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Dengue Antivirus Activity of n-hexane and Acetone Extract of *X. testudinaria*'s Rhizome From Spermonde Islands, South Sulawesi

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Abstract. DHF is a disease that continues to spread in Indonesia and often causes death to sufferers. DHF is caused by the dengue virus which is transmitted by *A. aegypty* and *A. albopictus* mosquitoes. Even though there is a vaccine available in Indonesia, its use is still very limited and requires a very large cost, so a solution is needed to find a drug that is effective in inhibiting the dengue virus. The research conducted was related to the assessment of dengue antiviral activity from the sponge extract *X. testudinaria* originating from the waters of the Spermonde Islands, South Sulawesi. The research stage included the extraction process with the maceration method using alternating n-hexane and acetone solvents, the extract phytochemical test, the extract's toxic activity test against vero cells and the dengue antiviral activity test (DENV2) from the extract. The results of the study obtained evidence that the extract contains steroid and flavonoid compounds. The extract of n-hexane and acetone *X. testudinaria* had toxic activity against vero cells where the acetone extract with a lower CC_{50} value of 2.1 µg/mL had stronger toxic properties to cells compared to n-hexane extract with a CC_{50} value of 21 µg/mL. While the n-hexane extract had very strong inhibitory activity against dengue virus with a low IC_{50} value of 3.11 µg/mL, and stronger than acetone extract with an IC_{50} value of 73.1 µg/mL. The SI values of n-hexane and acetone extract respectively showed that the n-hexane extract was more selective with an SI greater than 3, namely 6.75 compared to acetone extract with SI 0.028. This means that the n-hexane extract has more potential to be used as an antiviral drug than the acetone extract.

Introduction

Dengue hemorrhagic fever, DHF is a common disease in tropical and subtropical climates. This disease is usually characterized by high fever, hemorrhagic phenomena, hepatology and circulatory failure (WHO, 1997). DHF is caused by the dengue virus which is transmitted by female Aedes aegypty and Aedes albopictus mosquitoes.

Dengue virus is an RNA virus from the genus Flavivirus. The types of dengue virus (DENV) that infect several regions in the world consist of DENV1, DENV2, DENV3, DENV4 (Messina et al., 2014) and DENV5 (Mustafa et al., 2015).

Indonesia is one of the countries that is always infected by dengue fever every year, one of which is in the province of South Sulawesi. In 2017, South Sulawesi was in 12th place for the death rate from 34 provinces with a morbidity rate of 19.96 per 100,000 population (Ministry of Health, 2018).

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Dengue fever treatment options are still very limited due to the absence of effective dengue antiviral drugs (Cucucnawangsih and Lugito, 2017). Although the dengue vaccine is available in Indonesia, its use is still very limited and requires high costs. Therefore we need a solution to be able to cure the dengue disease, one of which is the search for a drug that is effective in inhibiting the dengue virus. The first step in the discovery of anti-dengue drugs is the search for secondary metabolites that can inhibit the dengue virus.

Indonesia's vast territorial waters make it a habitat for various marine biota which is rich in secondary metabolites as a priceless source of natural chemicals, one of which is sponges. Sponges are a source of secondary metabolites with certain bioactivity, one of which is anti-virus. Research conducted by Qureshi and Faulker (1999) showed that the secondary metabolites in the sponge extract Xestospongia sp. has activity in inhibiting the HIV-1 virus. Sponge Acanthostrongylophora sp. also contains secondary metabolites that have potential as antiviral HIV-1 (Peng et al., 2003), and antiviral HSV-1 (Palem et al, 2010).

The waters of the Spermonde Islands, South Sulawesi are a habitat for various types of sponges, including sponges from the petrosiidae family type *X. testudinaria*. The research conducted was to examine the potential for dengue virus inhibition from the extract of the sponge X. testudinaria in the waters of the Spermonde Islands, South Sulawesi as one of the sponges that are quite abundant in these waters

Experimental

Material and Methods

X. testudinaria sponge taken from the waters of Badi Island, Spermonde Islands, South Sulawesi, organic solvent n-hexane, acetone, phytochemical test reagents including Dragendroff, Wagner, iron (III)) chloride, Whatman filter paper, aquades, DENV-2 , Fetal Bovin Serum (FBS), MEM medium, Vero cell (African green monkey kidney cells), cell line C6/36, Phosphate-Buffered Saline (PBS), 1% L-glutamine, carboxymethyl cellulose (CMC), tetramethylbenzidine (TMB), $NaHCO_3$, H_2SO_4, aluminum foil, tissue.

The equipment used in this study were Glass tools, grinder, Buchner funnel rotary evaporator, digital scale, distillation device, vacuum pump, conical tube, T25 flask, centrifuge, freezer, CO_2 incubator, hemocytometer, microwell plate, inverted microscope, ELISA reader, luminator

Procedures

Preparation and Extraction Stage

First of all the sponge is cleaned, dried and ground until a sponge powder is obtained. The sponge powder is then macerated first using n-hexane solvent. The n-hexane extract was filtered and the remaining residue was macerated again with acetone solvent and then filtered. Each extract was dried using a rotary evaporator.

Phytochemical Screening Test

Normal-hexane and acetone extracts were taken partly for phytochemical tests. The phytochemical test of the extract was carried out using several reagents including Wagner, Meyer, Dragendorf to see the alkaloid content, Liebermann-Buchard to see the contents of steroids/terpenoids and $FeCl_3$ for flavonoids.

Antivirus testing

Cell and Virus Culture

The cell lines used in the study consisted of vero cells (African green monkey kidney cells) which were used as virus host cells and C6/36 cells from Aedes albopictus mosquito cells used to reproduce the dengue virus. Each cell was cultured in Minimum Essential Medium with nutrients in the form of 10% fetal bovine serum and 1% L-glutamine. Cell culture was carried out in 5% CO2 incubator at 37 0C and 28 0C. until the cells are confluent and washed with phosphate buffer saline twice

The dengue virus used in the study was DENV-2. Dengue virus is propagated in C6/36 cells. Culture was carried out by adding the dengue virus to C6/36 cells and then incubating it at 28 0C until it showed a cytopathic effect. The cells were then centrifuged and the viral supernatant was stored at -80 °C.

Cytotoxicity Test

Cytotoxicity test was carried out to determine the cytotoxic level of the extract against vero cells using the MTS method. First, vero cells were inserted into 96-well plates with a concentration of 3.5 x 105 cells/well with a final volume of 100 µl per well. Subsequently incubated at 37 0C at 5% CO2 for 24 hours. When the cell monolayer was confluent, cells from the cell culture medium were taken and washed with PBS. The extract was sequentially diluted with culture medium plus 2% serum to achieve various concentrations. Furthermore, each extract solution was added to the cells and then incubated at 37 0C with 5% CO2 for three days. After the incubation stage, 20 µl of MTS was added to the cells in each well and then incubated for 2 hours. The absorbance was measured using an ELISA reader at a wavelength of 490 nm. Data were calculated as percent toxicity. The percent toxicity value (y) with the concentration of the test solution (x) is then plotted into a

graph to obtain a regression equation, which is used to determine the CC50 value as the cytotoxic value of the extract (Karimi et al., 2016).

Inhibition Test Against Dengue Virus

The inhibition of the dengue virus was carried out using the ViralTox Glo method. Vero cells were put into 96 well plates and incubated for 24 hours to reach 80% confluent. 100 µl of virus stock was added to each test solution and left for one hour. After one hour, 100 µl of the test solution to which the virus had been added was then infected with the vero cells of each well. The cells were infected for 1 hour and shaken every 15 minutes. After 48 hours after infection, each cell was added with 100 µl of ToxGlo reagent and incubated for one hour. After one hour of incubation, the relative luminescence units (RLU) were measured using a luminator. The RLU number obtained for each test solution is used to determine the percentage of cell viability. The percentage of cell viability is then plotted on the graph as the y-axis and the concentration of the test solution as the x-axis. The line equation obtained is then used to determine the IC_{50} value (Villagomez, 2017).

Result and Discussion

Phytochemical Test

The phytochemical test results of the n-hexane and acetone extracts of the sponge X. testudinaria are presented in Table 1 below

Table 1. Phytochemical Test Results of n-Hexane and Acetone Extract of *X. testudinaria*'s Rhizome

Test	Reagent	Results	
		n-hexane	acetone
Terpenoid	Lieberman-Burchard	-	-
Steroid	Lieberman-Burchard	+	-
	Wegner	+	+
Alkaloid	Meyer	-	-
	Dragendrof	-	-
Flavonoid	Mg + amylalcohol	-	-
Phenolic	$FeCl_3$ + HCl	-	-

The data in Table 1 shows that the n-hexane extract contains steroid and alkaloid class compounds, while the acetone extract contains alkaloid group compounds. This is in accordance with previous research related to the genus Xestospongia sponge where the results showed that the Xestospongia genus sponge contains a class of compounds including steroids (Zhou et al., 2011; He et al., 2016; Sadarum et al., 2018) and alkaloids (Arai et al., 2016). The difference in the class of compounds contained in the extract is that the n-hexane extract contains steroids, but it is not found in the acetone extract. It is possible that the steroid class compounds in *X. testudinaria* dissolve well in the n-hexane solvent so that they are completely attracted.

Toxic activity of extracts against vero cells

Toxic test against vero cells was carried out by the MTS method ((5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl) tetrazolium). This method is based on the conversion of tetrazolium salt to color. formazan by mitochondrial activity from living cells (Aslantürk, 2018). Tests were carried out using six variations in the concentration of the test solution from each extract of n-hexane and acetone (6.25; 12.5; 25; 50; 100; and 200 µg/mL). The absorbance measurement results obtained for each test solution were then used to determine the level of toxicity. The toxicity level of each extract is presented in Figures 1 and 2 below:

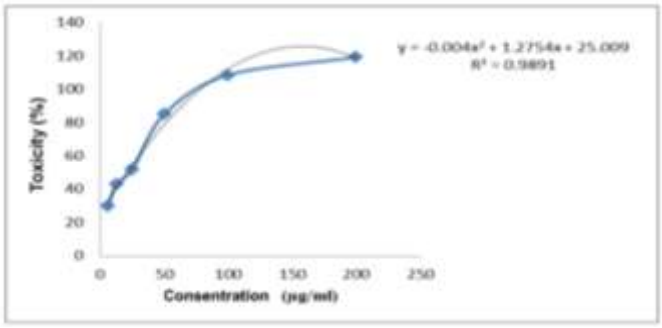

Figure 1. Graph of the relationship between the concentration of n-hexane extract of *X. testudinaria* (x) to the level of toxicity (y).

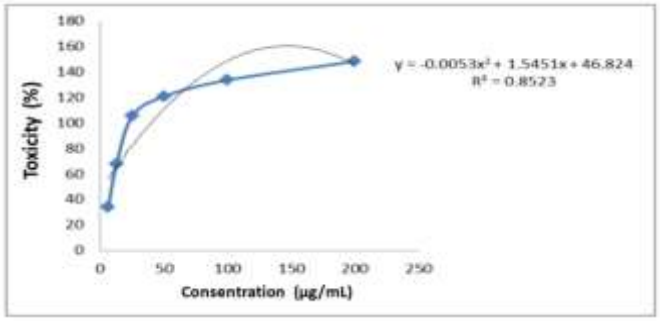

Figure 2. Graph of the relationship between the concentration of acetone extract of *X. testudinaria* (x) to the level of toxicity (y).

Figures 1 and 2 show that the higher the concentration of the extract tested, the higher the level of toxicity to vero cells. The line equation obtained from the graph of the relationship between the concentration and toxicity of each extract is then used to determine the CC_{50} value, which is the extract's minium concentration which can cause a toxic effect on vero cells by 50%.

The calculation results showed that the CC50 value of the n-hexane extract was 21 µg/mL and the acetone extract was 2.1 µg/mL. This means that the n-hexane and acetone extracts are highly toxic to vero cells with a low CC50 value, whereas the acetone extract is more toxic with a lower CC50 value than the n-hexane extract.

Antiviral activity of extracts against dengue virus

Dengue antiviral testing from the extract was carried out using the viralTox glo method. This method is based on cells that experience cytopathic effects due to virus infection which will experience a reduction in the amount of ATP, the amount of ATP is proportional to the level of cell viability (Promega, 2015). Tests were carried out using seven test solutions from each extract (100; 50; 25; 12.5; 6.25; 3.13; and 1.76 µg/mL). The test results are in the form of the lumensense measurement value of each extract test solution, which is then used to measure the level of viability of vero cells. The graph of the relationship between extract concentration and the level of viability of vero cells is presented in Figures 3 and 4 below:

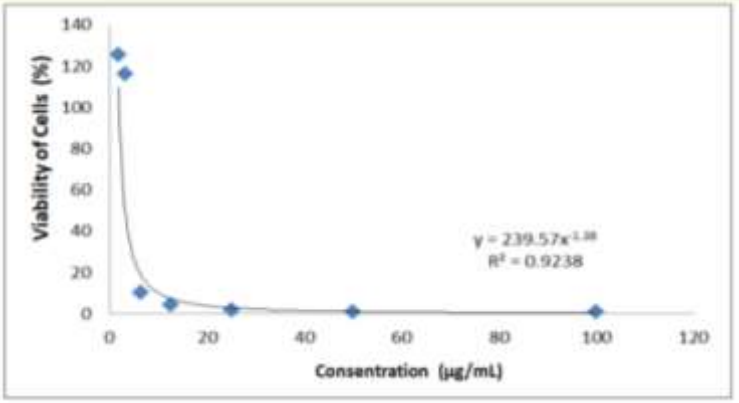

Figure 3. Graph of the relationship between the concentration of n-hexane extract of *X. testudinaria* (x) to the viability of vero cells (y).

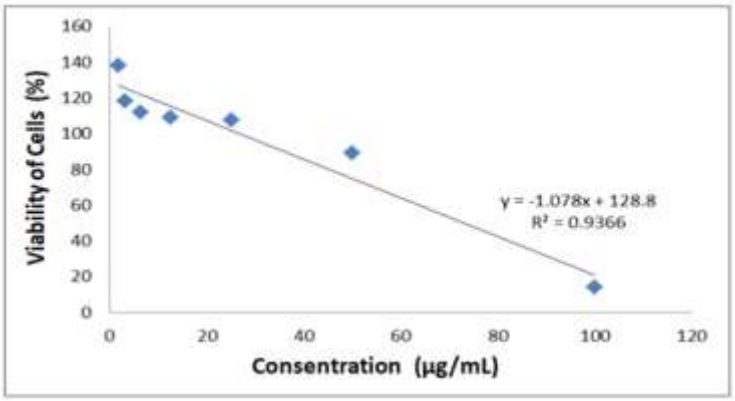

Figure 4. Graph of the relationship between the concentration of acetone extract of *X. testudinaria* (x) to the viability of vero cells (y).

The graph of each extract shows that the higher the extract concentration tested, the lower the cell viability level. This shows that apart from inhibiting the dengue virus, the extract is also toxic to host cells. The results of the calculation of the line equation obtained showed that the inhibitory activity of n-hexane and acetone extracts against dengue virus was strong seen from the low IC_{50} value. The n-hexane extract showed stronger inhibitory activity with an IC_{50} value of 3.11 µg/mL which was lower than that of acetone extract with an IC_{50} value of 73.1 µg/mL. This could be due to the fact that there were more types of compounds in the n-hexane extract as in the previous phytochemical test results, thus allowing more compounds to inhibit dengue virus.

The CC_{50} and IC_{50} values obtained were then used to calculate the selectivity index (SI) value of each extract. The calculation results show that the SI value of n-hexane extract is 6.75 and acetone extract is 0.028. This shows that n-hexane extract has a high selectivity to be used as a dengue antiviral drug as stated in Sutejo et al. 2016 stated that

extracts that have an SI value greater than 3 (SI> 3) have high selectivity. This shows that n-hexane extract has more potential to be used as a dengue antiviral drug with lower adverse effects on host cells than acetone extract.

CONCLUSION

The results showed that the acetone extract had stronger toxic activity against vero cells than the n-hexane extract. Meanwhile, n-hexane extract had greater inhibitory activity against dengue virus than acetone extract. The SI value of each extract indicated that the n-hexane extract had more potential as a dengue antiviral drug with a greater SI value (SI> 3) than the acetone extract.

Conflict of Interest

The authors disclose no conflicts.

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