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Stylotella sp. Acetone Extract from Selayar Island and Its Activity against Breast Cancer Cells MCF-7

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Abstract: Styletella sp. is a type of sponge in the Halichondriidae family which is rich in secondary metabolite compounds that have various bioactivities that have the potential as drugs in the future, one of which is anticancer. This study aims to determine the types of secondary metabolites found in *Stylotella* sp. from Selayar Island and to determine the secondary metabolite bioactivity of MCF-7 breast cancer cells. The method used was extraction using acetone, fractionation and purification. The purity test was carried out by testing three eluent systems at TLC, namely chloroform:ethyl acetate (9:1), *n*-hexane: acetone (8:2), chloroform: acetone (9:1) eluent. Then qualitatively and characterized by FTIR spectrophotometer. The results showed that pure isolates contained alkaloid compounds. The MCF-7 cell activity test showed an IC_{50} value for acetone extract of 1452.38 g/mL which has potential anticancer activity and an IC_{50} value for pure isolate acetone extract of 14983.52 g/mL.

Keywords: *Stylotella* sp, alkaloid, breast cancer cells MCF-7, IC_{50}

***Abstrak*:** *Stylotella sp. merupakan jenis spons dalam famili Halichondriidae yang kaya akan senyawa metabolit sekunder yang memiliki berbagai bioaktivitas yang berpotensi sebagai obat di masa depan, salah satunya adalah antikanker. Penelitian ini bertujuan untuk mengetahui jenis metabolit sekunder yang terdapat pada Stylotella sp. dari Pulau Selayar dan mengetahui bioaktivitas metabolit sekunder sel kanker payudara MCF-7. Metode yang digunakan adalah ekstraksi menggunakan aseton, fraksinasi dan pemurnian. Uji kemurnian dilakukan dengan menguji tiga sistem eluen pada KLT, yaitu eluen kloroform:etil asetat (9:1), eluen n-heksana: aseton (8:2), eluen kloroform: aseton (9:1). Kemudian secara kualitatif dan dikarakterisasi dengan spektrofotometer FTIR. Hasil penelitian menunjukkan bahwa isolat murni mengandung senyawa alkaloid. Uji aktivitas sel MCF-7 menunjukkan nilai IC_{50} untuk ekstrak aseton sebesar 1452,38 g/mL yang memiliki potensi aktivitas sebagai antikanker dan nilai IC_{50} isolat murni ekstrak aseton sebesar 14983,52 g/mL.*

Kata kunci*:* *Stylotella sp, alkaloid, sel kanker payudara MCF7, IC_{50}*

INTRODUCTION

In 2020, there were 2.3 million women diagnosed with breast cancer and 685000 deaths globally. As of the end of 2020, there were 7.8 million women alive who were diagnosed with breast cancer in the past 5 years, making it the world's most prevalent cancer (WHO, 2022). The World Health Organization (WHO) estimates that 84 million people died in the period 2005-2015. According to research conducted in Jakarta on April 2001-2003, it was found that from 2,834 people who had a lump in their breast checked, 2,229 of them (78%) were benign tumors, 368 people (13%) were diagnosed with breast cancer and the rest were infections and congenital abnormalities of the breast (Fahmy & Abdel-Tawab 2021). One alternative treatment that is trusted by the community to treat cancer is to use traditional ingredients which are none other than ingredients that have been provided by nature. Natural ingredients are materials that are generally processed and used by the community as ingredients for traditional medicine which are considered to be able to cure the disease (Han *et al*. 2019). However, based on the results of research on natural material processing carried out with isolation techniques, it is more effective in dealing with disease problems such as cancer and

other diseases. This is because the final product from the isolation process of natural ingredients is a pure product that can inhibit or even kill the source of the disease.

Marine biota has the potential as a source of bioactive materials that have only been studied in recent years, although not as much as research on land biota. The long evolutionary history of marine life causes marine biota to have a very high molecular diversity (Elissawy *et al*. 2021).

Sponges are the largest source of producing bioactive compounds among other marine invertebrates, as the results of research conducted Bian *et al*. (2020) states that of the many marine biota with various bioactivity, sponges are the most abundant source of bioactive materials. Based on research that has been done, it is known that sponges contain secondary metabolite, including alkaloid, which have various bioactivity compounds, including antibacterial (Bian *et al*. 2020), antiviral (da Silva *et al*. 2006), antifungal (Devi *et al*. 2013), anti-inflammatory (Ko *et al*. 2017), anticancer (Lim *et al.* 2014) and many compounds with unknown diversity of sponge bioactivity (Ko *et al*. 2017).

Stylotella sp. is one of the sponges that has a bright color, has an irregular shape resembling a coral, this type of marine sponge lives at a depth of 2, 4 and 9 meters (Haedar *et al*. 2016). Based on the results of the research, *Stylotella* sp. contains a variety of secondary metabolites with different bioactivities including antimicrobial compounds such as terpenes, alkaloids, and polyphenols (Hutagalung *et al*. 2014), antioxidant compounds (Asaf *et al*. 2018) and also anticancer compounds (Fahmy & Abdel-Tawab 2021). As the discovery of the sesquiterpene carbonimidic dichloride compound found from the acetone extract of Stylotella aurantium sponge on Iriomote Island, Okinawa which has a cytotoxic effect on tumor cells (Jomori *et al*. 2019).

This study aims to isolate secondary metabolites of *Stylotella* sp. from the Selayar Island and activity test against MCF7 breast cancer cells using acetone solvent which has semipolar properties because it is known that several bioactive compounds of sponge *Stylotella* sp. found in semipolar extracts.

MATERIALS AND METHODS

Materials

The materials used in this research are aluminum foil, distilled water, antibiotics, sulfuric acid Merck, acetone, iron(III) chloride, *cis*-platin, dimethyl sulfoxide (DMSO), ethanol, ethyl acetate, fetal bovine serum (FBS), calico fabric , filter paper, chloroform, TLC plate Silica gel 60 F_{254}, microtube, sodium hydroxide (NaOH), *n*-hexane, Dragendorff reagent, Liebermen-Burchard reagent, Mayer reagent, Wagner reagent, phosphate buffered saline, PrestoBlue cell viability reagent, Roswell park memorial medium (RPMI), MCF-7 breast cancer cells, silica G60 (230-400 mesh) merck no. catalog 7730, silica G60 (230-400 mesh) merck no. catalog 7733, silica G60 (230-400 mesh) merck no. catalog 7734, sponge Stylotella sp from Selayar Island, trypan blue, trypsin-EDTA tube, T-flask, and 96 well plate.

Instrumentation

This research uses the following tools: Fourier Transform InfraRed (FTIR) spectrophotometer (Prestige-21 Shimadzu), Biosafety Cabinet (BSC), inverted microscope, multimode reader, centrifuge, CO2 incubator, Kirin oven, UV lamp 254-336 nm, rotary evaporator Hanshin Scientific co. Hs model. 2000 NS, analytical balance, vacuum liquid chromatography column (KKCV), gravity chromatography column (KKG), vacuum pump, microplate, adapter, hotplate, condenser, steel head, 110°C thermometer, capillary tube, glassware.

Extraction

Samples were obtained from the waters of the Selayar Islands with a depth of about 3-10 m because the growth and community of coral reefs and sponges is optimum at that depth (Suharyanto & Utojo 2008). Samples obtained directly from the sea, and then cleaned by washing, after that the samples were stoned and dried in an open space that was not exposed to direct sunlight. The sample was 500 g and soaked in acetone for 24 hours. This process is carried out for 3 times or more until a clear filtrate is obtained. Furthermore, the macerate is concentrated with an evaporator to produce a crude extract.

Fractionation

The viscous extract obtained was analyzed using TLC and then followed by vacuum liquid column chromatography with a ratio of 1:40 between sample and silica (catalogue number 7730). In this process, the sample is fed with an eluent which polarity is increased so that several fractions are obtained. Fractions that have the same Rf value are combined.

Flavonoid Test

Test with concentrated sulfuric acid (H_2SO_4)

The sample was diluted with organic solvent then pipetted into a drop plate and dripped with concentrated sulfuric acid. After that, the color change from dark yellow to dark red was observed.

Test with 10% sodium hydroxide (NaOH)

The sample was diluted with organic solvent then pipetted into a drip plate and dripped with 10% sodium hydroxide. After that, the color change from dark yellow to light yellow was observed.

Test with 5% iron (III) chloride ($FeCl_3$)

The sample was diluted with organic solvent then pipetted into a drip plate and dripped with 5%

iron(III) chloride ($FeCl_3$). After that, the color change to blackish blue was observed.

Alkaloid Test

Test with Meyer's Reagent

The sample was diluted using several ml of acetone then pipetted into a drip plate and dripped with Meyer's reagent. The positive result is the formation of a white or yellow precipitate.

Test with Wagner's Reagent

The sample was diluted using several ml of acetone then pipetted into a drip plate and dripped with Wagner's reagent. The positive result is the formation of an orange precipitate.

Test with Dragendorff's Reagent

The sample was diluted using a few ml of acetone then pipetted into a drip plate and dripped with Dragendorff's reagent. The positive result is the formation of a brown solution.

Terpenoid and Steroid Test

The sample was diluted with organic solvent then pipetted into a drip plate and dripped with Liebermen Burchard reagent and observed for color changes. The positive result is that a red or purple solution is formed (terpenoids) and a blue or green color (steroids) is formed.

Purification

Purification was done by crystallization and recrystallization.

Characterization with FTIR

Sample of 1 mg of pure isolate was dissolved with KBr by grinding until homogeneous. The mixture was put into a pellet maker with a pressure of 74 atm and a time of 5 minutes to obtain a pellet with a thickness of approximately 1 mm. The plate is placed in the plate container and then measured using a Fourier Transform Infra Red (FTIR) spectrophotometer.

Anticancer Test

Media Preparation

A complete Roswell Park Memorial Institute Medium (RPMI) liquid culture medium (containing 10% Fetal Bovine Serum and 50 L/50 mL of antibiotics) was prepared. A positive control was prepared using, the positive control used in this test was Cisplatin. Dissolved the sample with a certain final concentration as stock. Use solvents that are not toxic to cells. Prepare the antiproliferative assay working solution. The working solution to be used is PrestoBlue™ Cell Viability Reagent.

Cell Preparation

Cells to be used have been confluent at least 70%. Discard the media on the dish, then rinse the cells twice with 1 mL of PBS. 1 mL of Trypsin-EDTA solution was added and then incubated for 5 minutes so that the cell layer was dispersed (under an inverted microscope the cells would appear to float. The cells were transferred to a tube containing the media. The cells were centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded, then pelleted and dissolved in a tube containing the media.

Seeding Cells into 96 well plates

Determine the number and viability of cells (with trypan blue exclusion), and resuspend cells with a final cell density of 170,000 cells/mL in the medium. (17,000 cells/well). Prepared 10 µL trypan blue in a sterile microtube. 10 µL of cell suspension was added to the trypan blue solution and then homogenized. Cleaned the hemacytometer and closed the slip using 70% ethanol then dried. Using a pipette, slowly add 10 µL of the trypan blue cell solution to one side of the chamber. Count the number of healthy cells and determine the number of (viable) cells per mL. Seeding/cell culture into 96 wellplates, then incubated for 24 hours (or until the cells are confluent at least 70%) at 37°C and 5% CO_2 gas.

Cell treatment with samples

Eight 1.5 mL microtubes were prepared, then each microtube was labeled with the appropriate dilution concentration, then the stock sample was diluted into eight concentration variants using a media solvent. Take out 96 well plates containing cells from the incubator. Labeled on the plate along the left margin for which rows will be treated by the standard and which rows will be sampled. Then removed the media from each well. Using a micropipette, 100 µL of each sample and positive control cisplatin were transferred from the microtube into each of the appropriate wells on a 96-well plate containing cells. Then incubated again for 24 hours.

Presto Blue reagent administration and absorbance measurement

Discarded media on each well. Prepare 9 mL of media in a tube, add 1 mL of "PrestoBlue™ Cell Viability Reagent" (10 µL of reagent for 90 µL of media), then add 100 µL of the solution mixture into each well of the microplate and then incubate for 1-2 hours until the color changes (Upon entering living cells, PrestoBlue® reagent is reduced from the blue compound resazurin without an intrinsic fluorescent value, to a resorufin compound which is red and highly fluorescent. The conversion value is proportional to the number of metabolically active cells and therefore can be measured quantitatively. To measure absorbance, an absorbance spectrum was used for resazurin and resorufin. Then the absorbance was measured at a wavelength of 570 nm (reference: 600 nm) using a multimode reader.

RESULTS AND DISCUSSION

Stylotella sp. macerated using acetone for 24 hours 3 times to produce 22.1 grams dark brown crude extract. *Stylotella* sp. acetone extracts then in the initial fractionation using vacuum liquid column chromatography (KKCV) with various eluent ratios. The resulting fractions were 19 fractions. From the TLC results, 13 main fractions were obtained, namely fractions I-XIII. Fraction III was then tested for purity by TLC test with three eluent systems, namely *n*-hexane: acetone (8:2), *n*-hexane: ethyl acetate (8:2) and chloroform: ethyl acetate (9:1). The results obtained from the three eluents showed one stain indicating that the isolate obtained was pure as shown in Figure 1. The pure isolates showed positive test for alkaloids as shown in Table 1.

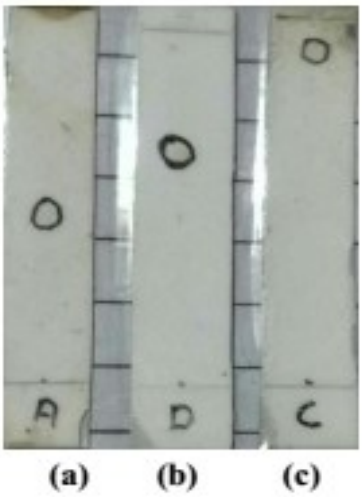

Figure 1. TLC results of three systems test Eluent (a) *n*-hexane eluent: acetone (8:2) with Rf 0.36 (b) *n*-hexane: ethyl acetate (8:2) with Rf 0.50 chloroform: ethyl acetate (9 :1) with Rf 0.74

Table 1. Results of the qualitative test of secondary metabolite compounds *Stylotella* sp.

Qualitative test		Result	
Group Compound	Reagen	Extract	Pure isolate
Flavonoid	H_2SO_4 pekat	-	-
	NaOH 10%	-	-
	$FeCl_3$ 5%	-	-
Alkaloid	Mayer	+	-
	Dragendorff	-	-
	Wagner	+	+
Terpenoid and steroid	Lieberman-Burchard	-	-
Saponin	Aquadest + heat + mixture	-	-

The formation of precipitates using Mayer and Wagner test showed that the acetone extract of the *Stylotella* sp. contains alkaloids. Positive results of alkaloids in the Mayer test were indicated by the formation of a white precipitate. It is estimated that the precipitate is a potassium-alkaloid complex. In the preparation of Mayer's reagent, a solution of mercury (II) chloride will react with potassium iodide to form a red precipitate of mercury (II) iodide. If potassium iodide is added in excess it will form potassium tetraiodomercurate (II). Alkaloids contain nitrogen atoms that have a lone pair of electrons so that they can be used to form coordinating covalent bonds with metal ions. In the alkaloid test with Mayer's reagent, nitrogen in the alkaloid will react with metal ions K^+ from potassium tetraiodomercurate (II) to form a precipitated potassium-alkaloid complex.

FTIR Spectrum

The wave numbers of the pure isolate functional group is presented in Table 2. The FTIR spectrum (Figure 2) shows the typical functional group absorption for alkaloid compounds. This is indicated by the presence of O-H stretching vibrations (hydroxyl groups) which are clear at a wave number of 3411 cm^{-1}. The presence of aliphatic C-H stretching vibrations at wave numbers 2982 cm^{-1} and 2854 cm^{-1}. Furthermore, the wave number 1738 cm^{-1} indicates the presence of an aliphatic C=O group (aldehyde). At the wave number 1638 cm^{-1} indicates the presence of aliphatic C=C bond vibrations. And at wavenumber 1466 and 1381 cm^{-1} indicates the presence of tertiary N vibrations and 1062 cm^{-1} indicates the presence of C-N bond vibrations.

Table 2. Functional group absorption value of FTIR

Wave number (cm^{-1})	Silverstein *et al*. (2005)	Functional Group
3411	3500-3200	O-H
2982 and 2854	2936-2916	C-H aliphatic
1738	1760-1665	Carbonyl group (C=O) aliphatic aldehid
1638	1900-1500	C=C aliphatic
1381	1384-1320	N tertiary
1062	1250-1020	C-N

Toxicity Test

Based on the absorbance measurement results of acetone extract and ethyl acetate isolate of *Stylotella* sp. at various concentrations, the IC_{50} value is presented in Table 3.

Tests on cell activity and cell propagation were carried out in several methods, one of which was

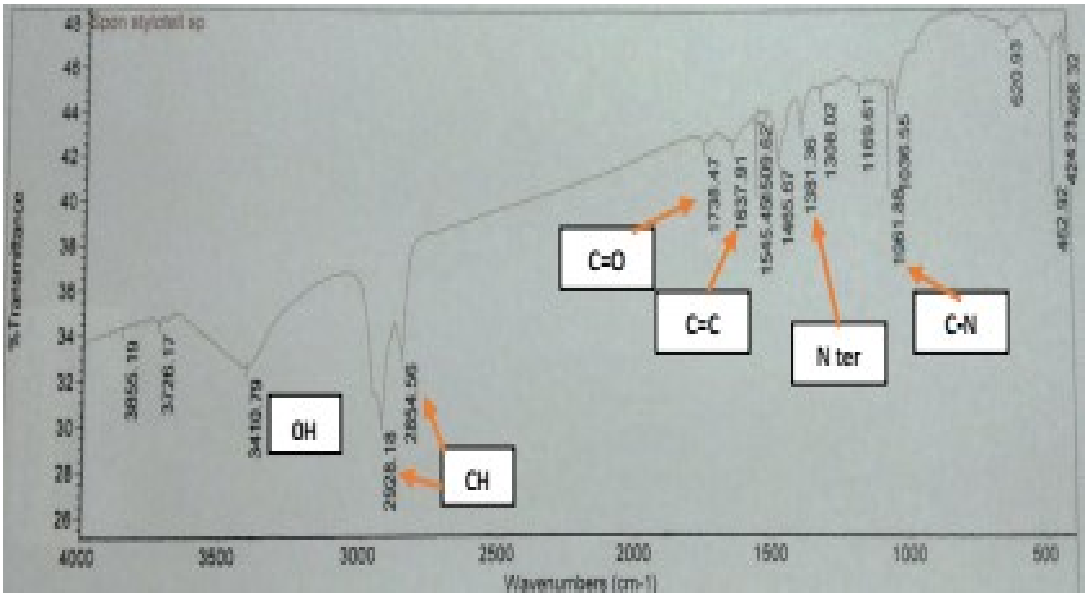

Figure 2. FTIR Spectrum of sample

Tabel 3. Result of antiproliferation assay

No.	Sampel	IC_{50} value
1.	*Cis*-platin	470 µg/mL
2.	Acetone extract	1452.38 µg/mL
3.	Purified isolate	14987.50 µg/mL

using the colorimetric method or color change due to an oxidation reduction reaction, where resazurin as a blue indicator was reduced to pink resorusin, a color change indicating cell activity. Cells that are still actively dividing carry out metabolic activities, resulting in enzymes derived from mitochondrial cell organelles causing a reduction in resazurin such as dihydrolipoamine dehydrogenase (Matsumoto *et al*. 1990).

Resazurin used is a presto blue reagent added to cells and the final result is absorbance measurement using a multimode reader. This test uses a solution of dimethyl sulfoxide (DMSO) as a negative control and as a sample solvent because it can dissolve well in various organic solvents, both polar and non-polar so that it can increase the solubility of the sample. As for cisplatin as a positive control which is a pure substance anticancer agent and is usually used as a comparison.

Stylotella sp. acetone extract made into eight concentration variations, namely concentrations of 1000.00 g/mL, 500.00 g/mL, 250.00 g/mL, 125.00 g/mL, 62.50 g/mL, 31.25 g/mL, 15.63 g/mL and 7.81 g/mL to see the relationship between concentration patterns and cell activity. The same treatment was carried out for pure isolates of *Stylotella* sp.

Based on Figure 3 can be concluded that the concentration of 1000.00 g/mL acetone extract of *Stylotell* sp. has an effect on the activity of MCF7 breast cancer cells, but does not have a cytotoxic effect to inhibit cancer cells. As for the pure isolates of *Stylotella* sp. showed no effect and cytotoxicity against MCF7 breast cancer cells. According to Prayong *et al*. (2008) cytotoxic activity is categorized into three depending on the IC_{50} value, namely IC_{50} < 100 g/mL including potential cytotoxic, 100 g/mL < IC_{50} < 1000 g/mL including moderate cytotoxic and IC_{50}> 1000 g/mL means has no cytotoxic to inhibit cancer cells. Judging from the color of the positive control produced, which is purple, it indicates a cytotoxic effect on MCF7 breast cancer cells as well as the administration of *Stylotella* sp sponge extract on cancer cells produces a purple solution which indicates an effect on decreasing the activity of cancer cells. As for the pure isolates of *Stylotella* sp. does not change color, which means that there is no inhibition of cell growth.

The decrease in cancer cell activity was influenced by the increase in the amount of concentration used. The following curve shows the relationship between concentration and absorbance of cells.

Based on the correlation curve between concentration and absorbance of acetone extract cells, it can be concluded that the greater the concentration of acetone extract used, the smaller the resulting cell absorbance. This shows that the smaller the absorbance, the smaller the cell growth.

The curve of the relationship between concentration and absorbance of pure isolates of *Stylotella* sp. Is presented on Figure 4.

Based on the correlation curve of concentration and absorbance of pure isolates of sponge *Stylotella* sp. it can be concluded that there is no effect of concentration on cancer cell growth.

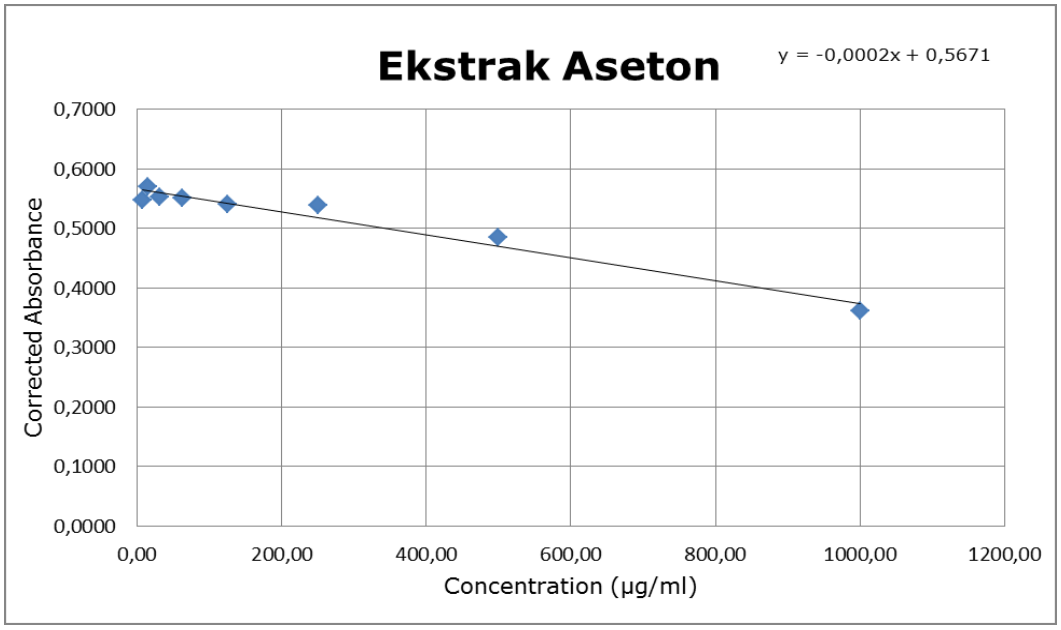

Figure 3. Acetone extract cell concentration and absorbance curve

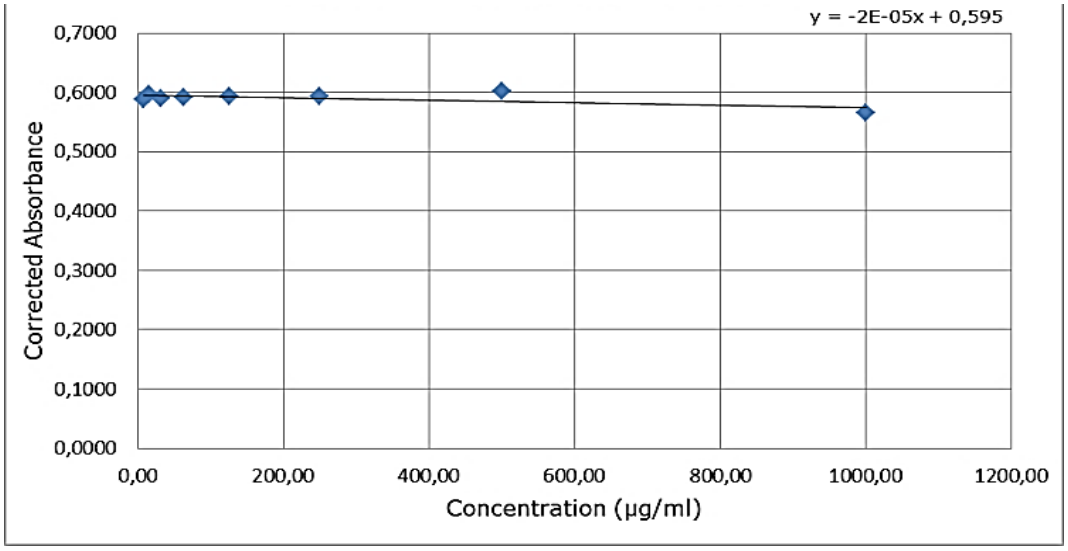

Figure 4. Pure cell isolate concentration and absorbance curve of acetone extract

MCF-7 cells are one of the models of breast cancer cells that are widely used in research. These cells were taken from the breast tissue of a 69-year-old Caucasian woman with blood type O, with Rh positive, in the form of adherent cells that can be grown in DMEM or RPMI growth media containing 10% fetal bovine serum (FBS) and penicillin-streptomycin antibiotics. 1%. MCF-7 cells have characteristics such as resistance to chemotherapy agents (Mechetner *et al*. 1998; Aouali *et al*. 2003) based on this, large concentrations are required to inhibit the growth of MCF7 breast cancer cells.

According to Thurston & Pysz (2021), alkaloids can be anticancer agents as antitubulin, alkaloids can bind to microtubule proteins in the formation of spindles, thereby inhibiting the cell division cycle, precisely at the metaphase stage. Microtubules are polymers of tubulin which are important in cell division.

CONCLUSION

Bioactive compounds of acetone extract isolate from Stylotella sp. is an alkaloid compound. The MCF-7 cell activity test showed an IC_{50} value for acetone extract of 1452.38 g/mL which has potential anticancer activity and an IC_{50} value for pure isolate acetone extract of 14983.52 g/mL.

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