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Determination of Anticancer from EtOAc Fraction of *Spatholobus Littoralis Hassk* and of Molecular Structure Prediction

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ABSTRACT

In recent years, the search for new anticancer drugs from local plants in Indonesia has become an interesting topic for research. One of the typical Indonesian local plants that have been used by the community as an anticancer traditional medicine (jamu) is *Spatholobus littoralis* Hassk (SLH) or better known as Bajakah. The native Dayak people of Kalimantan have used it for years for the treatment of breast cancer. In this study, the separation of single compounds from the ethyl acetate fraction of SLH stems was carried out, their molecular structure was analyzed, and anticancer bioactivity was tested. The isolation used the preparative HPLC method. The elucidation of the isolates from the ethyl acetat fraction used LCMS-MS, FTIR, and NMR. Anticancer bioactivity was determined using the MTT bioassay. The result is the acquisition of two isolates; it has been predicted that both are pseudobaptinin. The anticancer IC_{50} of the pseudobaptinin was 62.85 ppm, and Pseudobaptinin with impurities was 145.60 ppm.

Keywords: Anticancer; Cytotoxic Pseudobaptinin; Spatholobus Littoralis Hassk

INTRODUCTION

The World Health Organization (WHO) has reported an increase in the number of new cancer patients by 18.1 million, with 50% reported experiencing death in 2020 (Smaili & Boudjella, 2020). Data released by The International Agency for Research on Cancer (TIARC) in 2021 has also shown cases of cancer sufferers increasing to 19.3 million, followed by the number of deaths increasing to 10 million. (Sung et al., 2021). Likewise, in Indonesia, breast cancer in 2020 ranks first with a total of 65,858 cases with a mortality rate of 22,430 people (Gayatri et al., 2021). Facts show that 90-95% of cancers are caused by an unhealthy environment and lifestyle that trigger heavy metal pollution, continued oxidation, infectious bacteria, and excess amounts of free radicals. (Anand et al., 2008).

Treatment of cancer with anticancer drugs obtained from plants that contain secondary metabolites with high bioactivity is widely available, including vinblastine, vincristine, vindesine, medicine, vinorelbine, vinflunine, hydration, vinglycinate, vitriol, and others. (Khazir et al., 2014). These drugs are produced by pharmaceutical companies in America, France, and India from the *Catharanthus roseus* plant through isolation using Thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) purifies, characterizes, and quantifies the crude extract. (Kumar et al., 2022), (Dhyani et al., 2022), (Faller & Pandit, 2011). Not all anticancer drugs are produced in Indonesia. Indonesia is one of the countries that is highly dependent on imported raw medicinal materials.

Based on data released by the Ministry of Industry in 2021 shows that Indonesia's imports of medicinal raw materials reach 95 percent. This dependence can have an impact on increasing the price of anticancer drugs. Likewise, the cost of cancer treatment in Indonesia is quite expensive. Not even all cancer drugs and therapies are covered by the Health Social Security Administration Agency (HSCAA) (Effendi, 2021).

Based on these facts, the search for new anticancer drugs from local plants in Indonesia is an interesting topic for research. One of the typical Indonesian local plants that has been used by the community as an anticancer traditional medicine (Jammu) is Spatholobus littoralis Hassk, better known as Bajakah.

The native Dayak people of Kalimantan have used it for years for the treatment of breast cancer (Fitriani, 2019). However, so far, the use of S. littoralis Hassk as a herbal medicine for the treatment of breast cancer has not been reported

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regarding the bioactivity of pure secondary metabolites in this plant.

In a previous study, it was found that the ethyl acetate fraction from *Spatholobus littoralis Hassk* has the highest breast cancer anticancer bioactivity (IC50 7.4 mcg/mL) compared to the hexane fraction (IC50 20 mcg/mL) and the water fraction (not detected). Likewise, the selectivity index (SI) of the ethyl acetate fraction was higher (6.57) than the hexane fraction (10.75) (Iskandar et al., 2022). Researchers believe the SI fraction in the 10<SI<3 region to be a prospective anticancer (Weerapreeyakul et al., 2012).

Discovering new anticancer agents, complementing the understanding of old study results, and key ways to target them need to be explored in depth. In this study, a single compound was separated from the ethyl acetate fraction, its molecular structure was analyzed, and its anticancer bioactivity was determined.

MATERIALS AND METHODS

Plant Material

SLH was collected from Delang District, Lamandau Regency, Central Kalimantan and identified by the Biology Laboratory of FMIPA Tanjung Pura University.

Extraction and Isolation

A number of 2.4 Kg of wood powder was extracted using 15 L of 70% ethanol. the crude extract was concentrated, and 102,3 g of the extract was obtained. The extract was suspended in distilled water and partitioned by solvent-solvent extraction using n-hexane and ethyl acetate. Each fraction was tested for cytotoxic activity using the bioassay-guided isolation concept. The test results showed that the ethyl acetate fraction was the most active. The ethyl acetate fraction (4.9 g) was then purified using Preparative HPLC SYKNM S1125 (Acetonitrile, flow rate 1.0 mL/min, 15 bar), and two major fractions were obtained. The two fractions were 6.4 mg (retention time 4.08 min) and 10 mg (retention time 4.49 min). The two isolated compounds were analyzed using spectroscopic methods.

In Vitro cytotoxicity bioassay

The breast cell line was harvested at a concentration of 8 $x10^3$ cells/well and made dilutions with culture medium (MK), then implanted into a 96 well microplate of 100 µL/well and incubated for 24 hours in a 5% CO_2 incubator. Before being used for treatment, the media in the plate was discarded and then washed using PBS 1 times in the amount of 100 µL/well. Then the PBS was discarded and given a test solution (500; 250; 125; 62.5; and 31.25 ppm) of 100 µL/well. The cells were then incubated again for 24 hours. After incubation, washed with PBS and added 100 µL of MTT reagent/well and incubated for 3-4 hours at 37°C. After that, 100 µL/well of stopper solution (10% SDS in 0.01 N HCl) was added and incubated overnight at room temperature (25°C) in the dark, then read with an ELISA reader at λ 595 nm, and the absorption was obtained which stated the absorbance 4T1 cells. Single treatment absorbance data was converted into percent viability and used to calculate IC_{50} (Elufioye et al., 2017).

$$\% \text{ cell viability} = \frac{A_{sample}}{A_{control}} x100$$

DISCUSSION

Isolation and characterization

The purification steps of the ethyl acetate fraction isolate are based on the Preparative HPLC chromatogram (acetonitrile solvent), as presented in Figure 1. Based on Figure 1, the isolate of the major secondary metabolite SLH ethyl acetate fraction at peak A had a retention time (RT) of 4.08, and peak B had an RT of 4.49 minutes with a relative percent of 40% and 50%, respectively. The purification results using preparative HPLC components were isolate A of 6.4 mg (RT 4.08 minutes) and isolate B of 10 mg (RT 4.49 minutes). Then, the isolates were analyzed using analytical HPLC. Both showed the same retention time, 1,963 minutes (Figure 2). Moreover, the same RT data was also found in the LCMS-MS analysis results, both appearing at the highest RT of 15.36 minutes as presented in Figure 3.

Figure 4 also showed that both isolates A and B had the same $[M+H]^+$ value. To ensure that the two isolates were identical or isomers, the molecular ion [M+H]+ intensity ratio with a mass of 282.28 and the isotope of the two isolates with a mass of 283.28 was calculated. If all =1, then they were identical, and if >1, then they were isomers [201]. Based on the ratio of the intensity of the molecular ion ($[M+H]^+_A/([M+H]^+_B$ = 1) and the ratio of the two isotopic intensities ($([M+1+H]^+_A/([M+1+H]^+_B$ = 1) then the two isolates A and B were identical. The mass spectral pattern of this compound was also found in the pseudobaptigenin compound (Figure 5) which comes from a genus of Bajakah plants (*Spatholobus suberectus* Dunn) from the bark found by Yoon et al (Yoon et al., 2004). This compound was also discovered by Yanagihara et al as an anticancer (Yanagihara et al., 1993) and was discovered by Lee et al as an antioxidant (Lee et al., 2020). The structure of the compounds found is in accordance with Figure 6.

RESULT

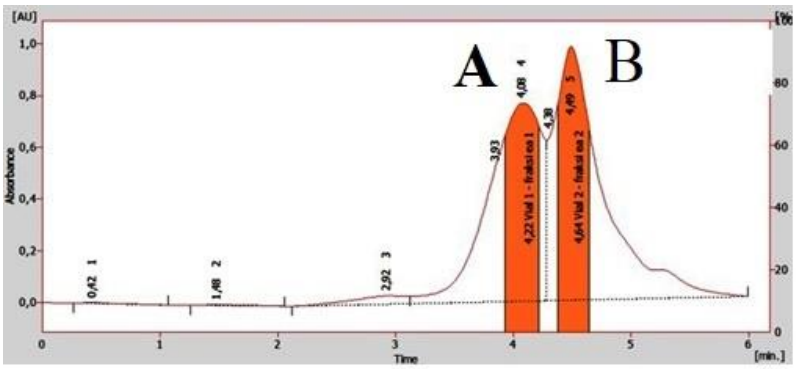

Figure 1. Preparative HPLC chromatogram of Major Isolates

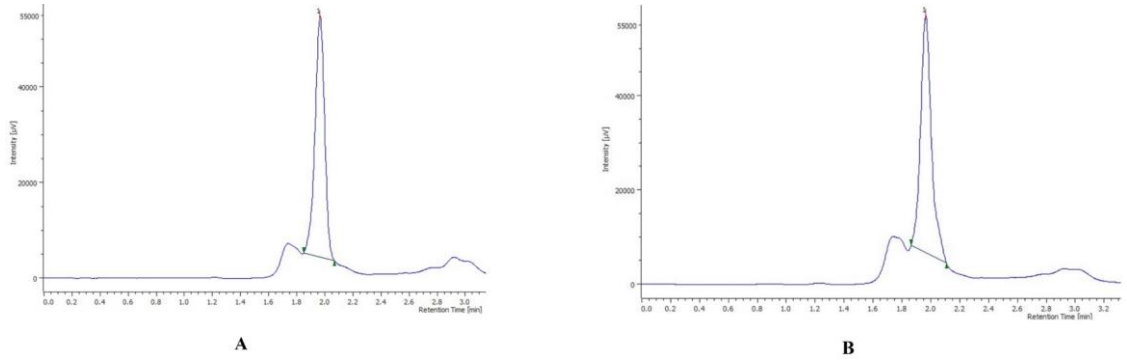

Figure 2. Analytical HPLC Chromatogram of Isolate A (RT 4.08 minutes) and B (RT 4.49 minutes)

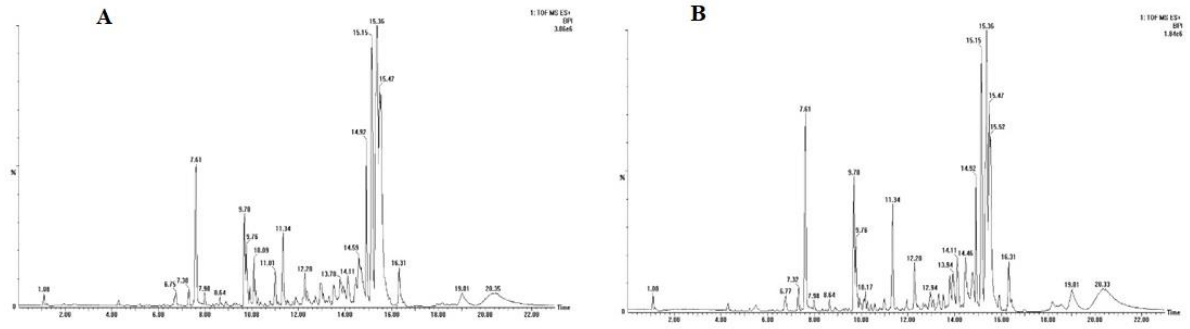

Figure 3. Chromatogram of Isolate A (RT 4.08 minutes) and B (RT 4.49 minutes)

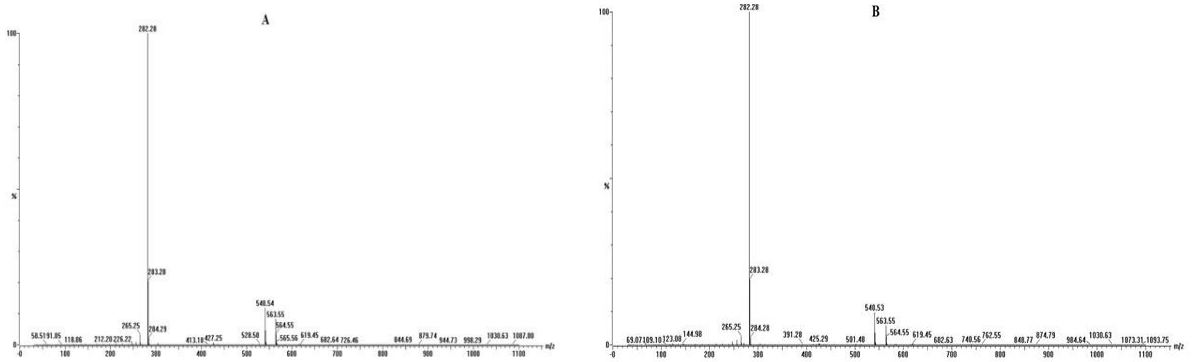

Figure 4. Mass spectra of Isolate A (RT 4.08 minutes) and B (RT 4.49 minutes)

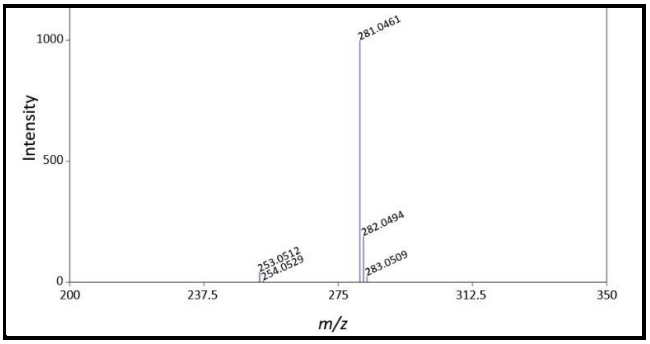

Figure 5. Mass Spectra of Pseudobaptigenin

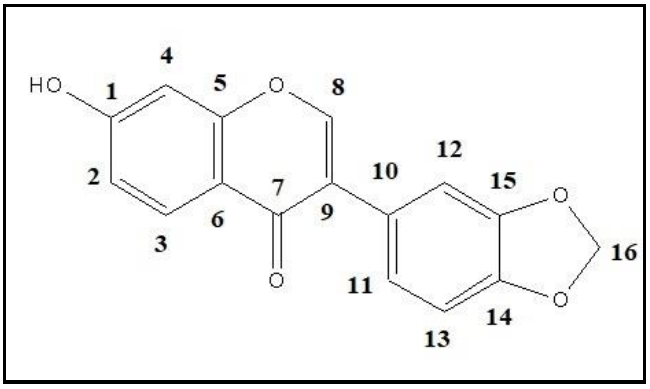

Figure 6. Pseudobaptigenin Compound Structure

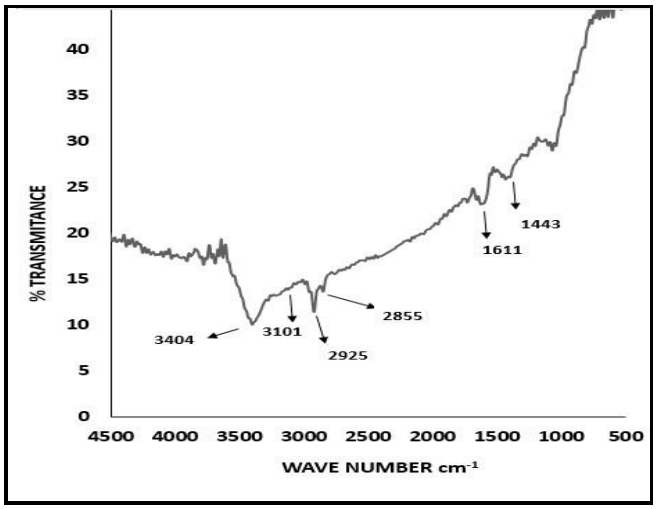

Figure 7. FTIR Spectra of Isolate A

The presence of OH groups, aromatic rings, and cyclic ethers is supported by FTIR data as explained in Figure 7. Based on the spectrum presented in Figure 7, absorption bands are found showing OH groups at wave number 3404 cm^{-1}, aromatic groups at wave number 3101 cm^{-1} and wave number 1661 cm^{-1}, tertiary carbon (-CH) at wave number 2925 and 2855 cm^{-1}, and the secondary carbon ($-CH_2$) is shown at wave number 1443 cm^{-1}. The presence of OH protons, benzene protons, ether protons, and protons on tertiary and secondary carbons, which are similar to the structure of the pseudobaptigenin compound, is explained by the 1H-NMR spectra as presented in Figure 8, Figure 6, and Table I.

The correlation between neighboring H atoms is explained in Figure 9 and 10. Protons on C no 2 atoms are neighbors to protons C no 3 atoms.

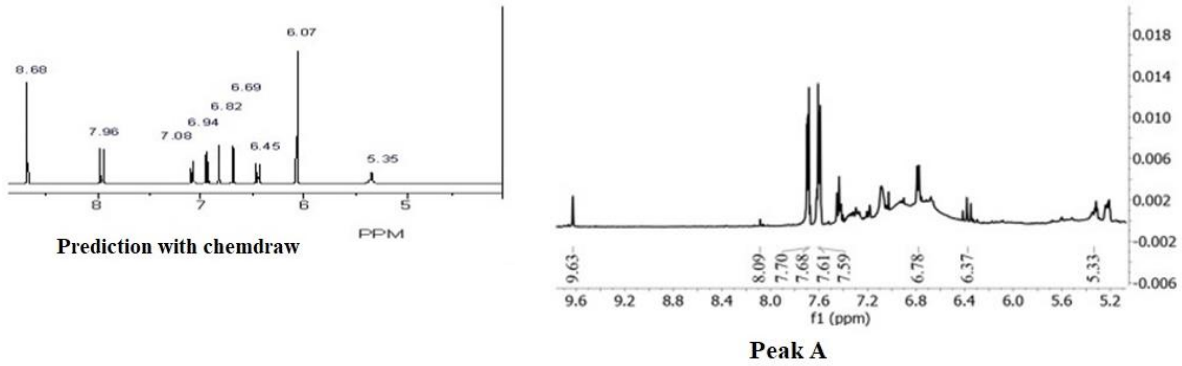

Figure 8. ^{1}D-^{1}H NMR Spectra of Pseudobaptigenin Chemdraw Prediction and Isolate A (RT 4.08 minutes)

Table I. Comparison of Proton Chemical Shifts of Pseudobaptigenin and Isolate A

No C	δH ppm		type of proton	multiplicity
	Pseudobaptigenin (ChemDraw prediction)	Isolate A		
1	5.35	5.33	-OH	Singlet
2	6.45	6.78	-CH aromatic	doublet
3	7.96	8.09	-CH aromatic	doublet
4	6.69	7.59	-CH aromatic	singlet
8	8.69	9.63	-CH ether	singlet
11	7.08	7.70	-CH aromatic	doublet
12	6.82	7.61	-CH aromatic	Singlet
13	6.94	7.68	-CH aromatic	doublet
16	6,07	6.37	-CH_2 ether	singlet

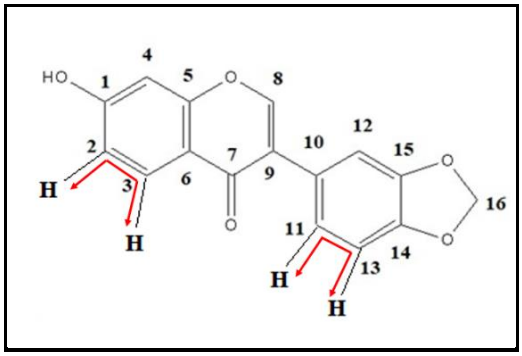

Figure 9. Correlation of H Atoms Neighboring Isolate A

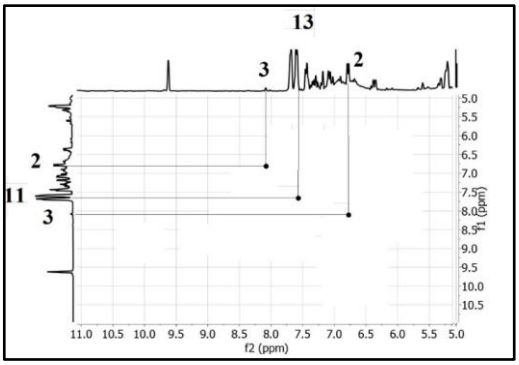

Figure 10. Cosy Spectrum of Isolate A

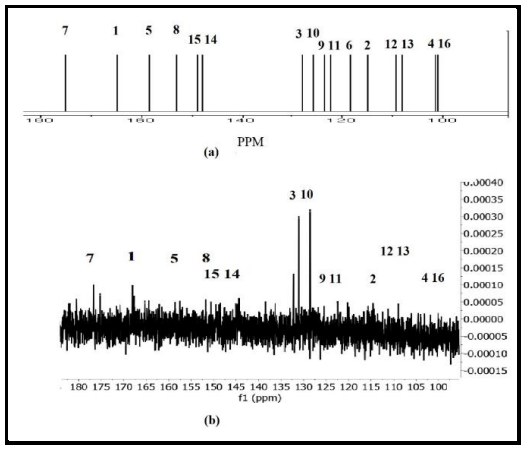

Figure 11. ChemDraw Predicted ^{13}C NMR Spectra of Pseudobaptigenin (a) and Isolate A (b)

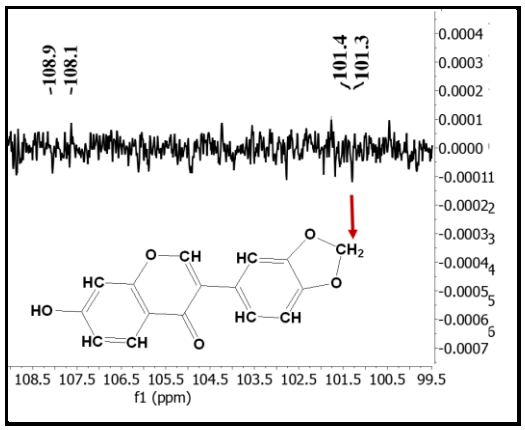

Figure 12. Spectra DEPT 135 Isolate A

Table II. Comparison of Pseudo-Baptinin and Isolate A Carbon Chemical Shifts

No C	δC ppm		Carbon Type
	Pseudobaptigenin (ChemDraw prediction)	isolate	
1	165.0	165.3	C quaternary
2	115.0	115.7	C tertiary
3	128.2	128.5	C tertiary
4	101.4	101.4	C tertiary
5	158.6	158.6	C quaternary
6	118.2	nd	C quaternary
7	175.3	175.3	C quaternary
8	153.2	151.8	C tertiary
9	123.5	120.2	C quaternary
10	125.8	125.5	C quaternary
11	122.4	122.4	C tertiary
12	109.4	108.9	C tertiary
13	108.0	108.1	C tertiary
14	148.0	148.0	C quaternary
15	148.7	148.7	C quaternary
16	101.2	101.3	C secondary

Likewise, protons on C no 11 atoms are neighbors to C no 13 atoms. The presence of secondary, tertiary, and quaternary carbons that are similar to the structure of the pseudobaptigenin compound is explained by the ^{13}C-NMR spectra as shown in Figure 11 and Table II. One secondary C atom (CH_2)

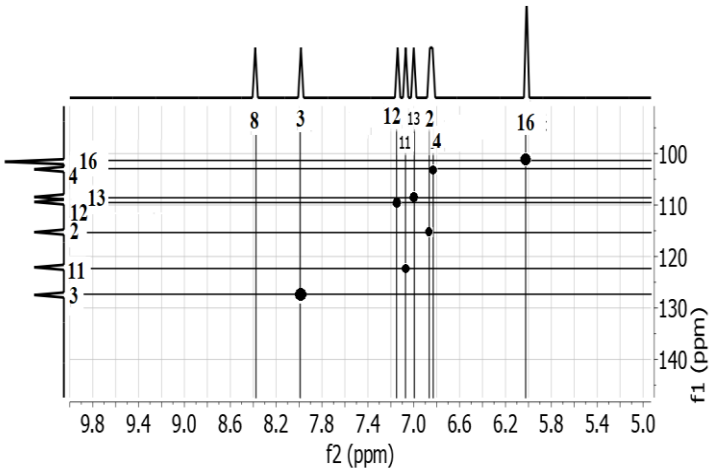

Figure 13. HSQC Spectra of Isolate A

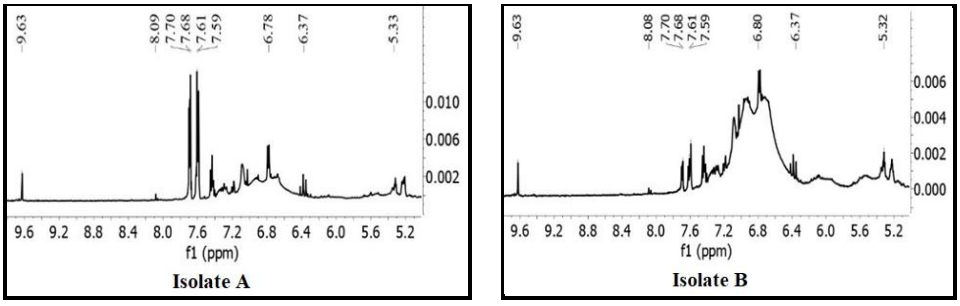

Figure 14. ^{1}H NMR spectra of Isolate A and B

Table III. Correlation of C and H isolate A

No C	δC ppm	δH ppm	Correlation
1	165.3	5.33	nd
2	115.7	6.78	exist
3	128.5	8.09	exist
4	101.4	7.59	exist
8	151.8	9.63	exist
11	122.4	7.70	exist
12	108.9	7.61	exist
13	108.1	7.68	exist
16	101.3	6.37	exist

Table IV. Comparison of 1H NMR Isolates A and B

No C	δH ppm	
	Isolate A	Isolate B
1	5.33	5.32
2	6.78	6.80
3	8.09	8.08
4	7.59	7.59
8	9.63	9.63
11	7.70	7.70
12	7.61	7.61
13	7.68	7.68
16	6.37	6.38

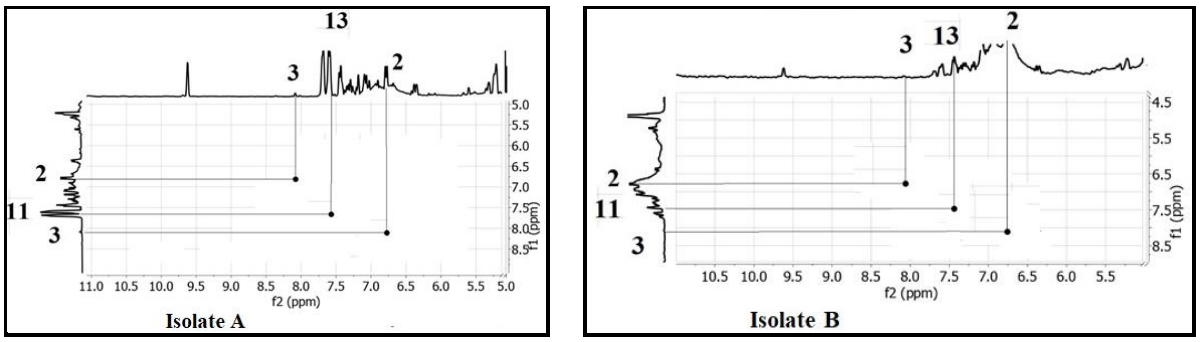

Figure 15. Spectra of Cosy Isolates A and B

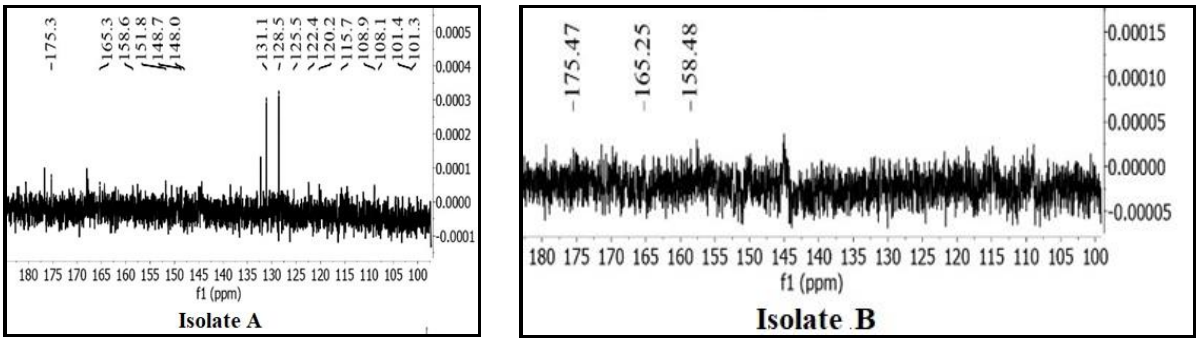

Figure 16. ^{13}C NMR spectra of Isolate A and B

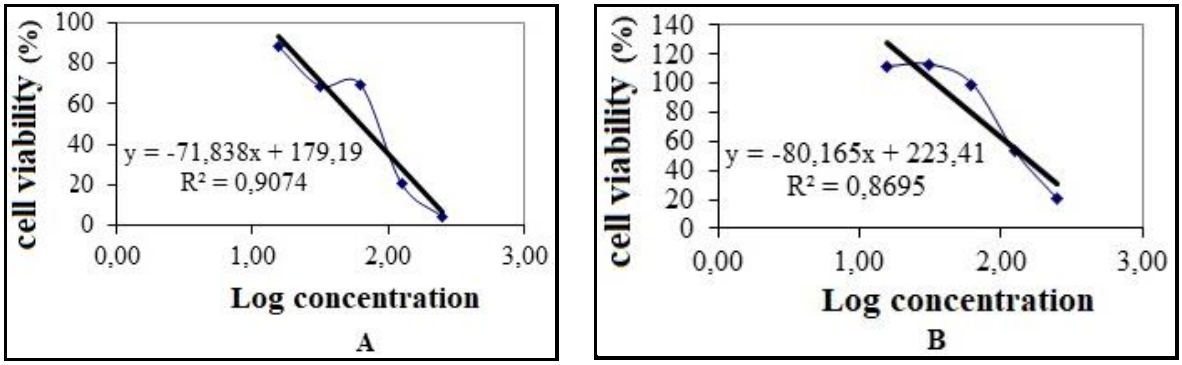

Figure 17. Linear relationship % viability and log concentration of isolate A and isolate B

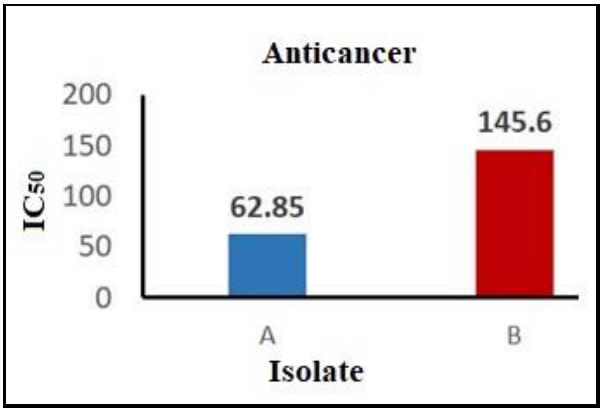

Figure 18. IC_{50} Anticancer Isolate A and Isolate B (ppm)

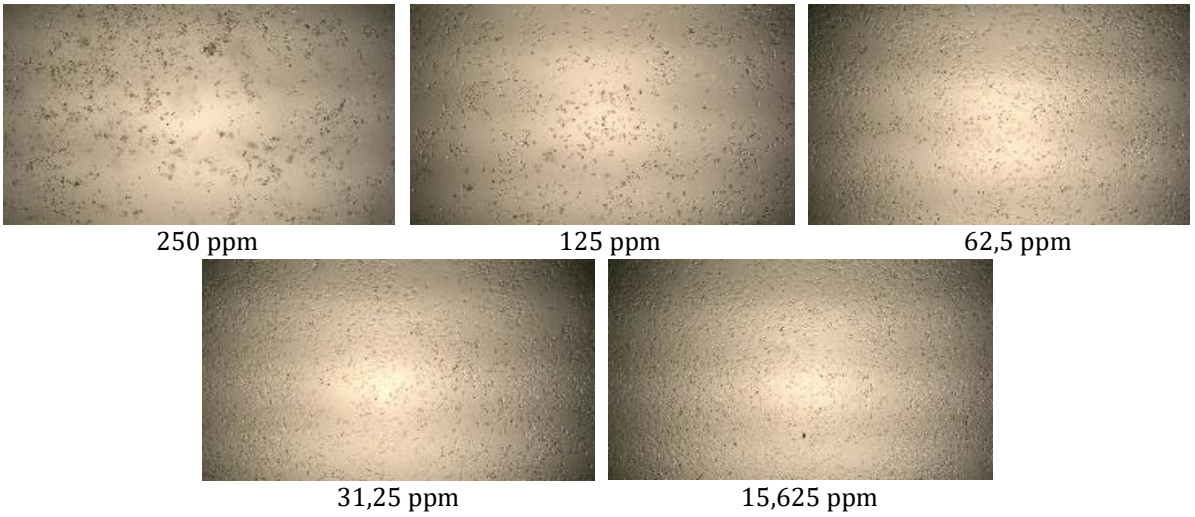

Figure 19. Photo of 4T1 Cell Viability Against Isolate A

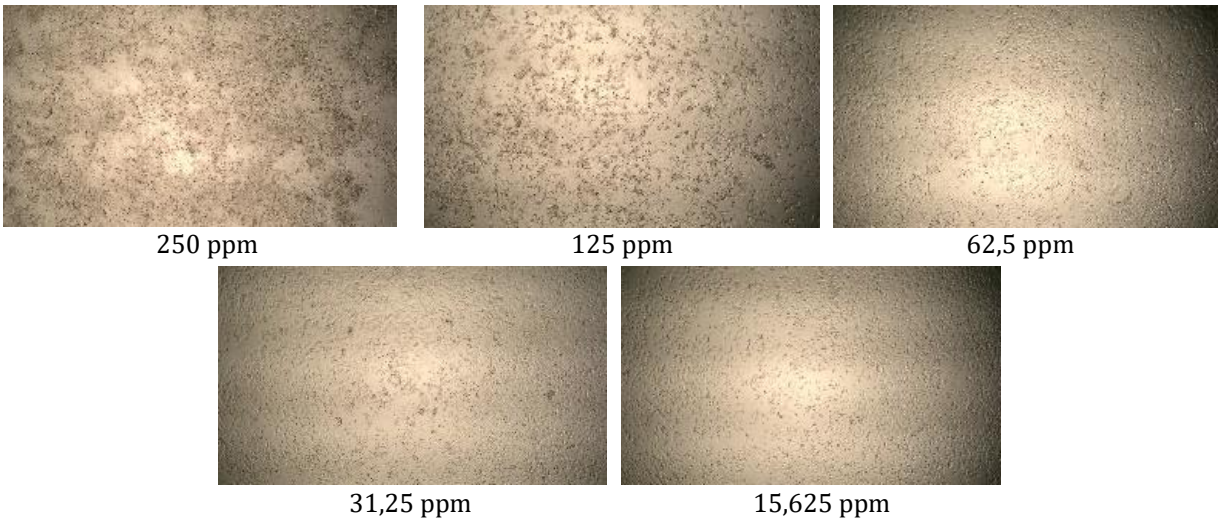

Figure 20. Photo of 4T1 Cell Viability Against Isolate B

can be seen in the ^{1}D-^{13}C NMR spectra as well as in the DEPT 135 spectra, as shown in Figure 12, with a chemical shift of 101.30 ppm. Proton and carbon correlations can be explained by the HSQC spectra of isolate A, as shown in Figure 13 and Table III. In addition to the similarity of the LCMS-MS and HPLC analytical data of isolates A and B, the similarities of the ^{1}H NMR spectra and comfort spectra of these two isolates were also obtained, as shown in Figure 14, Table IV, and Figure 15.

Thus, based on LCMS-MS data, analytical HPLC, ^{1}H NMR and Cosy it can be stated that isolate A and isolate B are the same compound. The difference in IC_{50} occurred, possibly caused by impurities that were still combined with isolate B. The ^{13}C NMR spectra of isolate B showed that many carbon signals did not appear due to the very minimal concentration of the isolate, so it could not be compared with the 13C NMR spectra of isolate A, as presented in Figure 16. Likewise, the spectra of DEPT 135 and HSQC Isolate B showed very weak signals, so they cannot be analyzed further. There is a strong suspicion that the ethyl acetate fraction isolate is a pseudobaptigenin compound containing OH, aromatic, cyclic ether, and ketone functional groups based on LCMS-MS, analytical HPLC, and NMR data. Apart from that, it is also confirmed by data from phytochemical test screening results, which show that the ethyl acetate fraction does not contain alkaloids. As is known, alkaloids are characterized by the presence of a cyclic nitrogen atom in their molecular structure (Kurek, 2019).

Anticancer Activity Test Results of Pure Isolate from Ethyl Acetate Fraction

The linear relationship between %viability and log concentration from the results of the

cytotoxic activity test against 4T1 cancer cells is presented in Figure 17. Based on the graph in Figure 17 above, the IC_{50} values obtained for isolate A (RT 4.08 minutes) and isolate B (RT 4.49 minutes) were 62.85 ppm and 145.60 ppm respectively (Figure 18). If this value is compared with the anticancer activity of the drug Capecitabine against 4T1 breast cancer cells (IC_{50} = 610,895 ppm) (Kaya Cakır & Eroglu, 2021), the anticancer power of the two isolates from the ethyl acetate fraction is still much stronger. Visually, the lower the concentration of the isolate, the higher the % viability as shown by the results of observing cancer cells from an inverted microscope in Figures 19 & 20.

CONCLUSION

Based on the results and discussion, it can be concluded that based on the LCMS-MS mass spectra, FTIR spectra, and 1D and 2D NMR data, isolates A and B from the ethyl acetate fraction are Pseudobaptigenin and the anticancer bioactivity of the compound Pseudobaptigenin from the ethyl acetate fraction isolate has an IC_{50} value of 62.85 ppm. On the other hand, isolate B, which is Pseudobaptigenin with impurities, has IC_{50} 145.6 ppm.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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