Isolation and Identification of Bioactive Proteins from Microsymbiont Algae *Eucheuma cottonii* and Their Potential as An Anticancer

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Abstract. The isolation and identification of bioactive proteins from the microsymbiont algae *Eucheuma cottonii* from Laikang district of Takalar in South Sulawesi have been carried out. This study aimed to isolate and identify the bioactive proteins from microsymbiont algae *E. cottonii* and their potential as an anticancer. The experiment method includes the stages of extraction, fractionation, and dialysis. The obtained bioactive proteins were identified by determining protein contents using the Lowry method. Furthermore, the preliminary anticancer test was conducted by testing Brine Shrimp Lethality Test (BSLT) method against shrimp larvae *Artemia salina* Leach. and the antimitotic test was also conducted against the sea urchin *Tripneustes gratilla* Linn. zygote cells. The result showed that the protein fraction of 40-60% saturation was the most toxic with LC50 with a value of 91.83 μg/mL and IC50 with a value of 74.13 μg/mL. This result suggested that the bioactive proteins of microsymbiont algae *E. cottonii* could be used as a potential anticancer agent.

1. Introduction
Indonesia is an archipelago country of 17,508 islands with a long coastline of 81,000 km [1]. The cancer cells have an ability to transform, hence they can change their form, nature, and kinetics. The growth becomes autonomous, wild, out of control from normal growth coordination resulted in dangerous diseases [2].

Cancer is the second largest cause of death globally after cardiovascular disease [3]. Several attempts have been done to prevent and cure the disease. One of the attempts that are being intensively
carried out was searching for antitumor compounds from natural products. The used natural products in this study are being researched to find active compounds; which are capable to suppress the proliferation of tumor cells, cytotoxic, antimitotic or induce apoptosis in tumor cells, and it is certainly safe to consume for the body [2].

According to [4], the sea is inhabited by various types of pathogenic organisms. Therefore, the seaweeds try to develop a defense mechanism against pathogenic organisms. One of the common mechanisms is to produce the chemical compounds which are toxic to predators, such as antibacterial, antifungal and anticancer compounds. The active compounds are probably the host of organism products or the symbiont products or possibly both host and symbionts products.

The microsymbiont algae contain several bioactive compounds that are effective as anticancer agents, for example, the halmon compounds of *Portieria hornemanii*, the halogenated monoterpenoid aldehyde compounds from *Placanum corallorhiza*, the piperazine compound and the epoxide groups of *Compsopogon helwani* compound, and hexapeptide compounds from *Ceratodictyon spongiosum* [5,6,7].

This study was conducted to isolate and identify the bioactive proteins from microsymbiont algae *E. cottonii* of Laikang Bay Puntondo Takalar, South Sulawesi. The bioactive proteins from microsymbiont algae were obtained through a series of processes of extraction, fractionation, and purification. The toxicity value of the obtained protein fractions was tested by using Brine Shrimp Lethality Test (BSLT) method against larvae shrimp *A. salina* Leach. The result would be confirmed by conducting antimitotic analysis using sea urchin *T. gratilla* Linn. zygote cells.

2. Materials and methods

2.1 Materials

The material used in this study includes; the colony of microsymbiont algae *E. cottonii*, buffer A (0.1 M Tris (hydroxymethyl) aminomethane pH 8.3; 2 M NaCl; 0.01 M CaCl2; ß-mercaptoethanol 1%, Triton X-100 0.5%); buffer B (0.1 M Tris (hydroxymethyl) aminomethane pH 8.3; 0.2 M NaCl; 0.01 M CaCl2), buffer C (0.01 M Tris (hydroxymethyl) aminomethane pH 8.3; 0.2 M NaCl; 0.01 M CaCl2); aquadest, BSA (Bovine Serum Albumin), ammonium sulphate, Lowry A (Follin-ciocalteus (phosphotungstate-phosphomolybdate acid solution) with aquadest 1:1), Lowry B (2% Na2CO3; 0.1 N NaOH; CuSO4.5H2O 1%, sodium potassium tartrate 2%), sterile seawater, *A. salina* Leach. shrimp eggs, the egg and sperm cells of sea urchin *T. gratilla* Linn., 10% KCl, vincristine and protozoa-free seawater.

2.2 Instruments

The instruments used in this study included the analytical balance, centrifuge, water bath, stirrer magnetic fisher, cellophane bags, sterile gauze, knife, blender, micropipette (100-1000 L), Eppendorf tubes, lamps for lighting, container hatchery fry shrimp, aquarium, refrigerator, syringe, microscope, spectronic 20D+, spray bottle and glass tools commonly used in laboratories.

2.3 Methods

2.3.1 Sample Preparation

The bioactive proteins from microsymbiont algae were isolated by a procedure modified from previous methods [8]. The selected micro-symbiont algae were cultured in 1000 mL of Nutrient Broth medium at optimum condition. The cultures of micro-symbiont algae were harvested by centrifugation to separate the filtrate and cells from the NB medium. Furthermore, 100 gram of The cell pellet was crushed and added with 200 mL of buffer A. Furthermore, the cell fractions were frozen/thawed 2-3 times and followed by sonication process to assist in solving the cell. The results of the cell fractions were centrifuged again at 5000 rpm, 4 ºC for 30 minutes to obtain supernatant as the crude extract.
2.3.2 Fractionation
The crude extract was fractionated by the ammonium sulphate at the rate of 0-20%, 20-40%, 40-60%, and 60-80% saturation.

2.3.3 Dialysis
The obtained precipitates after fractionation of each saturation level of ammonium sulphate were dissolved in buffer B and subsequently dialyzed in buffer C. The protein fraction was put into a cellophane bag which has been ensured that there was no leakage or damage. The cellophane that had been filled with protein that fractions were put into a beaker glass containing the Buffer C solution and it was stirred with a magnetic stirrer. The dialysis was continued until the buffer solution became colorless.

2.3.4 Determination of Protein Contents
The protein contents determination of each fraction that used the Lowry method [9] with Bovine Serum Albumin (BSA) solution as a standard.

2.3.5 Toxicity Test Using Brine Shrimp Lethality Test (BSLT) Method
2.3.5.1 Preparation of shrimp larvae. The shrimp eggs were put into containers which contained sea water for hatching, then they aerated under 40-60 watt incandescent lamp/neon. The hatching temperature was maintained in range of 25-30 °C and lightened for 48 hours. After the eggs hatched, the shrimp larvae were taken to be tested.

2.3.5.2 Implementation test. The Brine Shrimp Lethality Test (BSLT) method was used as the toxicity test. The test compounds were made in the concentration of 1 μg/mL, 10 μg/mL, and 100 μg/mL and they were placed in 3 vials. The sterile seawater contains 10 shrimp larvae that was added to the final volume of 5 mL, and it was stored under illumination for 24 hours. The dead larvae were observed and counted. The mortality percentage was obtained from the formula as follow[10]:

\[
\text{% Mortality} = \frac{(\sum \text{Dead larvae sample} - \sum \text{Dead larvae control})}{\sum \text{Total larvae}} \times 100\%
\]  

The LC₅₀ value was determined by the probit-log concentration graph. The same treatment was done on the buffer B solvent as a negative control and vincristine as a positive control.

2.3.6 Antimitotic Test Against Sea Urchin T. gratilla Linn. Zygote Cells.
2.3.6.1 Preparation of T. gratilla Linn. egg and sperm cells. The induction of male and female Sea urchin T. gratilla Linn. that was performed by 5 mL of 10% KCl injection into the gonad section laterally. The milky sperm and golden yellow egg cells were respectively accommodated in a different beaker and stored in the refrigerator for 15 minutes. A total of 1 mL sperm and 5 mL egg cells were fertilized in a beaker that contained 50 mL of protozoa-free seawater for 10 minutes.

2.3.6.2 Implementation test. The fraction of algae E. cottonii that had high toxicity in BSLT test was pipetted with volume certainly into the tube by a micropipette Eppendorf for concentrations of 1, 10 and100 μg/mL. The seawater was added based on the calculation into the sample inside the Eppendoff tube to suffice the final volume to 1 mL. Then, 100 μg/mL zygote was added into the tube after 10 minutes of fertilization. Each test sample and control were repeated for 2 times, then stored at a temperature of 15-20°C with interspersed shaking every 10 minutes. The observations of divided cells were carried out after 2 hours of incubation by using a microscope. The undivided zygote cells were observed and counted. The inhibition percentage was obtained from the formula as follow[11]:

...
\[
\% \text{Inhibition} = \frac{\sum \text{Cell dont divide sample} - \sum \text{Cell dont divide control}}{\sum \text{total cell}} \times 100 \%
\] (2)

The IC\(_{50}\) values were determined by probit-log concentration graph. The same treatment was done on the buffer B solvent as a negative control and vincristine as a positive control.

3. Results and discussion

3.1 Proteins isolation from microsymbiont algae

The bioactive proteins isolation from microsymbiont algae \textit{E. cottonii} that were conducted at the low temperature of 4°C as well as buffer A solvent addition. The isolation of bioactive proteins included three main things: lysis, separation (extraction), and purification. The cell lysis could be carried out by physically and chemically methods. While in chemically way, 100 g of cell pellet from 1000 mL of the selected culture from microsymbiont algae were pulverized in a blender with 250 mL of buffer A solvent. The extraction or separation of proteins from other compounds that were performed by the solubility properties of proteins. Furthermore, the additions of ammonium sulphate to precipitate the proteins on their isoelectric points that able to cause the reducing of protein solubilities. The results of these observations, the proteins could be separated by a precipitate method. This deposition only drew water hence it does not damage the structure of the proteins and cause the chemical changes. The protein purification was done by dialysis using a cellophane membrane. According to [12], another advantage of ammonium sulphate \((\text{NH}_4)_2\text{SO}_4\) beside it able to precipitate the proteins as well as it could be removed from the proteins by dialysis stage. The principle of the dialysis process was based on the principle of osmosis.

3.2 Determination of protein contents

The results of protein concentration measurements showed that the protein concentration of the crude extract from microsymbiont algae \textit{E. cottonii} was 2.6120 \(\mu\text{g/mL}\) with a total protein of 653 mg from crude extract volume of 250 mL. The total of protein in the fractionation of various saturation levels of ammonium sulphate from microsymbiont algae \textit{E. cottonii} could be seen in Table 1.

| Protein Fraction | Volume of Each Fraction(mL) | Protein Concentration(\(\mu\text{g/mL}\)) | Total Protein (mg) |
|------------------|-------------------------------|------------------------------------------|-------------------|
| Crude extract    | 250                           | 2.6120                                   | 653               |
| 0 – 20%          | 6.6                           | 0.6461                                   | 4.2643            |
| 20 – 40%         | 5.3                           | 0.3506                                   | 1.8582            |
| 40 – 60%         | 5.8                           | 1.1548                                   | 6.6978            |
| 60 – 80%         | 9.3                           | 1.4023                                   | 13.0414           |

Table 1 showed that the concentrations of proteins were different in each fraction. This indicated that the precipitated proteins from each fraction that were different. The proteins were precipitated by difference solubilities in water. The higher solubility in water, the more ammonium sulphate were added. The highest concentration of protein in the microsymbiont algae \textit{E.cottonii} was found in fraction 60-80% saturation and it was 1.4023 \(\mu\text{g/mL}\). While the lowest protein concentration was found in the fraction 20-40% saturation and it was 0.3506 \(\mu\text{g/mL}\).

3.3 Toxicity assay against \textit{A. salina} Leach.

In this research, toxicity assay of microsymbiont algae \textit{E. cottonii} proteins was conducted on shrimp larvae \textit{A. salina} Leach. The mortality of shrimp larvae \textit{A. salina} Leach was observed after 24 hours. The buffer B solution as the solvent of the sample was used as a negative control for the comparison. This could be proof that the larvae mortality that was only caused by the sample. The toxicity effect of
each sample could be determined based on the value of mortality data calculation as LC50 by the probit-log concentration graph. According to [13] that the death of *A. salina* Leach. was used as the parameter to indicate the presence of active substances which were cytotoxic. The level of toxicity of a test compound could be determined from the LC50 value by the probit-log concentration graph. If the value of LC50 < 1000 μg/mL that it is toxic. Otherwise, if the value of LC50 > 1000 μg/mL that it is not toxic. The smaller value of LC50 was the more toxic on the test compound. The calculation results of the LC50 were shown in Table 2.

**Table 2.** The calculation results of LC50 of shrimp larvae (*A. salina* Leach.) which were died toward protein fractions from microsymbiont algae *E. cottonii* and vincristine.

| Protein Fraction | LC50 value (μg/mL) |
|------------------|-------------------|
| Crude Extract    | 144.54            |
| 0-20%            | 208.11            |
| 20-40%           | 225.74            |
| 40-60%           | 91.83             |
| 60-80%           | 132.25            |
| Vincristine      | 0.22              |

![Figure 1](image)

**Figure 1.** The graph of LC50 value of some protein fractions from microsymbiont algae *E. cottonii* and vincristine as the positive control.

Table 2 showed that the fractions of the microsymbiont algae *E. cottonii* were in the toxic interval. The protein fraction of 40-60% gave the most toxic response against *A. salina* Leach shrimp larvae with the LC50 value of 91.83 μg/mL. The vincristine was used as a positive control with the LC50 value of 0.22 mg/mL.
3.4 Antimitotic test against sea urchin T. gratilla Linn. zygote cells.

The fraction that gave the most toxic response was further tested to determine its cytotoxic effect. The antimitotic test against sea urchin T. gratilla Linn. zygote cells that used the procedure of previous studies [11]. The following picture was the observation of sea urchin T. gratilla Linn. zygote cells after two hours of incubation.

According to [14] that the zygote cells of sea urchin had selective sensitivity toward drugs and experience the division of stages as well as cancer cells so that were widely used in anticancer researches. For example, in a study about the effect of a compound to inhibit the rate of cell growth which is referred to as antimitotic or cytotoxic properties. The following was a table of the observation results of the sea urchin T. gratilla Linn. zygote cells of the division of the percentage of inhibition by 40-60% saturation of protein fractions.

Table 3. The observation results of the sea urchin T. gratilla Linn. zygote cells division inhibition percentage by 40-60% saturation of protein fractions

| The concentration of 40-60% saturation of protein fraction (µg/mL) | % Inhibition |
|---------------------------------------------------------------|-------------|
| 1                                                             | 30.5        |
| 10                                                            | 40.0        |
| 100                                                           | 51.7        |

Table 3 showed that the protein fraction could inhibit cell divisions by 30.5% at the concentration of 1 µg/mL whereas at the concentration of 10 µg/mL and 100 µg/mL that was 40.0% and 51.7%, respectively. The higher the concentration of the used compound was the greater the value of the inhibition percentage. The method of cell inhibition was calculated as IC₅₀ which was determined by the probit-log of concentration graph. Table 4 was the results of a complete calculation of IC₅₀ values.
Table 4. The IC_{50} calculation results of the sea urchin zygote cells toward the protein fraction of 40-60% saturation and vincristine.

| Test compound                  | IC_{50} value (µg/mL) |
|--------------------------------|-----------------------|
| 40-60% saturation of protein fraction | 74.13                 |
| Vincristine                    | 0.24                  |

The results showed the protein fraction of 40-60% saturation was a potential compound as an antimitotic agent with IC_{50} values of 74.13 µg/mL. Based on the research results of [15] that the levels of cytotoxicity were IC_{50} values < 10 µg/mL (very strong), 10-100 µg/mL (strong), and 100-500 µg/mL (low). This suggested that the protein fraction of 40-60% saturation was in the strong cytotoxic interval. Therefore, it could be concluded that the protein fraction of 40-60% saturation of microsymbiont algae *E. cottonii* was potential as new anticancer material.

4. Conclusion
The conclusion of this study that the protein concentration of the crude extract of microsymbiont algae *E. cottonii* was 2.6120 µg/mL, 0-20% saturation fraction of 0.6461 µg/mL, 20-40% saturation fraction of 0.3506 µg/mL, 40-60% saturation fraction of 1.1548 µg/mL, and 60-80% saturation fraction of 1.4023 µg/mL. Furthermore, the 40-60% protein fraction from microsymbiont algae *E. cottonii* had the strongest anticancer activity with LC_{50} value of 91.83 µg/mL against shrimp larvae *A. salina* Leach. and IC_{50} value of 74.13 µg/mL toward sea urchin *T. gratilla* Linn. zygote cells. The protein fraction from microsymbiont algae *E. cottonii* was potential as new anticancer material.

Acknowledgements
The work was supported in part by Grant-in-Aid from Graduate School of Term Research (Research of Master’s Thesis 2019) with contract No. 1739/UN4.21./PL.01.10/2019. The authors also thank to the staff of the Biochemistry Laboratory of Faculty of Mathematics and Natural Science, Hasanuddin University.

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