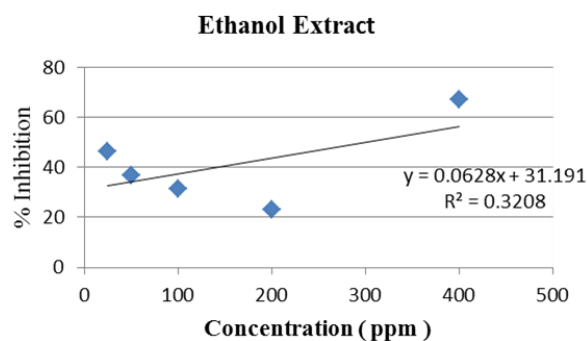


Figure 4: Effect of extracts on inhibition of cancer cell growth (HeLa Cell)

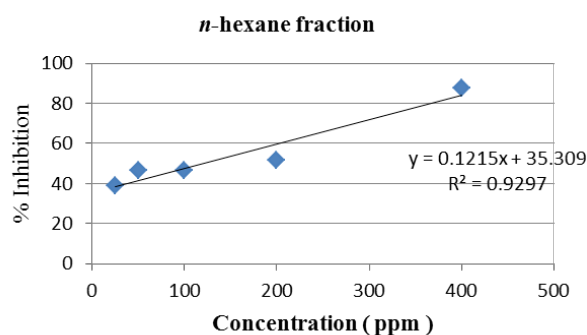


Figure 5: Effect of *n*-hexane fraction on inhibition of cancer cell growth (HeLa Cell)

Cytotoxic Activity Test for HeLa Cell breast cancer cells

Cytotoxic tests were carried out to determine the toxicity of Agarwood (*Aquilaria malaccensis* Lam) leaves on HeLa cells by the MTT assay. The principle of the MTT assay is a redox reaction in the cell. The reaction was carried out by reduced the tetrazolium salt (yellow) using an enzyme succinate dehydrogenase as a catalysator to produces formazan crystals (purple). Formazan crystals were absorbed by using an ELISA reader at a wavelength of 550 nm. On the curve (Figure 4, Figure 5, Figure 6, Figure 7),

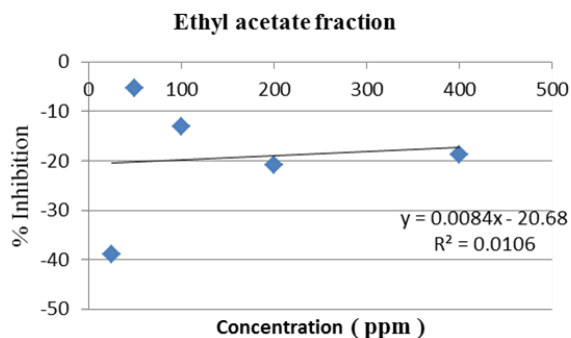


Figure 6: Effect of ethyl acetate fraction on inhibition of cancer cell growth (HeLa Cell)

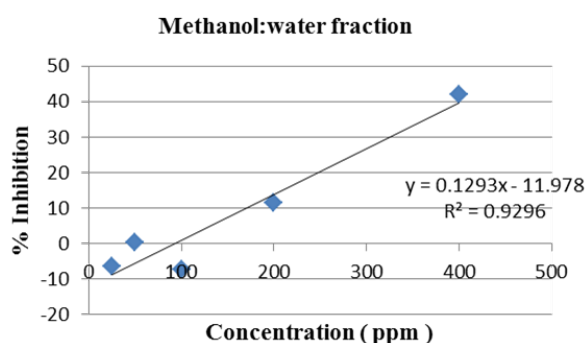


Figure 7: Effect of methanol: water fraction on inhibition of cancer cell growth (HeLa Cell)

the value of *y* shows the inhibition of cancer cell growth of cytotoxic activity of the extract, *n*-hexane fraction, ethyl acetate fraction, and methanol: water fraction of Agarwood leaves (*Aquilaria malaccensis* Lam) which then calculated the IC₅₀ value of each sample with a value of 50 (Table 3).

Based on the IC₅₀ data processing, it is obtained that the *n*-hexane fraction of Agarwood leaves (*Aquilaria malaccensis* Lam) has the best cytotoxic activity compared to ethyl acetate fraction, methanol: water fraction and extracts. The lower the IC₅₀ value produced, the better the cytotoxic activity test results. The *n*-hexane fraction falls into the moderate or quite active category refer to the cytotoxicity category that can be seen in the table below (Table 4).

CONCLUSION

The *n*-hexane fraction of Agarwood leaves (*Aquilaria malaccensis* Lam) has cytotoxic activity with an IC₅₀ value of 120.913 (μ g / mL) and is categorized as moderate or quite active against Serviks Cancer Cells that cause breast cancer cells.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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