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Isolation and Bioactivity of Steroids Isolates From Petroleum Ether Fraction Of *Chlorella sp.*

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Abstract. *Chlorella* sp. is a prospective steroids source which is potentially applied in pharmaceutical field. The aim of this research was to isolate and determine the toxicity and antioxidant activity of steroids compounds in petroleum ether (PE) fraction. *Chlorella* sp. was cultivated in 4% of sprout extract medium and extracted by maceration using methanol solvent. The methanol extract was partitioned with petroleum ether and the fraction was identified by LC-MS to know the type of steroids compound. The PE fraction contained steroids was separated with preparative thin layer chromatography (Preparative TLC) and column chromatography. The toxicity level and antioxidant assay of steroids isolates was determined by BSLT and DPPH method, respectively. According to phytochemical test and LC-APCI-MS identification, the PE fraction was positive for steroids compounds such as β-sitosterol, stigmasterol, campesterol and erythrodiol. Steroid isolates from TLC and column chromatography separation has toxicity and antioxidant properties. Based on toxicity test, LC_{50} value of steroid isolate by TLC separation was 19.69 ppm, whereas, by column chromatography separation, LC_{50} value of A10 and A12 isolate was 26.67 and 49.04 ppm, respectively. Antioxidant activity (EC_{50} value) of steroid isolate by TLC separation was 73.82 ppm. Percentage of antioxidant activity of A2, A8, A10 and A12 isolates (by column chromatography separation) at 50 ppm were 2.60, 4.09, 4.52 and 3.29 %.

INTRODUCTION

The number of diseases is rapidly increasing and it makes societies paying attention to their health. To prevent from the disease, people consume traditional medicine or natural supplement. Nowadays, consumption of medicine from natural product is more preferable than synthetic product. People believe consumption of synthetic drug in the long term may cause undesirable side effect to human body. The usage of herbal remedies offers a solution to reduce people concern to effect of synthetic drug. Exploration of natural product for pharmaceutical application become crucial and essential.

Indonesia's biodiversity provides unlimited natural sources especially from marine plant. *Chlorella* sp. is one type of microalgae easy cultivable and irrespectively seasonal condition [1]. Previous researches reported that *Chlorella* sp. can be potential biodiesel sources [2] dan has several bioactivities such as toxicity [3], antibacterial [4, 5, 6, 7] and antioxidant [8, 9].

The bioactivities of *Chlorella* sp. are correlated to its chemical compound. Some researches reveal the existence of steroid compound in the *Chlorella* sp. Steroids are secondary metabolites in natural product with various pharmacological activity against cancer. Diastuti and Warsinah [10] described steroid has cytotoxicity against cell myeloma (malignant tumor) and Sapar *et al.* [11] stated steroid can inhibit prostate cancer.

The initial screening to know *Chlorella* sp. has potency as anticancer is toxicity and antioxidant activity. In this study, toxicity level was determined by Brine Shrimp Lethality Test (BSLT) method with *Artemia salina* and

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antioxidant activity was measured by DPPH (1,1-diphenyl-2-picrylhydrazyl). The advantages of the both methods are simple procedure, inexpensive and easy treatment [12]. In addition, DPPH method is a common method for antioxidant activity with fast and high accuracy treatment.

Fasya *et al.* [13] performed steroids isolation from *Chlorella* sp. by maceration using methanol, hydrolyzed using hydrochloric acid and fractioned using organic solvent. Purification of the extract is conducted through separation of Thin Layer Chromatography (TLC) [14] and column chromatography [15]. Compared to TLC, column chromatography was able to produce a large number of pure compounds [16]. The objective of this research was to isolate suspected steroid compounds of *Chlorella sp*. and determined its bioactivity including toxicity and antioxidant activity.

EXPERIMENTAL DETAILS

Preparation of *Chlorella* sp. Biomass

A hundred of fifty milliliters of *Chlorella* sp. isolate were inoculated in Sprout Extract Medium (SEM) and incubated for 10 days with photoperiodicity 14 h of lightness and 10 h of darkness [17]. The harvesting of *Chlorella sp*. biomass was performed in the 10^{th} day. The biomass was analyzed its water content based on AOAC method [18].

Chlorella sp. Extraction

Thirty grams of dried biomass were macerated with methanol 150 mL for 24 h and were shaken at 120 rpm for 5 h at room temperature. The sample was separated using Buchner Funnel and the residue was extracted five times. All filtrate was collected and evaporated by rotary vacuum evaporator. Yield of extraction was calculated.

Methanol extract of 2.5 g was hydrolyzed with 5 mL of hydrochloric acid and stirred for 1 h at room temperature. The hydrolyzed extract was added with sodium bicarbonate up to neutral pH. The sample was then partitioned by petroleum ether 12.5 mL which is repeated five times. Organic phase was collected and concentrated by nitrogen gas. Yield of petroleum ether fraction was calculated.

Identification of Steroid

A small portion of petroleum ether fraction of *Chlorella* sp. was added 0.5 mL of chloroform and anhydrate acetic acid. The mixture was added with 1-2 drops of hydrochloric acid through the test tube wall. A brownish or violet ring at the border of two solvents indicates the presence of a triterpenoid, while a bluish green color indicates the presence of steroid.

Identification of Extraction by LC-APSI-MS

Petroleum ether fraction of 2 µL was injected to LC-MS. Mobile phase used gradient system with solvent A (0.01% of acetic acid in water) and solvent B (0.01% of acetic acid in acetonitrile) with flow velocity 300 µL/min. Solvent gradient used comparison solvent, 0% of A: 100% of B and 100% of A : 0% of B for 5 min. Column used was Hypersil Gold C-18 (1.9 µm x 2.1 mm x 50 mm)with 30°C of column temperature for running. Mass spectrophotometry (TSQ QUANTUM ACCES MAX, Thermo Finnigan) used APCI as ionization source with electric current of 4 µA [19].

Separation by Column Chromatography

This separation was conducted using wet column chromatography. Mixture of n-hexane: ethyl acetate (4:1) was used as mobile phase (eluent), while gel silica 60 (0.063-0.200 mm) was used as stationary phase. The bottom of column was filled with glass wool and eluent. Diameter of column was 1.5 cm and flow velocity for elution was 1.5 mL/min.

Preparation of stationary phase: 10 g of gel silica was activated at 110°C for 2 h, then cooled for 15 min in desiccator. Gel silica was added eluent and homogenized to form slurry. Gel silica slurry was put in treated column

for 24 h. Ethyl acetate fraction (0.1 g) was diluted in 1 mL of eluent. The sample was load in the treated column for elution process. Every 2 mL of eluate was collected in a vial as a fraction.

Monitoring of Separation by TLC

The separated fractions were monitored using TLC conducted in every 5 vials. The vial was classified based on the stain and retardation factor (Rf) value. A mixture of n-hexane: ethyl acetate (4:1) of eluent was used for monitoring. A 10x10 cm gel silica F_{254} was activated for 30 min and the eluent was saturated in a container for 1 h. Each fraction was spotted to the activated plate, afterward the plate was eluted. The stains in the plate were observed under UV at 254 and 366 nm after spraying with Lieberman-Burchard reagent. The Rf value was calculated and the suspected isolates contained steroid were gathered and evaporated it.

Toxicity Test of Steroids using BSLT Method

The positive steroid isolate was collected and dissolved in petroleum ether, then separated by centrifugation. The filtrate was taken to form isolate and the petroleum ether was evaporated. The isolate of 2.9 mg was dissolved in 5 mL of solvent to obtain a stock solution 580 ppm. Solvent was used according to solubility of the isolate. The variation of isolate concentration was 5, 10, 20 and 25 ppm. All samples put into different vials and the solvent was evaporated. A drop of yeast solution and 50 mL of DMSO were added to the vial. Homogenized mixture was put 10 larvae of *A. salina* shrimp and added sea water up to 5 mL of volume. There were two control solutions such as DMSO and sea water solution. All vials were monitored under a bulb for 24 h and observed the death of larvae. The determination of LC_{50} (Lethal Concentration) used MINITAB program 17.

Antioxidant Assay by DPPH Method

The isolates were varied into 15, 20 and 25 ppm of concentration. Each isolate of 4.5 mL was added 1.5 mL DPPH 0.2 mM. The mixture was incubated at 37°C for certain time. The absorbance of sample was measured using spectrophotometry at 515 nm. Percentage of antioxidant activity was calculated using Equation 1.

$$\text{Antioxidant activity} = \frac{\text{Absorbance of Control - Absorbance of Sample}}{\text{Absorbance of Control}} \times 100\%$$

DPPH control used 0.2 mM DPPH solution of 1.5 mL in 4.5 mL of 95% ethanol. Comparation solutions were BHT and carboxylic acid (vitamin C) treated as a sample. The determination of EC_{50} (Effective Concentration) values was performed by a program "prism 5 GraphPad software, Regression for analyzing dose response data".

RESULT AND DISCUSSION

Steroids Extraction from *Chlorella* sp.

Chlorella sp. was cultivated for 10^{th} days and the inoculum were discolored daily, from yellowish green to dark green (Figure 1). It indicated the increasing of cell density of *Chlorella sp*. The biomass was dried at room temperature to prevent destruction of the secondary metabolite. Dried biomass has greenish-black color at powder form. A 300.58 g of wet biomass produced a 5.10 g of dried biomass. Water content of the biomass was 10.25% (w/w).

Steroids compound was extracted by maceration using methanol. Steroids are an active compound in *Chlorella* sp. and bind naturally to polar group of glycoside compound. The polar group of glycoside makes steroids easy to extract with methanol. Yield of this extraction was 23.27% (w/w). Hydrolysis is one of steps in extraction to break glycoside bonds between steroids and glycoside compound. This process was conducted with an addition of hydrochloric acid to disrupt the bonds and a neutralization with sodium bicarbonate to stop the hydrolysis reaction. The hydrolyzed product was partitioned by petroleum ether solvent. Greenish-black concentrated extract was obtained with yield 52.85% (w/w).

FIGURE 1. Color changing of *Chlorella* sp. culture from 0^{th} to 10^{th} days

Identification of Steroids

Identification of the isolate using phytochemical test produced bluish green color. It indicated steroids presence in the isolate. Oxidation reaction by conjugated polyene formation caused discoloration of isolate. According to identification using LC-APCI-MS, the isolate contained steroids and triterpenoid compound (Table 1). The types of steroids compound were β-sitosterol, stigmasterol, and campaesterol, while that of the triterpenoid compound were erythrodiol. Stigmasterol has the highest peaks with retention time (Rt) 1.43. The retention time of campaesterol, β-sitosterol, and erythrodiol was 1.42, 1.52 and 1.92, respectively.

TABLE 1. Identification of isolate using LC-APCI-MS

Compounds	Retention time (min)	Molecule mass (g/mol)	Precursor ion (m/z)	Product ion (m/z)
Steroid				
β-sitosterol	1.52	414	397	161
Stigmasterol	1.43	412	395	81
Campaesterol	1.42	400	383	161
Triterpenoid				
Erythrodiol	1.92	442	425	177

Identification of separated compound was determined by preparative TLC (Thin Layer Chromatography) based on Rf value. Separation of petroleum ether fraction of *Chlorella* sp. was showed in the Table 2. There were 15 stains formed under UV 366 nm with various color. Aprelia and Suyanto [20] stated that a bluish green on *Christella arida* showed a steroids compound. Astuti *et al.* [21] studied that steroid of stem of *Spatholobus littoralis* Hassk produced bluish green on TLC plate. The stain number 12 was predicted steroid compound with 0.7514 of Rf value. The isolate formed needle-shaped crystals with 5.8 g of weight.

TABLE 2. Separation of petroleum ether fraction of *Chlorella* sp. by preparative TLC

No.	Rf Value	Colour stains	Predicted Compound
1.	0.0508	Pink	-
2.	0.0847	Pink	-
3.	0.1271	Brown	-
4.	0.1920	Pink	-
5.	0.2598	Pink	-
6.	0.2994	Light pink	-
7.	0.4576	Blackish red	-
8.	0.5197	Light pink	-
9.	0.6129	Blackish red	-
10.	0.6606	Light pink	-
11.	0.7146	Pink	-
12.	0.7514	Bluish Green	Steroid
13.	0.8135	Pink	-
14.	0.8757	Pink	-
15.	0.9519	Orange	-

Separation by column chromatography was carried out to get more purified steroids compound. Based on the monitoring, the isolate contained single and mixed spots. The spot with similarity of Rf value, color and shaped was collected into one fraction. The TLC data describe four single fractions (A2, A8, A10, A12) and the remain mixture fraction can be seen in the Table 3. The A2 fraction gave green color with 0.4375 of Rf. The other fractions gave red color with Rf 0.2125, 0.1250, and 0.0769 for A8, A10, and A12 fractions, for each.

TABLE 3. Monitoring column chromatography separation by TLC

Fraction	Vial	Spot Number	Color	Rf	W (mg)
A1	1 – 20	0	-	-	-
A2	21 – 26	1	Green	0.4375	1.70
A3	27 – 28	2	Green	0.4375	0,09
			Red	0.3750	
A4	29 - 30	3	Green	0.3125	0.60
			Red	0.2625	
			Red	0.2125	
A5	31 - 38	2	Green	0.2625	1.50
			Red	0.2125	
A6	39 - 43	3	Green	0.2625	1.19
			Red	0.2125	
			Red	0.1375	
A7	44 – 45	2	Green	0.2625	0.19
			Red	0.2125	
A8	46 – 57	1	Red	0.2125	1.10
A9	58 – 70	2	Red	0.2125	2.59
			Red	0.1375	
A10	71– 100	1	Red	0.1250	5.69
A11	101-140	2	Red	0.1392	1.50
			Red	0.0769	
A12	141-200	1	Red	0.0769	19.3

Bioactivity of *Chlorella* sp.

Toxicity assay was conducted for methanol extract, petroleum ether (PE) fraction, and steroid isolate after separations by TLC and column chromatography. Separation of TLC isolate by column chromatography was obtained two isolates, A10 and A12. Meyer *et al.* [12] stated that an extract was called very toxic if the extract inhibits 50% of test animals at concentrations less than 30 ppm. Table 4 showed LC_{50} of TLC isolate and A10 isolate gave lower value than 30 ppm. It indicated that TLC isolates and A10 isolate was very toxic extract with LC_{50} value 19.69 and 26.67 ppm, respectively (Table 4).

TABLE 4. LC_{50} values from *Chlorella* sp.

Sample	LC_{50}(ppm)
Methanol extracts	54.78
PE Fraction	38.88
TLC Isolates	19.69
A10 Isolates	26.67
A12 Isolates	49.04

Antioxidant activity of sample was determined by DPPH method. DPPH is a radical compound reduced by addition of hydrogen atom from steroid compound of *Chlorella* sp. This reaction was indicated by color changing, from purple DPPH to yellow 1,1-diphenyl-2-picrylhydrazin. The remaining DPPH was measured its absorbance to determine antioxidant potency of sample. The parameters of potential antioxidant is EC_{50} values and percentage (%) antioxidant activity. EC_{50} showed concentration of the sample reducing 50% of DPPH compound. The smaller EC_{50} value, the higher antioxidant activity [22].

Figure 2 showed that TLC isolate yielded the highest antioxidant activity with 73.82 ppm of EC_{50} values. Steroid compound in the TLC isolate might be responsible for the highest of antioxidant activity. The potency of antioxidant is divided three levels, strong (< 50 ppm), moderate (50-100 ppm), and low (151-200 ppm) [23]. Table 5 presented that A2, A8, A10 and A12 column chromatography isolate gave 2.60, 4.09, 4.52 and 3.29 % of antioxidant activity at 50 ppm of concentration, respectively. A10 fraction has highest antioxidant activity, whereas A2 fraction has lowest antioxidant activity.

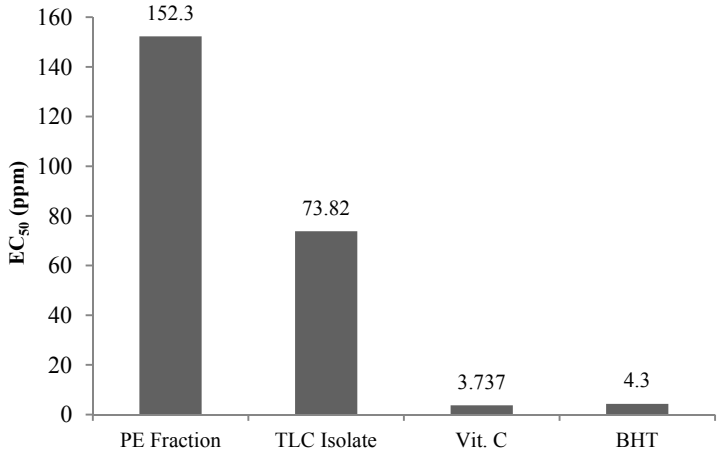

FIGURE 2. EC_{50} value of PE fraction and TLC isolate of *Chlorella* sp. vitamin C and BHT used as positive control of antioxidant activity.

TABLE 5. Antioxidant activity of column chromatography isolates

Isolate	Antioxidant Activity (%)
A2	2.60
A8	4.09
A10	4.52
A12	3.29

CONCLUSION

The PE fraction of *Chlorella* sp. contained steroids compound (β-sitosterol, stigmasterol, campaesterol and erythrodiol) based on phytochemicals test and LC-APCI-MS identification. The steroids isolated from TLC and Column Chromatography separation have toxicity and antioxidant activity. The LC_{50} value of TLC isolate was 19.69 ppm, while column chromatography isolates (A10 and A12) were 26.67 and 49.04 ppm, respectively. The antioxidant activity of TLC isolate was 73.82 ppm of EC50 value, whereas antioxidant activity of column chromatography isolates A2, A8, A10 and A12 at 50 ppm of concentration were 2.60, 4.09, 4.52 and 3.29 %, respectively.

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