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Research Article

BIOFUNCTIONAL PROTEIN FRACTION FROM RED ALGAE (*RHODOPHYTA*) *EUCHEUMA SPINOSUM* AS AN ANTIBACTERIAL AND ANTICANCER DRUG AGENT

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ABSTRACT

Protein fractions were isolated from red algae *Eucheuma spinosum*, this alga was derived from coast Takalar, Punaga Village, District Mangarabombang, Takalar. Protein fractions were macerated with Tris-HCl buffer. Ammonium sulfate was used to fractionation of proteins while determinations of protein content used Lowry method. The protein fractions were tested their activity against pathogenic bacteria. The fractions with the highest activity were combined with chloramphenicol and then was tested their activities back. The last test for protein fractions was screening toxicity; the method screening used Brine Shrimp Lethality Test (BSLT) method. The highest protein concentration on the crude extract was 96.76 mg/ml and the highest protein fraction concentration on a fraction of 20-40% (F2) was 88.42 mg/ml. The strongest protein fraction activity on a fraction 0-20% (F1) with inhibition zone is 9.09 mm to *Escherichia coli* bacteria and 9.56 mm to *Staphylococcus aureus* bacteria because the protein fraction F1 is highest so that it combined with chloramphenicol. All of a combination antibacterial activity was show an antagonistic effect against *E. coli* and *S. aureus* but did not for a combination of Protein Fraction F1: Chloramphenicol (95.35µg: 7.5µg), they shown additive effect (indifference) to *S. aureus*. The Protein fraction F1 from the *Eucheuma spinosum* algae was toxic to the *Artemia salina* Leach larvae; the value of LC_{50} was 7.252µg/mL. This fraction may be a potential anticancer drug agent, to prove it needs further research.

Keywords: *Eucheuma spinosum*, protein fraction, combination activity, antibacterial, anticancer, LC_{50}

INTRODUCTION

Cancer is the leading cause of death worldwide; in 2012 about 8.2 million cases of death are caused by this disease. The most common cancer causes deaths to include lung cancer (1.59 million deaths), liver cancer (745 thousand deaths), stomach cancer (723 thousand deaths), colorectal cancer (694 thousand deaths), cancer breast (521 thousand cases of death), and esophageal cancer (400 thousand cases of death)[1].

Many therapies have been performed to treat cancer diseases such as surgery, radiation, chemotherapy, immunotherapy, hormone therapy, and gene therapy[2]. Chemotherapy is the most common method removes cancer cells, prevents cancer recurrence, controls cancer and reduces symptoms of cancer; but, this therapy caused side effects[3]. This condition pushes to look for drugs did not cause side effects.

Most people believe herbal medicine as a drug with low side effects, even it is considered no side effects, and it can be obtained from natural resources that are in the sea or on land[4]. In recent years, marine organisms have become the new target drugs, because their potential produces bioactive compounds is greater than other organisms[5,6]. Marine organisms are a source of nutritious food components and are the place to find new bioactive components, especially bioactive proteins[5]. Some bioactive proteins have been reported to be as antibacterial, antifungal, and anticancer[6,7]. The advantages of protein are used as medicinal ingredients; they have well received by the body and have little side effects only[6].

Algae are one species marine organism with the highest protein content[8]. The protein content between algae varies, some reports showed that red algae contain the highest protein [47% (b/b) from dry weight], green algae contains moderate protein [9% -26% (w/w) from dry weight], while brown algae contains the lowest protein [3% -15% (b/b) from dry weight][5].

Eucheuma spinosum is red algae with the potential to trace because it contains primary metabolites and secondary metabolites such as hydrocarbons. It has been used commercially for example as organic fertilizer, cosmetics, textiles, and medicines. Based on the literature review it is known, *Eucheuma spinosum* is used because their carrageenan contents[9], but no one has discussed or explained the *Eucheuma spinosum* protein activity as an anticancer drug or as an ingredient of other medicines, when it is known that it contains the highest protein, therefore, in this research would be isolated protein fraction from red algae *Eucheuma spinosum* and then seeing the fraction is potential as anticancer drug agent.

MATERIALS AND METHODS

The materials were used UV-Vis spectrophotometer, cold centrifuge, magnetic stirrer, vortex, centrifuge tube, micropipette, aerator, incandescent lamp, magnifying glass, *Eucheuma spinosum* algae, dialysis pouch, Tris (hydroxymethyl) aminomethane, NaCl, $CaCl_2$, β-mercaptoethanol, Triton X-100, ammonium sulphate, *Artemia salina* leach eggs, *Escherichia coli*, *Staphylococcus aureus*, Lowry A and B reagents, and Bovine Serum Albumin (BSA).

Sampling and Identification

Samples red algae were collected from the coast of Takalar, they were washed with seawater until clean and put into samples bag, were labeled place and date, and they were placed in a cooler box. The samples brought to the laboratory[10,11]. In the laboratory, samples were rinsed with water to remove salt residues; species alga was identified and stored at -20 °C[12].

Protein isolation

Samples algae 500 g were cut into small pieces, the samples were homogenized with 300 mL Tris-HCl buffer solvent, the cell fraction was freeze-thawed 2-3 times, the mixture cell fraction and supernatant were centrifuged at 5000 rpm 4°C 30 minute to separates the mixture. The supernatant was fractionated with ammonium sulfate at saturation levels of respectively 0-20%, 20-40%, 40-60%, and 60-80%. The protein fraction was dialyzed with buffer C solvent[13].

Determination of Protein Content

The Lowry method was used to determination of bioactive protein content, the content protein was measured by UV-Vis spectrophotometer with Bovine Serum Albumin (BSA) as the standard[14].

Antibacterial activity test

The antibacterial activity test was carried based on the Siregar method, including this method is positive control test, negative control test, and antibacterial activity test of protein fraction. The positive control test was carried out with chloramphenicol antibiotics. The negative control test was carried out with a Tris-HCl buffer solvent, this test to identify the presence of the solvent effect to the zone inhibition diameter.

The Kirby-Bauer method was used in antibacterial activity test. Bacteria were inoculated at media with swap method, sterile disc paper 6.2 mm was immersed in the test sample and placed on agar medium. Petri dishes were wrapped with plastic and stored in an incubator at 37°C for 1-2 days.

Inhibition of microorganisms by antibiotics (protein fraction) was seen with clear areas around the disc paper. It is an indication of the sensitivity of microorganisms to antibiotic substances or compounds. The resistance zone was measured from the diameter of the clear area. The measurement of diameter inhibition zone was done by using the sliding term[15].

Combination Antibacterial Activity

The antibacterial combination test used the agar diffusion method by Kirby & Bauer, the same method on the first antibacterial activity test. The combination test varied the volume of protein fractions and chloramphenicol on blank disks. A variation volume of the protein fraction with a chloramphenicol sequentially is 0:0 (control negative), 100%:0, 75%:25%, 50%:50%, 25%:75% and 0:100% (positive control) with a maximum disk capacity of 20µL, the negative control used is distilled water, and the positive control used is chloramphenicol.

Brine Shrimp Lethality Test (BSLT)

Brine Shrimp Lethality Test (BSLT) according to Meyer procedure, the test compound was made in concentration 1, 10, 100 ppm with sea water. Ten Larvae *Artemia salina* leach were added in vials, they were added with a compound test. Yeast suspension 0.6 mg/mL was added one drop as food for the larva. After 24 hours larvae are live were counted with help by the magnifying glass. Observational data were tabulated and calculated with probity analysis to obtain Lethal Concentration 50 (LC_{50})[16].

RESULTS AND DISCUSSION

The *Eucheuma spinosum* algae were taken from Punaga Village, Mangarabombang District, Takalar (Figure 1), the protein was isolated with Tris buffer pH 8.3 at 4°C, buffers were used because all biochemical experiments such as isolation or extraction of organs or cell components should be performed on system buffer with a certain pH. pH buffers need to be maintained in order to the structure and activity of important compounds such as enzymes (proteins) unchanged[17]. Tris buffer at room temperature has a pH of 7.8; at a temperature of 4 ^{0}C having a pH of 8.4 and at 37 °C having a pH of 7.4, otherwise, a temperature of 4 °C is used to avoid the occurrence of protein denaturation[18].

The isolation proteins in this study used a combination of mechanical and non-mechanical techniques (mechanical techniques e.g., by grinding/ blending, non-mechanical techniques e.g., by freeze-thawed techniques and the use of Triton x-100 chemicals)[18]. The result of isolation was fractionated with ammonium sulphate salt, from this isolation is obtained some fraction with different solubility; this term is called salting-out. The salting-out effect occurs when the concentration of salt added is increased continuously, then the solubility of proteins is reduced at the highest salt concentrations, proteins will undergo deposition[19]. Fractions are obtained by centrifugation and dialysis. Centrifugation and dialysis are two techniques of protein separation or purification[18]. After the protein is pure, the protein content is measured.

The protein content measurement results of the crude extract 96.76 mg/ml, this is the highest protein content, this is fair because in the crude extract all of the protein is collected, the highest protein fraction content are at a fractions of 20-40% that is 88.42 mg/ml, the protein distribution pattern of crude extract and each fraction can be seen in Table 1. The table shows that its concentration varies in each fraction. This indicates the difference in protein solubility in water. The Proteins with less solubility in water precipitate earlier than proteins with higher solubility in water[20].

The protein fraction has been dialyzed and has been determined the protein content then tested it is antibacterial activity. On this study, the inhibitory test was conducted of two pathogenic bacteria *E. coli and S. aureus*. The method that uses is the diffusion method agar by using a paper disc. The positive control used is Generic chloramphenicol (30µg) while the negative control uses a Tris-HCl buffer.

The inhibitory zone results of protein fraction of the red algae *Eucheuma spinosum* on both test bacteria after 24 hours and 48 hours incubation obtained results as in Table 2, the table showed that all protein fractions can inhibit the growth of *E. coli* and *S. aureus* bacteria. This is indicated by the clear zone around the paper disc (Figure 2).

Table 1: Protein Distribution Patterns of *Eucheuma spinosum*

Protein Fraction	Protein concentration (mg/mL)	Faction Volume (mL)	Total protein (mg)
Crude extract	96.76	580	56120.8
0-20 %	19.07	8	152.56
20-40 %	88.42	8	707.36
40-60 %	76.50	9	688.5
60-80 %	23.25	10	232.5

Table 2: Diameter of Inhibition zone of Protein Fraction *Eucheuma sponisum* to *E. coli* and *S. aureus*

Code	Protein Fraction	Diameter of Inhibition zone (mm)			
		E. coli		*S. aureus*	
		24 hour	48 hour	24 hour	48 hour
F1	0-20 %	9.09	8.99	9.56	9.05
F2	20-40 %	8.26	8.06	8.05	8.00
F3	40-60 %	8.90	8.56	8.15	8.02
F4	60-80 %	8.66	9.26	8.57	8.13
EK	Crude extract	8.34	8.11	7.83	7.78
(+)	Chloramphenicol 30µg	27.08	27.08	23.05	23.05
(-)	Tris-HCl Buffer	6.20	6.20	6.20	6.20

Table 3: Diameter of Inhibition Zone Combination of Protein Fraction and Chloramphenicol to *Escherichia Coli* Bacteria

Diameter of Inhibition Zone (mm)					
Protein (ppm) / Chloramphenicol (ppm)	0	4767.5	9535	14302.5	19070
0					15.1
125				18.1	
250			20.2		
375		22.5			
500	23.5				

Table 4: Diameter of Inhibition Zone Combination of Protein Fraction and Chloramphenicol to *Staphylococcus aureus* Bacteria

Diameter of Inhibition Zone (mm)					
Protein (ppm) / Chloramphenicol (ppm)	0	4767.5	9535	14302.5	19070
0					9.2
125				16.7	
250			18.1		
375		21			
500	21.3				

Table 5: The LC_{50} Value of Shrimp Larvae to the *Eucheuma spinosum* Protein Fraction

Protein fraction	Code	LC_{50} (µg/ml)	Toxicity
Crude extract	EK	744.380	Weak
0-20 %	F1	7.252	Very toxic
20-40 %	F2	170.125	Medium
40-60 %	F3	146.363	Medium
60-80 %	F4	>1000	Not toxic

Figure 1: Red Algae (*Rhodophyta*) *Eucheuma spinosum*

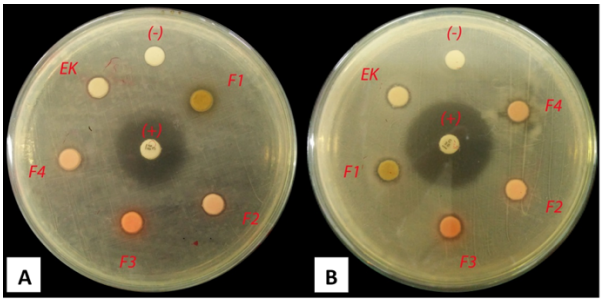

Figure 2: Diameter Inhibition zone of protein fraction on growth of *S. aureus* (A) and *E. coli* (B) at 24 hour incubation period

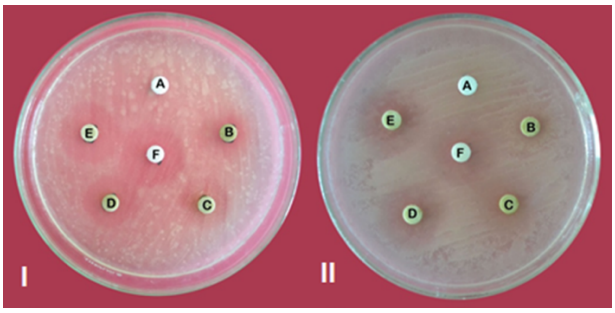

Figure 3: The results of the combination test of protein fraction and chloramphenicol against bacteria *Escherichia coli* (I) and *Staphylococcus aureus* (II), (Description Control negative distillated water, A; Protein Fraction 19070ppm, B; Protein Fraction 14302.5ppm combined chloramphenicol 125ppm, C; Protein Fraction 9535ppm combined chloramphenicol 250ppm, D; Protein fraction 4767.5ppm: chloramphenicol 375ppm, E and positive control Chloramphenicol 500ppm, F.)

The strongest antibacterial activity is found at a fraction 0-20% (F1) with diameter of inhibition zones 9.09mm to *Escherichia coli* bacteria and 9.56mm to *Staphylococcus aureus* bacteria for 24 hours incubation period, and After 48 hours incubation period all of the diameter inhibitory zones of protein fractions is decreases against *E. coli* and *S. aureus* bacteria. This is because the bioactive protein compounds contained in all of these fractions are bacteriostatic which can only to inhibit bacteria but did not to kill them; it is because the active protein compound has run out. According to Wattimena, an antibacterial is said to be bacteriostatic if the antibacterial is efficacious to inhibit the growth of test bacteria but did not kill bacteria[21].

The inhibitory activity of the protein fraction of the red algae *Eucheuma spinosum* is considered ineffective as an antibacterial agent; it is because the inhibitory zone diameter does not exceed the positive control (chloramphenicol) and does not exceed 14mm, whereas according to Cappucino an antibacterial agent is considered effective if having an inhibitory diameter ≥ 14mm[22].

The diameter inhibition zone of positive control of chloramphenicol (30µg) is 27.08mm to *Escherichia coli* and 23.05mm to *Staphylococcus aureus* for 24 hours incubation. The diameter inhibition zone remains after 48 hours incubation period. This proves that chloramphenicol is an effective antibacterial and broad spectrum to Gram-negative and Gram-positive bacteria, but the uses of chloramphenicol antibiotics are limited to some indications only and as an alternative treatment for serious infections. It is because chloramphenicol has side effects or toxic[23] and to avoid resistance to antibiotic chloramphenicol[24].

Combination of drugs with other compounds is one option to decrease the incidence of bacterial resistance, reduce toxicity or side effects, and increase the bactericidal activity of the combined compounds[22]. Now a combination of antibiotics with plant extracts is the target of the study because it can increase antibacterial activity to plant extracts as well as to antibiotics itself[23].

This study would have tested the antibacterial activity of a combination of protein fractions with chloramphenicol. This is done to observe whether there is a synergistic effect or an additive effect (indifference) between protein fractions with chloramphenicol. A compound of the combination test is said to be synergistic if the inhibition zone of the combination compound is greater than the inhibition zone of each compound, while, the test is said to be indifferent if the inhibition zone of the combination compound is not greater than the inhibition zone of each compound[22]. The protein fraction F1 (0-20%) is selected to combine with chloramphenicol because this fraction is the highest activity than other protein fraction.

The antibacterial activity test of protein fraction F1 individually, chloramphenicol individually, and a combination of protein fraction with chloramphenicol to *E. coli* and *S. aureus* bacteria for 24 hours incubation period can be seen in Figure 3. They all show activity excepting A code sample (Distilated water) which is a negative control.

Based on the data of the combination test results, the diameter inhibition zone does not increase when it is compared to the diameter inhibition zone single chloramphenicol, or in other words, this combination does not synergistic effect to *E. coli* and *S. aureus* except on the combination of Protein Fraction F1: chloramphenicol (4767.5ppm: 375ppm), this gives an additive effect (indifference) to *S. aureus*. Non-synergistic test results can be caused by several factors such as the longtime of fraction storage, the place of fraction storage, and the differences character of fractions in killing bacteria[23].

Jawetz and Gunnison explained that combinations of antibiotics can produce several effects, such as combinations of bacteriostatic antibiotics with other bacteriostatic antibiotics can cause additive effects; bactericidal antibiotics with other

bactericidal antibiotics can produce synergism effects, whereas bacteriostatic antibiotics with bactericidal antibiotics may be antagonistic effects[23], Siswandono explains the antagonistic effect is that bacteriostatic antibiotic will stop the growth of bacterial cells (microorganisms), while bactericidal antibiotics require the growth of microorganisms on process the mechanism of action[25].

The chloramphenicol is a bacteriostatic antibiotic[23], and from this study, we know the protein fraction F1 is a bacteriostatic antibiotic too. So the combination Chloramphenicol with protein fraction F1 means that two bacteriostatic antibiotics interact then the effect should be the additive effect (indifference), this case was showed on the combination of Protein Fraction F1: Chloramphenicol (4767.5ppm: 375ppm) with the individual chloramphenicol 500ppm to *S. aureus*.

Fraction and Crude Extracts of protein were further tested for their cytotoxic effects on the shrimp larvae of *Artemia salina*. Percent of death and LC_{50} counted after 24 hours. Meyer explains the category in indicating the presence of active substances that are toxic by looking at the LC_{50} value, if it <1000 µg/mL is said toxic, whereas, if it > 1000 µg/mL is said Not toxic, therefore, the smaller LC_{50} of a test compound, is the more toxic[16]. Based on this matter can be explained in the study there are four test compounds whose activities are categorized as toxic (Crude extract, fraction 0-20% (F1), fraction 20-40% (F2), fraction 40-60% (F3)) and there is one compound whose activities are categorized as non-toxic (fraction 60-80% (F4)).

These four toxic protein compounds can still be categorized in more detail by referring to the classification of LC_{50} toxicity values, if it <20 µg/mL is categorized as highly toxic, if it's at 20-100 µg/mL categorized as toxic, if it's at 100-500 µg/mL is categorized as medium, if it's at 500-1000 µg/mL is categorized as weak and if it's > 1000 µg/mL is categorized as non-toxic [26]. So that of four active protein compounds a weak activity compound that is EK with LC_{50} value 744.380 µg/mL, two compound of moderate activity that is F2 and F3 with value of LC_{50} respectively 170.125 µg/mL and 146.363 µg/mL, and its very toxicity high ie F1 with LC_{50} value 7.252 µg/mL (Table 5).

The toxicity value is related to the activity as an anticancer[27,28]. Based on the pharmacological point of view, there is a significant correlation between the death of shrimp larvae and the inhibition activity of cancer cell growth. This cytotoxic test has also been used as a pre-screening against antitumor drug research[26], however, this LC_{50} value cannot yet be a solid basis for ensuring that the protein fraction F1 (0-20% fraction) of *Eucheuma spinosum* can become a new anticancer agent, Further tests are still needed to confirm this.
The results of activity test for protein fraction of red algae *Eucheuma spinosum* to test bacteria and to shrimp larvae, *Artemia salina* leach is known that the strongest activity is at the same fraction of protein fraction 0-20% (F1), so it can be assumed this protein active whose protein with solubility is small in water. In addition, the antibacterial activity and cytotoxic activity of the activated 0-20% (F1) protein fraction simultaneously corresponds with Tyagi's opinion that the active compounds as anticancer are often found to be from compounds that are active as antibacterial[29]; however, to ensure further anticancer activity needs to be tested *in vitro* and *in vivo* further to prove it.

CONCLUSION

Based on the results of this study can be concluded protein fractions 0-20% (F1) isolated from algae *Eucheuma spinosum* active against pathogenic bacteria (*Escherichia coli* and *Staphylococcus aureus*); the combination of protein fraction F1 and chloramphenicol gives antagonistic effect to bacteria *E. coli* and *S. aureus* except in combination of Protein Fraction F1: Chloramphenicol (4767.5ppm: 375ppm) gives an additive effect (indifference) to *S. aureus*; protein fraction (F1) is also toxic to shrimp larvae *Artemia salina* leach with LC_{50} value of 7.252µg/mL; The fraction of the protein 0-20% (F1) of the *Eucheuma spinosum* algae may be potential as an antibacterial and anticancer drug agent, further research is needed to prove it.

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