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Toxicity Test of Column Chromatography Steroids Isolates from Ethyl Acetate Fraction of Hydrilla verticillata

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ABSTRACT
Hydrilla verticillata is one of the aquatic plants that contain some secondary metabolites, such as triterphenoids and steroids. The aim of this study was to determine the toxicity levels of steroids isolated from ethyl acetate fraction of *Hydrilla verticillata* separated by column chromatoghraphy. The biomass of *Hydrilla verticillata* was dried and powdered. *Hydrilla verticillata* powder is then extracted by maceration methods using ethanol solvent. The crude ethanol extracts were hydrolyzed using 2 N of HCl and partitioned using ethyl acetate solvent. The steroids compounds of ethyl acetate fraction were separated by Column Chromatography. The steroid isolates from Column Chromatography were identified by FTIR and UV-Vis spectrophotometer. The toxicity level of Column Chromatography steroid isolates was determined by BSLT method. The result of this research showed that extraction through maceration using ethanol produced 2.52 % yield, whereas the percent yield of the partition using ethyl acetate was 45.04 %. Separation by column chromatography resulted 3 steroids isolates B_1, B_2, and G_1. The toxicity test showed that column chromatography steroids isolate from ethyl acetate fraction of *Hydrilla verticillata* has toxicity properties. The LC_{50} value of B_1, B_2 and G_1 isolate was 14.58, 7.55 and 4.71 ppm, respectively.

Keywords: *Toxicity, Column Chromatography, Steroid, Ethyl acetate, Hydrilla verticillata*

1. INTRODUCTION

Indonesia has many natural resources that very potential to be explored. Indonesia is a tropical country with more than 75 % of the area is water, sea and fresh water. One of the aquatic plants that many advantages is *Hydrilla verticillata* which encountered a lot in Ranu Grati Lake. *Hydrilla verticillata* are found in reservoirs, rivers, and lakes [1].

Hydrilla verticillata has been used as organic fertilizer and fish food [2], detoxification agent and anti-aging [3], phytoremediation in waste degradation [4] and good absorbent to some heavy metal [5]. *Hydrilla verticillata* also has antioxidant activities [6], antimicrobial [7], antimalarial [8], anti-inflammatory [9] and antitumor [1].

Hydrilla verticillata contain the same secondary metabolites. *Hydrilla verticillata* contain some secondary metabolite compounds such as flavonoids, steroids, and alkaloids [10]; phenols, glicosides, alkaloids and steroids [11]. Petroleum fractions from the ethanol extract of *Hydrilla verticillata* are positive contain steroids [12, 6].

Steroids are secondary metabolites that have specific activities. Steroids antioxidant activities [13] and potential pharmacology [14] are known as potential compounds as medicine and drugs. Steroid has

cytotoxicity against cell myeloma (malignant tumor) [15] and can inhibit prostate cancer [16]. Stigmasterol and Stigmast-5-en-3β-ol steroids from *Dysoxylum alliceum* stem bark are potential as anticancer against breast cancer MCF-7 [17].

The active compounds of *hydrilla verticillata* can be extracted using maceration method with a polar solvent. Some secondary metabolites are bonded with sugar as glicosides, so can be extracted using polar solvent such as ethanol [18]. Separation can be done by hydrolysis using an acid solution to break glycosides bonds between glycon an aglycon [13], then partition using a semipolar or nonpolar solvent to separate the steroids [19]. More specific steroids separation can be done using column chromatography.

2. MATERIALS AND METHODS

2.1. Materials

The material in this research is *Hydrilla verticillata*, ethanol 95%, hydrochloric acid 2 N, ethyl acetate p.a., n-hexane 96%, Na-bicarbonate, sulfuric acid 98%, acetic anhydride p.a, dimethyl sulfoxide, seawater, *Artemia salina L*. eggs, bread yeast, silica gel 60 (0.063-0.200 mm), and silica gel F254 plate,

2.2. Methods

2.2.1. Preparation of Sample

Hydrilla verticillata is taken as much as 14 Kg from Ranu Grati Lake, then dried for 3-5 days. Then powdered and sieved ± 90 mesh.

2.2.2. Water Content Analysis

Hydrilla verticillata powder was weighed and put into the porcelain cup. The cup and the sample were heated for 15 minutes at a temperature of 100 - 105ºC. Then cooled down in a desiccator for 10 minutes and weighed until reached a constant weight.

2.2.3. Extraction of Secondary Metabolites

Secondary metabolites of *Hydrilla verticillata* is extracted by maceration methods using ethanol solvent. A 100 g of dry powder *Hydrilla verticillata* was put down into Erlenmeyer then added 500 mL of ethanol, then shaken for 24 hours at a speed of 120 rpm and soaked overnight. The filtrate and residue are filtered with a Buchner funnel. The filtrate is collected and the residue is extracted again with the new ethanol solvent. The treatment is repeated three times to get the maximum result. The macerat is concentrated using a vacuum rotary evaporator, then weighed and calculated the yield of maceration.

2.2.4 Hydrolysis and Partition using Ethyl Acetate

Two grams of concentrated *Hydrilla verticillata* ethanol extract was taken and placed into a beaker glass. The 4 mL of Hydrochloric acids 2N was added and stirred for 1 hour at room temperature. To end the hydrolysis process, saturated sodium bicarbonate is added to the mixture until neutral pH is reached. The mixture then partitioned in a separating funnel using 10 mL of ethyl acetate. The organic phase and water phase were separated. The organic phase is then concentrated using a rotary evaporator vacuum. The ethyl acetate fraction is weighed and calculated the yield.

2.2.5 Phytochemical Steroids Test

The ethyl acetate fraction of *Hydrilla verticillata* was tested phytochemically to know the steroids content using L-B reagent. The ethyl acetate fraction was put into a test tube, then dissolved in 0.5 mL of anhydrous acetic acid and 0.5 mL of chloroform. Then, 1-2 mL of sulfuric acid was added to the mixture slowly. The presence of steroid compounds was known by the formation of bluish-green colors.

2.2.6 Separation of Steroids Compounds by Column Chromatography

The 0.067 g of *Hydrilla verticillata* ethyl acetate fraction was dissolved in 1 mL of n-hexane: ethyl acetate (95:5) solvent and put into a column. Separation using column chromatography done with 50 cm length of the column, and 1 cm of column diameter. The stationary phase is silica gel 60. The mobile phase is n-hexane:ethyl acetate with a gradient comparison (95:5, 90:10, 85:15, 80:20, 75:25, and 70:30). The volume of each is 100 mL. The eluate was collected every 2 mL into several vials. The result of column chromatography was monitored using Thin Layer Chromatography.

2.2.7 Monitoring with Analytical Thin Layer Chromatography

The vials resulted from column chromatography separation will be monitored and grouped based on the same spots. The monitoring process using a F254 silica gel Thin Layer with n-hexane:ethyl acetate (17:3) as the mobile phase. The steroids isolates were known as blue or green color spots. The spots at the same Rf value are combined into one as the same isolates.

2.2.8 Toxicity Test

One milligram of steroid isolates was dissolved in 10 mL of n-hexane to get a 100 ppm of stock solution. Then, taken using micropipette 100, 200, 300, 400 and 500 μL respectively and put into five different vials then

evaporated. To each vial were added one drop bread yeast solution 60, 100 mL of DMSO, % and 2 mL of seawater. The mixed solution was put into a 10 mL volumetric flask to get was 1, 2, 3, 4, and 5 ppm concentration of the solutions. Each concentration of the solution was transferred into a vial and added 10 of *Arthemia salina* L. shrimp larvae. The mortality of *A. Salina* was observed after 48 hours.

2.2.9 Identification of The Steroid Isolates using Spectrophotometer

The steroids isolates were identify using Fourier Tranform- Infra Red (FT-IR) and Ultra Violet – Visible (UV-Vis) spectrophotometer.

3. RESULT AND DISCUSSION

3.1 Preparation of Sample

The sample of *Hydrilla verticillata* was dried and powdered to reduce the water content contained in it so that it saves to store and does not interfere the process of extraction. From 14 Kg of *Hydrilla verticillata,* wet biomass was obtained 1 Kg of dry powder.

3.2 Water Content Analysis

The water content of *Hydrilla verticillata* from this research was 6.53 %. The water content is below 10 %, indicate that the of *H. verticillata* water content fulfills the Indonesia standards of the Ministry of Health's [20].

3.3 Extraction of The Secondary Metabolites

The secondary metabolites of *Hydrilla verticillata* were extracted using ethanol solvent. The extraction was carried out three times. The color of the maceration filtrate was decreased from dark green into clear green (Fig.1). The maceration filtrate was concentrated and obtained 2.52 % of concentrated ethanol.

Figure 1 Color Changes of Maceration Filtrate

3.4 Hydrolysis and Partition using Ethyl Acetate

Hydrilla verticillata ethanol extract was hydrolyzed by the addition of HCl 2 N as a catalyst. The glycosides bonds between glycosides and secondary metabolites will be broken and the glycone and aglycone can be separated. The steroid compounds in *Hydrilla verticillata* ethanol extract will be extracted to the ethtyl acetate solvent, which is a semipolar solvent. The ethlyl acetate phase was taken and obtained 45.04% of yield.

3.5 Phytochemical Test of Steroid

The results of the phytochemicals test of steroid compounds in ethanol extract and ethyl acetate fraction of *H. verticillata* as showed the Green color when reacted with L-B reagent (Table 1). It is indicated that tethanol extract and ethyl acetate fraction of *H. verticillata* contain steroids compounds.

Table 1. The result of phytochemical test

Extract/Fraction	Color	Compounds
Crude Ethanol extract	Green	+ Steroid
Ethyl acetate Fraction	Green	+ Steroid

3.6 Steroids Isolation by Column Chromatography

Separation of steroids compounds using column chromatography done using silica as a stationary phase. The compounds in *hydrilla verticillata* will be eluted and separated corresponding to its polarity. The more polar compounds will interact with silica as stationary phase longer than semipolar and nonpolar compounds and will be separated. The steroids compound that is nonpolar compound will be eluated first and out from the column faster. Teh eluat will be collected for each 2 mL into the vials, resulted in 296 vials in this research. The eluate results of column chromatography separations contained in the vials are then monitored by Analytical TLC.

From the research, there are five single isolates obtained. Three isolates are presumed as steroid isolates because of its Dark green or Green color and two isolates are indicated as triterpenoid isolates because of its red colors (Table 2).

Tabel 2. Monitoring result of column chromatography

Isolates	Vials	color $UV_{254/366}$	*Rf*	presumed compounds
B1	**2-33**	**Dark Green**	**0.81**	**steroid**
B2	**40-45**	**Dark Green**	**0.80**	**steroid**
G1	**69-77**	**Green**	**0.77**	**steroid**
R1	151-169	Red	0.70	triterpenoid
R2	232-275	Red	0.66	triterpenoid

Based on Table 2, B1, B2 and G1 isolates are alleged steroids compounds.

3.7 Identification of steroid compounds using spectrophotometer

From identification using UV-Vis, base on UV-Vis spectra, there are peaks at maximum wave leght 205, 228 and 275 nm. Supported by the FTIR spectra that showed the presence of peak in wave number 1387 cm^{-1}. This is peak of dimethyl geminal groups that are typical group in steroids compounds.

3.8 Toxicity Test of Steroid Isolates

The Toxicity of steroids isolates B1, B2 and G1 isolates are showed in Tabel 3.

Table 3. LC_{50} of steroids isolates

Isolates	LC_{50}
B1	14.58 ppm
B2	7.55 ppm
G1	4.71 ppm

The toxicity test results in Table 3 indicate that steroids isolates from Column Chromatography of *Hydrilla verticillata* ethyl acetate fractions have a high level of toxicity. LC_{50} values of Bi, B2 and G1 isolates <30 ppm, these results indicate the steroids isloates are potential as an anticancer or antitumor.

4. CONCLUSION

The Column Chromatography steroid isolates of *Hydrilla verticillata* ethyl acetate fraction has toxicity properties. The LC_{50} value of steroid B1, B2 ad G1 was 14.58, 7.55 and 4.71 ppm. The isolates contained steroids compounds with maximum wavelength in UV-Vis 275 nm and have FTIR peak dimethyl geminal groups.

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