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Isolation, Identification and Bioactivity of Steroids Compounds from Red Algae *Eucheuma cottonii* Petroleum Ether Fraction

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Abstract. More than 75 % of Indonesia is covered by the sea. Indonesia has abundant natural resources that have potential to be explored as new renewable bioactive compounds. One of the natural resources is red algae *Eucheuma cottonii.* The aim of this research was isolating, identifying and determining the bioactivity (toxicity and antioxidant activity) of steroids compounds in Red Algae *Eucheuma cottonii*. Red Algae *Eucheuma cottoni* was extracted by maceration using methanol. The methanol extract was hydrolyzed with 2 N of hydrochloric acid and then partitioned with petroleum ether. Petroleum ether fractions were identified by phytochemical test and LC-MS to know the types of steroids compound contained. Then, the steroids compound was separated with Preparative Thin Layer Chromatography (TLC) and Column Chromatography. The toxicity level and antioxidant assay of steroids isolates were determined by BSLT and DPPH method. According to phytochemical test and LC-APCI-MS, petroleum ether fraction of Red Algae *Eucheuma cottoni* contained steroids compounds such as cholesterol, β-sitosterol, campesterol and stigmasterol. LC50 value of isolates of petroleum ether fraction was 26.70 ppm (A1), 16.30 ppm (A3) and 22.03 ppm (A5). EC50 value of steroid isolates of petroleum ether fraction was 13.07 ppm (A1), 3.9.1010 ppm (A4) and 50.92 ppm (A5).

INTRODUCTION

Red Alga *Eucheuma cottonii* contains secondary metabolites compounds, such as flavonoids, saponins, steroids, triterpenoids [1] and fluorotanine [2]. Among these bioactive compounds, steroids, have various pharmacological activities, which have the potential bioactivity as antiinflammation and analgesic agent [3]; antimicrobial [4]; antibacterial [5]; the activity of toxicity [6]; Antioxidant [7] and anti-breast tumour [8]. The aim of this research was isolating, identifying and determining the bioactivity (toxicity and antioxidant activity) of steroids compounds in Red Algae *Eucheuma cottonii*. Isolation of steroid compounds can be done using column chromatography. The basic principle of column chromatography is a separation based on the principle of adsorption. Separation can be done by placing the samples at the top end of the column and the solvent used is continuously flowed. The eluent/solvent will pass through the column in the presence of gravity or due to pressure [9]. Isolation of steroid compounds was carried out with wet loading technique. Eluent for *E. cottonii* separation was used n-hexane and ethyl acetate (18:2). The conditions of column chromatography were ratio sample: silica gel 1:150, diameter column 1 cm with flow rate 2 mL/min.

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The results of column chromatography isolates contained steroid compounds then tested for antioxidant activity and toxicity test. Antioxidants are a compound that can inhibit free radical so it can inhibit various diseases related to free radical. According to Hernani [10], the main use of antioxidant was to stop or break the chain reaction contained free radicals in the body. DPPH method was used for testing antioxidant activity. The method was simple, accurate, fast and could be done with a small sample. The DPPH method was also an unlimited method for measuring dissolved components in solvents used in the analysis (can measure total antioxidant activity in both polar and non-polar solvents) [11]. Toxicity test, using *Artemia salina* L. larvae or BSLT test, was an initial screening method to determine the cytotoxic properties of an extract or compound. The principle of the toxicity test was the toxic bioactive components given at high doses and become drugs at low doses [12]. Steroid isolates with the best antioxidant activity were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the type of steroids. Steroid isolates with the highest antioxidant activity were identified using UV-Vis and FT-IR spectrophotometers.

EXPERIMENTAL DETAILS

Sample Preparation

Red algae *Euchema cottonii* originating from the coast of Wonosorejo Banyuwangi. Artemia salina shrimp larvae. Red algae *Eucheuma cottonii* powder was weighed as much as 100 g and extracted by maceration using 99.9% methanol 500 mL solvent in Erlenmeyer and stirred using a shaker with a speed of 120 rpm (rotation per minutes) for 3 h and left for 24 h. Then filtered and the extract obtained was macerated again (3 times) with the same solvent and procedure until a faded colour filtrate. Then the filtrate was concentrated using a rotary evaporator. Then rendement value of the extract was calculated. Then it was hydrolyzed by adding 10 mL of 2N hydrochloric acid and stirred for 1 h at room temperature. The hydrolyzate obtained was neutralized with the addition of saturated sodium bicarbonate (NaHCO3), then the hydrolyzate was partitioned with 50 mL of petroleum ether solvent, three times. Partition extract was concentrated with a rotary evaporator. One milligram of petroleum ether fraction from *Euchema cottonii* was put in a test tube, dissolved with 0.5 mL of chloroform and added with 0.5 mL of anhydrous acetic acid. Next, the mixture was added with 1-2 mL H2SO4 concentrated through the tube wall. If the solution turned into bluish green indicating the presence of steroid compounds.

Isolation Steroids Compound with Column Chromatography

The steroids isolates were separated using by column chromatography. The stationary was silica gel 60 which was activated by heating the oven for 2 h at 1100C, then cooled in a desiccator for 15 min. before use. The column was wet slurry prepared and left for 24 h. 0.067 g of the extract was dissolved in 1 mL eluent and put into column chromatography using a pipette and waited until the sample dropped. Furthermore, eluent n-hexane: ethyl acetate (18:2) was kept at a flow rate of 2 mL/min and the eluate was accommodated every 2 mL in a vial bottle until approximately 250 vials obtained. The elution process is carried out by keeping the silica gel in the eluent submerged.

Monitoring with Analytical Thin Layer Chromatography

After obtaining several fractions from the column chromatography, first identification or monitoring was carried out by taking each of the 5 vials, vial no. 5, 10, 15, 20, 25 and the rest. The eluent used was a mixture of n-hexane: ethyl acetate 17: 3 and stationary phase silica gel F254. Then the stain was identified under UV 366 nm. Then the spot with green colour was identified and circled and the value of the Rf is calculated. Fractions that have the same or similar stains are made into one large fraction. The fraction group from the first monitoring was monitored by taking each 2 vials.

Antioxidant Activity Test Using DPPH

Samples were dissolved in ethanol with vary concentration (1, 2, 3, 4 and 5 ppm). Each concentration was put in a test tube and filled with 3 mL of isolates and DPPH 1 mL. The treatment was repeated 3 times, incubated at 37 ^{o}C

for 30 mins. The absorbance of each concentration is measured. The absorbance data for each concentration of each isolate calculated the% value of its antioxidant activity. This value is obtained by using equation 1:

$$\% \text{ Antioxidant Activity} = \frac{\text{A o}-\text{A1}}{\text{A o}} x100\% \quad (1)$$

After obtaining % antioxidant activity, the EC_{50} value was calculated by obtaining a nonlinear regression equation using the program GraphPad prism7 software, regression for analyzing dose-response data.

Toxicity Test by BSLT Method

250 mL of seawater was put in a hatching bottle, inserted 2.5 mg of egg *Artemia salina* Leach. Then aerated and the eggs hatch within ± 48 h and ready to be used as targets for toxicity tests. The treatment of tests was carried out in 5 replications for each sample of steroid isolates. The bottle prepared for testing, 5 bottles and 1 bottle as a control. The concentration is made 5 ppm, 4 ppm, 3 ppm, 2 ppm, 1 ppm, and 0 ppm as a control. The test solution is then inserted into the vial and evaporated by the solvent. After the solvent evaporates, it is added with 100 µL dimethyl sulfoxide (DMSO), a drop of bread yeast solution and sea water until the volume is 10 mL. The test solution was added with 10 shrimp larvae *Artemia salina* L. Control was made by adding 100 mL dimethyl sulfoxide, a drop of bread yeast solution, 2 mL of seawater into a beaker glass, then shaker until the extract dissolved in seawater. The solution is transferred in a 10 mL volumetric flask, then 10 *Artemia salina* shrimp larvae are added and seawater is added until the volume becomes 10 mL. Observations were carried out for 24 h on the death of shrimp larvae. Data analysis was performed to find the value of LC50 with probit analysis to show the LC50 value by calculating the value % mortality of shrimp larvae [5-6].

Identification Steroids Compound

The results of isolation of steroid compounds using column chromatography were identified using UV-Vis spectrophotometer at 200-800 nm, and the spectrum and λ max obtained. Then it was identified using FTIR. Steroid isolates with the best antioxidant activity were analyzed using LC-MS/MS. Steroid compounds were dissolved by 0.1% eluent formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). The mass analysis used is MS / MS Triple Q (Quadrupole) mass spectrometer TSQ QUANTUM ACCESS MAX from Thermo Finnigan with the SRM (Selected Reaction Monitoring) method and the source of APCI (Atmospheric Pressure Chemical Ionization) ionization controlled by TSQ Tune software which is operated in positive mode.

RESULTS AND DISCUSSION

Sample Preparation

Eucheuma cottoniii extraction was carried out by maceration using methanol after repeated up to 3 times or until the color of the filtrate turned clearer and the color of the sample had turned pale. It can be assumed that the active compound has been extracted from the sample *Eucheuma cottonii*. The obtained filtrate was combined and the solvent evaporated so the yield obtained was 15.59 %. Acid hydrolysis was done to break the glycoside bond between glycone (sugar) and aglycone (not sugar). The hydrolysis reaction was reversible, so it had to be neutralized to stop the reaction. The hydrolyzate obtained is then partitioned (liquid-liquid extraction) using petroleum ether. The partitioning process produced two liquid phases where there are water phases (polar compounds) found in the lower layer and organic phase (nonpolar compounds) in the upper layer. The extraction process was carried out three times to take the organic phase where the steroid compounds contained. The colour of the organic phase turned the blackish green colour into clear. From the results of petroleum ether partitioned yield was 9.25 %. The phytochemical test was conducted to make sure the steroid contained and showed a positive result (green colour solution).

Isolation of Steroid Compound with Column Chromatography and Monitoring with Analytical Thin Layer Chromatography

The collected vial from column chromatography was monitored using Analytical TLC. The monitoring results were shown in Table 1. The presence of steroid was detected using Liebermann-Burchard reagent and the colour spots

changed into blue or green. It can be seen that there were blue and green spots that indicated those compounds were steroids. There were five single stain isolates suspected of being steroid compounds, in isolates A1, A2, A3, A4 andA5. The single stain isolate was then determined the toxicity test and antioxidant activity.

TABLE 1. The results of steroid separation using column chromatography

Vial	Spots	Rf	Colour	Weight (mg)
	-	-	-	0
14-16	**1**	**0,7000**	**Green**	**0,9 (A1)**
23-38	1	0,6250	Bluish Green	-
39-49	-	-	-	3,4
50-52	**1**	**0,5500**	**Green**	**0,7 (A2)**
53-57	-	-	-	0,4
58-65	1	0,4500	Red	1,3
66-76	1	0,4870	Red	5,4
78-79	2	0,4125	Green	0,8
		0,3370	Blue	
80-96	**1**	**0,3625**	**Green**	**6,2 (A3)**
97-99	-	-	-	1,1
100	**1**	**0,2870**	**Blue**	**0,5 (A4)**
101-120	2	0,2750	Blue	9,3
		0,1870	Red	
122-128	1	0,1750	Red	3
132-210	1	0,1250	Red	18,7
212-224	**1**	**0,0625**	**Green**	**1,5 (A5)**
225-258	2	0,1250	Red	5,7
		0,3000	Blue	
260-264	1	0, 2875	Blue	-
265-270	2	0,1250	Red	0,9
		0,3000	Blue	

Toxicity Test and Antioxidant Test of Steroid Isolates

Toxicity test was done on five isolates of steroids from secondary isolation by column chromatography (A1, A2, A3, A4 and A5). A compound could be categorized as toxic if the LC50 value when it values less than 1000 ppm. Based on the toxicity value (Table 2) against *Artemia salina* L. each shrimp larvae, each isolate had very toxic properties because the LC50 value was less than 30 ppm. A compound was said to be very toxic if the value of LC_{50} <30 ppm [12]. Secondary metabolite compounds were toxic to the body of *Artemia salina*. These secondary metabolites will be toxic by inhibiting the enzyme RNA polymerase and Na + / K + ATPase contained in the body of *Artemia salina*. As a result, the RNA polymerase enzyme cannot work in the separation of DNA strands so that protein synthesis cannot be formed and Na + / K + ATPase cannot work to transport ions so that integral membrane proteins bulge and break. As a result of the inhibition process, it can cause damage to the body of *Artemia salina*, causing *Artemia salina* to die [13]. Antioxidant activity or the EC50 value of steroid isolates were shown in Table 3. Based on Table 3 shows that the steroid fraction of petroleum ether macroalgae fraction *Eucheuma cottonii* in isolates A1 and A5 has the ability of antioxidant activity to inhibit free radicals.

Table 2. LC_{50} of steroid isolates

No	Sample	LC_{50} (ppm)
1	Isolate A1	26.70
2	Isolate A2	-
3	Isolate A3	16.30
4	Isolate A4	-
5	Isolate A5	22.03

Table 3. EC_{50} of steroid isolates

No	Sample	EC_{50} (ppm)
1	Isolate A1	13.07
2	Isolate A2	-
3	Isolate A3	-
4	Isolate A4	$3.9\ 10^{10}$
5	Isolate A5	50.92

Identification of Steroids using UV-Vis Spectrophotometry

Isolate A5 had the highest absorption at a λ maximum at 203.0 nm indicating a transition π-π* which indicates the presence of a conjugated C=C double bond. The resulting spectra pattern was similar to that of Etika and Suryelita [14], which identified UV-Vis steroid compounds from isolation from *Morinda citrifolia* L. and obtained absorption at a wavelength of 203 nm and was believed as stigmasterol then comprehended with other identification such as FTIR, 13C-NMR and 1H-NMR. In addition, Sari [15] has also isolated steroid compounds from the red algae *Eucheuma cottonii* and produced a wavelength of 203.9 nm from the identification using UV-Vis, and LC-MS / MS proved that the most dominant type of steroid β-sitosterol and alongside with other compounds such as campesterol, stigmasterol, and cholesterol.

Identification Steroids using FT-IR Spectrophotometry

The presence of functional group alcohol (OH) at a wave number between 3600-3450 cm-1. There is also absorption at wavenumbers between 2970-2850 cm-1, which indicates the presence of Csp3-H groups. Besides that, in the three isolates, the absorption appeared at wave number 1732-1728 cm-1, which showed the presence of a group C=O. There is also a buckling C-H vibration which indicates the presence of dimethyl geminal groups. The buckling of C-H vibration is in the absorption band 1460-1380 cm-1, which is a typical uptake of steroid and the uptake at wave numbers 1160-1100 cm-1 which indicated absorption from the functional group C-O secondary alcohol. 1645-1635 cm-1 appeared in isolates B and E, which are absorption from the C=C nonconjugated groups and wave numbers 1080-1020 cm-1 appear in isolates E and F, which are the primary C-O alcohol functional groups. This absorption was supported by the results of the Mamahit [16] study which isolated steroid compounds from Gedi leaves and identified using FTIR showed Csp3-H 2920 and 2850 cm-1 groups, CH bending (methylene) 1462 cm-1 and CH bending (methyl) 1375 cm-1 which is a gem-dimethyl group and supported by identification with 13C-NMR and 1H-NMR stated that the steroid compound that was isolated was β-sytosterol.

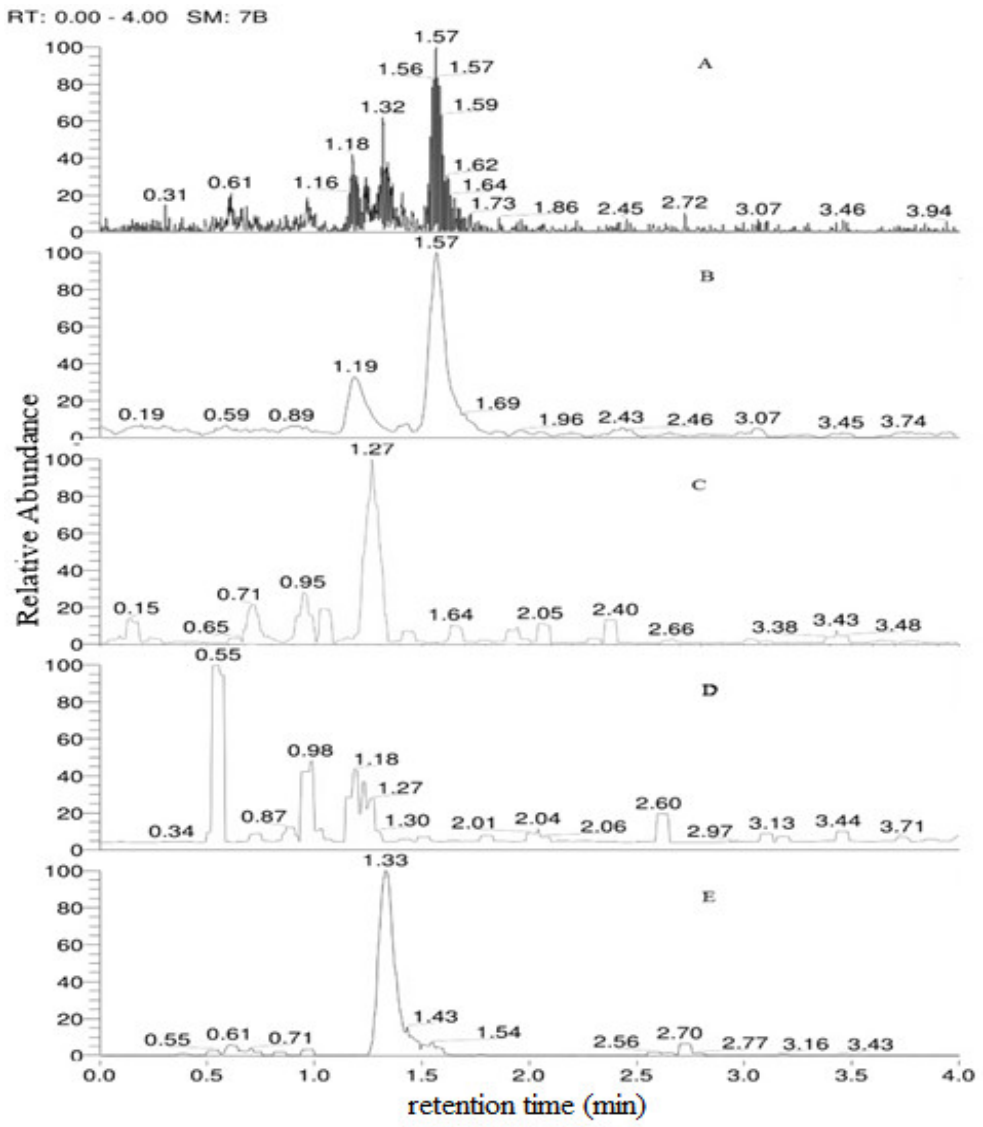

FIGURE 1. Chromatogram of steroid isolates of petroleum ether fractions

Identification Steroid Compound using LC-MS/MS

Identification using LC-MS/MS aimed to determine the type of steroid macroalgae *Eucheuma cottonii* Petroleum ether fraction. The targeted types of steroid compounds, the results of the identification of the four targeted compounds: cholesterol, campesterol, β-sitosterol and stigmasterol. The chromatogram could be seen in Fig. 1. Samples were analyzed using the MS/MS method which only detects targeted compounds. In the ionization process, the ions detected in MS are positive in the form of precursor ions or called the parent mass and in the second MS will produce the production form as a mass daughter (Table 4).

Table 4. Results of identification of LC-MS/MS

Steroids type	Time retention (min)	Mr (g/mol)	Parent mass (m/z)	Daughter mass (m/z)
Cholesterol	1.19	386	369	94,5-95,5
Campesterol	1.27	400	383	160,5-161,5
Stigmasterol	1.27	412	395	80,5-81,5
β-sitosterol	1.93	414	397	160,5-161,5

SUMMARY

This research was conducted to isolate, identify and determine the bioactivity (toxicity and antioxidant activity) of steroids compounds in Red Algae *Eucheuma cottonii.* Isolation of steroid in *Eucheuma cottoni* red algae using column chromatography resulted in 5 isolates from petroleum ether fraction (A1, A2, A3, A4 and A5). Based on phytochemicals test and LC-APCI-MS/MS identification showed that PE fraction of *Eucheuma cottonii.* Contained steroids compound (cholesterol, campesterol, stigmasterol, and β-sitosterol). Steroids isolates from Column Chromatography separation had toxicity and antioxidant activity. LC50 value of steroid isolates were26.90 ppm (A1); 16.297 (A3) and 5.146 ppm (A5) and Antioxidant activity of steroid isolate were 13.07 ppm (A1); 39.1010 (A4) and 50.92 ppm (A5).

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