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Anti coagulant activities of Brown Seaweed *Sargassum cristaefolium* Extract

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Abstract. Brown algae, *Sargassum cristaefolium* is grown abundant in Indonesia especially in South Sulawesi Province. In vitro anticoagulant effect of the extract has been shown. Therefore a study of *Sargassum cristaefolium*, used to inhibit blood coagulation in vivo was done. In this study, effect of flavonoid compounds from *Sargassum cristaefolium* to elongate bleeding and blood clotting time and also (Prothrombin Time (PT) and Activated Thromboplastin Time (APTT) tests were carried out. Moreover, histology of mice stomach was also studied. From this study we found, that effect on bleeding time after 14 days treatment of *Sargassum* extract in vivo prolonged the clotting and bleeding time significantly ($p < 0.05$). Prothrombin time (PT) and activated partial thromboplastin time (APTT) tests on plasma confirm these research. From this study we conclude, that the flavonoid compound in *Sargassum cristaefolium* exhibited an anticoagulant activity.

1. Introduction

The mortality rate of cardiovascular disease is still the highest with the number of deaths as much as 30% of all deaths in the world. In 2012 an estimated 17.5 million deaths were caused by cardiovascular disease or three times more than deaths due to HIV/AIDS, tuberculosis and malaria when combined together. Anticoagulant drugs are the standard treatment for thromboembolic disease. Anticoagulant compounds are widely used as a therapy for treating cardiovascular disease, and heparin is the first choice and has been used for more than 60 years (Aldredge et al, 2013). High risk of clotting (thrombosis) or [bleeding (hemorrhage) can be caused by disorders in blood coagulation (Guerra-Rivas, 2011, Nurdin, 2018) Anti-coagulants are substances that prevent blood coagulation by inhibiting blood from clotting (Desai, 2004). De Zoysa *et al* reported the isolation and characterization of fucose containing sulfated polysaccharide as an anti-coagulant agent from *Sargassum fulvellum*. Hot water extracts from *Sargassum horneri* showed potent anticoagulant activity because of its high effect on activated partial thromboplastin time (APTT) (Athukorala et al, 2007).

One abundant source for obtaining new anticoagulants is the polysaccharide from marine algae. Marine algae are the main source of obtaining sulfate polysaccharides (SPs) from non-mammal groups. SPs (for example fucoidan, laminaran, carrageenan, ulvan) and fluorotanine from marine algae show potent anticoagulant activity based on several studies (Jing Li, 1993; Subhash et al, 2014). In the last few decades, there is a tremendous growth in the area of herbal medicine. It is popular in developing as well as in the developed countries due to its natural origin treatment of internal diseases which are considered to be stubborn and incurable by other system of medicines. This study is to prove effect of *Sargassum cristaefolium* extract as anticoagulant by measuring blood clotting and bleeding time and also APTT and PT.



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2. Material and Methods

2.1. Drugs and Chemicals

All reagents used were analytical grade.

2.2. Seaweed Collection

Sargassum cristaefolium was collected from Jeneponto region and authenticated in Marine Biology Laboratory. Sample was washed with sea water to remove impurities, as well as to avoid evaporation from the sample (Meenakshi et. al., 2009). The sample was then rinsed twice using running water to remove the remaining salt and impurity and the next step the rinsing was done using distilled water to ensure no impurity of the sample (Hidayat, 2018). The seaweed was dried and then crushed into fine powder by using homogenizer. Then samples were sieved using mesh number sieves 18 to obtain coarse powder.

2.3. Extraction

To extract flavonoid compound, sample was extracted by infuse method, namely 100 grams of sample was added 1000 ml aquadest then heated to a temperature of 90 ° C. After the temperature was reached the sample was heated for 15 minutes. Water extract was then lyophilized using freeze dryer for 2 x 24 h (Hidayat, 2018).

2.4. Analisis of Total Flavonoid Content

Ten milli grams of sample was dissolved in 100 µl of AlCl₃ 2% and then sufficed withethanol p.a after that sample was incubated for 30 minutes and was analysed using spectrophotometer Uv-Vis (Corpuz et.al. 2013).

2.5. Animal Preparation

Wistar rats (*Rattus norvegicus*) were purchased in Sleman Jogjakarta, They were devided into 4 groups, namely group of negative control received NaCMC as vehicle, Group I received extract concentration of 200 mg/kg, Group II of 400 mg/kg, Group III of 600 mg/kg. The rats received extract for 14 days and observation was done at day 0, 7 and 14. This research has received an ethical agreement from the ethics commission of the Faculty of Medicine, Hassanuddin University.

2.6 Blood Clotting Time

Blood clotting time was done by take rat blood through the tail by cutting the tail by 2 mm, then put 2-3 drops of blood above the glass object and observe the formation of fibrin threads from the blood specimen using lancet (Kartiningasih et al., 2018).

2.7 Bleeding Time

The bleeding time test was carried out by taking rat blood through the tail by cutting the tail by 2 mm, then observed bleeding that occurred every 30 seconds using tissue paper (Kartiningasih et al., 2018).

2.8 Prothrombin Time (PT)

PT testing can be done by adding 2.7 ml of wistar rat blood into a blue tube containing sodium citrate, then centrifuged at a speed of 3500 rpm for 15 minutes. Entering the still board into the cuvette, adding a plasma sample of 50 µl into the cuvette. The sample was incubated for 1 minute in the stago diagnostic tool, then added 100 µl of neoplastin reagent and mixed into the sample, then the sample was moved to the place of analysis of dialectic diagnostic (Vogel, 2002).

2.9 Activated Partial Thromboplastin Time (APTT)

APTT testing can be done by adding 2.7 ml of wistar rat blood into a blue tube containing sodium citrate, then centrifuged at a speed of 3500 rpm for 15 minutes. Inserting the still board into the cuvette added 50 µl of plasma samples into the cuvette. The sample was incubated for 1 minute in the stago diagnostic tool, added 50 µl of cephalin kaolin Prest reagent and mixed into the sample and then incubated for 110 seconds, added CaCl₂ reagent, then the sample was transferred to the analysis site in the stago diagnostic tool (Vogel, 2002).

3. Data Analysis

The result was analysed using One Way Anova (p <0,05). To define the significancy, every sample group was analysed using post hoc methode, namely Least Significantly Difference (LSD) (p<0,05).

4. Results and Discussion

4.1 Analysis of total flavonoid content

Analysis using spectrophotometer uv-vis to determine the total flavonoid content in Liophilisate of *Sargassum* Sp. according to Corpuz (2013) using quersetin as control and methanol and also $AlCl_3$ 2% as eluent showed that total flavonoid content in brown seaweed was 1.65 mg/g sample. The results of qualitative analysis using silica gel plates GF 254 and as solvent we used toluene: ether: acetic acid (10: 10: 2), obtained spots with a value of Rf 7.8 on the plate with sitroborat spray reaction, which glowed yellow at UV 254 nm and 366 nm. This is in compare with the total flavonoid levels in *Sargassum* sp. from Takalar Regency are $1.428\% \pm SD$ 0.168% (Ruslin, M et al, 2018).

4.2 Blood Clotting Time

From the study of the activity of the extract on the platelet aggregation by measuring the blood clotting time on extract given to male rat within 14 days, can be shown in Table 1 and 2. Data analysis using Tuckey showed significant difference between Na CMC treatment as negative control, and brown seaweed extract with concentration of seaweed. We can show that treatment of wistar rat with 200 mg/kg extract has significant effect against NaCMC as negative control. From statistical analysis using repeat measured Anova with p-value $0.000 < \alpha$ (0.05) on bleeding and clotting time, it can be concluded that sample extract is significant against negative control. By using post hoc p value was < 0.05 .

4.3 Bleeding Time

From the study of the activity of the extract on the platelet aggregation by measuring the bleeding time.

4.4 Activated Partial Thromboplastin Time (APTT) and Prothrombine Time (PT)

Study of Activated Partial Thromboplastine Time (APTT) and Prothrombine time (PT) was carried out using the instrumental stag diagnostic. PT is used to determine inhibition of extrinsic factors (Factor VII) and other pathway factors (Factors X, V, II, fibrinogen). APTT is used to determine inhibition of intrinsic factors (Factors XI, XII, IX, VIII) and other pathway factors (Factors X, V, II, fibrinogen). Activated partial thromboplastin time (aPTT) and prothrombin time (PT) are 2 major methods of screening patients for bleeding tendency. Heparin is an anticoagulant commonly used for various clinical conditions and will thus affect the coagulation profile.

APTT reagents contain activators (silica, elagic acid or kaolin) and phospholipids but do not contain calcium chloride (Selby et al., 2013). Most probably the active compound, or compounds of the algal species were related to high molecular weight polysaccharide, or a complex form with carbohydrate and protein (proteoglycan) (Athukorala, 2007). More over another research showed that chemical analyses from seaweed *Dictyopteris delicatula* showed that all polysaccharides contain heterofucans composed mainly of fucose, xylose, glucose, galactose, uronic acid, and sulfate. The 2,4-O-sulfated fucose branch is the key structural factor of fCSs for prolonged APTT/TT and thrombin inhibition, whereas the inhibitory effect of fCSs on factor X, XII stimulation and thrombus production was assigned to the overall structure of fCS polysaccharide.

In this study it can be shown, that extract of *Sargassum cristaefolium* with concentration of 200 mg/kg was very potent to prolong the bleeding and blood clotting time in compared to concentration of 400 and 600 mg/kg, so it was continued to study its effect on APTT and PT, as can be seen in Table 3:

In prothrombin time experiments, the inhibitions of prothrombin were identified in the *Ecklonia cava*, *E. stolonifera*, *Eisenia bicyclis* (Kjellman) Setchell, *Ishige foliacea*, *I. okamurai*, *Sargassum confusum* and *Ishige foliacea*. In a PTT experiments, the seaweeds with blood coagulation inhibition factors were *E. cava*, *E. stolonifera*, *E. bicyclis* (Kjellman) Setchell, *I. foliacea*, *I. okamurai*, *S. confusum* and *Hixikia fusiforme Okamura*. These anticoagulant effect from seaweed were from flavonoid substance.

Table 1. Blood Clotting based on time treatment

Treatment	Day of treatment	Average (second)	Accuracy value of Analysis	
			Min.Value(sec.)	Max. Value (sec.)
Natrium CMC	Day 0	56.2 ± 5.12	47.12	65.28
	Day 1	47.8 ± 14.82	38.72	56.88
	Day 7	49.6 ± 3.20	40.52	58.68
	Day 14	55.4 ± 6.02	46.32	64.48
200 mg/kg	Day 0	38.2 ± 4.60	29.12	47.28
	Day 1	67.4 ± 15.18	58.32	76.48
	Day 7	62.0 ± 9.82	52.92	71.08
	Day 14	90.6 ± 8.35	81.52	99.68
400 mg/kg	Day 0	48.4 ± 10.14	39.32	57.48
	Day 1	66.2 ± 14.11	57.12	75.28
	Day 7	64.4 ± 8.73	55.32	73.48
	Day 14	60.8 ± 11.77	51.72	69.88
600mg/Kg	Day 0	46.2 ± 8.98	37.12	55.28
	Day 1	52.2 ± 14.6	43.12	61.28
	Day 7	53.4 ± 3.78	44.32	62.48
	Day 14	67.0 ± 5.83	57.92	76.08

Table 2. Effect of extract on bleeding time

Treatment	Day of Treatment	Average	Accuracy value of Analysis	
			Min. Value (sec)	Max. Value (sec)
Natrium CMC	Day 0	82.60 ± 9.40	62.44	102.76
	Day 1	83.0 ± 10.61	62.84	103.16
	Day 7	67.0 ± 1.41	46.84	87.16
	Day 14	76.40 ± 1.52	56.24	96.56
200 mg/kg	Day 0	92.20 ± 20.77	72.04	112.36
	Day 1	121.8 ± 24.64	101.64	141.96
	Day 7	149.6 ± 13,58	129.44	169.76
	Day 14	281.2 ± 6.69	261.04	301.36
400 mg/kg	Day 0	75.40 ± 9.94	55.24	95.56
	Day 1	160.6 ± 66.37	140.44	180.76
	Day 7	140.8 ± 10.47	120.64	160.96
	Day 14	291.2 ± 4.49	271.04	311.36
600 mg/kg	Day 0	81.60 ± 28.50	61.44	101.76
	Day 1	119.6 ± 33.87	99.44	139.76
	Day 7	79.2 ± 9.86	59.04	99.36
	Day 14	155.6 ± 3.36	135.44	175.76

Table 3. Study of APTT dan PT

Treatment	APTT	PT
NaCMC	9.73 ± 0.29	18.9 ± 0.32
Extract 200 mg/kg	11.33 ± 0.31	22.8 ± 0.45

5. Conclusion

We can conclude some points from the results, as follows:

1. Flavonoid content can be identified in the water extract of *Sargassum cristaefolium* treatment with concentration of 1.65 mg/g of extract.
2. Measuring of bleeding and clotting time showed that doses of 200 mg/kg has the same effect with aspirin of 325 mg/kg
3. Extract with dosage of 200 mg/kg is very potent to prolong the bleeding and blood clotting time in compared to concentration of 400 and 600 mg/kg.

Acknowledgments

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