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Cytotoxic activities of extract and fraction of agarwood leaves (*aquaria malaccensis* Lam) as herbal medicine on MCF-7 cancer cells

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ABSTRACT

MCF-7 cell is a cell line widely used in studied breast cancer. Breast cancer is one type of cancer that has a high prevalence in the world, including Indonesia. Agarwood leaves (*Aquilaria malaccensis Lam*) is one of the plants known to possess antioxidant activity. This study aims to determine the cytotoxic activity of extracts and fractions of Agarwood leaves (*Aquilaria malaccensis Lam*) against MCF-7 cancer cells. Agarwood leaves (*Aquilaria malaccensis Lam*) were extracted using the maceration method with 96% ethanol and followed by fractionation using *n*-hexane, ethyl acetate, and methanol: water as a solvent. Phytochemical screening test results Agarwood leaves extract (*Aquilaria malaccensis* Lam) contains alkaloid compounds flavonoids, quinones, tannins, saponins, steroids and triterpenoids. The results of thin-layer chromatography monitoring showed that the extract and fraction of Agarwood leave hexane had active flavonoid compounds. The extracts and its fractions were tested for cytotoxic activity against MCF-7 cell line using *MTT Assay* [*3- (4,5 dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide*]. The results showed IC_{50} values for extract, methanol:water fraction, ethyl acetate fraction, and *n*-hexane fraction were 728.412 μg/mL, 505.026 μg/mL, 1881.482 μg/mL, and 144.458 μg/ mL, respectively. The *n*-hexane fraction of Agarwood leaves (*Aquilaria malaccensis Lam*) has the best cytotoxic activity against MCF-7 cancer cells and has the potential to be developed as an anti-cancer herbal medicine.

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INTRODUCTION

There were 18.1 million new cancer cases worldwide in 2018, where 11.6% of the cases were lung cancer, and 10.2% of the cases were breast cancer (Global Cancer Observatory, 2018). Breast cancer ranks first in women contributing about 24.2% and causes the death of approximately 15.2% (Sobin, 2009).

Cancer treatments can be done with surgery, chemotherapy, or radiation. Chemotherapy is conduct by giving anti-cancer drugs (cytotoxic) to destroy cells that cause cancer. Ethanol extract of Agarwood leaves (*Aquilaria malaccensis* Lam) has antioxidant activity with an IC_{50} value of 25.71 μg/mL (Khalil *et al.*, 2013).

This study aimed to investigate the cytotoxic activity of MCF-7 cell from extracts and fractions of Agarwood leaves (*Aquilaria malaccensis* Lam) using *MTT Assay*.

MATERIALS AND METHODS

The labware used in this study were Blender, glassware (Erlenmeyer, pellets, beaker glass (Pyrex), oven, rotary evaporator (Buchi), micropipette 100-1000 μL, 96-Well Plate, pH meter, vortex mixer, CO_2 incubators, inverted microscope, petri dish, and ELISA reader.

The materials used in this study were Agarwood leaves (*Aquilaria malaccensis* Lam). Technical grade solvents used were 96% ethanol, *n*-hexane, ethyl acetate, methanol: water, aqua dest, 25% ammonia, chloroform, 10% hydrochloric acid, 2N HCl, 1N NaOH, amyl alcohol and ether. *Dragendorff* reagent (pa), *Mayer* reagent (pa), *Liberman-Bauchard* reagent (pa), Fe (III) chloride (pa), *Steasny* reagent (pa), magnesium powder (pa), formaldehyde (pa), gelatin (pa), Dimethyl Sulfoxide (DMSO), MTT reagent, *Phosphate Buffer Saline*, MCF-7 breast cancer cells.

Determination of Agarwood Leaves

Agarwood leaves were obtained from Palembang and were determined by the Indonesian Institute of Science (LIPI Bogor) by certificate number of SK:2438 / IPH.1.01 / II.07 / XI / 2018.

Simplicia Characterization

The Simplicia characterization includes,

Determination of total ash content

A total of 2 grams of Simplicia powder was put into a silicate crucible that had been heated and weighed. Then slowly heated until the charcoal run out, cooled, and weighed. If in this way, the charcoal cannot be removed, then hot water is added, filtered through ash-free filter paper, then the remains of filtering and filter paper is heated in the same crucible. The filtrate was put into the crucible, evaporated, and heated to a constant weight. Ash content was measured against the weight of the simplicia powder and was expressed in % w/w (Harborne, 2013).

Determination of acid-insoluble ash content

The ash obtained from the determination content was boiled with 25 mL of dilute sulfuric acid for 5 minutes. The parts which were not soluble in acid were collected by filtering through sintered glass funnels or by ash-free filter paper, washed with hot water, heated until obtained a constant weight. Acid insoluble ash content was measured against the weight of the simplicia powder and expressed in % w/w (Harborne, 2013).

Determination of water-soluble extract

A total of 5 grams of dried simplicia powder was macerated for 24 hours with 100 mL of saturated chloroform water using a clogged pumpkin while being shaken repeatedly for the first 6 hours and then allowed to stand for 18 hours. The macerate was filtered, then 20 ml of the filtrate was taken to be evaporated to dryness in a round, flat-bottomed dish that has been weighed. Drying was carried out at 105°C until obtaining a constant weight. The water-soluble extract was calculated in per cent by the number of compounds that dissolve in water to the weight of initial simplicia (Harborne, 2013).

Determination of ethanol-soluble extract

A total of 5 grams of dried simplicia powder was macerated for 24 hours with 100 mL of 95% ethanol using a clogged pumpkin while repeatedly shaking for the first 6 hours and then allowed to stand for 18 hours. The macerate was filtered rapidly by avoiding the evaporation of ethanol, then 20 mL of filtrate was taken to be evaporated to dry in shallow flat dishes that have been weighed. Drying was carried out at 105°C until fixed weight. Levels were calculated in per cent of compounds dissolved in ethanol 95% against the weight of initial simplicia (Harborne, 2013).

Determination of drying shrinkage

The drying shrinkage of the *Aquilaria malacensis* Lam simplicia were determined using the *Moisture Balanc*e 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, 2013)

Phytochemical Screening

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

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 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, 1996).

Flavonoid Test

Many 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, 1996).

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, 1996).

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, 1996).

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 hydroxide. When it remains yellow means the presence of quinones (Farnsworth, 1996).

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, 1996).

Extraction

Agarwood leaves (*Aquilaria malaccensis* Lam) were made to fine powder first before the extraction process was carried out. The extraction was done using the maceration method with repetition three times each 24 hours using 96% ethanol solvent.

For each repetition, a 96% ethanol solvent was replaced. The extract obtained was then concentrated using a rotary evaporator until a thick extract was obtained. Then calculate the extract yield obtained (Indonesian Herbal Pharmacopoeia, 2008).

Fractionation

The thick extract obtained was dissolved in methanol: 20% water, then fractionation was carried out using the liquid-liquid extraction method (-hexane, ethyl acetate, and methanol: water) by threefold repetition.

The fractionation results from *n*-hexane, ethyl acetate, and methanol: water obtained was collected and concentrated using a rotary evaporator.

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 (-hexane: ethyl acetate (9:1, v/v)) as mobile phase. The spot on TLC surface was sprayed with 5% $AlCl_3$, 10% $FeCl_3$, and 10% H_2SO4 (Indonesian Herbal Pharmacopoeia, 2008).

Test of MCF-7 breast cancer cell cytotoxic activity using MTT Assay method

Cell culture media was made, growing cells, cell subculture, and cell counts. 100 μL of cells were put into 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 CO_2 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 *ELISA reader* at a wavelength of 550 nm. Furthermore, absorbance graphs were made after subtracting media control. Calculate live cell percentage and IC_{50} 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 quality 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

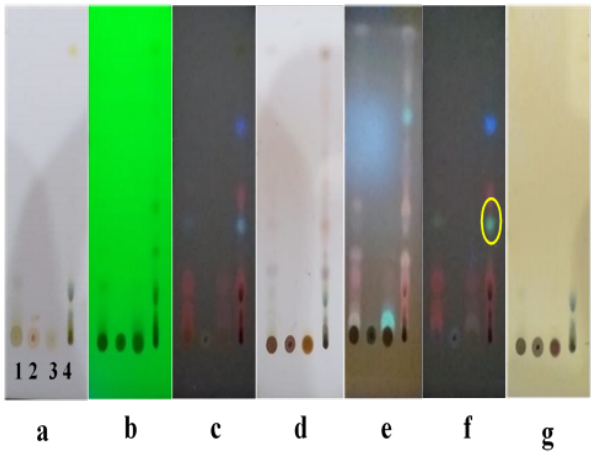

Figure 1: TLC of extracts and fractions by using n-hexane: ethyl acetate(9:1, v/v) as mobile phase

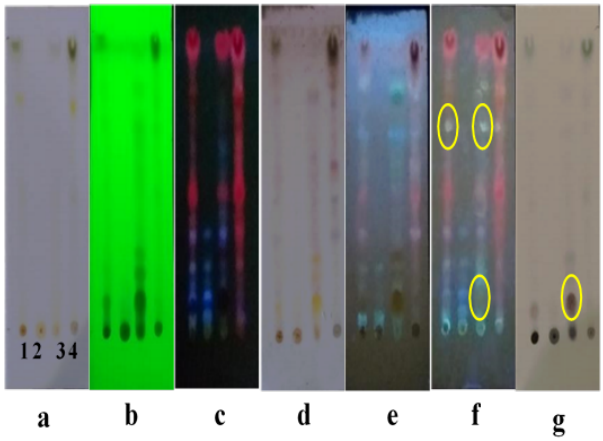

Figure 2: TLC of extracts and fractions by using chloroform: methanol (9:1, v/v) as mobile phase

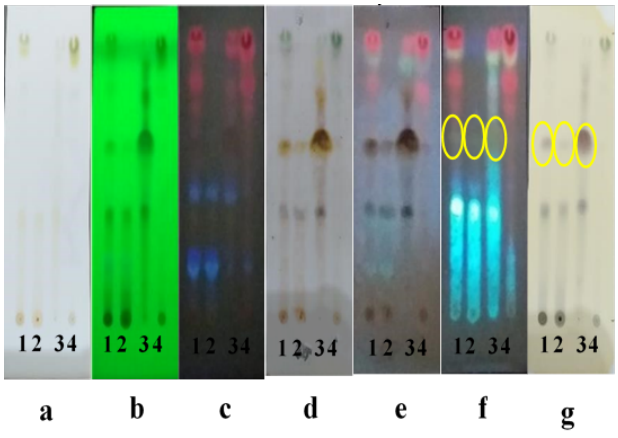

Figure 3: TLC of extracts and fractions by using ethyl acetate: methanol: water (8:1:1, v/v) as mobile phase

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 (Shoeb *et al.*, 2010).

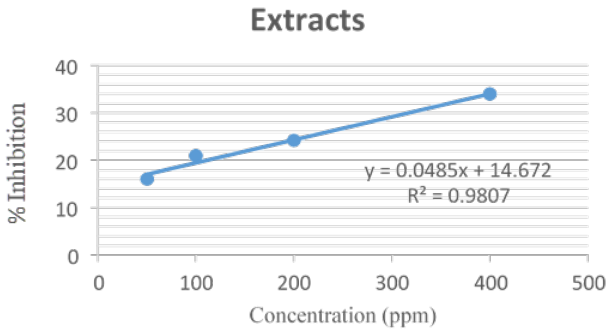

Figure 4: Effect of extracts on inhibition of cancer cell growth (MCF-7)

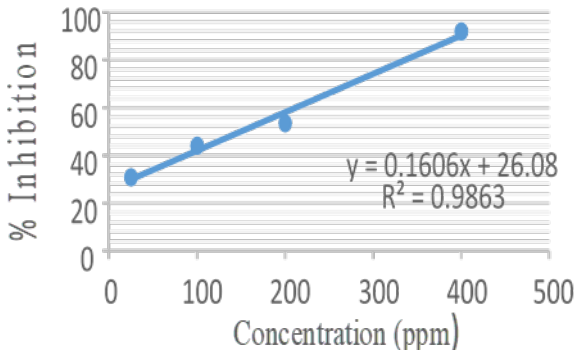

Figure 5: Effect of n-hexane fraction on inhibition of cancer cell growth (MCF-7)

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

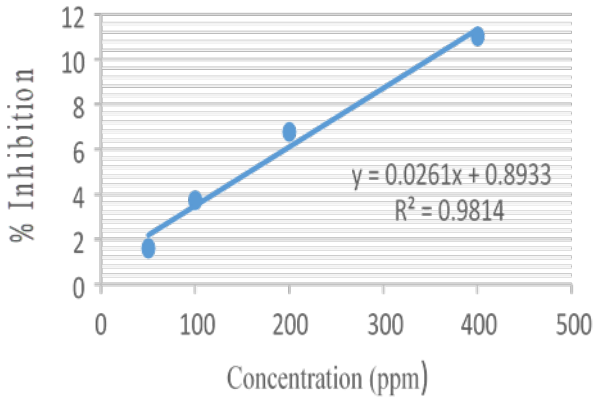

Figure 6: Effect of ethyl acetate fraction on inhibition of cancer cell growth (MCF-7)

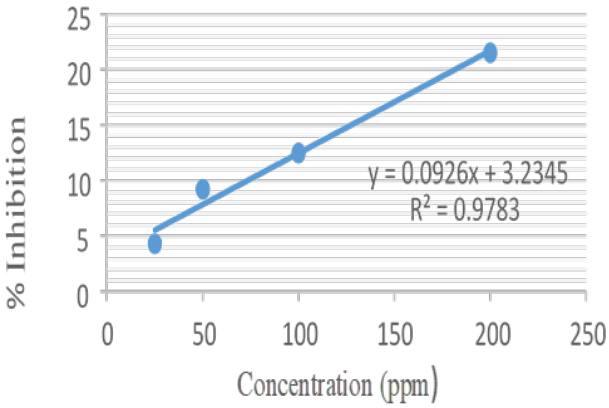

Figure 7: Effect of methanol: water fraction on inhibition of cancer cell growth (MCF-7)

as antioxidants.

Extraction

The extraction was carried out using the maceration method, which yielded 8.54% extract yield from 256.3 g thick extract and Simplicia weighing 3 kg.

Fractionation

The thick extract was dissolved in 20% methanol and fractionated using a liquid-liquid extraction method with different polarity properties using *n*-hexane, ethyl acetate, and methanol: water to obtain compounds that are soluble in non-polar, semi-polar and polar solvents to obtain compounds that are non-polar, semi-polar and non-polar.

Fraction yield obtained from the *n*-hexane solvent was 20.26%, the yield of ethyl acetate fraction was 12.46%, and the methanol: water fraction of 60.26%, which was obtained from 200 gr thick extract. The result of fraction yield showed many compounds from Agarwood (*Aquilaria malaccensis* Lam) leaves are dissolved in the methanol: water solvent and may contain many compounds that have polar properties.

Table 1: Simplicia characterization results and extract density

Test	Result (%) w/w
Total ash content	3.92
Acid insoluble ash content	1.17
Ethanol-soluble extract	9.27
Water-soluble extract	8.95
Drying shrinkage	9.85
Density	0.885 w/v

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 (Figures 1, 2 and 3). The TLC system was carried out by using silica gel as stationary phase and polar solvent as mobile phase (ethyl acetate: 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.

TLC spot evaluation of extracts, *n*-hexane fraction, ethyl acetate fraction and methanol: water fraction of Agarwood leaves (Aquilaria malacensis) 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 $AlCl_3$ 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 $FeCl_3$ did not show black spots when observed under visible light (Figure 2).

TLC spot evaluation of extracts, *n*-hexane fraction, ethyl acetate fraction and methanol: water fraction of Agarwood leaf (*Aquilaria malacensis*) by using silica gel as stationary phase and a semi-polar [chloroform: methanol (9:1, v/v)] as mobile phase and sprayed using $AlCl_3$ 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 $FeCl_3$ showed black spots when observed under visible light, which shows the presence of active phenolics (Figure 3).

TLC spot evaluation of extract, *n*-hexane fraction, ethyl acetate fraction, and methanol: water fraction of Agarwood leaves (*Aquilaria malacensis*) 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 $AlCl_3$ showed

Table 2: Phytochemical screening results

Compound	Test	Results	Description
Alkaloids	Filter Paper	Orange Precipitate	+
	Mayer	White Precipitate	+
	Deggendorf	Brick-red Precipitate	+
Flavonoids	+ Amyl Alcohol	Yellow-orange in the amyl alcohol layer	+
Tannin	FeCl3	Blackish green	+
	Gelatin	White precipitate	+
	Stasny	Pink after heating	+
Quinones	NaOH	Red	+
Saponins	2 N HCl	Stable foam	+
Steroids/ Triterpenoids	LiebermanBuchard	Bluish-green	+

Note:+ (detected)

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 $FeCl_3$ and observed under visible light showed green spots, which means the presence of active phenolics.

Table 3: IC_{50} values of MCF-7 cancer cells forextract and its fraction

Samples	IC_{50} Value (μg/mL)
Extract	728.412
Methanol-water fraction	505.026
Ethyl acetate fraction	1881.482
-hexane fraction	144.458

Note: IC_{50} value = sample concentration needed to inhibit 50% of MCF-7 cancer cells

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

	IC_{50} (μg/mL)	IC_{50} value (μg/mL)
1	≤ 20	Very toxic
2	21 < IC50 < 200	Moderate / quite active
3	201 < IC50 < 500	Weak
4	>500	Not toxic

Cytotoxic Activity Test for MCF-7 breast cancer cells

Cytotoxic tests were carried out to determine the toxicity of Agarwood (*Aquilaria malaccensis* Lam) leaves on MCF-7 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 *ELISA reader* at a wavelength of 550 nm. On the curve (Figures 4, 5, 6 and 7), 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 IC_{50} value of each sample with a value of 50 (Table 3).

Bed on the IC_{50} 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 IC_{50} 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).

CONCLUSIONS

The *n*-hexane fraction of Agarwood leaves (*Aquilaria malaccensis* Lam) has cytotoxic activity with an IC_{50} value of 144.458 μg / mL and is categorized as moderate or quite active against MCF-7 cancer cells that cause breast cancer cells.

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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