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CHARACTERIZATION OF SECONDARY METABOLITES AND CYTOTOXIC ASSAY OF *Haliclona* sp. SPONGE AGAINST T47D BREAST CANCER CELLS

AJUK SAPAR[1*], MILLENIA[1], ANTHONI BATAHAN ARITONANG[2], RUDIANSYAH[1], AND WINDA RAHMALIA[1]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Tanjungpura, Prof. Dr. H. Hadari Nawawi Street, Pontianak, Indonesia

*Corresponding author email: ajuk.sapar@chemistry.untan.ac.id

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Abstract

Characterization of secondary metabolites and cytotoxic testing of *Haliclona* sp. against T47D breast cancer cells were conducted in this study. The objective was to assess the cytotoxicity of T47D cancer cells and identify the functional groups involved. The research methods employed included maceration, partitioning, phytochemical testing, toxicity testing using the BSLT method, separation through flash column chromatography (FCC), cytotoxic testing using the MTT method, and characterization using FTIR. The partition results of methanol extract consist of n-hexane, ethyl acetate, and methanol-water fractions. The methanol extract demonstrated high toxicity, with an LC_{50} of 5.21 ppm. Among the fractions, the ethyl acetate fraction exhibited the highest toxicity compared to the n-hexane and methanol-water fractions, with LC_{50} values of 25.76 ppm, 42.71 ppm, and 55.26 ppm, respectively. Phytochemical testing of the ethyl acetate fraction yielded positive results for terpenoids, steroids, alkaloids, and phenolic compounds. The ethyl acetate fraction was further separated using flash column chromatography, resulting in ten combined fractions (M1-M10). The cytotoxicity tests of the M3 fraction against 747D breast cancer cells showed non-toxic effects, with an IC_{50} value of 1382.29 ppm. The FTIR analysis of the M3 fraction revealed the presence of functional groups such as O-H, =C-H, C-H aliphatic, C=O, and C=C, which is indicative of the presence of terpenoids, steroids, and esters.

INTRODUCTION

Sponges are multicellular animals that inhabit coral reef ecosystems and have developed defense mechanisms similar to those of terrestrial and aquatic plants [1][2]. Sponge developed a biodefense system that produces secondary metabolites [3] or antipredator chemical defenses [4]. Sponges serve as a valuable source of secondary metabolites, which exhibit chemopreventive and tumor chemotherapeutic properties [5]. Among secondary metabolites synthesized by sponges are alkaloids, terpenoids [6][7], steroids, polyketides, and various other compounds [7], which have shown potential applications in the pharmaceutical field as an antioxidant, antimicrobials, anti-inflammatories, and anticancer agents [8].

The toxic effect of the methanol extract of the sponge Haliclona sp from Lemukutan Island, Indonesia, on the larvae of Artemia salina Leach is 70.1 ppm [9]. A preliminary test of anticancer activity was carried out using a brine shrimp lethality assay using Artemia salina [10]. The ethyl acetate extracts *Haliclona amboinensis* sponge from South Sulawesi, Indonesia shows anticancer activity against T47D breast cancer cells [11]. Another *Haliclona* (Soestella) *mucosa* sponge is also cytotoxic against human melanoma cell lines (MEWO and A375 cells [5]. An acetylenic compound was isolated from a Red Sea sponge, Haliclona Sp potent selective antitumor activity towards MCF-7 cells [12] Therefore, it is necessary to determine the cytotoxic activity of the M3 fraction from the ethyl acetate fraction Sponge *Haliclona* sp against T47D cancer cells and characterization of its secondary metabolites.

EXPERIMENT

Materials

The materials used in this study were H_2O (technical), HCl (p.a Merck), H_2SO_4 (p.a Merck), CH_2Cl_2 (p.a Merck), C_2H_6OS (p.a Merck), $C_4H_8O_2$ (technical), metal Mg, CH_3OH (technical), C_6H_{14} (technical), $FeCl_3$ (p.a Merck), Dragendorf reagent (p.a Merck), Lieberman Burchard reagent (p.a Merck), Mayer reagent (p.a Merck), MTT reagent (3-(4,5-dimethiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (p.a Merck), Salkowski reagent (p.a

Merck), 5% Cerium sulfate reagent (pa Merck), Vanillin sulfate reagent (p.a Merck), Wagner reagent (p.a Merck), silica gel (70-230 mesh), Sponge sample with code LMK-4 (*Haliclona* sp). and eggs of *Artemia salina* Leach.

Instrumentations

The instrument used in this study was the Fourier Transform Infrared Spectroscopy (FTIR, IRPrestige 21 Shimadzu).

Procedures

Sample Preparation

Sponge samples were taken on Lemukutan Island, Bengkayang Regency, West Kalimantan using SCUBA diving techniques at a depth of 6 m. A sample code LMK-4 of 117.172 grams was cleaned of impurities, then cut into small pieces.

Maceration and Partitioning

Samples were extracted using the maceration method. The sponge is inserted into the macerator tube, then soaked using methanol solvent for 24 hours until the solvent is clear again. The sample was filtered using Whatman filter paper and concentrated using a rotary evaporator with a temperature of 48 °C until a viscous methanol extract was obtained, then weighed the mass. Then the viscous methanol extract is partitioned using *n*-hexane and ethyl acetate solvents. Then it is concentrated using a rotary evaporator with a temperature of 48°C until a viscous fraction is obtained [10].

Phytochemical Test

Terpenoids dan steroids test

The extract and each fraction were added three drops of Lieberman Burchard reagent for a steroids test, and for a terpenoids, test added three drops of Salkowski reagent. Positive results of steroids are characterized by a red, orange, or bluish-green color, while terpenoids are characterized by a red or brown tint [13].

Alkaloids test

The extract and each fraction were added three drops of HCl 2N. Then three drops of each specific reagent were added namely Dragendrof, Mayer, and Wagner. The positive result of alkaloids is characterized by the formation of deposits of orange color (Dragendrof), yellowish-white precipitates (Mayer) and brown precipitates (Wagner) [13].

Saponin Test

The extract and each fraction are put into a test tube, then add aqueducts in a ratio of 1:1 and shaken for 1 minute. If the foam is formed, then add two drops of HCl 2N. Positive results of saponins are characterized by the formation of stable foam for 10 minutes with a height of 1-10 cm [13].

Tannin test

The extract and each fraction are added to three drops of 5% $FeCl_3$ reagent. The positive result of tannins is characterized by a blackish-green or dark blue color [13].

Toxicity Test Brine Shrimp Lethality Test (BSLT) Method

Artemia salina Leach eggs are hatched in a container containing artificial seawater (38 grams of non-iodized salt in 1000 mL of aqueous), in a dark section that has been partitioned. The bright part is given an aerator and a 25-watt lamp as a light source. The mother liquor with a concentration of 2000 ppm is diluted to 10; 100 and 1000 ppm in a volume of 5 mL. All concentrations and blanks are made on a triple basis and during testing are given one drop of yeast suspension. Observations were made after 24 hours [13].

Characterization of Secondary Metabolite Compounds of Ethyl Acetate Fraction

Characterization of the functional groups of the ethyl acetate fraction using the FTIR instrument.

Extraction and Fractionation

Extraction and fractionation are initiated with orientation using thin-layer chromatography (TLC). The silent phase used is the silica plate and the mobile phase used includes *n*-hexane, dichloromethane, ethyl acetate, chloroform, and methanol, as well as the ratio of the mixture. The eluent with the best separation is selected as the mobile phase in the fractionation process using flash column chromatography (FCC) and the stationary phase is silica gel. Eluat is accommodated in a volume of 5 mL and separation with the same stain pattern will be combined. The fractionation results obtained were tested for cytotoxic activity in vitro using T47D cancer cells and the characterization of their secondary metabolites using the FTIR instrument.

Characterization of the FTIR Spectrum

Characterization of the functional groups of the M3 and M4 fractions using the FTIR instrument.

Cytotoxic test of the MTT Method

The M3 fraction of 10 mg was dissolved in 100 µL of DMSO and homogenized with a vortex. Then made seven series of concentrations 10; 50; 100; 250; 500; 1000 and 1500 mL, all concentrations are made on a triple basis. Cells were transferred into a 96-well plate of 100 µL, then incubated overnight. The next day the condition of the cells was observed and seven concentration series were added. Then it is incubated overnight. The next day the condition of the cells was observed, then added 100 µL of MTT reagents and incubated for 4 hours until purple formazan crystals formed. The reaction is stopped in increments of a 100 µL SDS stopper and stored at room temperature. After 24 hours, observe the absorbance value using an ELISA reader at a wavelength of 570 nm [14].

RESULTS AND DISCUSSION

Sample Preparation

Sponge samples taken on Lemukutan Island, Bengkayang Regency, West Kalimantan were identified taxonomically to determine the type of species. The following are the results of the identification of sponge samples:

Figure 1. Sponge Sample

The results of the taxonomic identification of the sponge sample in **Figure 1** are as follows:

Kindom : Animalia
Phylum : Polypheric
Class : Demospongia Sollas, 1885
SubClass : Heteroscleromorpha Cárdenas, Pérez - Boury-Esnault, 2012
Order : Haplosclerida Topsent, 1928
Family : Chalinidae Gray, 1867
Genus : *Haliclona* Grant, 1841
Subgenus : *Haliclona* (Haliclona) Grant, 1836
Species : *Haliclona* (Haliclona) sp.

Maceration and Partitioning

A total of 117.17 g of *Haliclona* sp. sponge were macerated using methanol as a solvent for 10 cycles of 24 hours each, resulting in crude methanol extract weighing 8.792 g with a yield of 7.51%. To precipitate the extracted salts, 6.696 g of crude methanol extract was dissolved in 2 mL of water and 98 mL of methanol. The mixture was then partitioned using *n*-hexane and ethyl acetate solvents to separate the non-polar and semi-polar compounds. The obtained results showed a mass of 0.116 g for the *n*-hexane fraction with a yield of 1.73%. The ethyl acetate fraction weighed 1.183 g with a yield of 17.67%. The methanol-water fraction weighed 0.987 grams with a yield of 14.74%.

Phytochemical Test

Phytochemical tests were carried out to determine the content of its secondary metabolite compounds in each extract and fraction shown in **Table 1**. Test steroids using anhydrous acetic acid and concentrated sulfuric acid. The use of anhydrous acetic acid aims to form acetyl derivatives and concentrated sulfuric acid as catalysts to accelerate the occurrence of discoloration reactions [15]. Terpenoids assays using chloroform and concentrated sulfuric acid. The alkaloids test used specific reagents like Dragendrof, Mayer, and Wagner. The addition of hydrochloric acid (HCl) 2N is to form alkaloid salts or free base [16]. A positive result of the alkaloids will give an orange-red precipitate formed, indicating the presence of alkaloids [17]. Saponin test using the Forth method in the form of aqueous. Foam formation is caused by a combination compound between non-polar sapogenin chains and polar side chains that form micelles during shaking [9]. The tannin test showed a positive reaction to $FeCl_3$ when a color change from brownish to blackish was formed [18].

Table 1. Phytochemical test results of *Haliclona* sp. sponge.

Compound	Sample			
	Methanol Extract	*n*-hexane fraction	Ethyl acetate fraction	Methanol-water fraction
Steroids	++	+++	+	-
Terpenoids	+	+++	+	-
Alkaloids	+++	+++	++	++
	+++	+++	++	+
	+++	+++	++	++
Saponin	-	+	-	-
Tannins	++	-	+++	++

Response description: (+) low, (++) medium, (+++) high, (-) undetectable.

Toxicity Test Brine Shrimp Lethality Test (BSLT) Method

A toxicity test of the BSLT method was carried out as an initial screening to see the toxic activity of the extract and fraction of *Haliclona* sp sponge. The concentrations used are 10, 100, and 1000 ppm as shown in **Table 2**. The blank solution contains DMSO and seawater.

Table 2. A toxicity test result of *Haliclona* sp. sponge.

Sample	Concentration (ppm)	Number of larvae	Average larval mortality	LC_{50} (ppm)
Blank solution	0	30	0	-
Methanol Extract	10	30	14	5,21
	100	30	30	
	1000	30	30	
n-Hexane fraction	10	30	10	42,71
	100	30	17	
	1000	30	26	
Ethyl acetate fraction	10	30	10	25,76
	100	30	20	
	1000	30	28	
Methanol-water fraction	10	30	8	55,26
	100	30	19	
	1000	30	24	

Based on the LC_{50} value calculation obtained, it shows compound polarity also affects toxicity values such as methanol extract and ethyl acetate with an LC_{50} value of 5.21 ppm and 25.76 ppm, respectively are highly toxic compared to the *n*-hexane 42.71 ppm. Toxicity test results show that ethyl acetate fraction has a greater potential to continue cytotoxic tests *in vitro* so that the fraction is continued to the separation and characterization stages of its secondary metabolites.

Characterization of Secondary Metabolite Compounds of Ethyl Acetate Fraction

The FTIR spectrum of ethyl acetate fraction is shown in **Figure 2**. The spectrum indicates a compounds complexity and causes strain vibrations of the N-H group (3300-3500 cm^{-1}), the =C-H group (3100-3000 cm^{-1}), an aliphatic C-H group (3000-2850 cm^{-1}) only forms a shoulder, since overlapping occurs with along O-H group. But the presence of the N-H stretching group is represented by the presence of the N-H bend. group (1516.05 cm^{-1}) and C-N stretching (1201.65 cm^{-1}). The presence of a cluster =C-H str. represented by the cluster C=C stretching (1641.42 cm^{-1}). The existence of an aliphatic C-H group is represented by the -CH_3 bending group (1338.60 and 1402.25 cm^{-1}), -CH_2 bending (1338.60 cm^{-1}), and CH_2 rock. (740.67 cm^{-1}). FTIR interpretation results correlate with phytochemical test results, where alkaloid compounds are characterized by the N-H bend. absorption (1516.05 cm^{-1}) and C-N streching (1201.65 cm^{-1}). Phenolic or tannin compounds are

characterized by a broad band of O-H stretching (3446.79 cm^{-1}), C=C stretching (1641.42 cm^{-1}), and CH_2 bending (1402.25 cm^{-1}), CH bending (1338.60 cm^{-1}) and C-O stretching (1118.71 and 1045.42 cm^{-1}). Terpenoid compounds are characterized by C=C stretching (1641.42 cm^{-1}) and CH_2 bending (1402.25 cm^{-1}). Steroid compounds are characterized by O-H stretching (3446.79 cm^{-1}), C=C stretching (1641.42 cm-1), CH_3 bending (1463.97 cm^{-1}), CH_2 bending (1402.25 cm^{-1}), and CH_2 rocking (740.67 cm^{-1}).

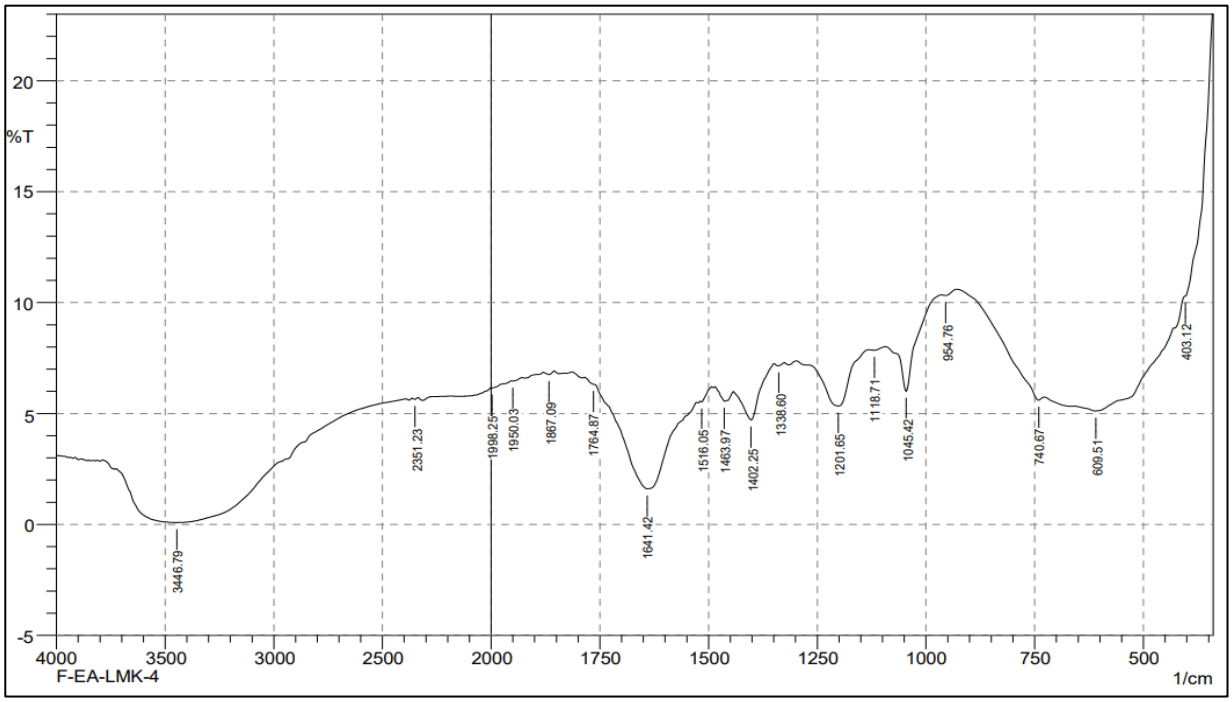

Figure 2. FTIR spectrum of ethyl acetate fraction.

Extraction and Fractionation

The separation of ethyl acetate fractions uses a ratio eluent of *n*-hexane: dichloromethane in the elucidation process, and orientation using TLC shows an occurrence of separation due to the increase in the polarity of the eluent. Gradient eluent compositions used in the flash column chromatography (FCC) method include *n*-hexane 100%, *n*-hexane: dichloromethane (90:10 for 2 times, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90), dichloromethane 100%, dichloromethane: methanol (95:5, 90:10, 50:50), methanol 100% and methanol: water 90:10.

The elution yield on an ethyl acetate fraction yielded 381 vials. The combined fractionation results were seen based on the similarity of the stain patterns obtained by the TLC and then viewed under UV 254 nm (**Figure 3**) and UV 366 nm (**Figure 4**) so that 10 combined fractions were obtained as shown in **Table 3**.

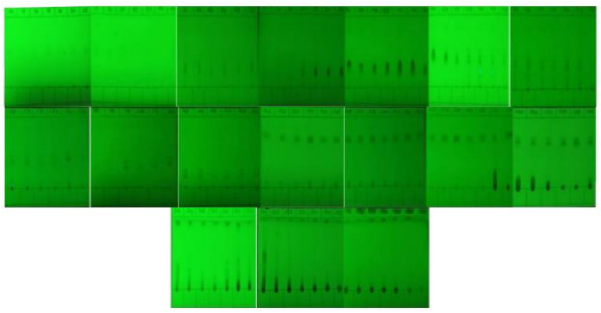

Figure 3. Chromatogram profile of TLC at UV 254 nm.

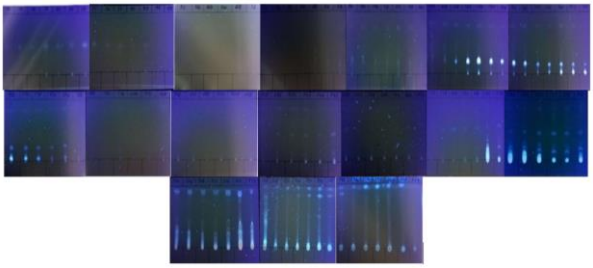

Figure 4. Chromatogram profile of TLC at UV 366 nm.

Table 3. The TLC of fractions combine.

Sample Code	Vials combine
M1	10-51
M2	52-83
M3	84-127
M4	128-191
M5	192-247
M6	248-271
M7	272-289
M8	290-299
M9	300-329
M10	330-381

The combined fraction of M1-M10 was monitored with TLC and sprayed using vanillin sulfate and 5% cerium sulfate reagents.

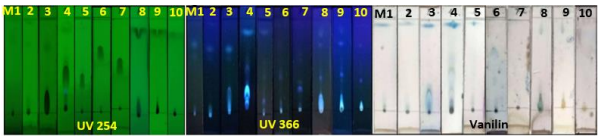

Figure 5. Combined fraction (M1-M10) under UV254 and 366 nm are sprayed with vanillin sulfate reagent.

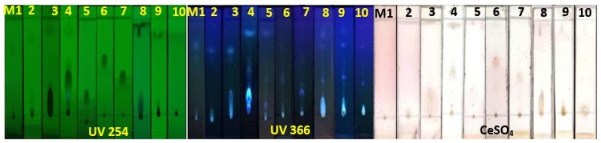

Figure 6. The combined fraction (M1-M10) under UV254 and 366 nm are sprayed with $CeSO_4$ reagent.

The combined fraction TLC plate sprayed with cerium sulfate reagent showed a purplish-blue discoloration of the stain patches as shown in **Figure 5** and **Figure 6**. Terpenoids showed a positive reaction to cerium sulfate reagent with the formation of a brownish-red color [19]. In addition, combined compounds include alkaloids, steroids, terpenoids, and phenolics [13]. The mass of each of the combined fractions is shown in **Table 4**.

Table 4. The mass of the combined fraction.

Sample code	Mass fraction (mg)
M1	13,8
M2	13,3
M3	42,3
M4	24,3
M5	20,3
M6	10,9
M7	9,1
M8	43,8
M9	297,9
M10	909,9

The M3 and M4 fractions were selected to be continued to test their cytotoxic activity *in vitro* and FTIR analysis, where the fractions have a strong luminescence under UV 254 nm and 366 nm and also show a brownish orange spot against cerium sulfate and blue against vanillin sulfate. A compound can fluoresce due to the presence of a chromophore group responsible for the absorption of radiation rays. A chromophore group was suspected that it can bind to the T47D cancer cell receptors strongly through covalent bonds in inhibiting T47D cancer cells.

Characterization of the FTIR Spectrum

The results of the FTIR spectrum analysis of the M3 fraction are shown in **Figure 7**. The presence of a widened absorption of the O-H stretching group (3450.65 cm^{-1}), supported by the presence of strain vibrations of the C-O group (secondary alcohol) stretching (1124.50 cm^{-1}) and C-O (primary alcohol) stretching (1076.28 cm^{-1}). The presence of a C=O group (ester) str. (1735.93 cm^{-1}) is supported by the presence of a C-O group (ester) stretching (1280.73 cm^{-1}). The presence of a cluster =C-H stretching (3072.46 cm^{-1}), is supported by the presence of the cluster C=C stretching (1637.56 cm^{-1}) and cluster =C-H bending (966.34 cm^{-1}). Absorption of the stretching aliphatic C-H group (2956.87, 2924.09, and 2854.65 cm^{-1}), supported by the presence of a CH_2 bending group (1382.96 cm^{-1}), cluster CH_3 bending (1462.04 cm^{-1}) and CH_2 rocking (966.34 cm_{-1}). The presence of these functional groups when associated with phytochemical test results is suspected to be related to the terpenoid, steroid, and ester groups.

The results of the FTIR spectrum analysis of the M4 fraction are shown in **Figure 8**. The presence of a widened absorption of the O-H stretching group (3435.22 cm^{-1}), supported by the presence of strain vibrations of the C-O group (secondary alcohol) stretching (1126.43 cm^{-1}) and C-O (primary alcohol) stretching (1076.28 cm^{-1}). The presence of a C=O group (ester) str. (1734.01 cm^{-1}) is supported by the presence of a C-O group (ester) stretching (1282.66 cm^{-1}). The presence of a cluster =C-H stretching (3072.60 cm^{-1}), supported by the presence of the C=C (olefin) stretching group (1635.64 cm^{-1}) and cluster =C-H bend. (968.27 cm^{-1}). Absorption of the stretching aliphatic C-H group (2956.87, 2926.01, and 2854.65 cm^{-1}), supported by the presence of a bending CH_2 cluster. (1382.96 cm^{-1}), cluster CH_3

bending (1462.04 cm^{-1}) and CH_2 rocking (968.27 cm^{-1}). The presence of such groups indicates the presence of terpenoids, steroids, and esters.

Based on TLC monitoring of M3 and M4 fractions, it can be seen that the separation pattern has similarities. The results of the interpretation of FTIR on the M3 and M4 fractions have similarities of suspected compounds, namely terpenoids, steroids, and esters. The difference in the spectrum of the M3 and M4 fractions is seen in the absorption of the wave number, where the M4 fraction gives a stronger peak uptake than the M3 fraction.

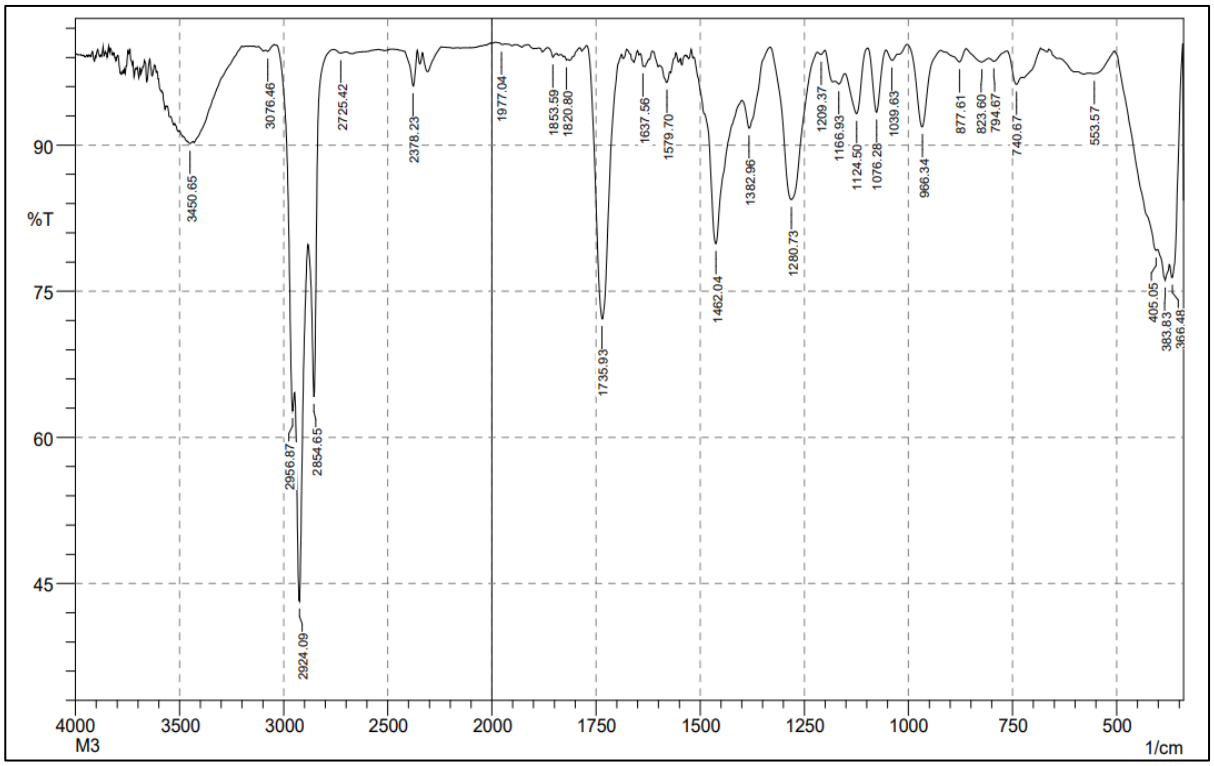

Figure 7. FTIR spectrum fraction M3.

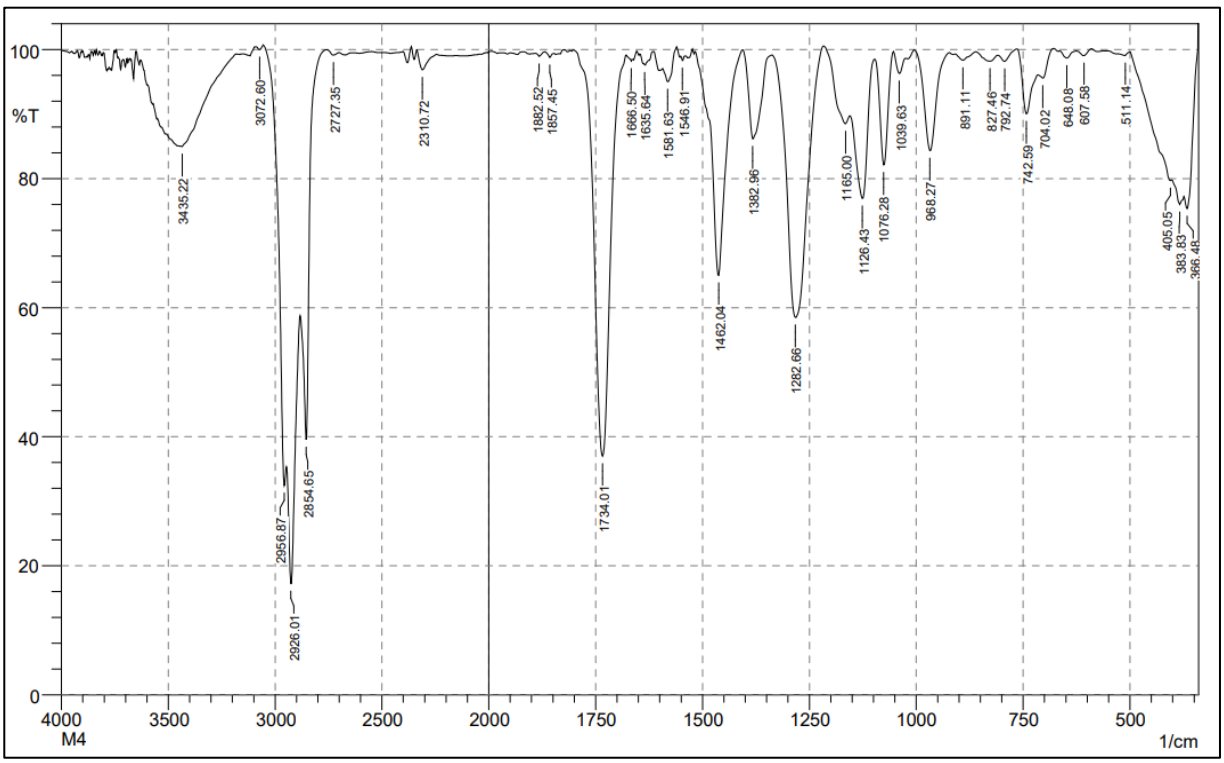

Figure 8. FTIR spectrum of fraction M4.

Cytotoxic test of the MTT method

One type of cancer cell that is commonly used for cytotoxic tests is 747D breast cancer cells [20]. Cytotoxic testing on M3 fraction using seven concentration series of 10; 50; 100; 250; 500; The 1000 and 1500 ppm listed in **Table 5**, as well as as a positive control in the form of 5-fluorouracil made seven series of concentrations, namely 10; 50; 250; 500; 750 and 1000 ppm listed in **Table 6**.

Table 5. Cytotoxic test results of fraction M3.

Concen-tration	Living cell (%)			The average	IC_{50} value
	R1	R2	R3		
10	79.86	86.21	83.96	83.35	
50	80.74	70.77	84.26	78.59	
100	78.69	76.63	78.20	77.84	
250	71.75	74.68	67.84	71.42	1382.28
500	67.25	64.02	61.87	64.38	
750	52.59	54.44	56.10	54.38	
1000	50.63	52.00	52.29	51.64	

Table 6. Cytotoxic 5-Fluorouracil test results.

Concen-tration	Living cell (%)			The average	IC_{50} value
	R1	R2	R3		
10	72.29	60.13	59.20	63.87	
50	77.13	47.97	37.37	54,15	
100	68.86	64.65	67.77	67.10	
250	51.87	50.46	34.87	45.73	267.31
500	43.13	37.83	35.65	38.87	
750	30.97	24.74	15.38	23.70	
1000	22.24	22.08	16.78	20.37	

Based on cytotoxic test results, it shows that the M3 fraction is non-toxic against T47D cells by an IC_{50} value of 1382.286 ppm. These results are different from the ethyl acetate extract of *H. amboinensis* which showed anticancer activity against 747D cells with an IC50 value of 29.0 ± 1.5 [11] and the ethyl acetate extract of *Haliclona fascigera* against 747D cells with an IC50 value of 67,1 [21]. This is presumably due to differences in species or the synergistic effect of the compounds contained in the ethyl acetate fraction of *H. amboinensis species*. Meanwhile 5-fluorouracil used as a positive control indicates moderate toxicity properties by an IC_{50} value of 267.314 ppm. A comparison of cytotoxicity test results between M3 and 5-fluorouracil showed that the cytotoxic activity of the M3 fraction was much smaller than that of 5-fluorouracil because the M3 fraction is not a pure compound. 5-Fluorouracil is usually used as a positive control in colorectal cancer cells [22] and as a chemotherapeutic agent for the treatment of solid tumors [23]. The M3 fraction still contains several compounds such as terpenoids, steroids, and esters as identified from the FTIR spectrum. The presence of compounds combination can interfere with the cytotoxic activity of the fraction [24]. Inactive cytotoxic activity can be caused by internal and external factors. Internal factors such as impurity compounds are thought to be a combination of toxic and non-toxic compounds so these toxic compounds cannot work effectively in inhibiting the growth of cancer cells. [25]. External factors, such as too long a time lag in the handling process, so internal and external factors greatly affect the final result of the analysis [24].

CONCLUSION

The conclusion in this study was that the ethyl acetate fraction was very toxic in toxicity tests using *Artemia salina* Leach larvae with an LC_{50} value of 25.76 ppm. The fraction with the code M3 is non-toxic in cytotoxic tests using T47D cancer cells with an IC_{50} value of 1382,285 ppm and the presence of terpenoid compounds, steroids, and esters was identified.

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