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Isolation and Cytotoxic Activity Test Methyl Gallate Compounds from *Shorea Singkawang* Bark in P388 Murine Leukemia Cell

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ABSTRACT

In this research we has isolated and tested the activity of phenolic compounds methyl gallate group of ethyl acetate fraction from the bark of Shorea singkawang. Cytotoxic test was conducted using Brimp Shrimp Lethality Test (BSLT). Analysis and structure determination by spectroscopic methods UV, IR, GC-MS and NMR (1H-NMR, and ^{13}CNMR). From the test results showed cytotoxic ethyl acetate extract is toxic with LC_{50} = 2.05 µm/mL and bioactivity in P388 murine leukemia cell, IC_{50} value of 1.66 µm/mL. Meanwhile, test cyto-toxicity using fry shrimp Artemia salina Leach using Brine Shrimp Lethality Bioassay methyl gallate showed LC_{50} = 1.81 µm / mL is a compound that is toxic and can inhibit P388 murine leukemia cell with IC_{50} values below the compound its standard 4 µm/mL.

Keywords: phenolic, methyl error, cytotoxic, Brimp Shrimp Lethality Test (BSLT), P388 murine leukemia cells

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INTRODUCTION

Traditionally chemical nature of materials related to the isolation, structure determination and synthesis of organic compounds derived from natural sources. However, the isolation, structure determination, and not the end of the synthesis of chemical activity of natural materials. With the development of spectroscopic techniques at this time, the determination of the structure of natural bioactive compounds as a starting point. Empowerment and research chemical plants as a major source models, new bioactive molecules is one of the alternatives that can address and solve problems in the field of health in the present and the future. Chemical research plant who carried out a systematic, sustained and concerted should receive top priority in order to meet the needs of new bioactive materials in the fields of health, agriculture, bioindustry and others.

One of the research studies of the plant is Family *dipterocarpaceae*. This is due to a variety of chemical compounds, including terpenoids, arilpropanoid, flavonoids, phenolic and oligostilbenoid, contained therein. Oligostilbenoid, stilbene derivative which is one of the major polyphenols and unique and a lot of shows bioactivity useful, such as a chemopreventive, hepatoprotective, anti-inflammatory, antibacterial, antifugal, insecticidal and cytotoxic, HIV and anticancer [1].

Today has been believed that the formation of secondary metabolites in plants related to ecological function as the embodiment of that plant interactions with the environment. The durability of wood *Shorea singkawang* and tallow oil produced from the fruit/seeds showed their interactions. One of them is the bark of *Shorea singkawang* thought to contain secondary metabolites that function as an antibacterial, and anticancer antifugal.

Singkawang or red meranti Shorea is the largest genus of plants in the genus 16 *dipterocarpaceae* family. About 25 species have been studied chemical content since 1983 until 2010, producing 38 types of oligomer compound resveratrol. The diversity of the resveratrol molecule partly due to the difference in the amount of resveratrol constituent units, which include dimers, trimers, and tetramers resveratrol. In addition, the oligomers of resveratrol resveratrol with the same number of units, diversity of structures can occur due to differences in stereochemistry. Some oligoresveratrol, such as ε-viniferin [dimer] is active as an antibacterial, for example against Staphylococcus oxford and Escherichia coli [2] can induce apoptosis of HL-60 [3] and as an inhibitor of rat liver 5α-reductase. In addition, α-viniferin [trimer] is also active as antifungal, antioxidants [4] antibacterial against S. aureus [3], and as anti-inflammatory cytotoxic [4; 5], hepatoprotective and inhibit the enzyme acetylcholine esterase. In addition, some of the oligomer compound resveratrol also reported to inhibit the enzyme 5α-reductase and the enzyme acetylcholinesterase. Hopeafenol [tetramer] as antimicrobial active against Myicobacterium smegmatis and S. aureus and antioxidants as well as anti-inflammatory against leukotriene B4, anti-cancer.

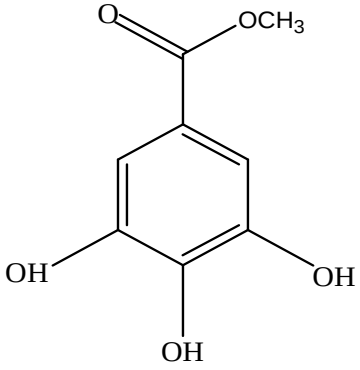
Bergenin compounds that exist in Shorea sumatrana also an antibacterial against P. aeruginosa bacteria [6]. Vatikanol B is in Shorea sumatrana also can inhibit its antibacterial S. aureus and Propionibacterium sp C. albicans and is an antioxidant. It has been reported also that *dipterocarpaceae* is the source of chemical compounds polyphenols that shows the bioactivity, as kemopreventive for cancer, antioxidant, cytotoxicity against human tumor cell, hepatoprotective, anti-inflammatory, inhibiting the spread of histamine STP-ase hull and topoisomerase II, antibacterial, antifungal, and anti-HIV [1]. Some oligoresveratrol are inhibitors of the activity of tyrosinase and turns compounds decreased with increasing the size of the oligomer [7].

While Hirano (2000) resulted in a patent that vatikanol A, ampelopsin C, melapinol melapinol A and B, of plant Shorea spp shows the nature of the 5α-reductase inhibitor, which is useful as a medicinal cosmetics, hair loss and acne. Based on the literature review is done, of course, there are many more other research that still needs to be done to plants or tallow Shorea singkawang the *dipterocarpaceae* family related to the functioning and its bioactivity, because they mirror the economic aspects of the active compound, which is very useful.

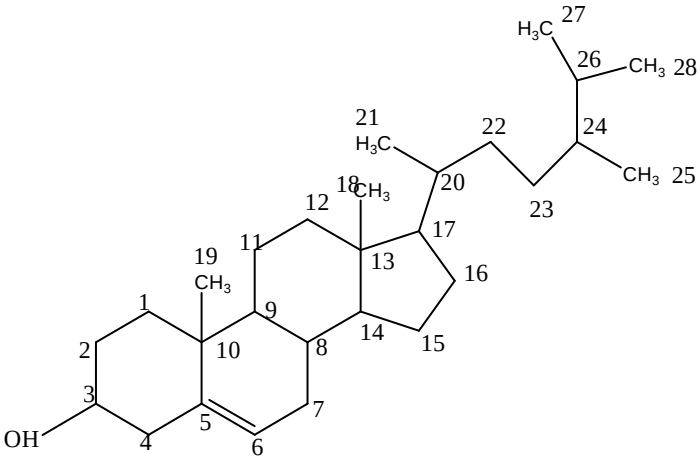
From the results of the preliminary study of the phytochemical bark plant Shorea singkawang identified postif contains flavonoids (orange, +), phenolic (purple black, +), coumarin stain of fluoroscence blue (+), triterpenoids and steroids (red H_2SO_4 anhydride, +).

Given the use of the bark of this diverse genus Shorea of bioactivity, it is very necessary scientific information for safety. For the safety of the use of the bark of *Shorea singkawang*, it is necessary to do the testing of cyto-toxicity. Testing extract, fractions and compounds bark of *Shorea singakwang* been done in vitro to larvae *Artemia salina* Leach with methods Brine Shrimp Lethality Bioassay (BSLB).

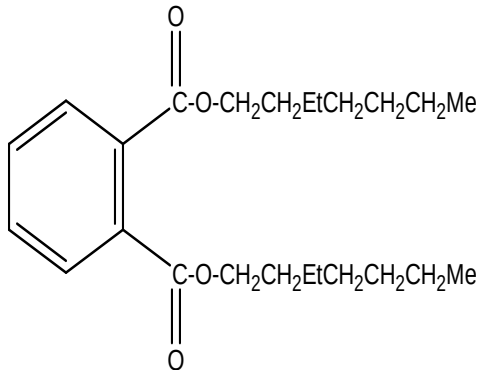
From the research results (as shown in Figure 1), was obtained methyl gallate (1) of ethyl acetate fraction; Campesterol (ergost-5-en-3-ol (3βeta) (2); bis (2-ethylhexyl) phthalate (3) of the fraction of n-hexane, and compound bergenin (dihidroisocoumarin) (4) of the fractions dichloromethane. Testing activity of the compound methyl gallate as anticancer against P388 murine leukemia cell values obtained LC_{50} = 2.05 the μm/mL and IC_{50} in the testing of murine leukemia cell, IC_{50} = 1.611 μm/mL that methyl gallate was highly active against murine leukemia cell, because under the standard values 4 μm/mL.



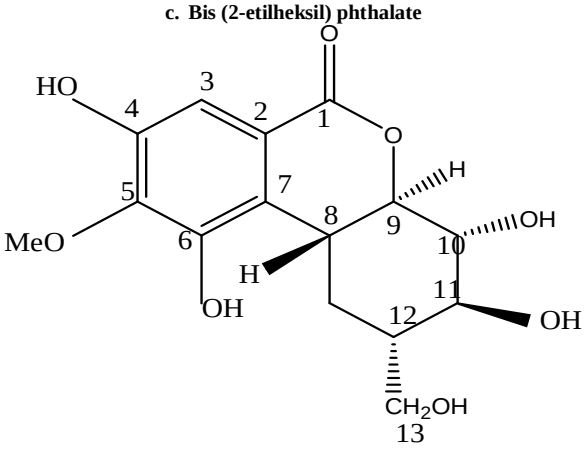
a. Methyl Gallate



b. Campesterol (ergost-5-en-3-ol (3βeta)



c. Bis (2-etilheksil) phthalate



***d.* Bergenin**

Figure 1. The resulting compound of Shorea Singkawang bark extract, among others, (a) methyl gallate, (b) campesterol, (c) Bis (2-ethylhexyl) phthalate and (d) Bergenin

MATERIALS AND METHODS

The methods used in isolating the plants are extracted by maceration. Maceration is a simple search process by soaking the powdered bark of Shorea singkawang with organic solvents for 3-5 days. The advantages of this maceration process is working techniques are simple and can be used for all types of samples, both wet and dry and are thermostable (8).

Then proceed with fractionation, which is the second stage of the process of separation of the compound. Fractionation is a separation technique and grouping extract chemical constituents by polarity. In the fractionation process used two solvents Incompatible have different levels of polarity. The compounds contained in extracts separated according to polarity (9), followed by thin-layer chromatography examination to know the group of compounds contained in the results of fractionation, separation and elution buffers suitable stain (10). Then, followed by recrystallization of the compound using a solvent that dissolves and a solvent that can not dissolve the sample, while the determination of the purity level using melting point, where the compound which has been pure has a difference of 2°C temperature difference. Characterization followed by measurement of UV-Vis spectroscopy, FTIR, GC-MS, ^{1}HNMR, ^{13}CNMR and DEPT. And as anticancer bioactivity testing, using the P388 murine leukemia cells.

Extraction and Isolation

The bark of the *Shorea singkawang* (5.0 kg), macerated with methanol (20 L) 3 x 24 hours. After the solvent was evaporated at low pressure, gained as much as 380 g thickened extract, in the form of red-brown colored residue. Then about 250 g of condensed methanol extract fractionation performed successively by using an organic solvent n-hexane, dichloromethane and ethyl acetate. Further, the solvent evaporated and the fraction of n-hexane 15 g, fraction dichloromethane (DCM) 6 g and 8 g of ethyl acetate fraction, the remaining fraction 216.8 g.

Isolation begins by sampling the bark of *Shorea singkawang* in the District Seling village Rantau Panjang Merangin District, then the sample is dried after aerating and finely chopped dried and smoothing with a grindstone. Perajangan aims to expand the sample surface so that the area of contact between the sample with a solvent more widely, thereby facilitating the penetration of the solvent into the cell membrane and will accelerate the process of dissolution of the compounds contained in the sample into the solvent used, and also to prevent the occurrence of thermolysis.

Extract the sample done by maceration. Maceration techniques have been selected because they have not known the nature of the compounds that are in the sample. Maceration is done by soaking the samples in organic solvents Salama 3-5 days, during which the sample immersion occasionally shaken to accelerate the penetration of the solvent into the cell membrane and chemical components will be dissolved. Maceration process is carried out in a place protected from the light in order to avoid the possibility of degradation of the structure, especially the compounds nonpolar groups and less stable to light. Then, after three days the extract was filtered using filter paper, and obtained samples of red-brown color.

Methanol used as a solvent in this maceration because methanol is a universal solvent that dissolves almost all organic compounds in plants, both polar, semi-polar and non-polar. The boiling point of methanol was relatively quite low (64.57^{o}C) so easily evaporated without using high temperatures. This is to prevent damage to the compounds present in these plants due to the temperature, in addition to the price of this solvent is relatively cheap compared with other polar solvents, such as ethanol having a boiling point (78.5^{o}C) and the price is relatively more expensive.

The methanol extract obtained solvent is evaporated in vacuo, because in a vacuum solvent vapor pressure will be off and the solvent will boil at of temperature lower than the boiling point. It aims to reduce the risk of damage to thermolabile compounds contained therein. Obtained from the extraction of 580 g methanol extract thick, so that the yield of methanol extract obtained to the weight of the sample is 9.666%. Total yield is affected by the extraction process, in which the extraction process is carried out, not missing the red color of the pulp bark of *Shorea singkawang* (Miq). Miq, in other words, not all of the extracted compounds. 580 g of viscous extract taken half (1/2) to do fractionation, as many as 250 g.

Fractionation is done by using a liquid-liquid extract using a separating funnel. The separation of the chemical components of the second phase of the solvent that is not intermingled in which most of the components dissolved in the first phase and in part late in the second phase, the second phase containing a dispersion fractionated, and then allowed to stand until there is complete separation and formed two layers of liquid phase, and component chemistry will be separated into the phases according to the degree of polarity.

Starting from nopolar solvent to the polar solvent. It aims to separate the compounds exist based on the nature of polarity in which these substances are easily soluble in solvents that have this level of polarity that is the same or

nearly the same, the solvent n-hexane will attract non polar compound because of its non-polar, dichloromethane (DCM) will attract semi-polar compounds because they are semi-polar, ethyl acetate will attract semipolar compounds because they are semipolar, while the polar compounds will be in the remaining fraction (fraction of water) which is polar. The fraction obtained was concentrated by using an evaporator, so that the viscous extract obtained respectively. It aims to determine the weight and calculate the yield of each fraction.

The fraction obtained was concentrated by using a rotary evaporator. The use of a rotary evaporator and the evaporation would extend the presence of a heat source that helps the process of evaporation. Solvents will be accelerated by the reduction of air pressure that causes a decrease in the vapor pressure of the solvent so that the solvent boils at a temperature much lower than its boiling point. Each fraction was evaporated to produce viscous mass so that in can weight of each fraction.

After concentrated by rotary evaporator showed fractionation n-hexane from the stem bark of Shorea singkawang 18.6456 g of sample size fractionated, while the yield fraction of dichloromethane (DCM) is 8.8792 g *Shorea singkawang* bark, of a number of samples were fractionated, and the ethyl acetate fraction of the 10.0374 g bark of sample size fractionated. Highest number of fractions which is the remaining fraction 223.7845, then continued fraction of n-hexane, ethyl acetate fraction onwards and dichloromethane (DCM).

Ethyl acetate fraction due by Thin Layer Chromatography techniques to determine the composition of its chemical content. Thin-layer chromatography is the separation of the chemical components based on differences in absorption and partitions as well as the solubility of the chemical components that will move to follow the polarity of the eluent (mobile phase), therefore the absorption of adsorbent against chemical components are not the same, then the components move at different speeds so this is what caused the separation. Thick n-hexane fraction, the fraction viscous dichloromethane (DCM), ethyl acetate, thin-layer chromatographed to determine the levels of the chemical composition, spotting the plate thin layer chromatography (TLC) was monitored under a 254 nm UV lamp. Spotting active in UV marked. Some eluent mixture of different polarity after a try in the TLC. Eluent used include n-hex, n-hexane -Ethyl acetate with a ratio of 9: 1, 8: 2, 7: 3, 6: 4 and 1: 1 ethyl acetate, methanol.

The separation of ethyl acetate fraction of thick bark of *Shorea singkawang* using column chromatography with a stationary phase silica gel 60 and eluted GSP by the mobile phase n-hexane, n-heksan- eteil acetate, ethyl acetate and methanol, as much as 4 g viscous fractions of ethyl acetate was made into powder pe-re-absorption by adding silica gel as the sample weight. First sample is dissolved by the solvent n-hexane after it had added silica gel. Then the solvent was evaporated in vacuo to obtain a mixture of silica gel and a sample of dry sebuk brownish-colored cat. Columns are made by inserting a 400 g silica which has been suspended by emulsifying liquid is n-hexane, into the glass column while (shaked) is not clogged and homogeneous. Samples that have been finished powder pe-re-absorption are carefully placed and evenly, then the sample was eluted with the mobile phase solvent n-hexane 100% through the column wall bit by bit so it makes all. Eluent exit accommodated in a vial, a tube-shaped rings on the new column elution exchanged with n-hexane: ethyl acetate, ethyl acetate, methanol, any fraction of the solvent is evaporated to obtain a thick fraction, then monitored with TLC plate. The fraction that has the same Rf combined. From the 87 fractions can result in a combined form of 7 sub fraction.

Isolation of chemical compounds from the ethyl acetate fraction as much as 100 mg of the compound obtained by using thin layer chromatography. Discloser stain reagent reagent $FeCl_3$ solution is formed this dark blue, indicating that positive type phenolic compounds from the fraction of ethyl acetate.

RESULTS AND DISCUSSION

Characterization of these compounds was obtained the following data: the white needle-shaped crystals as much as 85.8 mg, melting point of 188-190 °C, UV-vis (MeOH) λ max (log ε) 218 nm and 275 nm, (MeOH + NaOH) λ max : 205 nm; 233 nm and 280 nm, (MeOH + $AlCl_3$) λ max 218 nm, 275 nm; (MeOH + $AlCl_3$ + HCl) λ max: 218 nm, 274 nm; (MeOH + NaOAc + H_2BO_3) λ max; 206 nm; 300.nm, IR (KBr) 3408.74, 3053.65 cm^{-1} (OH), 2934 cm^{-1} (CH) aliphatic, 1678.56 cm-1 (C = O conjugated), 1612.59, 1540.99, 1465.05, 1439.26, 1312.04 (C = C benzene), 1262.35, 1204.14, 1100.02, 1035.75, 1003.33, 1053.33, 917.22, 879.80, 806.22, 773.62, 752.84, 737.83 (CO oxy-aryl. Spectrum) 1 H NMR (acetone -d6 400 MHz) δH (ppm): 3,772 (3H, s OCH_3) δ 7.102 (2H, s, H2, H6): 13 C NMR (Acetone-d 6 220 MHz) δ5 1.9 (OCH_3 C-6), δ 109.7 (C-2) δ 121.7 (C-1) δ 138.7 (C-4), δ 146.0 (C-3, C-5), δ 167.2 (C = O). GC-MS were compared with data that existed before this (11).

Data analysis FTIR spectrum showed the presence of carbon-carbon bonds: 3408.74, 3053.65 cm^{-1} (OH), 2934 cm^{-1} (CH) aliphatic, 1678.56 cm^{-1} (C=O conjugated), 1612.59, 1540.99, 1465.05, 1439.26, 1312.04 (C=C benzene), 1262.35, 1204.14, 1100.02, 1035.75, 1003.33, 1053.33, 917.22, 879.80, 806.22, 773.62, 752.84, 737.83 (CO oxy-aryl). Their absorption bands at two wavelengths are 218 nm and 275 nm and identify the occurrence of UV absorption due to electronic transitions of electrons π to π antibonding, which identifies the type of uptake of the compound benzene is substituted with a substituent polar (Suratman Unang, 2009). It means this type of uptake methyl gallate cromotor identify phenolic compounds.

Based on MS spectrum as shown in Figure 2, the methyl gallate compounds known to have a molecular weight of 184 with a base peak at m/z 153 (100) derived from $C_7H_5O_4$ + caused by removing OCH_3• of molecular ions. With the release of OCH_3 formed $C_7H_6O_4$ which formed the base peak at m/z 153 is the typical peak gallate compounds which looked at the height of m/z 153 (12). These ions then release CO •$C_6H_5O_3$ + formed at the peak of m/z 125. These ions then release $C_6H_3O_2$ + H_2O molecules formed at the peak of m/z 107. Thus, these ions that form releasing CO •C_5H_3O + at peak m/z 79, ion this release CO •C_5H_3+ molecules that form the peak of m/z 51.

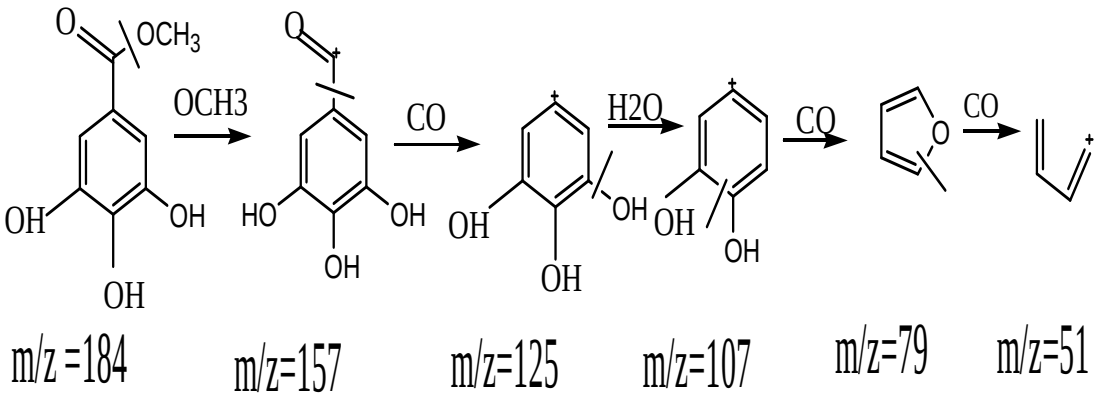


Figure 2. The pattern of fragmentation methyl gallate

Analysis of the structure using NMR spectra covering ^{1}HNMR and ^{13}CNMR. Based on the data of the spectrum there are eight carbon ^{13}CNMR and five proton bound to carbon, on this spectrum appears five signals that is: C x5, CH and CH_3 x2 x 1. By comparing the existing data before these compounds have the molecular formula is $C_8H_8O_5$ or known as methyl gallate (1) or 3,4,5 trihydroxy-methoxy benzoate (11).

Spectrum analysis ^{1}HNMR dan ^{13}CNMR (as shown in Table 1) shows a molecule that symmetry which has two protons aromatic δ 7.102 (2 H, s, H-2, H-6), three hydroxyl groups δ at 146.0 (C-3, C-5) and δC 121.78 (C-4), a methyl δ 3.78 (3H-, s, OCH) and an ester carbonyl δC 16.

Table 1. ^{13}CNMR chemical shift data of methyl gallate and Reference data of compound methyl gallate compounds (12)

No	Chemical Shifts	Type	methyl gallate	type
1	121.7	(C-1)	120.3	(C-1)
2	109.7	(C-2)	108.9	C-2)
3	146.0	(C-3)	145.3	(C-3)
4	138.7	(C-4)	138.8	(C-4)
5	146.0	(C-5)	145.3	(C-5)
6	108.9	OCH_3 (C-6)	108.9	(C-6)
7	167.2	(C=O)	167.9	(C=O)

Bioactivity

Cyto-toxic Activity with *Brine Shimp Lethal* Method

Cytotoxicity activity test of *Shorea singkawang* bark extract in *Artemia salina* Leach shrimp larvae, the larvae obtained by using egg for 48 hours. To dissolve the compound ethyl acetate extracts and pure compounds used DMSO. The addition of DMSO into the vial of test aims to help dissolve poorly soluble compounds in seawater so distributed evenly. DMSO is added up to 50 mL for the above mentioned values DMSO can cause the death of shrimp larvae. Addition of DMSO to the vial control is to control the effects of DMSO on shrimp larvae *Artemia*

salina Leach. Results of control no larvae mortality, so it can be concluded that DMSO at a concentration of 50 mL did not cause the death of larvae shrimp *Artemia salina* Leach.

The toxicity of some plant extracts against the larvae of shrimp *Artemia salina* Leach, can be determined based on the value of extreme these plants can be toxic if LC_{50} <1000 ppm, with the meter an extract is considered highly toxic when it had LC_{50} values below 30 ppm, is considered toxic if it has a value of LC_{50} 30 -1000 ppm and is not considered toxic hem LC_{50} values above 1000 ppm. While the pure compounds declared toxic if it has a value of LC_{50} <200 ppm (13; 14). The toxicity level of giving meaning to the activity as a potential anti-tumor/anticancer.

Cytotoxicity testing activities ethyl acetate extract of the bark of *Shorea singkawang* is 2:05 LC_{50} values obtained with a concentration of 125 ppm means ppm resulted in the death of 50% of the number of larvae shrimp *Artemia salina* Leach (2:05 LC_{50} ppm). Toxicity of some plant extracts against the larvae of shrimp *Artemia salina* Leach can be determined based on the value of LC_{50} <1000 ppm. Ethyl acetate fractions of bark extract is obtained Shorea singkawang 2.05 ppm LC_{50} value is less than 125 ppm, which limit toxic to extract. From this it can be said that the fraction of ethyl acetate extract from the bark of Shorea singkawang showed cytotoxic activity, because LC_{50} fraction of ethyl acetate extract of the bark of Shorea singkawang 2:05 ppm less than 125 ppm, then the fraction of ethyl acetate can be catergories very toxic (14). The test results from the activity of ethyl acetate fraction bark extract Shorea singkawang 2.05 ppm LC_{50} values obtained resulted in the death of 50% of the number of larvae *Artemia salina* Leach (2.05 LC_{50} ppm).

Ability of cytotoxic ethyl fraction of acetate extract from the bark of *Shorea singkawang* allegedly associated with the content of secondary metabolic owned *Shorea singkawang* bark. based on the results of checks on the positive fraction containing phenolic compounds characterized by dark blue color when sprayed on a plate using $FeCl_3$ stain penampak. Activity of phenolic compounds in nature due strong inhibitors in the DNA chain cleavage and her ability as a binder oxygen radicals and also due to the potential in forming chelate with metal, and phenolic compounds, tannins and flavonoids have potential as cytotoxic (Peteros, NP and Myelene, 2010).

Cyto-toxic Activity due P388 Murine Leukemia Cells
Results of tests on P388 murine leukemia cell to Compound Phenolic (methyl gallate) shows the IC_{50} 1.66 μm/mL, while the test sitoksisitas using fry shrimp Artemia salina Leach using Brine Shrimp Lethality Bioassay compound Methyl gallate showed LC_{50} 1.81 μm/mL is a compound that is toxic and can inhibit P388 murine leukemia cells with IC_{50} values below the value of the standard compound which is 4 μm/mL, it can be concluded these compounds are toxic to P388 murine leukemia cells.

CONCLUSION

Ethyl acetate extract of the bark of Shorea singkawang in the outcome of an methyl gallate compound molecular formula $C_8H_8O_5$. The test results of activities with Artemia salina fraction of ethyl acetate extract from the bark of Shorea singkawang 2.05 ppm LC_{50} values obtained resulted in the death of 50 % of the number of larvae *Artemia salina* Leach (2.05 LC_{50} ppm). The results of the test against P388 murine leukemia cells to the compound methyl gallate showed IC_{50} 1.66 μm/mL, while methyl gallate cyto-toxicity test using fry shrimp *Artemia salina* Leach using Brine shrimp Lethality Bioassay showed LC_{50} 1.81 μm/mL methyl gallate is very active against *Artemia salina* shrimp larvae and active against P388 murine leukemia cells.

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