

In Vitro Citotoxicity Assays of Seagrass (*Enhalus acoroides*) Methanol Extract from Soropia Coastal Waters in Southeast Sulawesi Province

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Received: February 11, 2020

Revised: March 13, 2020

Accepted: April 13, 2020



Abstract

The studies analysing the use of natural ingredients as an alternative treatment in the field of pharmacology are developing very rapidly. One of researches that is quite promising in the pharmaceutical industry is the application of marine materials. Marine materials that are frequently used consist of shellfish, algae, sponges and seagrass. Several studies on toxicity tests have shown that the methanol extract of seagrass (from species *Enhalus acoroides*) is more toxic than the other seagrass family. This study aims to test the toxicity level of Seagrass (*E. acoroides*) extract from Soropia coastal waters. The research method in this study was an experimental laboratory using *E. acoroides* Seagrass as a sample that was obtained from Soropia Coast, Konawe Regency, Southeast Sulawesi Province. The sample was extracted using methanol as a solvent by macerating it and was tested for its toxicity using the Brine Shrimp Lethality Test (BSLT) method. Toxicity test results showed that the samples with a concentration of 10 ppm, 100 ppm and 1000 ppm in leaves extracts produced an LC₅₀ value of 404.88 ppm, while the stem and root extracts has a value of LC₅₀ > 1000 ppm. The test was continued with higher concentration of leaves extracts consisted of 250 ppm, 500 ppm, and 1000 ppm. The toxicity test showed an LC₅₀ value of 0.7309; which means that it was very toxic. The methanol extract of Seagrass (*E. Acoroides*) is potential to be used for further analysis and anticancer formulations.

Keywords

Cytotoxicity, *Enhalus acoroides*, soropia coastal waters, southeast regency

INTRODUCTION

The research and development on new compounds with unique structures and interesting pharmacological activities have been isolated from marine biota. In general,

these compounds are beneficial for humans as it contains high-value bioactive compounds that can be used in both the research and industrial setting. A very complex and dynamic interaction of marine

ecosystems trigger marine biota to produce secondary metabolites that has a function to defend their lives against competitors, predators and parasite.

Since the last ten years, the study of marine natural products has began to be carried out in Indonesia (1). Corals, sponges, algae and seagrasses are marine organisms that are often used as research materials to find new raw material for medicines. However, among these organisms, seagrass is still relatively new in the development of raw materials of new drugs. Seagrass is a group of closed seeded plants (angiosperms) and single piece plants (monocots) that are able to live permanently below the sea level. Because seagrasses live permanently below the sea level, seagrass is classified as benthic organisms. These organisms produce secondary metabolites to maintain their survival from external disturbances both physicochemically and biologically (2).

Several studies have shown that seagrass contains compounds that are potential as an anticancer (3), antioxidants (4), and antibacterial agents (5). The research on the potential of some seagrasses as anticancer have been carried out in several palces in Indonesia including the Pramuka Island waters, Lampung Waters, Morotai and Spermonde waters. The results declared that it has acute cytotoxic potential which can be applied as a new raw material for anticancer drug (6). The toxicity tests intend either to

evaluate the safety of a compound or to detect the anticancer activity of a compound.

In the Soropia Waters of Konawe Regency, Southeast Sulawesi Province, the data on the distribution of seagrass has been extensively studied, but the research on the level of toxicity of active compounds contained in seagrass is still lacking. The Soropia sub-district is a coastal area where natural products in the form of marine materials can be studied and utilized for various sectors including the health sector. The reason of choosing *Enhalus acoroides* as a research sample is based on Aprilyani et al's (15) research which analysed the seagrass distribution in Southeast Sulawesi waters, where *E. acoroides* ranks first in the seagrass distribution in all stations (7).

This study tests the cytotoxicity of seagrass in Soropia waters by using the Brine Shrimp Lethality Test (BSLT). This test is usually used in screening natural marine bioactive compounds because it shows a correlation within in vitro cytotoxic methods and a specific anticancer test (8). Some methods can also be employed in this test, such as Lemna Assay, Potato disc as well as sell culture methos (Microculture Tetrazolium Salt/MTT). Among the four methods, BSLT is highly recommended by Anderson in the toxicity test because it has a correlation of up to 95% confidence level in specific anticancer tests. Although MTT also gets the same results as BSLT, BSLT is

easier, faster, cheaper and practical method (9). This study used methanol as an extraction solvent based on the results of previous studies by Aulia Fajarullah on the Extraction of Secondary Metabolites. Seagrass extraction process uses several types of solvent, where methanol solvent yields the highest extract (10.09%) and secondary metabolite compounds namely tannins, saponins, triterpenoids and steroids (10).

MATERIALS AND METHODS

This research was conducted at the Biochemistry Laboratory of Hasanuddin University for approximately one month. This research was an analytical study with a laboratory observation approach.

Chemical and Reagents

Methanol, *E. acoroides*, *Artemia salina* larvae were purchased in Laboratory of Poltekkes Kemenkes Kendari, Indonesia. Fresh leaves, root and rhizome of *E. acoroides* were used for experiments. It was collected from Soropia District, South Sulawesi Province, Indonesia.

Sample Preparations

The wet weight of seagrass samples was collected and was immediately placed in plastic bags containing sea water in order to prevent evaporation. Then, it was transported to the laboratory under cool condition. The seagrass samples of *E. acoroides* was washed thoroughly in deionized water to removed the biota that attached to it. In addition, the

samples were separated into the roots, stems and leaves and each parts was arranged in aluminum trays. The *E. acoroides* samples were dried using oven dryer with a temperature of 50°C for 4 days. Drying process was conducted until the samples reached a moisture content below 10%. Furthermore, the samples were removed from the oven and were stored in a jar then was put in a dry place (Total Solid). The dried samples were milled by using grinder machines and the powder was then sieved manually by using mesh filter with size 30 mm. The fresh samples used in this experiment were 2 kg and it yielded 800 g powder after the drying and grinding process. Weight shrinkage in the seagrass samples were 60%. The powdered samples were stored in a refrigerator for further use.

E. acoroides Extract

Extraction were conducted by maceration method, namely by immersing the sample in a solvent within a few days. The maceration method was chosen for extraction because it is easy to do and only uses tools that are simple and easy to obtain (10,11). The dried seagrass sample was weighed as much as 50 g and was soaked in a 250 mL methanol solvent in a glass bottle and was macerated for 24 hours. The sample solutions were then filtered using filter paper through glass funnel and Whatman No. 42. The filtrate was concentrated to dryness by

rotation using evaporator under reduced pressure at temperature of 4°C. Finally, the crude extract from the seagrass sample was obtained in the form of a paste. Then, it was weighed to get the percentage of yield, and it was kept at -20°C prior to further analysis.

Preparation of The *Artemia salina* Larvae

A. salina eggs was prepared by soaking it in sea water for 10-15 minutes. Good eggs would be settle while bad eggs would be float. As much as 50 mg of *A. salina* eggs were hatching in a container filled with sea water for 10-15 minutes. Then, the eggs at the bottom of the container were taken and were hatched in a container that also contained sea water under a 25-watt lamp and was equipped with an aerator. *A. salina* eggs hatched and became larvae after 24 hours. *A. salina* larvae that were good for the BSLT test were those that have a lifetime of 48 hours. If it died more than 48 hours, it was feared that *A. salina*'s death was not caused by extract toxicity but rather by limited food supply (12).

Toxicity Analysis

The toxicity of *E. acoroides* crude extract was analyzed by using the Brine shrimp Letality Test (BSLT). This test were applied on *A. salina* L. This test aims to determine the level of toxicity of a natural material (13). BSLT test was carried out by describing a total of 10-15 shrimp larvae in each test bottle

which was then added with a crude extract of seagrass from each tests. Test solutions with concentration of 250 ppm, 500 ppm and 1000 ppm were inserted into the bottle. Incubation was carried out for 48 hours and it was aerated. This test was carried out 3 times in each concentration series. The observation was run for 24 hours. The number of shrimp larvae death to determine the Lethal Concentration 50 (LC₅₀) value was counted. LC₅₀ is an assessment of the level of toxicity of a substance against 50% of larval deaths. Shrimp larvae defined have died while they were motionless for 10 seconds. The data were analyzed using Probit analysis to obtain the value LC₅₀ by Microsoft Excel (2009) for Windows. Analysis were conducted by comparing the LC₅₀, if the value of LC₅₀ was higher than 1000 ppm, it would be categorized as toxic (13).

RESULTS

Extract of *E. acoroides* were found to inhibit the growth of *A. salina* on BLST test (Table 1). Based on the results of the toxicity test of seagrass extract (*E. acoroides*) which was divided into leaf extract, stem extract and root extract, leaf extract gave an LC₅₀ value of 404.88 ppm (low toxic) while stem and root extract gave an LC₅₀ value of >1000 which means it's not toxic.

Table 1. Toxicity Test Results of Seagrass (*E. acoroides*) Extract with BSLT Method

Sample	Conc (µg/mL)	Log Conc	Replication 1 (larvae)		Replication 2 (larvae)		Replication 3 (larvae)		% Dead	% Corrected	Probit	LC ₅₀ (ppm)
			Dead	Life	Dead	Life	Dead	Life				
<i>E. acoroides</i> (leaf)	100	3	5	5	7	3	5	5	56.67	56.67	5.17	404.88
	10	2	4	6	4	6	4	6	40	40.00	4.75	
	1	1	2	8	2	8	2	8	20	20.00	4.16	
<i>E. acoroides</i> (stem)	100	3	2	8	2	8	3	7	23.33	23.33	4.27	>1000
	10	2	2	8	1	9	3	7	20	20.00	4.16	
	1	1	0	10	0	10	3	7	10	10.00	3.72	
<i>E. acoroides</i> (root)	100	3	2	8	2	8	4	6	26.67	26.67	4.38	>1000
	10	2	2	8	1	9	0	10	10	10.00	3.72	
	1	1	1	9	0	10	1	9	6.67	6.67	3.50	

Note: LC₅₀ <1 (very toxic), >1 and <100 (moderate toxic), >100 (low toxic) (16).

The toxicity test on leaf extracts with several levels of concentration including 250

ppm, 500 ppm and 1000 ppm represents in Table 2.

Table 2. Toxicity Test Results of Seagrass (*E. acoroides*) Extract with BSLT Method

No.	Concentration (µg/ mL)	Total Larvae Dead	Precentation of Larvae Dead (%)	LC ₅₀ (µg/ mL)
1	250	11	37	0,7309
2	500	21	70	
3	1000	20	100	

Note: LC₅₀ <1 (very toxic), >1 and <100 (moderate toxic), >100 (low toxic) (16).

DISCUSSION

In this study, researchers chose seagrass species *E. acoroides* as research samples. The selection of this sample type was guided by Aprilyani et al's (15) research on mapping of seagrass distribution in Southeast Sulawesi waters, where *E. acoroides* ranks first in seagrass distribution in all stations (7). *E. acoroides* seagrass was then extracted using methanol as a solvent. Among the various types of solvents used in screening the secondary metabolites of the seagrass *E. acoroides*, methanol has been shown to have very high rendering activity and has the highest secondary metabolite content of four compounds namely tannins, saponins, triterpenoids and steroids (10). Methanol is a

universal solvent that has polar (-OH) and nonpolar deciduous (-CH₃) groups so that it can attract polar and nonpolar compounds (14).

Bioactive compounds are usually having toxic at high doses. Therefore, in this study, the acute toxicity test used BSLT. The mechanism of death of *A. salina* was thought to be related to the function of compounds dissolved in seagrass extracts which can inhibit the feeding power of larvae (antifeedants/food dodgers). Therefore, toxicity analysis was performed to know the toxicity level of the *E. acoroides* extracts in order to determine their toxicity level. The way these compounds work is by acting as stomach poisoning (stomach poisoning).

Thus, if these compounds enter the larva body, the digestive apparatus will be disrupted. In addition, these compounds can inhibit the taste receptors in the mouth area of the larvae. This results in larvae failing to get a taste stimulus so they are unable to recognize their food until finally the larvae starve and die (13). The results of BSLT on leaf extract samples (*E. acoroides*) with dilution of 1 ppm, 10 ppm and 100 ppm gave LC_{50} values of 404.88 ppm with low toxicity interpretation, while stem extracts and root extracts gave LC_{50} values of >1000 ppm with non-toxic interpretation. Because only leaf extract that gave an LC_{50} value of 404.88 ppm, we conducted further tests on leaf extract with dilution variations of 250 ppm, 500 ppm and 1000 ppm. The calculation results show LC_{50} of 0.7309 ppm with a very toxic interpretation. Tannin is one of the secondary metabolites contained in the methanol extract of *E. acoroides* that can be used as an anticancer compound.

Anti-cancer tannin activity occurs through the mechanism of inhibiting the work of enzymes, prevention of mutagenesis of cells that can cause cancer, and activation of cancer macrophage cells. The mechanism of

action of this tannin uses histone deacetylase inhibitors (HDAC) (15). The role of Saponin as an anticancer has been known to inhibit the formation of Bcl-2 which is expressed too high, induce caspase-3 protein that is expressed too low, and can trigger G1 cell cycle arrest (16). Whereas, triterpenoids and steroids act as anti-cancer by activating apoptosis as well as making anti-proliferation (17).

CONCLUSIONS

The finding of this study revealed that *E. acoroides* methanol extract had bioactivity potentials. The extracts with a concentration of 250 ppm, 500 ppm and 1000 ppm has an acute toxic potential againsts *A. salina* larvae, which is indicated by LC_{50} values. LC values of *E. acoroides* methanol extract is 0.7309 ppm, thus proving the existence of BSLT method. It is necessary to do further study to identify and isolate the active compounds that serve as anti-cancer agents to maximize the benefits of *E. acoroides* to human medicine.

CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

1. Permana AH, Husni A, Budhiyanti SA. Antioxidant Activity and Toxicity of Seagrass *Cymodocea* Sp. Journal of Agricultural Technology. 2016 Apr;17(1):37-46.
2. Jaafarzadeh N, Hashempour Y, Angali KA. Acute Toxicity Test Using Cyanide on *Daphnia magna* by Flow-Through System. Journal of Water Chemistry and Technology. 2013;35(6):281-286.
3. Satria D, Silalahi J, Haro G, Ilyas G, Hsb PAZ. Antioxidant and Antiproliferative Activities of an Ethylacetate Fraction of *Picria Fel-Terrae* Lour. Herbs. Asian Pacific Journal of Cancer Prevention. 2017;18(2):399-403. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5454734/>

4. Ahmad A, Usman H, Natsir H, Karim A. Isolation and Characterization of Bioactive Protein from Green Algae *Halimeda macrobala* Acting as Antioxidant and Anticancer Agent. 2014;2(5):134-140.
5. Fajarullah A, Irawan H, Pratomo A. Ekstraksi Senyawa Metabolit Sekunder Lamun 4. *Thalassodendron Ciliatum* Pada Pelarut Berbeda. Repository UMRAH. 2014.
6. Puspitasari R. Evaluation of the Use of Seagrass Extract as Active Antifouling Material for Aquatic Producers. *Jurnal Segara*. 2016 Mar 5;12(1).
7. Amudha P, Jayalaksmi M, Pushpabharathi N, Vanitha V. Identification of Bioactive Components in *Enhalus acoroides* Seagrass Extract by Gas Chromatography–Mass Spectrometry. *Asian Journal of Pharmaceutical and Clinical Research*. 2018;11(10):131-137.
8. Widiastuti E, Arfiani R, Kairani IA, Christyanto Y, Ara NF, Maharani HW. Antioxidant Effect of *Clerodendrum* sp and *Acanthus illicifolius* Methanol Extraction on Blood Profile of Male Mice Induced by Benzo(α)pyrene. The 4th International Conference on Biological Science and Biotechnology. 2019 July:1-9
9. Permana AH, Husni A, Budhiyanti SA. Antioxidant Activity and Toxicity of Seagrass *Cymodocea* sp. Extracts. *Journal of Agricultural Technology*. 2016 Apr 23;17(1):37-46.
10. Kurnia Y, Widiastuti EL, Nurcahyani E. Effect of Antioxidant Taurine, Seagrass (*Enhalus acoroides* L.), and Red Algae (*Eucheuma cottonii* L.) on Oxidative Stress in Some Organ Homeostasis in Which Infected by Glifosat. *Proceedings of the National Seminar on Indonesian Medicinal Plants* 55th. 2019;1(1):296-303.
11. Baehaki A, Widiastuti I, Herpandi H. Antioxidant Activity of Extracts of *Halodule pinifolia* Seagrass from Solvents with Different Polarities. *Oriental Journal of Chemistry*. 2017;33(1):181-185.
12. Wardani G, Farida N, Andayani R, Kuntoro M, Sudjarwo SA. The Potency of Red Seaweed (*Eucheuma cottonii*) Extracts as Hepatoprotector on Lead Acetate-induced Hepatotoxicity in Mice. *Pharmacognosy Research*. 2017;9(3):282-286.
13. Rengasamy KRR, Arumugam R, Thangaradjou T, Anantharaan P. Phytochemical Constituents, Antioxidant Properties and P-Coumaric Acid Analysis in Some Seagrasses. *Food Research International*. 2013;54(1):1229-1236.
14. Wang XB, Sun ZH, Fan LX, Liu YY, Feng J, Ma GX, Chen DL. Two novel diterpenes from the stems and leaves of tropical seagrass *Enhalus acoroides* in the South China sea. *Natural product research*. 2019 Aug 19:1-9.
15. Aprilyani V, Hamdi A, Arami H. Types of Diversity and Seagrass Distribution Patterns in the Waters of Holimombo Village, Buton Regency. *Journal of Water Resource Management*. 2018;3(4):309-317.
16. Wagner JG. *Pharmacokinetics for The Pharmaceutical Scientist*. Technomic Pub., Lancaster-Basel. 1993.