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Phytochemical Constituent and Cytotoxic Activity of *Eusideroxylon zwageri* Teijsm & Binn Extract and Sub-Fraction on Human Breast Cancer Cell Line T47D

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ABSTRACT. *Eusideroxylon zwageri* Teijsm. & Binn has been reported to have bioactivity, one of which is the potential as an excellent cytotoxic agent using the BSLT method. So far, stage tests using cancer cells and analysis of the content of secondary metabolites have not been reported. Therefore, it is essential to analyze the content of secondary metabolites using Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS) and determination of cytotoxic activity using Brine Shrimp Lethality Test (BLST) method with various solvent polarities. Then separation by bioassay-guided isolation using column chromatography of the active extract assay BSLT. Furthermore, the cytotoxic activity of the active extract and the active isolate was tested against T47D cancer cells with Microculture Tetrazolium (MTT) method. The results of the analysis of the content of secondary metabolites using LC-MS/MS identified senbusine B and 6,7-sdroartemisinic acid on hexane extract, while the ethyl acetate extract produced 4 compounds, and 15 compounds were identified in the methanol extract. The results of the cytotoxic activity test using the BSLT method on each extract showed that the hexane extract was the most active, with an LC_{50} of 17.56 μg/mL. Furthermore, the cytotoxic activity of hexane extract against T47D cells showed weak activity with an IC_{50} value of 237.5 μg/mL, while the AB1 sub-fraction showed moderate activity with an IC_{50} value of 138.4 μg/mL. The results of the analysis using HPLC indicated that the active isolate in the form of sub-fraction AB1 contained the compound senbusine B.

Keywords: Cytotoxic, *Eusideroxylon zwageri* Teijsm. & Binn, secondary metabolites

INTRODUCTION

Data reports from the Global Cancer Observatory (GLOBOCAN) in 2020 showed there was an increase in cases of people living with cancer in the world, as many as 19.3 million people, and the number of people who died as many as 9.9 million people (Sung et al., 2021). Cancer patients in Indonesia in 2020 were 396914, and the number who died was 183368. The highest incidence occurred in breast cancer, with 65858 (16.6%) with a mortality rate of 22430 (9.6%), and cervical cancer, with 36633 new cases (9.2%) and a mortality rate of 21003 (9%) (The Global Cancer Observatory, 2020). So with the increasing number of cancer patients, it is necessary to make efforts to find cancer treatment solutions that are safe, efficient, and affordable. One of them is by using medicinal plants.

Eusideroxylon zwageri Teijsm. & Binn belongs to the Lauraceae family and Spread in Bangka Belitung, Kutai, Jambi (Muara Bulian Regency), Musi Rawas South Sumatra (Aiso-Sanada et al., 2020). It's a plant often used in traditional medicine, and is one of the forest commodities that the people have long known of Indonesia (Badariah, 2013). The community uses all parts of the plant as traditional medicine, including the decoction of the bark of the stems used by the Kutai community as a medicine for diabetes, toothache, stomachache, antipyretic, gynecological problems, fever, and ironwood which is burned and made into powder, which is applied to wounds caused by scorpion poison (Ajizah et al., 2018)

In previous studies, it was reported that ironwood bark was used as a diarrhea medicine with its activity in inhibiting the growth of *Staphylococcus aureus* bacteria (Darussalam, 2016), It can also inhibit the growth of bacteria *Aggregatibacter actinomycetemcomitan, Escherichia coli* and *Salmonella typhi* (Mariam et al., 2018). In addition, this stem bark has also been reported for its antioxidant activity using the 1,1-difenil-2-pikrililhidrazil (DPPH) method and superoxide anion (Kusuma et al., 2018), antidiabetic activity by inhibiting the action of □-Amylase and □-glucosidase enzymes (Kusuma et al., 2018), antifeedant activity by increasing termite mortality (Raharjo et al., 2020). Furthermore, activity as a biological insecticide against caterpillars (Johari, 2010) and against ladybugs (Badariah, 2013).

Abdullah et al (2013) reported that acetone extract from the bark of *E. zwageri* had a toxicity activity using

the BSLT method with an LC_{50} value of 0.8 μg/mL. (Abdullah et al., 2015). These results indicate that the bark of *E. zwageri* has the potential as a natural ingredient that acts as an anti-cancer agent. Because advanced testing using cancer cells and analysis of compound content have never been reported on the bark of *E. zwageri*, in this study, cytotoxic activity was tested using the microculture tetrazolium (MTT) method on T47D breast cancer cells against the active extract and the active sub-fraction assay. BSLT obtained from bioassay guided isolation. Furthermore, analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) was carried out to determine the content of active compounds in the bark of *E. zwageri* and analysis using high-performance liquid chromatography (HPLC) on active isolates.

EXPERIMENTAL SECTION

Material

Solvents : hexane (merck), dichloromethane (merck), ethyl acetate (merck), methanol (merck). Mayer dan Dragendroff reagent, Liebermann Burchad reagent, Mg powder and HCl (Merck, Gemany CAS 7664-93-9), iron (III) chloride (Merck, Gemany CAS 10025-77-1), sea water, *Artemia salina* shrimp larvae, DMSO, silica gel 60 (0.063-0.200 mm/Merck) and TLC plate (DC-Alufolien Kieselgel 60 F254 Merck), sterile aquadest, dimethyl sulfoxide (DMSO) (Merck), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) (Merck), trypsin-EDTA, phosphate buffer saline (PBS) (Merck), tryphan blue (Merck), Penicillin-streptomycin (pen-strep), reagen 3-(4,5-dimetilthiazol-2-il)-2,5-difeniltetrazolium bromide (reagen MTT) (Merck). 0.1% formic acid in acetonitrile (HPLC grade), 0.1% formic acid in aquabides (HPLC grade) and acetonitrile (HPLC grade).

Apparatus

Maceration bottles, mortar and pestle, hot plate, glass box for shrimp breeding containers, a set of distillation equipment, micropipette, analytical balance, oven, rotary evaporator, chromatographic column (various size), UV lamp (λ 254 nm & 365 nm). Equipment used for characterization LC-MS/MS (XEVO G2S Q-TOF) and HPLC (LC 1200 series) as well as glassware commonly used in laboratories. Cytotoxic test equipment using the MTT method, including the micropipette (Ecopipette), analytical balance (Kern Abj), 37°C incubator with 5% CO_2 (Thermo Scientific), inverted microscope (Zeiss), well plate-96 (Iwaki), laminar air flow safety cabinet (Thermo Scientific), and microplate spectrophotometer (xMark).

Sample Preaparation and Extraction of *E. zwageri* Bark

E. zwageri was collected in Jambi Province, Indonesia, and identified at the Biology Laboratory of Andalas University. The dry bark of *E. zwageri* was macerated in stages using 3 types of solvents with different polarity levels starting from hexane, ethyl acetate, and methanol.

Phytochemical Analysis

E. zwageri extract of hexane, ethyl acetate, and methanol was analyzed for compound content by LC-MS/MS based on the procedures that have been carried out by Molina et al (2018). Column C18, column temperature 40 °C, flow rate 0.6 mL/min; injection volume 10 L, Eluent (A) acetonitrile 99.9/ formic acid 0.1[v/v] and (B) water (HPLC grade) 99.9/formic acid 0.1 [v/v] with a series of MS analyzer XEVO G2S Q-TOF (quadrupole-time of flight) and positive and negative Electrospray Ionization (ESI) sources (Molina-Calle et al., 2017).

Cytotoxic Test with Brine Shrimp Lethality Test (BSLT) Method

Cytotoxic test with BSLT method, extract of hexane, ethyl acetate, and methanol *E. zwageri* with various concentrations (3.125-500 μg/mL) taken as much as 5 mL and then evaporated the solvent. Then added, 50 μL DMSO and 2 mL of sea water. Controls only included DMSO and seawater. Ten shrimp larvae of *A. salina* Leach that had been incubated for 48 hours were put into the test and control solution. The test and control solutions volume was made up to 5 mL with seawater. The number of dead larvae was counted after 24 hours. The test was carried out in triples. The data were used to calculate the LC_{50} value obtained from the regression equation of the probit value with the concentration log (Ova et al., 2016).

Column Chromatography of Active Hexane Extract against BSLT Test

Separation by column chromatography of hexane extract with eluent hexane: dichloromethane (10:0-0:10). The separation results obtained 4 main fractions (fraction A-D) and the BSLT test. Fraction A and fraction B weighed 1.1089 grams and 2.8786 grams, respectively, because these fractions were active fractions against the cytotoxic test using the BSLT method and had almost the same stain pattern and compound components. The AB fraction was further separated by column chromatography starting from the eluents of hexane: dichloromethane (10:0–0:10) and dichloromethane: ethylacetate (10:0–5:5). The results of the separation obtained 4 sub-fractions (AB1-AB4) which were then carried out by the BSLT test. The active sub-fraction AB1 was the most active against the BSLT test, so it was continued with HPLC and cytotoxic activity tests against T47D cancer cells using the MTT method.

Testing Using HPLC on Subfraction AB1

Sub-fraction AB1 was dissolved with methanol. Then 10 μL was injected into the HPLC instrument. The mobile phase used was A (water (0.1% formic acid) and B (acetonitrile). Gradient elution system. Flow rate 1 mL/min, column temperature 40°C (Tan et al., 2011).

Cytotoxic Test of Hexane Extract and Sub-fraction AB1 using the MTT Method

The human breast cancer line (T47D) was a cell culture from the Biomedical Laboratory, Faculty of Medicine, Andalas University. Cells were cultured in an incubator at 37 °C with 5% CO_2 in a DMEM medium containing 10% FBS (Fetal Bovine Serum). Furthermore, hexane extract, sub-fraction AB1, and control were added and incubated for 24 hours. The concentrations used were 1000, 500, 250, 125, 62.5, and 31.25. T47D breast cancer cells were incubated on plates for 12 hours. Furthermore, hexane extract, sub-fraction AB1, and control were added and incubated for 24 hours. The concentrations used are 1000, 500, 250, 125, 62.5 and 31.25 □g/mL. Well plate 96 was removed from the incubator and washed with PBS. Then added 25 μL MTT 0.5 mg/mL and incubated again for 6 hours at 37 °C. The supernatant was then discarded, and DMSO was added to dissolve the formazan. The absorbance was measured at 550 nm. Absorbance data were converted into a percentage of cell viability using the formula:

$$\% \text{ Cell viability} = \frac{(\text{sample absorbance - negative control absorbance})}{(\text{positive control absorbance - negative control absorbance})} X\ 100\%$$

The test was carried out 4 times and the IC50 determination was carried out using the Graph Pad Prism 9.0 software (Ismail et al., 2018).

RESULTS AND DISCUSSION

Extraction

Stratified extraction of *E. zwageri* bark resulted in 10.01 grams of thick hexane extract (yield 1.82%), ethyl acetate extract 21.90 grams (yield 3.98%) and methanol extract 120.46 grams (yield 21.9%).

Phytochemicals Analysis

Analysis of the content of secondary metabolites contained in hexane-ethyl acetate and methanol extracts using LC-MS/MS can be seen in **Table 1**.

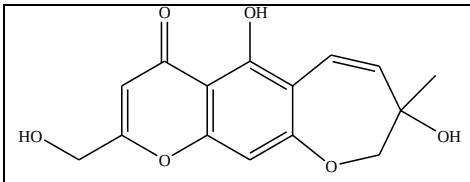
The results of the analysis showed that the most identified methanol extracts were 15 compounds (2 terpenoid compounds, 4 alkaloid compounds, and 8 flavonoid compounds), followed by ethyl acetate extract with 4 compounds (1 terpenoid group and 3 flavonoid groups) and hexane extract with 2 compounds (1 terpenoid and 1 group of alkaloids). As a substitute for the similarity index there are 4 factors or conditions that must be met by an analyte to be declared positive (similar to the Library) among them Mass error ≤ 5 ppm, Isotope match MZ RMS PPM ≤ 6 ppm & Isotope match MZ RMS % ≤ 10 %, Intensity/Response ≥ 300, and Fragment match ≥ 1 mass fragment. Results in (**Figure 1**) can be used as a reference and basis for the separation process for the targeted compounds.

Cytotoxic Activity Using the BSLT Method

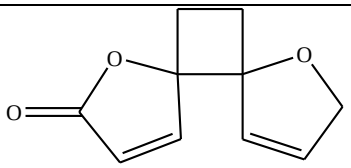
Cytotoxic test using *A. salina* Leach is often used in research on natural ingredients and is a preliminary test to find anticancer compounds by determining the LC_{50} value after exposure to the extract for 24 hours (Sandrawati et al., 2019). The cytotoxic activity of hexane, ethyl acetate, and methanol extracts was seen to have varying results (**Figure 2**).

Table 1. Results of LC-MS/MS analysis of hexane, ethyl acetate, and methanol extracts

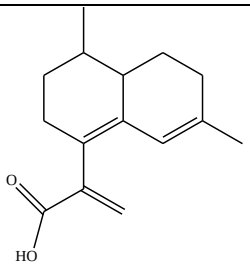
Extract	Compound name	Retention time (min)	Coumpound mass (m/z)
Hexane	6,7-Dehydroartemisinic acid	15.66	233.1530
	Senbusine B	16.14	424.2681
Ethyl acetate	Mururin A	7.16	449.0866
	Anemonin	12.66	193.0497
	3',5-Dihydroxy-7,4'-dimethoxy flavone	15.67	315.0870
	3-(4'-Hydroxy-benzyl)-5,7-dihydroxy-6-methyl-8-methoxy-chroman-4 one	16.41	331.1183
Methanol	(-)-Epigallocatechin	4.83	305.0652
	Tiliroside	4.90	595.1440
	Undulatoside A	5.14	353.0870
	7-O-Isopentenyl-8-fagarine	6.04	312.1231
	Cnidimol F	6.43	289.0707
	Coclaurine	6.44	286.1442
	d-Isoboldine	6.60	326.1382
	Procyanidin B7	7.16	577.1352
	Sinomenine	7.28	330.1702
	Anemonin	8.21	191.0347
	Fibraurin	8.67	371.1111
	2,5-Dimethyl-7-hydroxychromone	9.66	180.0550
	Cinchonain I b	11.51	451.1039
	Mururin A	12.79	447.0713



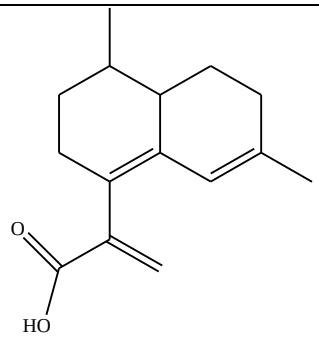
Cnidimol F



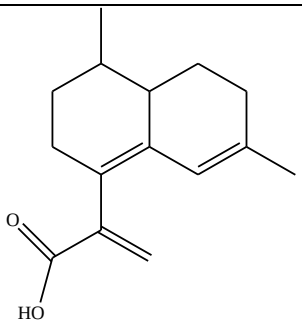
Anemonin



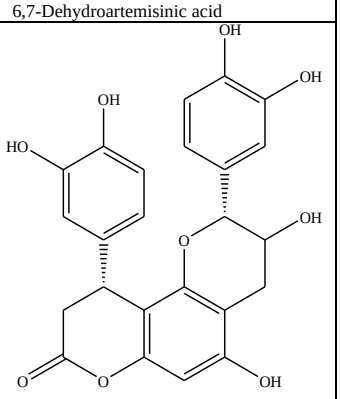
6,7-Dehydroartemisinic acid



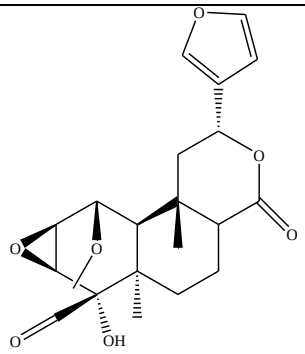
6,7-Dehydroartemisinic acid



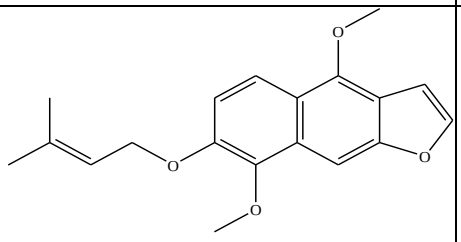
6,7-Dehydroartemisinic acid



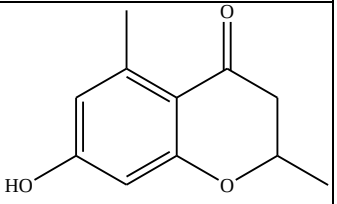
Cinchonain Ib



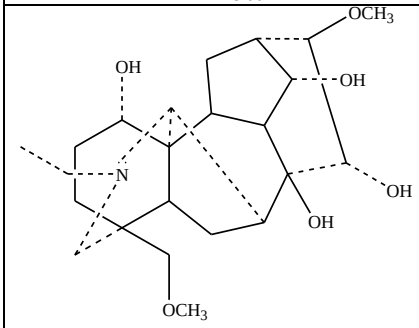
Fibraurin



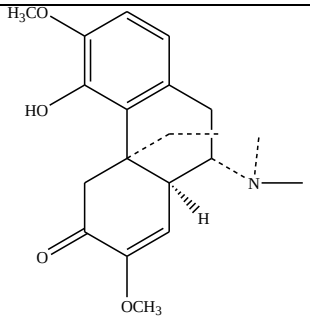
7-O-Isopentenyl-8-fagarine



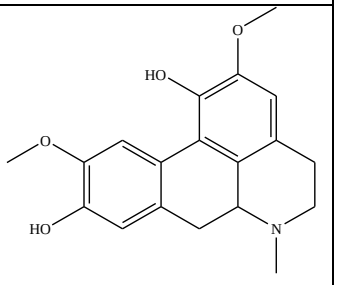
2,5-Dimethyl-7- hydroxychromone



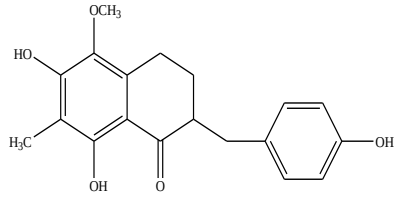
Senbusine B



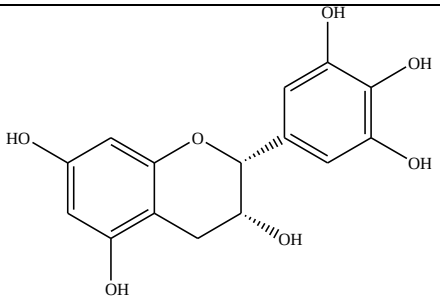
Sinomenine



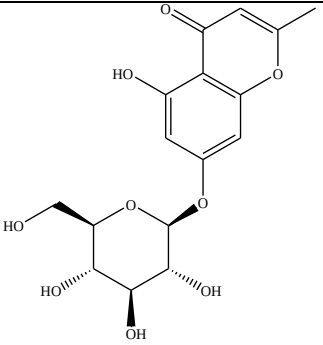
d-isoboldine



3-(4'-Hydroxy-benzyl)-5,7 dihydroxy-6-methyl-8-methoxy-chroman-4 one



(-)-Epigallocatechin



Undulatoside A

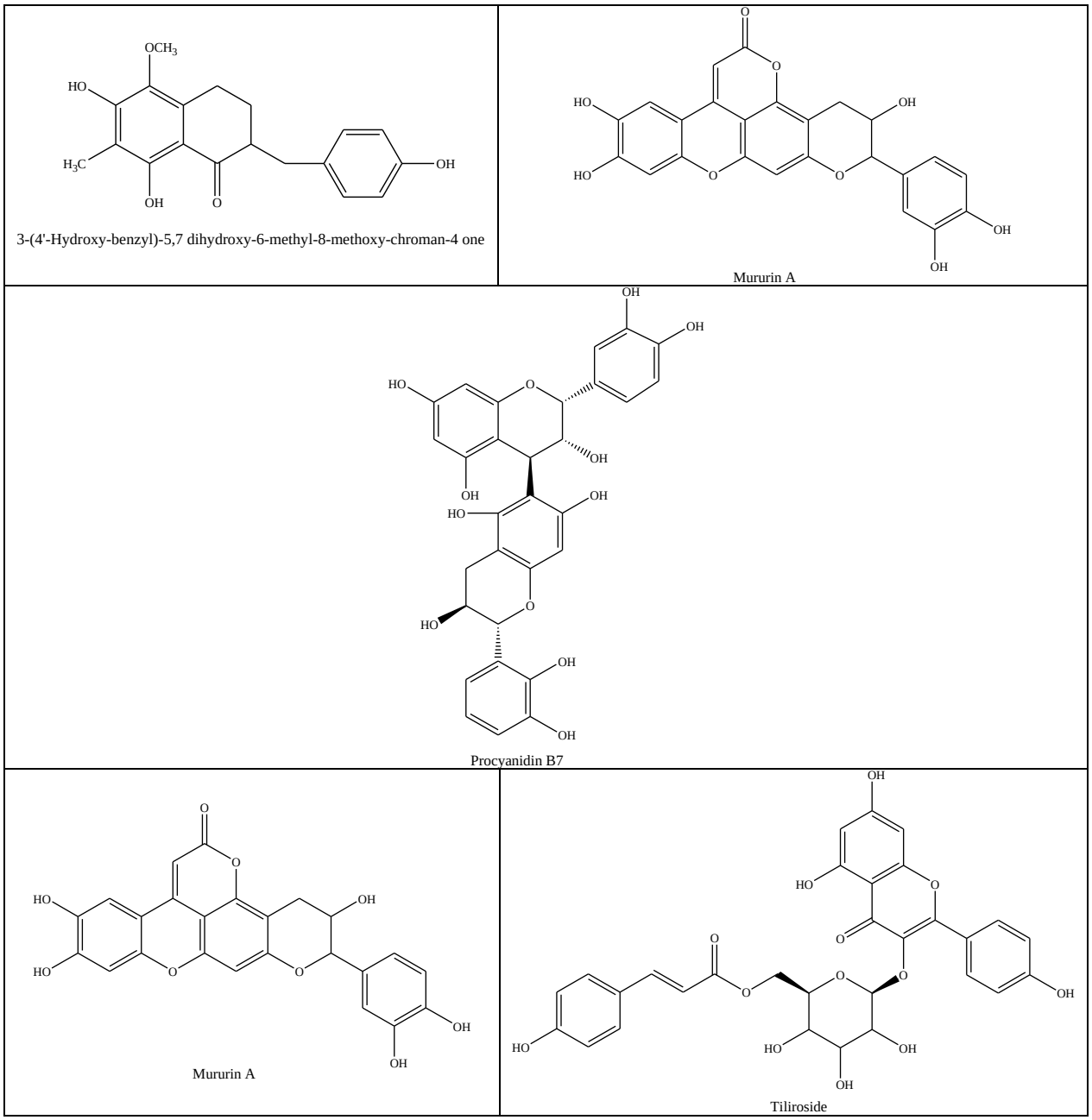


Figure 1. The structure of compound was identified in *E. zwagery* stem bark using LC-MS/MS

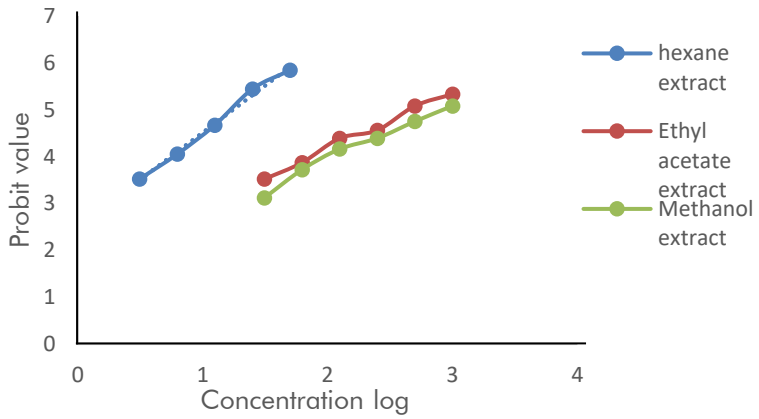


Figure 2. The relationship between the concentration log and the probit value of hexane, ethyl acetate and methanol extracts

Clarkson (2004) classifies the toxic level of extracts based on LC_{50} values in the following order extracts with LC_{50} above 1000 μg/mL are non-toxic, LC_{50} 500-1000 μg/mL are weak, extracts with LC_{50} 100-500 μg/mL are moderate, while extracts with LC_{50} 0-100 μg/mL is very strong. (Clarkson et al., 2004). Based on the LC_{50} value, hexane extract was highly toxic to Artemia salina shrimp compared to ethyl acetate and methanol extracts which had very weak toxicity with LC_{50} values of 17.567, respectively; 491.6092; and 771.081 μg/mL.

Separation of Secondary Metabolite Compounds by Column Chromatography from *E. zwageri* Hexane Bark Extract

The results of the separation obtained 4 fractions, including fractions A (1.1089 g), B (2.8786 g), C (9.6847 g), and D (6.7672 g). Four fractions in the BSLT test. The results also show differences in probability values that vary with the same concentration log value (**Figure 3**).

Results LC_{50} values obtained based on fractions A and B had potent cytotoxic activity against Artemia salina shrimp compared to fractions C and D, which had feeble toxic levels with LC_{50} values of 73.25, respectively; 41.5; 171.791 and 319.154 μg/mL. Fractions A and B were combined because they had potent cytotoxic activity, and from the TLC results, fraction A had almost the same stain pattern and compound components.

Fractions A and B, which have been combined with a total weight of 3.8 grams, were further separated using column chromatography with the same method as before and obtained 4 sub-fractions. Sub-fractions AB1 (0.213 g), AB2 (0.843 g), AB3 (0.754 g) and AB4 (0.567 g). Four sub-fractions in the BSLT test.

The LC_{50} value obtained in (**Figure 4**), the AB1 sub-fraction is highly toxic compared to the AB2, AB3, and AB4 sub-fractions, which have moderate toxicity with LC_{50} values of 85.684, 254.331, 174.462, and 456.983 μg/mL.

Analysis Using HPLC on AB1 Sub-fraction

Based on the results of the analysis of hexane extract using LC-MS/MS produced senbusine B and 6,7-dehydroartemisinic acid. HPLC testing of the AB1 sub-fraction to see if there is a possibility of containing senbusine B and 6,7-dehydroartemisinic acid compounds by looking at the peaks that appear at a specific retention time and then compared with previous research.).

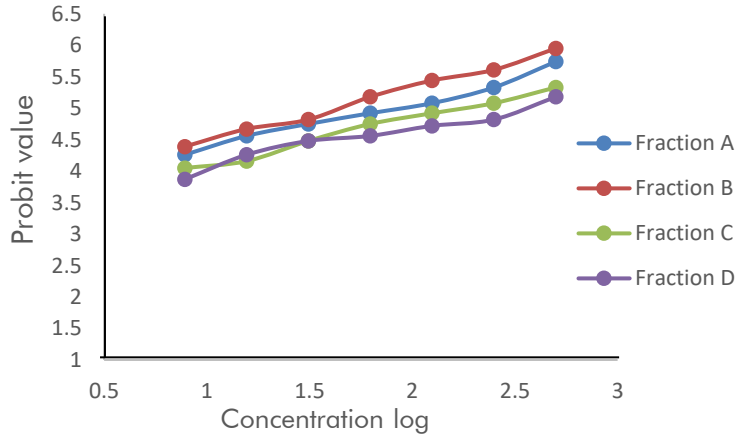


Figure 3. Relationship of concentration log with probit value of cytotoxic test fractions A, B, C, and D

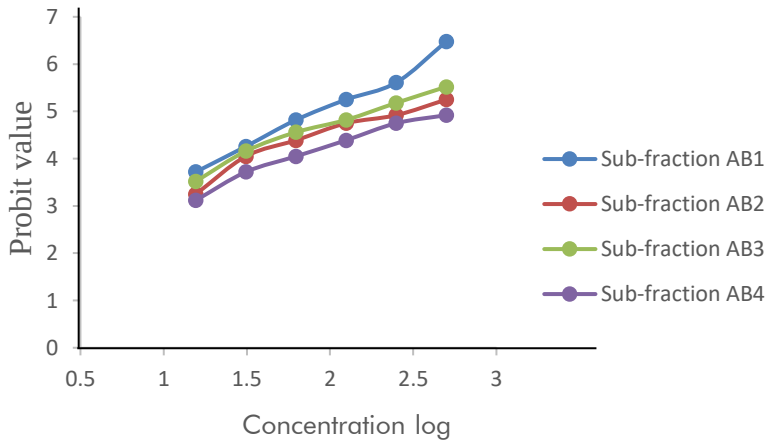


Figure 4. The relationship between the concentration log and the probit value of the cytotoxic test sub-fractions AB1, AB2, AB3 and AB4

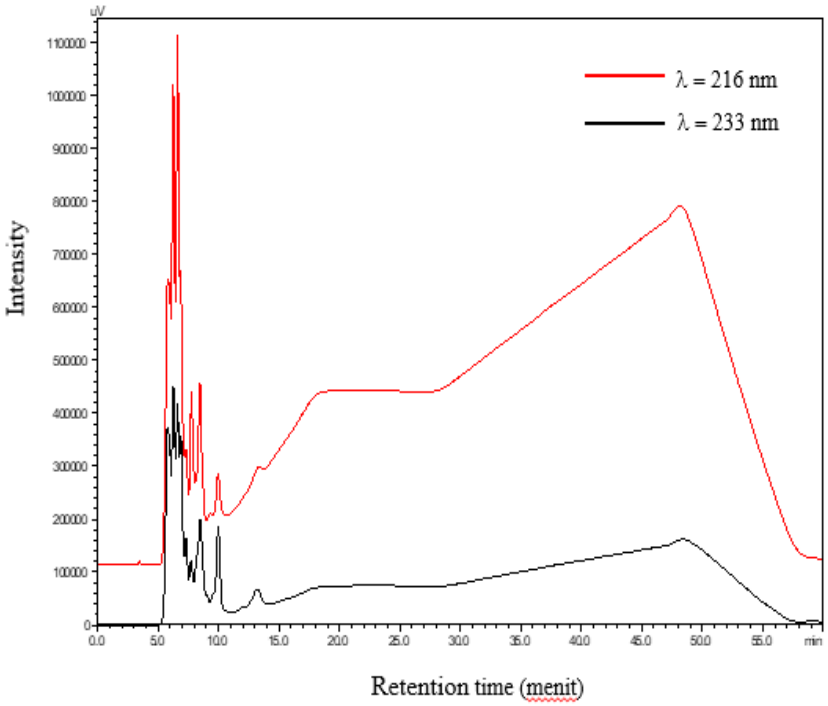


Figure 5. HPLC chromatogram at wavelengths of 216 nm and 233 nm

Based on (**Figure 5**), it was found that peak 1 with retention time at wavelengths of 216 nm and 233 nm was 6.843 and 6.884 minutes, respectively, with a percent area of 1.465% and 3.809%. Based on the research conducted by Tan et al (2011), senbusine B was identified at a retention time of 6.93 minutes (Tan et al., 2011). These results indicate that peak 1 can be indicated as the peak of senbusine B compound. Furthermore, the compound 6,7-dehydroartemisinic acid could not be identified. Because, no retention times were found that were close to the compound 6,7-dehydroartemisinic acid which had been identified in previous studies.

Cytotoxic Activity of Hexane Extract and Sub-fraction AB1 against T47D Breast Cancer Cells

Test the cytotoxic activity of hexane extract and sub-fraction AB1 using the MTT method. Following are the results of observations on the morphology of T47D cancer cells after being incubated for 24 hours (control). The morphology of cancer cells after growing for 24 hours can be seen in (**Figure 6)**, which shows that living cells are round and oval. The results of the observation of cancer cell death can be seen on a microscope after 24 hours of administration of hexane extract, and sub-fraction AB1 can be seen in (**Figure 7**).

The results of the observation of cancer cell death can be seen in a microscope after 24 hours of administration of hexane extract and sub-fraction AB1, and it can be seen in the picture that the dead cells will turn black and appear shriveled. The shape and number of cells can be visually compared with cells in the control (**Figure 5**) which are predominantly oval. Under the microscope, the viable cancer cells appear to decrease with increasing concentrations.

In (**Figure 8**) it can be seen the relationship between the concentration log and % cell viability, in **Figure (8a)** the hexane extract, and in **Figure (8b)** the AB1 sub-fraction used with the percentage of live T47D cells. The percentage of living T47D breast cancer cells tended to decrease with increasing concentrations of hexane extract and sub-fraction AB1. IC_{50} values of hexane extract and AB1 sub-fraction were 237.5 μg/mL hexane extract and 138.4 μg/mL AB1 sub-fraction, respectively. Based on the IC_{50} value, the extract can be categorized into four, namely strong for compounds having IC_{50} < 20 μg/mL, moderate for compounds having IC_{50} = 21-200 μg/mL, weak for compounds having IC_{50} = 201-500 μg/mL and not cytotoxic for compounds having IC_{50} 500 μg/mL (Sajjadi et al., 2015). This classification shows that the hexane extract is classified as weak cytotoxic, and the AB1 sub-fraction is classified as moderately cytotoxic.

The results of the cytotoxic activity test using the MTT method depended on the content of the compounds contained in the hexane extract and the AB1 sub-fraction. The hexane extract contains the compound senbusine B, which has a structural framework of diterpenoid alkaloids, and 6,7-dehydroartemisinic acid is a compound of the sesquiterpene lactone group (Konno et al., 1982). Meanwhile, the AB1 sub-fraction is estimated to contain only senbusine B compounds. In recent decades, the diterpenoid alkaloid group of compounds has been reported to have excellent cancer activity (Liang et al., 2018). Senbusine B has a similar structure to senbusine A and C, and the three

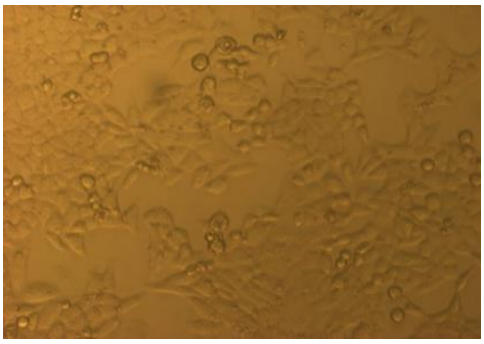


Figure 6. Morphological changes of the T47D cells after 24 h of incubation (control)

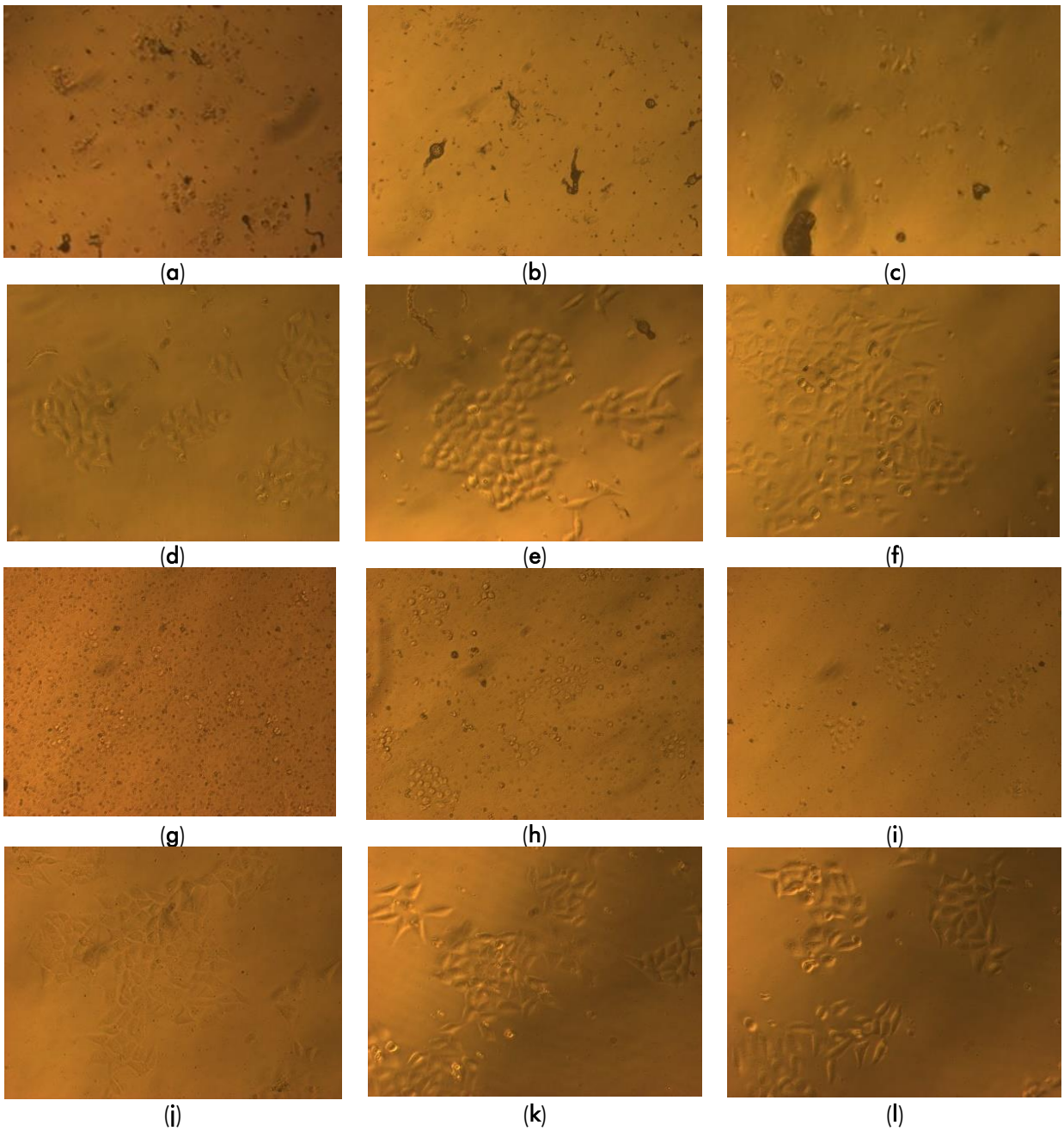


Figure 7. Morphology of T47D cancer cells after being given the hexane extract test solution at various concentrations ((a) 1000 μg/mL; (b) 500 μg/mL; (c) 250 μg/mL; (d) 125 μg/mL; (e)) 62.5 μg/mL; (f) 31.25 μg/mL) and Sub-fraction AB1 ((g) 1000 μg/mL; (h) 500 μg/mL; (i) 250 μg/L; (j) 125 μg/L; (k) 62.5 μg/L; (l) 31.25 μg/mL)

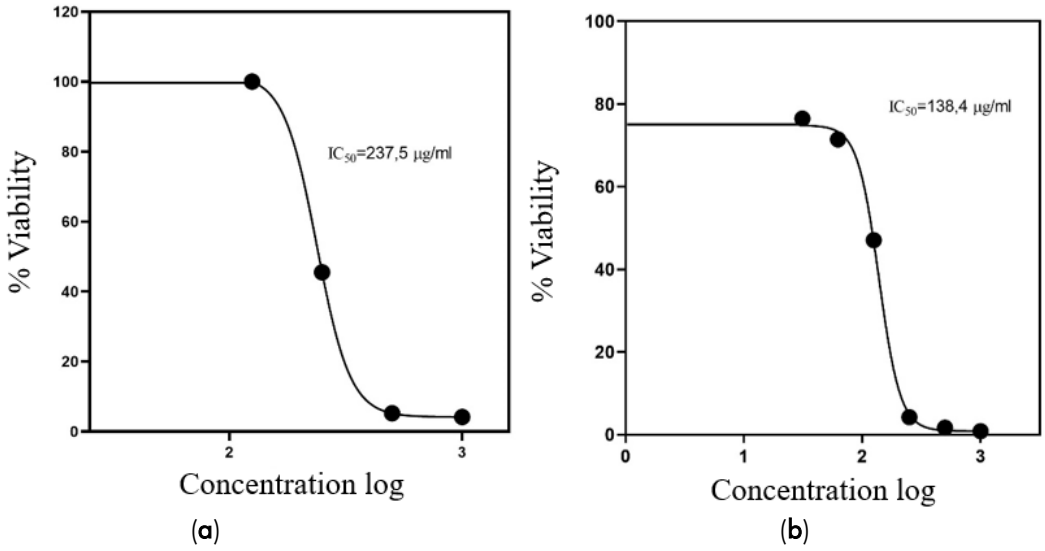


Figure 8. Correlation of concentration log with % viability test on T47D cancer cells; (**a**) hexane extract; (**b**) sub-fraction AB1

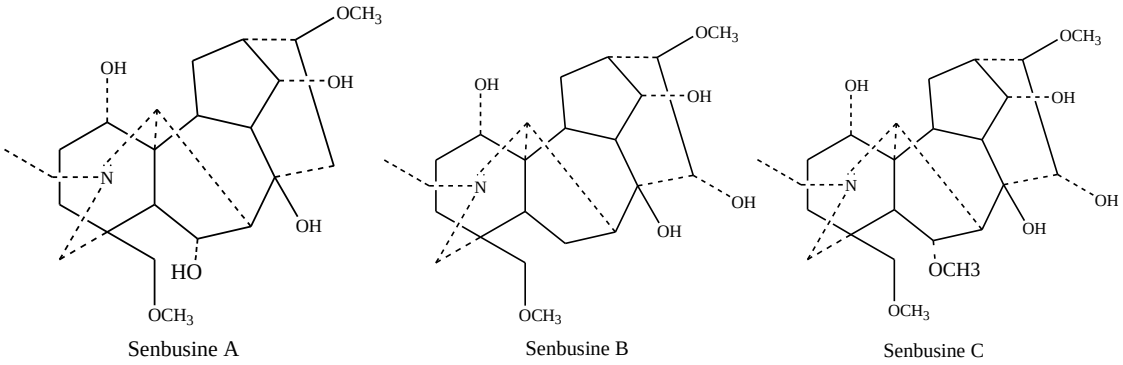


Figure 8. Compound structure senbusine A, senbusine B, and senbusine C

compounds are derivatives of isotalatizidine compounds. The difference between the three compounds lies in some of the substituents. The differences in the three compounds can be seen in (**Figure 8**), where there are differences in the number and position of substituents (hydroxyl, methoxy). In senbusine A and senbusine B, they have the same number of hydroxyl groups and methoxy groups, namely (which distinguishes the position of the substituent), whereas in senbusine c has more hydroxyl groups and methoxy groups, namely 4 hydroxyl groups and 3 methoxy groups.

Senbusine A and C have been widely tested pharmacologically with various cancer cells. One of them is MCF-7 breast cancer cells. Senbusine C has an IC_{50} value of 75.2 ± 6.8 μM and senbusine A > 100 μM (Kamil et al., 2017). Tests for the compound senbusine B have not been reported, but by looking at the ability of senbusine A and C to inhibit cancer cell growth, senbusine b contained in hexane extract and sub-fraction AB1 of *E. zwageri* stem bark can be indicated as an active compound in inhibiting cell growth T47D cancer. Because the number of hydroxyl groups and methoxy groups, senbusine A and B are the same, while senbusine C has better activity than senbusine B because it has more hydroxyl groups and methoxy groups (Arwansyah et al., 2014; Lestari et al., 2018).

The compound 6,7-dehydroartemisinic acid is a derivative of the compound dihydroartemisinic. Hydroartemisinic acid group compounds based on research conducted by Yan zhu (2019) showed endometrial cancer activity, which generally occurs in women. (Zhu et al., 2019). These results indicate that the compound hydroartemisinic acid can inhibit the growth of cancer cells in general, so it can be indicated that the compound 6,7-dehydroartemisinic acid contained in hexane extract also plays an active role in inhibiting the growth of T47D cancer cells.

The IC_{50} values obtained from the hexane extract and the AB1 sub-fraction differed significantly from the IC_{50} values of 237.5 and 138.4 μg/mL, respectively. The AB1 sub-fraction showed better results after the hexane extract's separation process by column chromatography. This is because the separation process produces a simpler number of components to reduce the antagonistic effect between compounds. This antagonist effect is caused by a compound that

can reduce the activity of other compounds. If we look at the previous research conducted by Yuliastri et al (2022) regarding the cytotoxic test using MDA-MB-251 breast cancer cells against fraction A-G, it showed that fraction A had the best toxic level with an IC_{50} value of 187.89 μg/ml (Yuliastri al., 2022). in this research we have a better results with IC_{50} value 138.7 μg/mL towards sub-fraction AB1

CONCLUSIONS

The hexane extract was the most active in the BSLT test, with an LC_{50} of 17.567 μg/mL. The hexane extract and the AB1 sub-fraction obtained from the separation have activity in inhibiting the growth of T47D cancer cells at an incubation time of 24 hours with an IC_{50} value of 237.5 μg/mL for hexane extract and AB1 sub-fraction 138.4 μg/mL. The results showed that the hexane extract was classified as weakly cytotoxic. The AB1 sub-fraction was classified as moderately cytotoxic. Analysis of the content of secondary metabolites of hexane extract using LC-MS/MS obtained compounds senbusine B and 6,7-dehydroartemisinic acid indicate these compounds play an active role in inhibiting the growth of T47D cancer cells, and sub-fraction AB1 is also indicated to contain senbusine B compounds from the analysis results using HPLC.

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