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Phenolic Content, Antioxidant, Cytotoxic of Fractions of Spatholobus Littoralis Hassk from Kalimantan, Indonesia

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Abstract: Spatholobus littoralis Hassk (SLH) plant grows wild in the forests of the island of Kalimantan. This plant has the potential as an antioxidant and anticancer. The current research was to quantify the total phenol content, measure the antioxidant and anticancer power against breast cancer cells in non-polar (hexane), polar (water), and semi-polar (ethyl acetate) fractions from Hassk littoralis spatholobus wood. Phenol content was determined with gallic acid as standard. Antioxidant activity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, the IC_{50} value was determined. Anticancer strength against breast cancer cells was determined using 4T1 cell culture. Toxicity to normal cells was analyzed using Vero cells. The selectivity index (SI) was calculated by dividing the IC_{50} of Vero cells by the IC_{50} of 4T1 cells. The results showed that total phenol for ethanol extract and water fraction were 350 mg and 146.9 mg equivalent to gallic acid mg/50 g SLH. The IC50 values of antioxidant were 198.76 ppm, 349.89 ppm, and 2.17 ppm for the hexane, water, and ethyl acetate fractions respectively. Breast anticancer assay with cell line 4T1 showed that the IC50 values of the hexane fraction and fraction of ethyl acetate in sequence were 20.0 mcg/mL and 7.4 mcg/mL. Toxicity to Vero cells (cell no cancer) showed the IC50 value for the hexane and ethyl acetate fractions were 131.52 mcg/mL and 79.55 mcg/mL respectively. SI for hexane fractions and ethyl acetate fractions were 6.57 and 10.75 respectively. In summary, the SLH plant has the potential as an antioxidant and anticancer of the breast and can continue in further research. The novelty of this study is the quantification of phenol content, the strength of antioxidant activity, and breast cancer in vitro.

Keywords: spatholobus littoralis hassk, phenol content, breast anticancer, selectivity index.

来自印度尼西亚加里曼丹的石鳖组分的酚类含量、抗氧化剂和细胞毒性

摘要：石鳖植物生长在加里曼丹岛的森林中。这种植物具有抗氧化和抗癌的潜力。目前的研究是量化总酚含量，测量来自山楂木的非极性（己烷）、极性（水）和半极性（乙酸乙酯）级分中对乳腺癌细胞的抗氧化和抗癌能力。以没食子酸为标准测定苯酚含量。采用2,2-二苯基-1-苦基肼法测定抗氧化活性，测定半数最大抑菌浓度值。使用4T1细胞培养物确定针对乳腺癌细胞的抗癌强度。使用维罗细胞分析对正常细胞的毒性。通过将维罗细胞的半数最大抑菌浓度除以4T1细胞的半数最大抑菌浓度计算选择性指数。结果表明，乙醇提取物和水馏

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分的总酚分别为350毫克和146.9毫克，相当于没食子酸毫克/50公克山楂。对于己烷、水和乙酸乙酯馏分，抗氧化剂的半数最大抑菌浓度值分别为198.76百万分之几、349.89百万分之几和2.17百万分之几。4T1细胞系乳腺癌抗癌试验表明，己烷组分和乙酸乙酯组分的半数最大抑菌浓度依次为20.0微克/毫升和7.4微克/毫升。对维罗细胞（无癌细胞）的毒性显示，己烷和乙酸乙酯部分的半数最大抑菌浓度值分别为131.52微克/毫升和79.55微克/毫升。己烷馏分和乙酸乙酯馏分的SI分别为6.57和10.75。总之，山楂植物具有作为抗氧化剂和抗乳腺癌的潜力，可以继续进行进一步的研究。这项研究的新颖之处在于对苯酚含量、抗氧化活性强度和体外乳腺癌的量化。

关键词：山楂、酚含量、乳腺癌抗癌、选择性指数。

1. Introduction

Plants have been used as medicine by humans for thousands of years. People still use medicinal plants as a source of antioxidants, anticancer, antiviral, antibacterial, and antidote to other diseases [1]. In developing countries, 80 percent of people still depend on medicinal plants to treat diseases and health problems [2]. People are increasingly aware of maintaining health by consuming ingredients from nature that contain antioxidant compounds, antivirals, immune boosters, anticancer, and other active compounds. This awareness has increased sharply, especially during the COVID-19 coronavirus pandemic [3]. The number of herbal purchases during the corona pandemic in Indonesia has increased. This is evidenced by a study conducted in the province of South Kalimantan, Indonesia. The results stated that during the pandemic, 88% of the people consumed products from medicinal plants, namely jamu gendong. Likewise, the production of jamu gendong has increased by 100% [4]. Likewise, in general, in the whole of Indonesia, public consumption of herbal medicines increased by 41% [5]. The popularity of herbal medicines can be boosted by people's experience after consuming and feeling the benefits, besides that it is also supported by scientific data and clinical trials [6]. Although herbal medicines are widely used, the absence of information about the toxicity of herbal medicine can lead to misuse. Thus, the presence of toxicity data will make users feel safe [7].

One of the plants that can be used as traditional medicine is the genus spatholobus. Plants of the genus spatholobus have many bioactivities. Several publications mention that this genus of plants has efficacy as a hypoallergenic [8], antimicrobial [9, 10], antituberculosis [11], radical scavenging [12], antioxidant [13], cytotoxic [14], antimicrobial [10, 15] and anticancer [16]. Antioxidants are substances in small amounts that can delay or block the oxidation of the substrate[17]. Antioxidants play an important role in the prevention and treatment of cancer. Cancer therapy using drugs can cure. However, the drug's effectiveness is limited to a few deleterious side effects. Therefore, to erode the negative effects of a drug, the consumption of antioxidants is often necessary. In addition, antioxidants can also reduce the active substance and minimize persistent oxidative damage [18]. However, the exact number of doses given to patients with cancer must be taken seriously [19]. Medicinal plants with high phenolic content are thought to provide antioxidant activity and make a significant contribution to the fight against pathological conditions such as cancer, diabetes, aging, cardiovascular, and other degenerative diseases [20].

Breast cancer is a disease caused by tumors that are the most common in women and the most malignant, and the highest cause of death in more than 100 countries in the world [21]. Based on Globocan 2020, breast cancer patients in Indonesia reached 65,858 new cases and 22,430 deaths [22]. The process that causes failure in treatment due to breast cancer and the death of cancer sufferers is metastatic. Metastatic breast cancer is influenced by two factors, which include intrinsic and extrinsic. Intrinsic are MYC, PI3KA, and TP53 mutations. Extrinsic factors include hypoxia, oxidative stress, and acidosis [23]. To determine the efficacy of a plant drug against metastatic breast cancer, it can be done by in vitro assay using cell lines such as 4T1 [24]. Cell line 4T1 is one of the cell models that is widely used as a representative of breast cancer cells [25].

One species of the genus Spatholobus in Indonesia is Spatholobu littoralis Hassk (SLH). This plant grows wild in the forests of the island of Kalimantan. Previous research stated that the ethanol extract of this plant has antioxidant activity with an IC50 value of 8.25 g/ml using the DPPH method [26]. In addition, anticancer test of water extract of spatholobus littoralis hassk wood using several cell cultures showed IC_{50} 1,063.28 g/mL (MCF7), 53.34 g/mL (HepG2), 150.63

g/mL (T47D), 114.38 μg/mL (WiDR), 97.50 μg/mL (HTB), 182.95 μg/mL (HeLa), and 710.10 μg/m (Vero) [27]. In this study, we have carried out total phenol assays, antioxidant, anticancer, and toxicity tests in vitro in three groups of non-polar, semi-polar, and polar solvents.

2. Materials and Methods

2.1. Sample Collection

SLH plants were obtained from the Lamandau district, Central Kalimantan Province, Indonesia and identified in the taxonomy laboratory of the Department of Biology at the University of Tanjungpura on March 3, 2021. The sample used is the wood part. The wood is chopped and made into powder at the Materia Medica laboratory in Batu, Indonesia.

2.2. Chemicals

Materials with analytical grades used include distilled water, methanol, dragendorff, mayer, wagner, salkowski, froath, ferric chloride, gelatin alkaline, lead acetate, modified borntrager, molisch, benedict, fehling a&b, ninhydrin, ciulei, gallic acid, Folin–Ciocalteu Reagent, Sodium carbonate, diphenyl picrylhydrazin (DPPH) and ethanol. Technical solutions include ethanol, ethyl acetate, and hexane.

2.3. Preparation of SLH Extracts

Spatholobus littolaris Hassk Plant identification was carried out in the Biology laboratory of Tanjung Pura University (0.0606° S and 109.344056° E, West Borneo, Indonesia) on March 3, 2021. 2.7 kg SLH wood powder was macerated with technical ethanol (96%), concentrated with a rotary evaporator, and dried in a water bath at 50 Celsius. Obtained dry crude extract as much as 97 grams. The dry extract was crushed in a mortar, dissolved in distilled water, and sonicated into a suspension. Then in the first step, 30 grams of the dry extract was fractionated using the liquid-liquid partition method using water to hexane ratio of 3:1. Then in the second stage, the water fraction was partitioned with ethyl acetate with a ratio of 3:1. In the third stage, the water fraction is obtained. Each fraction was concentrated and dried and obtained 3.7 g, 4.9 g, and 7 g of hexane, ethyl acetate, and water respectively.

2.4. Analysis

2.4.1. Qualitative Phytochemical Screening Tests

The method used was adapted with slight modifications [28]. These qualitative tests include alkaloids, phytosterol, saponins, phenols, tannins, flavonoids, glycosides, carbohydrates, protein & amino acids, and triterpenoids.

2.4.2. Total Phenol Content Test

The method used was adapted with slight modifications [29].

2.4.2.1. Standard Calibration Curve

Weighed 50 mg of gallic acid, added 1 mL of 96% ethanol, then added distilled water until the final volume was 50 mL so that a concentration of 1 mg/mL was obtained. From the main solution of gallic acid with a concentration of 1 mg/mL pipette 1 mL, 2 mL, 3 mL, 4 mL, and 5 mL respectively and then diluted with distilled water to a final volume of 10 mL to produce concentrations of 100, 200, 300, 400 and 500 ppm. gallic acid, respectively. From each concentration of the gallic acid solution, 0.1 mL was pipetted and then 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent were added and shaken until homogeneous and allowed to stand for 8 minutes. 1.5 mL of 10% Na2CO3 solution was added then shaken homogeneously and then allowed to stand for 2 hours at room temperature. Measure the absorption at a maximum absorption wavelength of 765 nm, then a calibration curve was made for the relationship between gallic acid concentration (μg/ml) and absorption.

2.4.2.2. Total Phenol Content

Each ethanol extract and water extract weighed 100 mg and dissolved to 10 mL with distilled water to obtain a concentration of 10 mg/mL. 1 mL was taken and diluted with distilled water to 10 mL to obtain an extract concentration of 1 mg/mL. Dipiper 0.2 mL of extract and add 15.8 mL of distilled water and 1 mL of Folin-Ciocalteu reagent, then shaken. Let stand for 8 minutes and then add 3 mL of 10% NaCO3 to the mixture. Allow the mixture to stand for 2 hours at room temperature. Absorption was measured using a UV-Vis spectrophotometer at a wavelength of 765 nm for 3 replications to obtain the phenol content as mg gallic acid/mg material.

2.4.3. In Vitro Antioxidant Activity Test

The method used was adapted with slight modifications [30].

2.4.3.1. DPPH Preparation

Weighed 2 mg of DPPH, dissolved in 50 ml of ethanol, incubated for 30 minutes in a dark room at room temperature, and measured at a wavelength of max.

2.4.3.2. Sample Preparation

The hexane, ethyl acetate, and water fractions were weighed 10 mg each., dissolved in 10 ml of ethanol, made with concentrations 62.5 ppm, 125 ppm 250 ppm, 500 ppm, and 1000 ppm. Furthermore, incubated for 30 minutes in the dark at room temperature. Each

fraction was added DPPH solution with a ratio of 1:1, absorbance was measured using a spectrophotometer at a wavelength of max (712 nm) and calculated % inhibition using with this equation:

$$\%\text{Inhibition} = \frac{A_{control} - A_{sampel}}{A_{control}} x100\% \quad (1)$$

The standard linear curve is created y=ax+b and calculated IC_{50} value (y=50).

2.4.5. In Vitro Cytotoxicity Assay

The method used was adapted with slight modifications [31]. Breast cancer cells (line 4T1) and Vero cells were cultured using RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% fungal zone. Then, the cells were inoculated in a 5% CO_2 incubator for 24 h (37 C; 95% relative humidity). Next, 8×103 cells in 100 L of the compound were isolated in the test solution (31,25-500 g/mL), then incubated for 24 hours. Next, the cells were washed with PBS and added 100 L of MTT reagent/well, incubated for 4 hours at 37 °C. After that, 100 L of 10% SDS solution was added/well and incubated overnight at room temperature in a dark room, then determined. absorbance with a microplate reader at 595 nm. Single treatment data were converted to percent viability and used to calculate IC50. Percent cell viability is calculated using the following equation:

$$\%\text{Cell viability} = \frac{A_{sampel}}{A_{control}} x100\% \quad (2)$$

2.4.6 Selectivity index (SI)

SI value is determined based on the following equation [32, 33]:

$$SI = \frac{IC_{50}^{\text{no cance cell}}}{IC_{50}^{\text{cancer cell}}} \quad (3)$$

2.5 Statistical Analysis

Quantitative experiments used measurements three times and the average value was taken. Linear regression equations and graphs using Microsoft Excel tools.

3. Results and Discussion

3.1 Qualitative Phytochemical Content and Total Phenol Content

Table 1 shows Qualitative phytochemical screening test results where (-) = absent, (+) = present, SLH = *Spatholobus littoralis Hassk* Bark. Table 2 and Fig. 1 show standard calibration curve.

Table 1 Qualitative phytochemical screening test results

no	Metabolites	test	SLH extract			
			hexane	Ethyl acetate	ethanol	aquades
1	Alkaloids	dragendorff	(-)	(-)	(-)	(-)
		Mayer	*(-)*	*(-)*	*(-)*	*(-)*
		wagner	*(-)*	*(-)*	*(-)*	*(-)*
2	phytosterol	salkowski	*(-)*	*(+)*	*(-)*	*(-)*
3	Saponin	Froath	*(-)*	*(-)*	*(+)*	*(+)*
4	Phenol	Ferric chloride	*(-)*	*(-)*	*(+)*	*(+)*
5	Tannin	gelatin	*(-)*	*(-)*	*(-)*	*(-)*
6	Flavonoid	alkaline	*(-)*	*(-)*	*(-)*	*(-)*
7	Flavonoid	lead acetat	*(-)*	*(-)*	*(+)*	*(+)*
8	Glycosides	Modified borntrager	*(-)*	*(-)*	*(-)*	*(-)*
9	carbohydrates	Molisch	*(-)*	*(-)*	*(-)*	*(-)*
		Benedict	*(-)*	*(-)*	*(-)*	*(-)*
		Fehling A&B	*(-)*	*(-)*	*(-)*	*(-)*
10	Protein & amino acids	Ninhydrin	(-)	(-)	(+)	(+)
11	triterpenoids	Ciulei	(-)	(-)	(+)	(+)

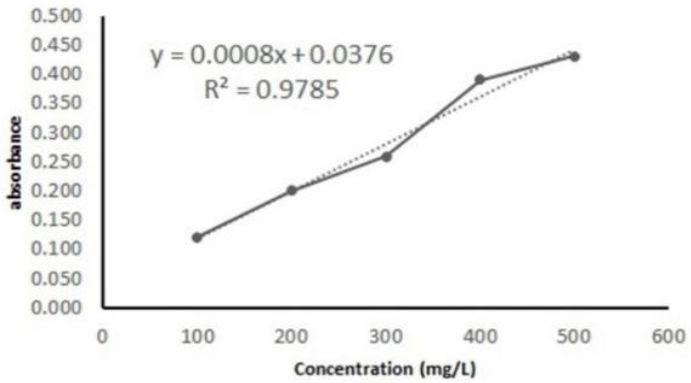

Fig. 1 Standard calibration curve

Table 2. Standard calibration curve

ppm	Absorbance
100	0.121
200	0.200
300	0.259
400	0.390
500	0.430

The absorbance of the ethanol extract sample was measured three times, namely 0.317; 0.318, and 0.317. So the average absorbance of the ethanol extract sample was 0.317. Measurements of water extract absorbance were 0.133; 0.133 and 0.134. So that the average absorbance of the aqueous extract. The sample was 0.133.

3.2. Antioxidant Activity

Fig. 2 and table 3 elaborate antioxidant activity.

Table 3 Antioxidant test of fractions

Fractions	IC_{50} (ppm)
Hexane	198.76
Water	349.89
Ethyl acetate	2.17

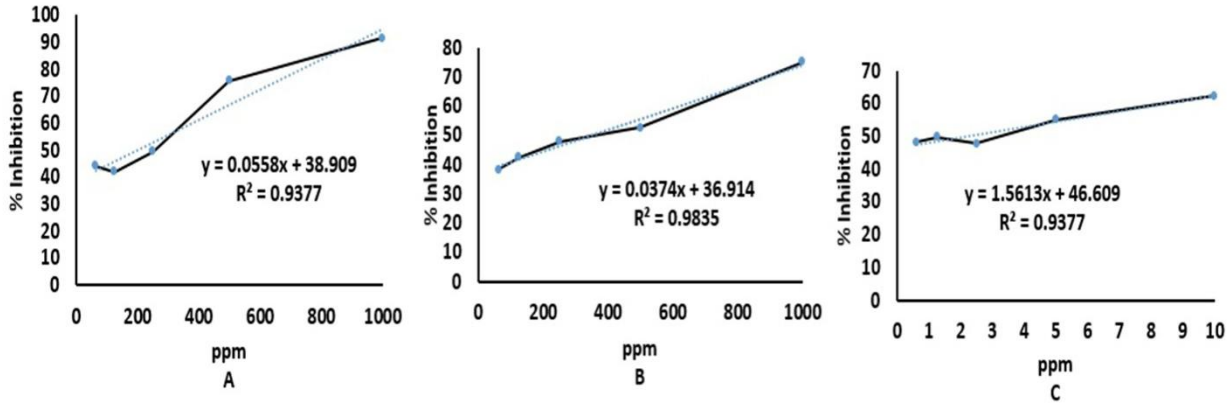

Fig. 2 DPPH Standard curve of A (hexane fraction), B (water fraction), and C (fraction of ethyl acetate)

3.3. Breast Anti-Cancer Bioactivity

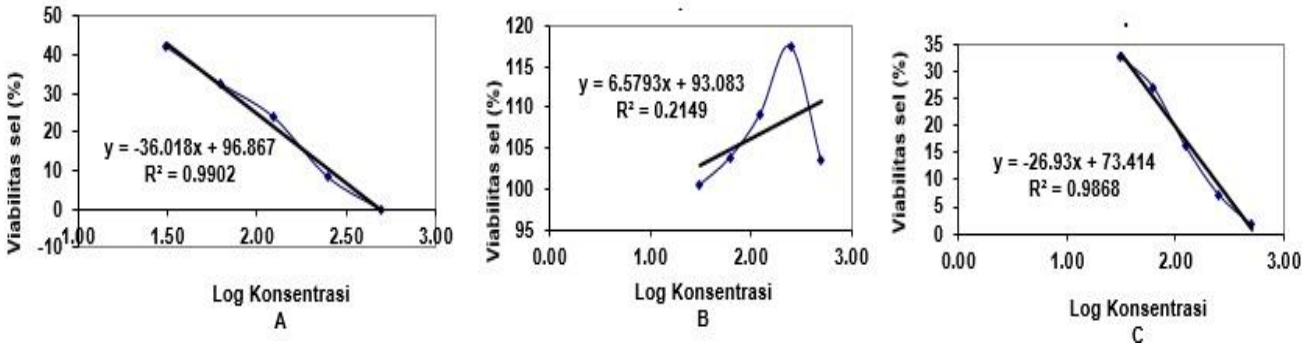

Fig. 3 Breast anti-cancer test of fractions (A: hexane; B: water; C: ethyl acetate) against 4T1 cell line

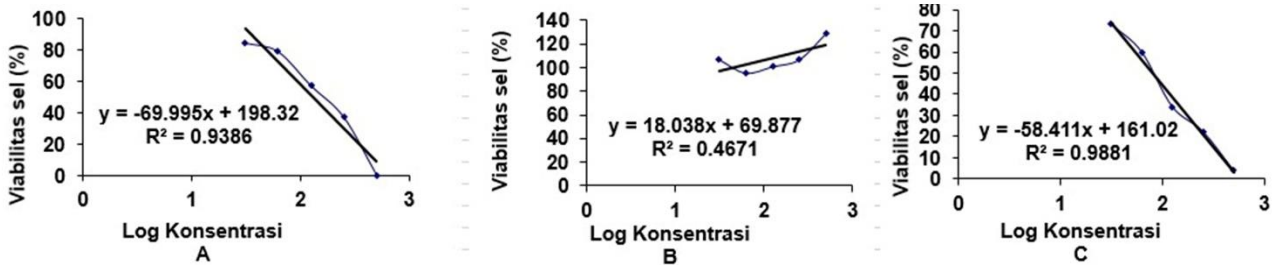

Fig. 4 Cytotoxic test of fractions (A: hexane; B: water; C: ethyl acetate) against Vero cell line.

Table 4 Breast anticancer bioactivity of fractions

Fractions	IC_{50} (mcg/mL)		Selectivity Index
	4T1 Cell line	Vero cell	
Hexane	20.0	131.52	6.57
Water	—	—	—
Ethyl acetate	7.4	79.55	10.75

Fig. 3-4 and table 4 elaborate breast anti-cancer bioactivity. Extraction can be done from solid to liquid, from liquid to liquid, and so on. This process transfers a collection of compounds from one phase to another that is physically separated. This is where further process analysis can be carried out [34]. Ethanol and hexane are miscible and indistinguishable from each other.[35]. For this reason, it is not possible to obtain a non-polar fraction such as hexane if the dry extract is first dissolved in ethanol. Thus, by taking advantage of the water's reluctance to mix with hexane, the dry extract of SLH was first dissolved in water to obtain the non-polar (hexane) fraction easily. Furthermore, after the hexane fraction is separated, the water fraction can be partitioned with ethyl acetate solvent which is semi-polar and easily separated from the water fraction, although it requires a long separation time than the separation of the water fraction and hexane fraction.

SLH wood extract qualitatively contained phytosterols in the fraction of ethyl acetate, saponins in the (ethanol and water) fraction, phenol in the ethanol and water fractions, proteins & amino acids in the ethanol and water fractions, and triterpenoids in the ethanol and water fractions. Based on a qualitative phytochemical test, only the ethanol extract and water fraction contained phenol. By using equation y = 0.0008x+0.0376 (Fig.1) and inserting the absorbance values, total phenol for ethanol extract and water fraction are 350 mg and 146.9 mg equivalent to gallic acid mg/50 g SLH respectively. many accept the opinion that a high total phenol content does not necessarily have a high antioxidant activity as well. This is because each extract has a different capacity of electron donor [36]. The absence of a relationship between the total phenol content and antioxidants can be understood from several perspectives. First, the total phenolic fraction has not combined all the antioxidant compounds. Second, the synergistic interaction between antioxidant compounds in the mixture does not have to depend on the concentration of the mixture but can depend on the structure and properties of the antioxidants themselves. Third, the phenolic content of plants depends on several genetic, environmental, and processing factors [37].

By observing the DPPH standard curve, the hexane and water fractions were able to detect the absorbance value of the solution with concentrations between the ranges of 63.5 – 1000 ppm. In contrast to the fraction of ethyl acetate, it can only be detected in the range of 1-10 ppm. Entering 50 as y from the regression equation for each curve, the IC_{50} can be obtained. The IC_{50} values were 198.76 ppm, 349.89 ppm, and 2.17 ppm for the hexane, water, and ethyl acetate fractions respectively (Fig.2). Based on the IC50 value, the antioxidant power can be grouped into 5 categories. First, the activity is very strong if it is < 10 ppm, the activity is strong if it is between 10-50 ppm, the activity is moderate if it is between 50-100 ppm, less active if it is between 100-250 ppm, and inactive if it is > 250 ppm [38]. Thus, the hexane fraction has less active antioxidant activity, the water fraction is not active and the fraction of ethyl acetate is very strong. It is different from SLH ethanol extract, its antioxidant activity is very strong with an IC50 of 8.25 ppm [26].

In the breast anticancer test using the 4T1 cell line, the greater the concentration of the fraction, the lower the % viability. Because the more active substances in the fraction, the more 4T1 cancer cells will die. This trend can be seen in the experimental results of the hexane fraction and fraction of ethyl acetate. However, this does not apply to the water fraction. It may be that the anticancer substances in the ethanol extract have all shifted to non-polar (hexane) and semi-polar (ethyl acetate) fractions (Fig 3). Breast anticancer assay with cell line 4T1 showed that the IC_{50} values of the hexane fraction and fraction of ethyl acetate in sequence were 20.0 mcg/mL and 7.4 mcg/mL. several categories of extract cytotoxicity strength based on its IC50 value. very strong <10 mcg/mL, strong between 10-100 mcg/mL, moderate between 100-500 mcg/mL. There are three categories of extract cytotoxicity strength based on its IC50 value (very strong <10 mcg/mL, strong between 10-100 mcg/mL, and moderate between 100-500 mcg/mL) [39]. From these data (Table 4), the cytotoxicity of the hexane fraction is strong and the fraction of ethyl acetate is very strong. Toxicity to Vero cells (cell no cancer) showed the IC_{50} value for the hexane and ethyl acetate fractions were 131.52 mcg/mL and 79.55 mcg/mL respectively. The selectivity index is defined as the ratio of the toxic concentration of a sample to its effective bioactive concentration [40]. To evaluate the anticancer activity of a sample, the cytotoxicity to non-malignant cell lines must be calculated [32, 33]. SI for hexane fractions and ethyl acetate fractions were 6.57 and 10.75 respectively. Some researchers stated that a sample with SI value ≥ 10 then as the chosen one for further research [32]. Others argue that samples with SI values less than 10 and ≥3 are considered prospective anticancer [41]. Thus, both fractions deserve to be studied further and are prospective as herbs in terms of breast cancer anticancer perspective.

4. Conclusion

Measurable phenol content in ethanol extract and water fraction from SLH wood was 350 mg and 146.9 mg equivalent to gallic acid mg/50 g SLH. The strongest antioxidant activity was the fraction of ethyl acetate (IC50 2.17 ppm) followed by the hexane fraction (IC50 198.76 ppm) and the water fraction (IC50 349.89 ppm). Meanwhile, the greatest strength of breast anticancer activity in vitro was the fraction of ethyl acetate (IC50 7.4 mcg/mL) followed by the hexane fraction (IC50 20.0 mcg/mL). the toxicity of the

hexane and ethyl acetate fractions were 131.52 mcg/mL and 79.55 mcg/mL. so that the selectivity indices for both hexane fractions and ethyl acetate fractions were 6.57 and 10.75 respectively. Both the ethyl acetate and water fractions have anticancer activity in the breast that deserve to be studied further and in more depth.

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