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Abstract. *Hydrilla verticillata* contains some active compounds that potential as an antioxidant, antibacterial, anticancer, antimicrobial and antitumor. One of the active compounds in *Hydrilla verticillata* is steroids. This research aimed to isolate, to identify and to determine the toxicity and antioxidant activity of steroid compounds in petroleum ether (PE) fraction of *Hydrilla verticillata. Hydrilla verticillata* biomass powder was extracted by maceration using ethanol solvent. The ethanol extract was hydrolyzed with 2 N of hydrochloric acid and then partitioned with petroleum ether solvent. The steroid compounds from petroleum ether fraction were separated with Preparative Thin Layer Chromatography (TLC) and Column Chromatography. The steroid isolates were identified by UV-Vis and FTIR spectrophotometer. The toxicity level and antioxidant assay of steroid isolates were determined by BSLT and DPPH method, respectively. The result of the study showed that extraction through maceration produced 4.54% yield, whereas the product yield of the partition using petroleum ether was 65.41%. The steroid isolates from TLC and Column Chromatography separation has toxicity and antioxidant properties. The LC_{50} value of steroid TLC isolate was 1.41 and 12.2 ppm. The LC_{50} value of steroid Column Chromatography isolates (H_2, H_4 and H10) were 4.32, 8.24 and 10.35 ppm. The EC_{50} value of H_2 Column Chromatography isolate was 23.00 ppm.

1. Introduction

Hydrilla verticillata are aquatic plants that are often found in Indonesian waters [1]. This plant is found in rivers, reservoirs and lakes [2]. According to Hasanah [3], Lake Ranu Grati Pasuruan is a lake dominated by *H. verticillata*, but there has not been a maximum utilization due to the lack of exploration from the community.

Utilization of *H. verticillata* in Developing Countries such as India has been used as fish food and organic fertilizer [4], whereas in Indonesia *H. verticillata* is still used as an aquarium decoration. The *H. verticillata* has various antioxidant and antimicrobial activities [5], antitumor [2], antimalarial [6], antiaging, detoxification agent [7], and anti-inflammatory [8]. These diverse activities are due to the presence of secondary metabolites in *H. verticillata*. The secondary metabolite compounds contained in *H. verticillata* include alkaloids, flavonoids, saponins, triterpenoids and steroids [9].

Secondary metabolites are known as potential compounds as drugs. Steroids are secondary metabolites that have certain activities, such as the algal extract of Tydemnia expeditions containing

steroids which can inhibit the growth of prostate cancer [10]. According to [11], stigmast-5-en-3β-ol steroids and stigmasterol from *Dysoxylum alliceum* stem bark which can be used as anticancer in MCF-7 breast cancer. The potential of steroid compounds as a drug can be the basis for the importance of isolating steroid compounds from *H. verticillata*.

H. verticillata in this study were taken from Ranu Grati Pasuruan Lake. The presence of *H. verticillata* in the lake is very abundant, but its utilization is still not optimal. *H. verticillata* in this study will be isolated steroid compounds using ethanol maceration, hydrolized than partitioned using petroleum ether, separated using TLC and column chromatography which will then be tested for toxicity to shrimp larvae of Artemia Salina L and tested for antioxidant activity against DPPH. The results of the steroid isolate obtained can be identified by UV-Vis and FTIR.

2. Methods

2.1. Materials

The material used in this study was *H. verticillata* from Ranu Lake Pasuruan were n-hexane 96%, sulfuric acid 98%, chloroform 96%, ethanol 95%, HCl 2 N, saturated Na-bicarbonate, ethyl acetate p.a, acetic anhydride p.a, ethanol p.a. dimethyl sulfoxide, bread yeast, seawater, silica gel F254 plate, silica gel 60 (0.063-0.200 mm), DPPH, and *Artemia salina L*. eggs.

2.2. Methods

2.2.1. Sample Preparation. H. verticillata is taken from the surface of Ranu Grati Lake water. Samples taken as much as 10 Kg, then dried for 7 days. Furthermore, the dry sample was mashed in the Materia Medika Batu and sieved with a size of ± 90 mesh.

2.2.2. Analysis of Water Content. Porcelain cups are heated in an oven at 100 – 105 ºC for 15 minutes to remove the water content. The plates were stored in a desiccator for 10 minutes, then the plates were weighed and the same treatment was repeated until they reached a constant weight. The 5 g of *H. verticillata* powder was put into the cup and heated at a temperature of 100 – 105 ºC ± 15 minutes. The cup was put in a desiccator for 10 minutes then weighed and the same treatment was repeated until it reached a constant weight.

2.2.3. Extraction of Hydrilla verticillata Active Compounds. Extraction of active components in the sample is done by maceration extraction or immersion of the sample with ethanol solvent. A total of 100 g of H. verticillata fine powder was added with 500 mL ethanol and then shaken using a shaker for 24 hours at a speed of 120 rpm and soaked for 24 hours. The immersion extract is filtered with a Buchner funnel to produce filtrate and residue. The filtrate is collected in an Erlenmeyer and the residue is extracted again with the same solvent and the treatment is repeated 5 times. The five extracts from the extraction are combined into one, then concentrated using a rotary evaporator vacuum. The concentrated ethanol extract obtained was then weighed and the yield calculated.

2.2.4. Hydrolysis and Concentrated ethanol Extract Partition. Concentrated ethanol extract of *H. verticillata* was taken as much as 10 g and placed into a beaker, then added 10 mL HCl for hydrolysis. The hydrolysis process is carried out for 1 hour and homogenized with a magnetic stirrer at room temperature, then added saturated sodium bicarbonate to neutral pH. The hydrolysis results are then partitioned with 50 mL of petroleum ether in a separating funnel and allowed to stand until two layers are formed, the organic layer and the water layer. Each layer formed is then separated. The organic layer is taken then concentrated with a vacuum rotary evaporator. The results of the petroleum ether fraction obtained can be weighed and the yield calculated.

2.2.5. Phytochemical Steroids Test for ethanol Extract and Petroleum Ether Fraction. Steroid phytochemical tests are carried out on the results of extraction and hydrolysis-partition results. The results of ethanol extract and petroleum ether fraction *H. verticillata* were tested phytochemically by using Liebermann Burchard reagent, each sample was put into a test tube, then dissolved in 0.5 mL chloroform and 0.5 mL acetic acid anhydrous. The mixture is then added to 1-2 mL of sulfuric acid in the tube wall, if a bluish-green is formed it indicates the presence of steroid compounds in the extraction and hydrolysis-partition results.

2.2.6. Separation of Steroid Compounds by Preparative Thin Layer Chromatography. Samples were taken as the result of partition 10 mg and then diluted with 10 mL of petroleum ether. Separation P-TLC used steroids with silica gel plate F_{254} is activated by heating in an oven at a temperature of 100-105 °C for 30 minutes. Each plate with a size of 10 x 20 cm. petroleum ether fractions which have been reconstituted with the solvent spotted at a distance of 1 cm from the bottom edge of the plate with the capillary tube, then drain and eluted using the eluent n-hexaneethyl acetate (4:1). The stain of separation results then observed under UV light at wavelengths of 254 and 366 nm.

2.2.7. Separation of H. verticillata petroleum ether fraction by Step Gradient Polarity (SGP) Column Chromatography. The 0.067 g petroleum ether fraction of *H. verticillata* was initially dissolved in 1 mL of n-hexane: ethyl acetate (95:5) solvent. Several samples of dissolved petroleum ether fraction are then put into a column. Separation of n-hexane fraction compounds using column chromatography with a stationary phase in the form of silica gel 60, column diameter of 1 cm, and column length of 50 cm. The elution process is carried out in a gradient with a comparison of n-hexane: ethyl acetate mobile phase (95: 5, 90:10, 85:15, 80:20, 75:25, and 70:30) with a volume of 100 mL each. The results in the form of eluate from column chromatography will be accommodated into vials every 2 mL [12].

2.2.8. Monitoring of Steroid Isolate Results with ATLC. Even-numbered vials on the results of the separation of column chromatography will be grouped based on the comparison of their mobile phase and monitored by Analytical Thin Layer Chromatography (ATLC). The monitoring process uses a 10x10 cm F254 silica gel plate which has been activated at 110 °C for 30 minutes. The isolates from each vial were then bottled on a silica plate that was marked ± 1 cm from the bottom edge and top edge. The bottling of isolates was carried out with capillary tubes 10 times periodically by drying, then eluted with the mobile phase of n-hexane: ethyl acetate (17:3) [13].

The next process is to analyze the results of the spot on the plate, if a bluish-green colour is formed then it shows the presence of steroids [14]. Stains on plates that have the same *Rf* value and the same spots, then combined into one as the same fraction, then the results of these isolates can be tested for toxicity to Artemia s and tested its antioxidant activity against DPPH.

2.2.9. Toxicity Test of Steroid Isolates. Toxicity tests on the steroid isolates resulting from TLC and column chromatography began with the hatching of *Artemia salina L.* eggs. *A. salina* eggs were hatched in hatching containers filled with seawater which was aerated and irradiated with fluorescent/incandescent lamps. Eggs of *Artemia salina L.* as much as 2.5 mg were put into a container containing 250 mL of seawater, then aerated for ± 48 hours with a hatching temperature of 25-30 °C. Hatched shrimp larvae are ready to be used as test animals.

The results of steroid isolates in each vial weighing 1 mg were dissolved with n-hexane as much as 10 mL to make a stock solution of 100 ppm. The stock solutions obtained were then pipetted 100, 200, 300, 400 and 500 µL respectively, which were each put into 5 vials and evaporated by the solvent. After the solvent in each vial evaporates 100 mL of dimethyl sulfoxide (DMSO) is added, one drop of 60% bread yeast solution, 2 mL of seawater, is shaken until mixed. The mixed solution was put into a 10 mL volumetric flask and then demarcated with seawater and homogenized. After each solution was marked, the concentration of the solution was 1, 2, 3, 4, and 5 ppm, then each concentration of the solution was transferred into a vial bottle and 10 larvae of *Artemia salina* shrimp were added for observation.

2.2.10. Steroid Isolate Activity Test against DPPH

2.2.10.1 Determination of Maximum DPPH Waves. Ethanol 95% pipetted as much as 4.5 mL then added a 0.2 mM DPPH solution of 1.5 mL, put into the cuvette until full. Furthermore, λmax DPPH is searched and the λmax measurement results are recorded for use in the next step [15].

2.2.10.2 Measurement of Antioxidant Activity in Samples. Each isolate was dissolved in a 95% ethanol sailor with a concentration of 50 ppm and its antioxidant activity was tested using a UV-Vis spectrophotometer at the obtained λmax. Then the best antioxidant activity test results vary in concentrations of 1, 2, 3, 4, and 5 ppm. Isolate each concentration pipetted 3 mL and added 1 mL DPPH 0.2 mM then incubated at 37 for 90 minutes, then absorbance was measured using a UV-Vis spectrophotometer at λmax that was obtained. The absorbance data obtained from each concentration of each sample was calculated as a per cent (%) of its antioxidant activity.

2.2.11. Identification of Steroid Compounds. Identification of isolated compounds using UV-Vis, FT-IR. Steroid isolates from column chromatography were identified by UV-Vis at a wavelength of 200-800 nm. Steroid isolates were also identified using FT-IR by mixing the evaporated steroid isolates with 0.2 g KBr pellets.

3. Results and discussion

3.1. Sample preparation

Hydrilla verticillata in this study obtained from Ranu Grati Lake. *H. verticillata* is dried to reduce the water content contained in it so that it does not interfere with the extraction process. *H. verticillata* dried obtained as much as 1.1 Kg and then mashed in Materia Medika Batu with a size of 90 mesh. Refinement of the sample is intended to enlarge the surface area of the particles to facilitate contact of the solvent at the time of extraction [16]. Dry samples of *H. verticillata* were obtained in the form of 950 g of powder.

3.2. Analysis of water content

The results of measurement of *H. verticillata* water content in this study were 6.53 %. These results indicate that the water content of *H. verticillata* meets the Ministry of Health's Republic of Indonesia standards [17] with a required maximum limit of 10%. These results indicate that the water content in this study did not interfere with the extraction process because the results obtained did not exceed the specified maximum limit.

3.3. Maceration extraction

The extraction process in this study was carried out as many as 4 replications to produce sediment and filtrate. Repetition is stopped when the maceration filtrate is clear green which indicates that the compound has been extracted. The maceration filtrate from several replications was concentrated with a vacuum rotary evaporator so that a concentrated ethanol extract of 4.54 % was obtained.

3.4. Hydrolysis and partition

Concentrated ethanol extract is then hydrolyzed to break the glycone and aglycone bonds in secondary metabolites which are still in the form of glycosides. Hydrolysis is carried out by the addition of HCl 2 N as a catalyst, as for the alleged glycoside termination reaction. The hydrolysates obtained are then partitioned (liquid-liquid extraction) using petroleum ether solvents which are nonpolar to take steroid compounds which are also nonpolar. The partition process produces two liquid phases, namely the organic phase which contains steroid compounds and the aqueous phase which contains the salt resulting from the hydrolysis reaction. The organic phase was taken on each partition result which was carried out 3 times and obtained a concentrated petroleum ether fraction of 65.41%.

3.5. Phytochemical steroid test of ethanol extract and petroleum ether

The results of the identification of steroid compounds in ethanol extract and petroleum ether fraction of *H. verticillata* as shown in Table 1.

Table 1. Liebermann Burchard Test Results.

Sample	colour	Information
ethanol extract	Green	+ Steroid
petroleum ether Fraction	Green	+ Steroid

3.6. Separation by thin layer chromatography

The steroid compounds in *H. verticillata* petroleum ether fraction was separated using Preparative TLC with n-hexane: ethyl acetate (3.75:1.25) eluent. Identification of compounds separated on TLC plates using a stain that looked and value of *Rf*. The results were shown in Figure 1. From Figure 1, there are two stains with green or bluish-green colour that alleged as steroid compounds.

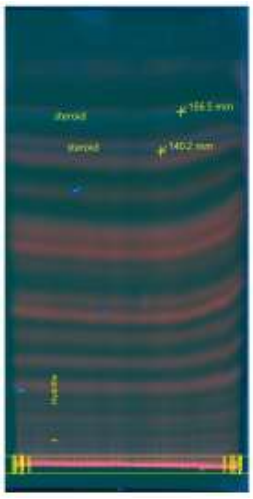

Figure 1. Result of P-TLC of *H. verticillata* petroleum ether fraction.

3.7. Separation by Column Chromatography

Separation of compounds by column chromatography the elucidation process begins with a variation of the nonpolar eluent, n-hexane: ethyl acetate (95: 5, 90:10, 85:15, 80:20, 75:25, and 70:30). It is assumed that nonpolar steroid compounds will be eluted and separated first. Nonpolar samples will pass through the stationary phase while those that are polar will be retained in the stationary phase. The silanol hydroxyl group contained in silica will form hydrogen bonds with polar compounds in the sample. The eluate from the separation is collected every 2 mL/min into the vial.

3.8. Monitoring Column Chromatography Results with ATLC

The eluate results of column chromatography separations contained in the vials are then monitored by Analytical TLC for further grouping. Vial grouping is based on the similarity of the chemical content profile of the stains formed at TLC and the same *Rf* value. The compounds contained in the sample have different speeds when passing through the column so that the migrated compounds are first shown with a high *Rf* value. The results of grouping isolates in this study obtained 12 combined isolates.

The monitoring results showed the presence of isolated compounds with a single stain and mixture, a single stain formed in TLC showed a good level of separation of a compound. Six single isolates obtained in this study were thought to be 3 steroid isolates and 3 triterpenoid isolates as in Table 2 below.

Table 2. Results of Monitoring and Incorporation of Vials.

Isolate	Vial	colour $UV_{254/366}$	*Rf*	alleged compound
H2	5-11	Black	0.80	Steroid
H4	15-61	Black	0.77	Steroid
H6	64-66	Red	0.70	Triterpenoid
H8	76-79	Red	0.66	Triterpenoid
H10	83-96	Green	0.75	Steroid
H12	100-110	Red	0.47	Triterpenoid

Based on the results in Table 2, isolate H2, H4 and H10 which is a suspected steroid compound will be continued with a toxicity test against *Artemia salina* shrimp larvae. In addition, there is also the petroleum ether fraction whose toxicity is tested to determine the potential of the sample before and after it is isolated by column chromatography.

3.9. Toxicity Test of Steroid Isolates

Toxicity tests in this study were carried out on TLC and Column Chromatography isolates that were suspected as steroid compounds resulting from column chromatography. Test results in the form of mortality data were calculated using MINITAB to obtain LC_{50} values that indicate the level of sample toxicity. The results of LC_{50} TLC and Column Chromatography isolates can be seen in Table 3.

Table 3. LC_{50} of TLC and Column Isolates.

Sample	LC_{50} value
TLC Isolates	1.41 ppm
	12.2 ppm
Column Isolates H2	4.32 ppm
H4	8.24 ppm
H10	10.35 ppm

Based on these results indicate that TLC and Column Chromatography isolates have a high level of toxicity. The toxic activity of each sample in this study was due to the presence of secondary metabolites found in the fraction of petroleum ether and isolate B. Secondary metabolite compounds such as steroids that have -OH groups can bind to integral proteins in cell membranes. According to Budaraga et al. [18] in his research on *Cinnamomum burmanni* stated that the -OH group in flavonoid compounds can bind to the integral protein in the larval cell membrane. This can inhibit the active transport of Na^+/ K^+ ions so that it will cause integral proteins in the cell membrane to swell because Na^+/ K^+ ions accumulate in the cell and rupture. This is what can cause shrimp larvae to die. LC_{50} values of the two test samples in this study each <30 ppm, these results indicate the potential as an antitumor and anticancer.

3.10. Antioxidant Activity Test against DPPH

The antioxidant activity test in this study was carried out on isolate which was suspected as a steroid compound from column chromatography. The test results in the form of data% of antioxidant activity were calculated using GraphPad PRISM to obtain an EC_{50} value indicating the level of antioxidant activity of the sample. The results of EC_{50} in steroid isolate can be seen in Figure 2 and Table 4.

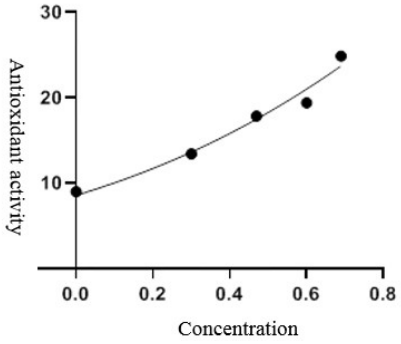

Figure 2. Antioxidant activity of H2 Isolates.

Table 4. The yield of % antioxidant activity and EC_{50} H2 Isolate.

concentration (ppm)	per cent (%) antioxidant activity
1 ppm	8.98 %
2 ppm	13.41 %
3 ppm	17.83 %
4 ppm	19.38 %
5 ppm	24.87 %
EC_{50} = 23.00 ppm	

The results of the calculation of per cent (%) of antioxidant activity in Table 4.2 show that the isolate steroid *Hydrilla* has antioxidant activity. The value of per cent (%) of antioxidants increases with the increase in the concentration used. The highest value of steroid isolates was at a concentration of 5 ppm. This shows that the more concentration used, the more DPPH radicals are stabilized by antioxidant compounds found in the sample. Data obtained from the calculation of the value of per cent (%) of antioxidant activity is used to calculate EC_{50} which is the main parameter in the measurement of antioxidant activity. EC_{50} results from all *Hydrilla verticillata* steroid isolates were obtained at 23.00 ppm. Antioxidants are very strong if the EC_{50} value is less than 50 [19].

3.11. Identification of steroid compounds

The identification with UV-Vis obtained the maximum wavelength in the petroleum ether fraction 203, 206.9 and 275 nm. The maximum wavelength obtained in steroid isolates was 275 nm. These results were further identified by FTIR and showed the presence of dimethyl geminal groups as a typical steroid group in both samples.

4. Conclusion

The steroid isolates from TLC and Column Chromatography separation has toxicity and antioxidant properties. Based on toxicity test, LC_{50} value of steroid TLC isolate was 1.41 and 12.2 ppm, whereas the LC_{50} value of Column Chromatography isolates (H2, H4 ad H10) was 4.32, 8.24 and 10.35 ppm. EC_{50} value of H_2 Column Chromatography isolate was 23.00 ppm.

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