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Cytotoxic activity of bioactve compounds from Hydroid *Aglophenia cupressina Lamoureoux* against Hela Tumor Cells

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Abstract

Hydroids are marine invertebrates living on sponges, which belong to the phylum Coelenterata. They also contain several chemical compounds, including alkaloids, steroids, terpenoids, and histamine, which can be used as medicinal raw materials, such as antimicrobials and anticancer. Therefore, this study aimed to assess the working mechanism of active compounds obtained from the extract and the fraction of Hydroid *Aglaophenia cupressina Lamoureoux* against Hela ttumor cell line, as a solvent used n-hexane. The cytotoxic activity was carried out by using MTT method. The results showed both extract and fraction activities against Hela tumor cells. They were also categorized as moderate cytotoxic activity based on the IC_{50} values of the extract and fraction, namely 0.31726 µg/mL and 0.32712 µg/mL, respectively.

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Keyword
Bioactive compounds;
Cytotoxic;
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Introduction

Cancer is a severe disease characterized by disorder or failure of the multiplication regulation mechanism, which causes uncontrolled cell behavior. It is also characterized by uncontrolled division and the ability of cells to invade surrounding tissues (Bray F. et al., 2012; Costa J. 2019). Based on WHO (2018), data, cancer is the second leading cause of death in the world after cardiovascular diseases, and it accounts for 13% of the global mortality cases. Furthermore, it affects an average of 12 million people annually with 7.6 million death cases. Several studies predicted that the number of people infected can rise to 26 million with a total of 17 million deaths by 2030 when preventive measures are not implemented. Another study revealed that poor and developing countries are at more risk of increased cancer prevalence (Fitzmaurice C, 2015; Moudi, M. et al., 2013). The treatment of the disease can be carried out through surgery, radiotherapy, and adjuvant therapy, such as hormonal therapy, chemotherapy, and immunotherapy (Bishayee, A. et al., 2011; Bray, F.et al., 2012; Ostapiuk, A., et al., 2019). In most cases, patients are not cured of the disease, hence, various efforts to isolate anticancer compounds from active marine biota materials, such as algae, sponges, and corals are being intensified (Greenwood. P.G., 2009; Sjafaraenan, Johannes E. 2017). Previous studies explored the use of bioactive compounds from Hydroid *Aglaophenia cupressina* Lamoureoux for cancer treatment (Harada H. et al., 2002; Johannes, E. et al., 2017).

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Reported that hydroids belong to the Cnidaria or Coelenterata phylum, and they secrete a nematocyst through their body to fight predators around them. The nematocysts released cause skin irritation after contact with humans (Jain S. et al., 2016; Pisa D.,and Stefania P., 2017). Revealed that hydroids contain several chemical compounds, including alkaloids, steroids, and carboxylic acids, which can be used as antimicrobials. Another study by (Chudzik M., 2015; Johannes, E., et al., 2015; Maiyo F. et al., 2016) on the toxic activity of their methanolic fraction against Artemia salina and HeLa tumor cells obtained LC50 values of 19.70 µg/mL and 9.11 µg/mL, respectively. These results indicate that the fractions obtained have toxic effects on HeLa cells growth. Therefore, this study aims to assess the cytotoxic activity and apoptosis of hydroids' extracts and n-hexane fractions against HeLa cells.

Materials and Methods

Materials

Hydroid *Aglaophenia cupressina* Lamoureoux samples were obtained from Lae-Lae Island, Ujung Pandang District, Makassar City, South Sulawesi. They were then aerated, dried, chopped, mashed, and 500 g of the sample was weighed. Furthermore, HeLa cells culture was collected from the Cancer chemoprevention Research Center of Gadjah Mada University, Yogyakarta. Other materials used for this study include n-hexane, Ethyl-acetate, Rosewell Park Memorial Institute (RPMI) media 1640, 10% Fetal Bovine Serum (FBS); 1.5% Amphotericin B, 2% Penicillin-streptomycin, 10% Sodium dodecyl sulfate (SDS); Phosphate buffered saline (PBS), 1.5% NaHCO3, MTT, 70% alcohol, Trypan blue, Duran bottle, Round bottom flask, Conical tube rack, conical tube, Micropipette; 96-well microplate, Tissue culture dishes, Rotary evaporator, Cell culture incubator, Laminar Air Flow (LAF), and ELISA reader.

Instruments

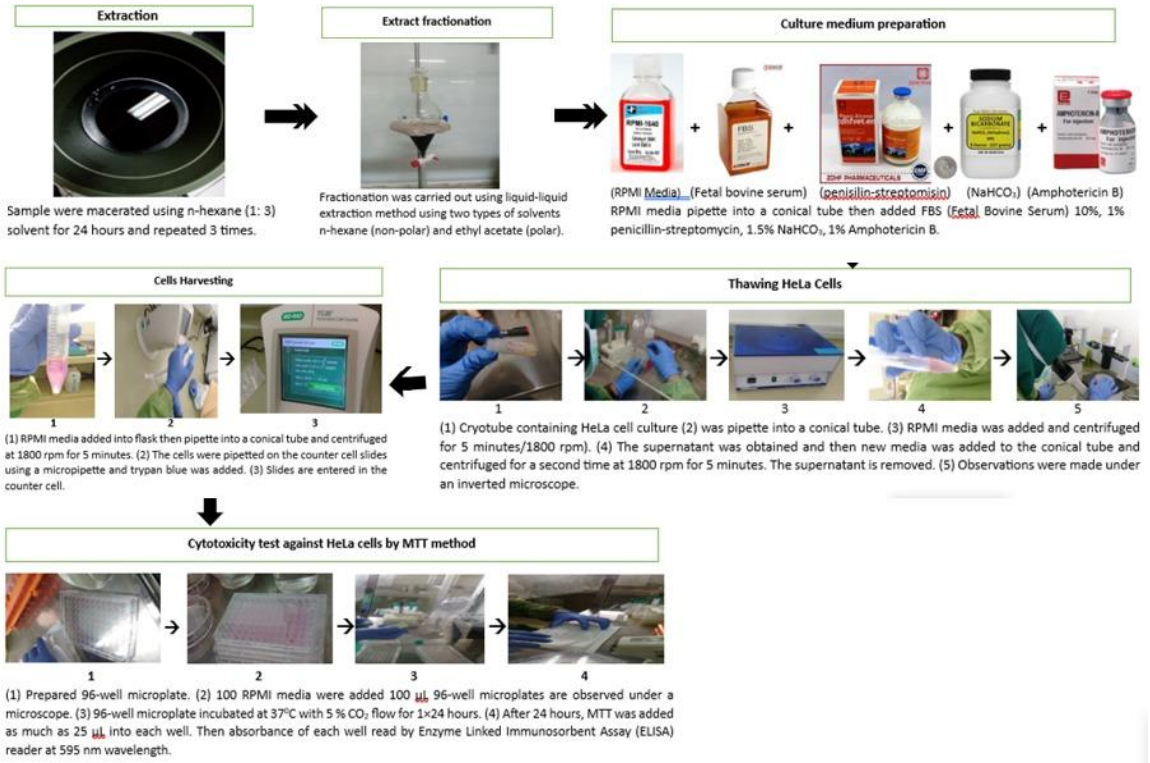

Figure 1. Study procedure

Method

The isolation of Hydroid Aglaophenia cupressina Lamoureoux samples was carried out through maceration using n-hexane (1:3) solvent for 24 hours with 3 replications. The extract obtained was then evaporated using a vacuum rotary evaporator.

Extract fractionation

Fractionation was carried out with the liquid-liquid extraction method using two types of solvents, namely n-hexane (non-polar) and ethyl acetate (polar). The thick macerate obtained was dissolved using 50 mL of n-hexane solvent, and then mixed with 50 mL of ethyl acetate (1:1). Subsequently, it was placed in a separating funnel with occasional shaking. The fractionation process was stopped when 2 phases were formed, namely n-hexane and ethyl acetate fractions. The product obtained was then evaporated using a rotary evaporator.

Culture medium preparation

The culture medium used was the RPMI media (Roswell Park Memorial Institute). It was pipetted into a conical tube, followed by the addition of 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, 1% Amphotericin B, and 1.5% $NaHCO_3$. The preparation of the culture media was carried out at room temperature. After all the ingredients were added, the conical tube was shaken repeatedly.

Thawing HeLa cells

Cryotube containing HeLa cells culture was liquefied at room temperature and pipetted into a conical tube. RPMI media was then added and the mixture was centrifuged for 5 minutes at 1800 rpm. Subsequently, the supernatant was collected and a new media was added to the conical tube, followed by centrifugation at 1800 rpm for 5 minutes. The supernatant produced was then removed. Observations were made under an inverted microscope, after which the tissue culture dishes were incubated at 370^{o}C along with the addition of 5% CO2. When the color of the media changes to yellow, it is replaced and the cell count is calculated.

Cytotoxicity test against HeLa cells by MTT method

Cytotoxicity test was carried out using 3 experimental samples, namely cell, treatment, and media controls, which consist of 100 µL cells + 100 µL media as the positive control, 100 µL cells + 100 µL extracts of test compounds, and 200 µL of culture media as negative control. Approximately 80-90% of the live confluent cells were used for the MTT testing. A 96-well microplate was prepared, and 6 cell pipettes with concentrations of 10^3 were placed in each well. Subsequently, 100 µL of RPMI media were added and observed under a microscope. It was then incubated at 37^{0}C with 5 % CO_2 flow for 1×24 hours. The treatment of cell culture was carried out by extracting the test compounds with various concentrations in the 96-well microplate. The cell and media controls were then added to the mixture, followed by incubation at 37^{0}C with 5% CO_2 flow for 1 × 24 hours. After 24 hours, 25 µL of MTT was added into each well. The microplate was re-incubated in a CO_2 incubator for 4 hours, after which the MTT reaction was stopped by adding 50 µL of 10% sodium dodecyl sulfate (SDS). The absorbance of each was read using Enzyme-Linked Immunosorbent Assay (ELISA) reader at a wavelength of 595 nm.

Data Analysis

Data analysis was carried out by calculating the IC50 value using the percentage of cell viability. The higher the viability, the fewer the number of dead cells. The percentage of viability was calculated using the formula below:

$$\%\ Cell\ Viability = \frac{(A - B)}{(C - B)} \times 100\%$$

Where A, B, and C are the absorbance of the treatment, medium, and cell controls, respectively.

Results and Discussion

Cytotoxicity test against HeLa cells

A Cytotoxic test is an in vitro assay, which is often carried out with cell culture to determine the toxicity level of a compound based on the number of cell deaths. The RPMI media was used as the growth media because it is suitable for growing cancer cells within a short period (Novotny L. et al., 2017; Sajjadi, S.E. et al., 2013). It also contains an adequate amount of growth nutrients, namely amino acids, vitamins, inorganic salts, and glucose. It also contains an adequate amount of growth nutrients, namely amino acids, vitamins, inorganic salts, and glucose (Graidist, P. Et al., 2015; Khan Z. et al., 2022). The serum added to the culture contains hormones that can stimulate cell development. Albumin serves as a transport protein, while lipids are needed for cellular growth. Furthermore, the mineral content function as enzyme cofactors (Jain, S. et al., 2016; Sjafaraenan, Johannes E. 2017). The 10% FBS added serves as a growth factor, while 1% penicillin-streptomycin and Amphotericin B prevent the culture from bacterial and fungal contamination, respectively. The 1.5% NaHCO3 helps to maintain the pH of the media after the addition of strong acids and bases as well as dilution. The MTT test results are presented in Figure 2. The figures of cells before and after treatment with the extract and n-hexane fractions showed similarities.

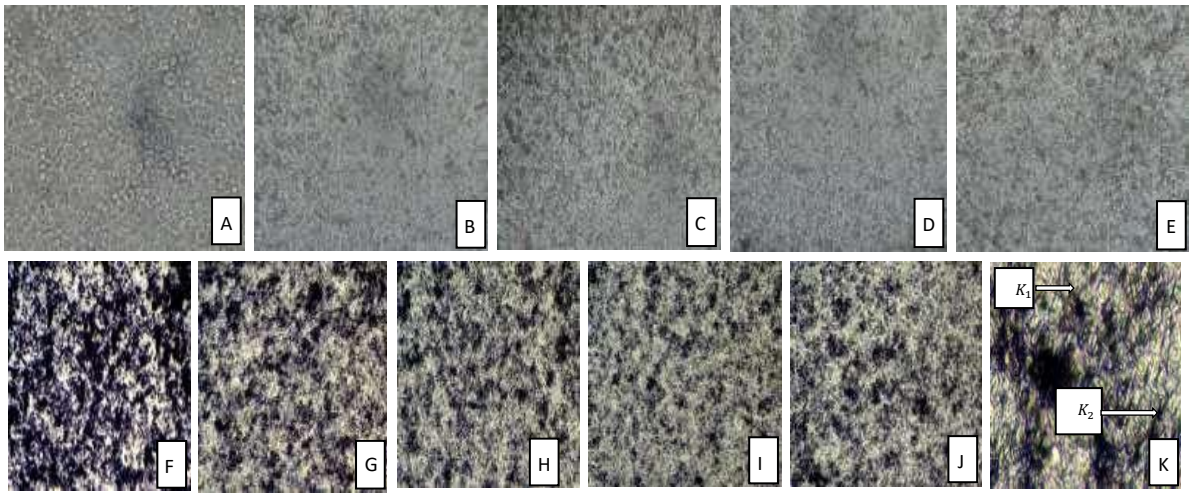

Figure 2. (A) Cell control before MTT addition. (B), (C), (D), and (E) Treatment control with concentration of 2.44 µg/mL, 4.88 µg/mL, 9.76 µg/mL, 19.53 µg/mL before MTT addition. (F) Cell control after MTT addition (G), (H), (I), and (J) Treatment control with concentrations of 2. 44 µg/mL, 4.88 µg/mL, 9.76 µg/mL, and 19.53 µg/mL after MTT addition. Cell magnification, (k1) Dead cells, (k2) Living cells.

Figure 2. (A), (B), (C), (D), and (E) illustrate the samples before the addition of MTT, which show that there is no color difference between all of them, hence, it is impossible to distinguish between living and dead samples. Figure (F) is the cell control, and it reveals the presence of several living cells, which are marked by the intense purple formed. Figs. (G), (H), (I), and (J) illustrate the treatment controls. The higher the concentration of the test compound, the lesser the number of living cells marked by the purple coloration. The formation of color is due to the ability of mitochondrial reductase enzymes to reduce salt methyl thiazol tetrazolium. These enzymes are produced by living cells during metabolism.

Furthermore, they react with MTT to form purple crystal formazan (Graidist P. et al., 2015; Habli Z. et al., 2017; Maiyo F. 2016). The higher the number of deaths, the fewer the formazan crystals formed (Sliwka Sliwka L. et al., 2016; Upadhyay, R.K. 2014). The mortality caused by the treatment led to a decrease in the amount of mitochondrial reductase enzymes produced, hence, MTT did not change to formazan crystals after its addition. Morphological changes caused by exposure to active compounds or certain chemotherapy agents are a reflection of biochemical conditions that can lead to apoptosis or necrosis (Bishayee A. et al., 2011; Kaliberov, S.A., and Buchsbaum, D.J.; Maiyo, F. Et al., 2016).

Table 1. MTT Results data in the measurement of cytotoxicity for hexane extract of Hydroid *Aglaophenia cupressina Lamoureoux* against HeLa Tumor Cells

Concentration (µg/mL)	Living cells (%)	Concentration log (X)	Death (Y)
2.44	59.15	0.564348497	15.77
4.88	61.98	0.557957385	17.26
9,76	82.74	0.521453777	38.02
19.59	84.23	0.519354579	40.85

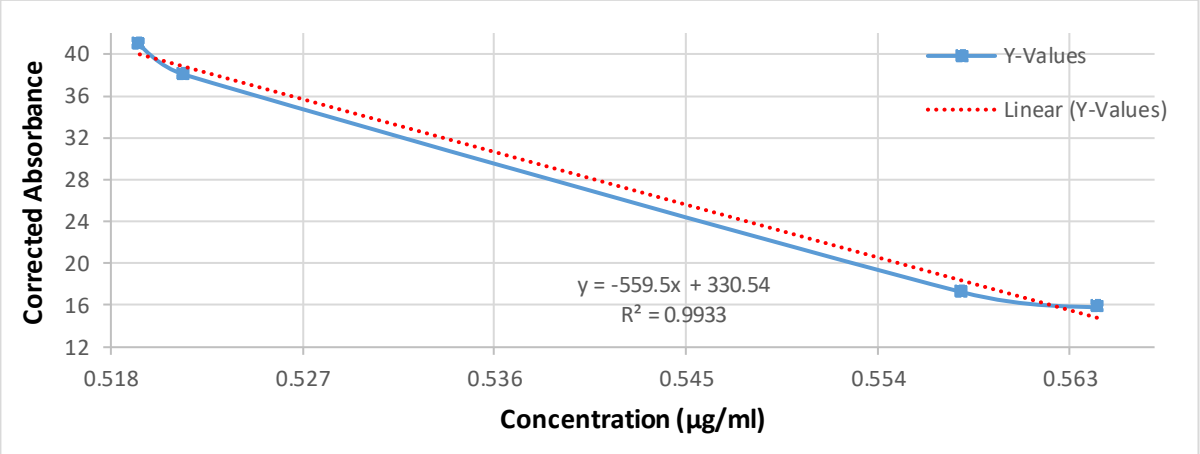

Figure 3. Graph of the logarithm equation between the average absorbance and the concentration of extract in determining the IC_{50} value of n-hexane Hydroid *Aglaophenia cupressina Lamoureoux* extract against HeLa tumor cells IC_{50} = 0.31726 µg/mL

Table 2. MTT Results data on cytotoxicity measurement For N-Hexane Fraction of Hydroid *Aglaophenia cupressina Lamoureoux* against Hela tumor cells

Concentration (µg/mL)	Living Cells (%)	Log concentrate ion (X)	Death (Y)
156.25	64.42161	0.45582	35.57838
78.125	67.84627	0.52832	32.15372
39.06	69.08295	0.62824	30.91704
19.53	71.42314	0.77477	28.57680
9.76	78.04414	1.01066	21.95586
4.88	81.64003	1.45260	18.35996

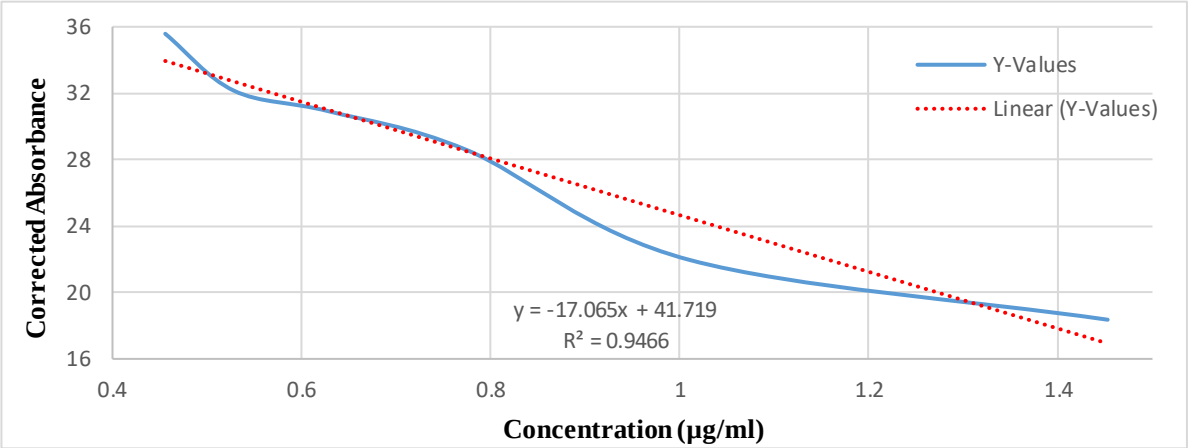

Figure 4. Graph the logarithm equation between the average absorbance and the concentration of extract in determining the IC50 value of the n-hexane fraction, IC value 0.32712 µg/mL

The MTT test revealed that the extract and fraction of n-hexane have IC_{50} values of 0.31726 µg/mL and 0.32712 µg/mL, respectively. Furthermore, the percentage of cell viability decreased along with an increase in their concentration. The data analysis using a linear regression equation was used to calculate the IC_{50}.

The results showed that the extract and n-hexane fraction of hydroid *Aglaophenia cupressina* Lamoureoux can inhibit the growth of HeLa tumor cells by 50%. (Graidist, 2015; García M.T., et al., 2011) stated that an extract and compound are cytotoxic when they have IC50 values of <20 µg/mL and <4 µg/mL, respectively. This finding indicates that the extract and n-hexane have anticancer activity against HeLa tumor cells. This activity is due to the presence of secondary metabolites in Hydroid *Aglaophenia cupressina* Lamoureoux, including flavonoids, saponins, tannins, and steroids, hexadecanoic acid, and steroids (Ostapiuk A. at al., 2019; Pisa D, and Stefania P. 2013; Ravi L. and Kannabiran K., 2017).

Revealed (Johannes E. et al., 2015; Su-Wen L. et al., 2015) that the content of compounds produced by Hydroid *Aglaophenia cupressina* Lamoureoux fraction using n-hexane includes hexadecanoic acid, Aglao E. unhas, and β-sitosterol. Hexadecanoic acid is a carboxylic acid with high toxicity and an LC_{50} value of 29.54µg/mL (Sangpairoj K. Et al., 2022; Sayeed, M.S.B. et al., 2016) stated that fatty acid derivatives, namely hexadecanoic and

octadecanoic acids act as anticancer compounds. Furthermore, Aglao E. Unhas is an alkaloid compound with high toxicity and an LC_{50} value of 133 µg/mL. (Gupta E. 2021; Kaliberov, S.A., and Buchsbaum 2012). Reported that alkaloids can be used as antitumor drugs because they induce apoptosis through their bond with DNA and topoisomerase I, thereby stabilizing the truncated topoisomerase-DNA complex (García MT et al., 2011; O'Donovan, M. A. 2012; Ravi L., and Kannabiran K. 2016). This stability causes permanent damage to the double-strand DNA, which leads to apoptosis (Habli Z. et al., 2017; Khan Z. Et al., 2022; Upadhyay, R.K. 2014). This stability causes irreversible damage to double-stranded DNA, leading to apoptosis, and also induces the expression of LC-3, a marker of autophagic cell death at Cytotoxic (Chudzik M et al., 2015; Nikita A.M. et al., 2015; Novotny L. et al., 2017; Bahuguna A. et al., 2017). These compounds can also regulate p2 WAF1/C1P1 as well as induce apoptosis in breast cancer cells through the p53-dependent (MCF-7) or p53-independent pathways (MDA-MB-648) (Kristanti AN. Et al., 2022; Fitzmaurice C. 2015; Sangpairoj K. Et al., 2022). Revealed that alkaloids can inhibit the formation of microtubule components in mitotic coils, which leads to the stoppage of metaphase (Costa J. 2019; Kristanti AN. et al., 2022; Maiyo F. et al., 2016). β-sitosterol is a steroid derivative and it has been reported to have anticancer activity (Novotny L. et al., 2017). (Baskar A.A. et al., 2010; Bishayee, A. et al., 2011; Bray F. et al., 2012) stated that triterpenoids can inhibit the division mechanism as well as trigger cancer cell apoptosis (Mondal A. et al., 2019). Therefore, it is assumed that the toxic properties of HeLa cells line are caused by these compounds.

Conclusion

Based on MTT test results of the extract and n-hexane fraction of Hydroid *Aglaophenia cupressina* Lamoureoux, the tested compounds had cytotoxic effects on HeLa tumor cells with IC_{50} values of 0.31726 µg/mL and 0.32712 µg/mL, respectively. They were also classified as very effective anticancer agents based on the inhibition values obtained.

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