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Activity of Red Algae (*Eucheuma Cottonii*) against some Bacteria and Fungi

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Abstract

Aim: to know the potential extract and fractions of the red algae as natural antimicrobial

Methods:

The sample was extracted by maceration using ethanol 96% and fractionated by liquid-liquid extraction (LLE) using n-hexane, ethyl acetate, and water. The antimicrobial test was done by agar diffusion and microdilution against bacteria *Staphylococcus aureus*, *Methicillin-Resistant Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, and *Pseudomonas aeruginosa Methicillin-Resistant* (PAMR), *Bacillus subtilis*; and fungi *Candida albicans* and *Aspergillus niger*.

Results: The results showed that *E. cottonii* extract was active against *S. aureus*, *B. subtilis* and *S. epidermidis* at a concentration of 30, 40, 50 and 60 % (w/v). The fractions of LLE at concentration 50 % (w/v) showed active against some bacteria.. n-hexane fraction only active against *B. subtilis.* Ethyl acetate fraction was active against bacteria *S.aureus, S. epidermidis, B. subtilis* and *P.aeruginosa*. Water fractions were active against *S.aureus* and *B. subtilis*. However, the extract and all n- fractions were not active against fungi.

Conclusion: Red algae (*E.cottonii*) has the potential activity and can be developed as a natural antimicrobial

Keywords: Red Algae, *Eucheuma cottonii*, natural antimicrobial

INTRODUCTION

Various diseases arise in the community, one of which is a disease caused by infection by microorganisms such as *Staphylococcus aureus*. However, over time some microbes are resistant to existing synthetic ingredients [1,2].

Indonesia has an area of sea waters of 7.9 million km2 (including the Exclusive Economic Zone) is an archipelagic country that is heading for a maritime country [3]. Marine organisms have the potential as a bioactive source of secondary metabolites in the development of pharmaceutical raw materials [4]. One of these marine organisms is a marine algae plant, or commonly called seaweed or seaweed.

Algae produce various bioactive secondary metabolites as antimicrobial, anthelmentic, and cytotoxic substances. Macroalgae are considered a producer of several bioactive compounds because of its high activity [4]. Therefore research on marine algae is of great concern in its utilization.

Bioactive compounds of secondary metabolites from marine algae are developed through various studies to use as alternative drugs. Some algae from Indonesian waters are found to have active compounds, such as alkaloid compounds, flavonoids, steroids/triterpenoids, and tannins which are antimicrobial against pathogenic bacteria [5]. One of the marine algae that come from Indonesian waters is red algae (*Eucheuma cottonii*).

Until now various types of infectious diseases have been treated with antibiotics. In recent years there have been several cases of *S. aureus* bacteria that are resistant to methicillin antibiotics and β-lactam class antibiotics. This group is known as *Methicillin-Resistant Staphylococcus aureus* (MRSA). Antibiotic resistance experienced by *S. aureus* requires the use of antibiotics and specialized therapies for healing. However, it feared that this could cause poisoning during treatment, in addition to expensive costs for treatment. This condition causes the need for individual subscribers to MRSA infections and encourages efforts to find new drugs as antibacterials [6,7].

MATERIALS AND METHODS

Materials

The red algae (*E. cottonii*) collected from Pangandaran Beach, West Java. The material was soaked, dried then cut into small pieces and powder. The plant identified at the Plant Taxonomy Laboratory, Biology Department, Mathematics and Natural Sciences Faculty, Universitas Padjadjaran.

Chemicals

The chemicals used in this study were distilled water, amyl alcohol, ammonia, anhydrous acetic acid, hydrochloric acid, concentrated sulfuric acid, iron (III) chloride, dimethyl sulfoxide (DMSO), ethanol 96%, ether, ethyl acetate, gelatin, potassium hydroxide 5%, chloroform, lugol, n-butanol, n-hexane, sodium hydroxide, Mayer reagent, Dragendorff reagent, Liebermann-Burchard reagent, magnesium powder, spirtus, and toluene. The medium used for bacterial growth is Mueller Hinton Agar, Mueller Hinton Broth, Manitol Salt Agar, and physiological NaCl.

Apparatus

The tools used in this study include autoclave (Hirayama HL 42AE), stirring rod, glass beaker, chromatographic vessel, petri dish, separating funnel, toluene (Barstead) distillation tools, desiccator, scissors, incubator (Sakura

IF-4), term shovels, cotton, matches, Erlenmeyer flasks, 254 nm UV lamps and 366 nm (Camag), refrigerators, macerators, microtiter plates 96 wells, volume 1 micro pipets 1 µl - 10 µl, volume 10 micro pipets 10 µL - 100 µL, micropipette volume 10 µL - 1000 µL, microplate, analytical balance (Mettler Toledo AL 204), ose, oven (Memmert 200 and Memmert 400-800), GF 254 silica gel plates, Bunsen burners, water baths (Memmert), 6 mm diameter perforators, tweezers , knives, rotary evaporator (Buchi Rotavapor, R-300 and IKA® RV10 Basic), furnaces, micropipette tips, and vortex mixers (Health® HVM-300).

Microorganism Test

The test bacteria used in this research was *Staphylococcus aureus* Clinical Isolates obtained from the Health Polytechnic of the Rock Mountain Health Ministry. *Methicillin-Resistant Staphylococcus aureus* (MRSA), obtained from the Faculty of Medicine, University of Indonesia. The other bacteria *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa Methicillin-Resistant* (PAMR), *Bacillus subtilis*; and fungi *Candida albicans* and *Aspergillus niger* were available in Microbiology Laboratory, Faculty Pharmacy Universitas Padjadjaran.

Methods

Extraction and Fractionation

Powdered of red algae (*E. cottonii*) extracted by maceration for 3 x 24 hours using 96% ethanol. Filtrate concentrated using a rotary evaporator obtained the crude extract. The crude extract fractionated by Liquid-Liquid Extraction (LLE). Dried red algae extract dissolved with aquadest 200 mL. The extract solution put into a separating funnel, then added with n-hexane solvent with the same volume (ratio of 1: 1). The mixture is shaken for a while, then left until the two phases are formed and separated. The n-hexane phase stored in a glass beaker. Fractionation continued until n-hexane remains clear mean almost solute extracted. After that, water phase in a separating funnel added with ethyl acetate fractionation process continue in the same way as n-hexane phase. The ethyl acetate phase stored in beaker glass. N-hexane and ethyl acetate phases were concentrated using a rotary evaporator to obtain n-hexane and ethyl acetate fractions. While water phase concentrated using freeze dryer to become water fraction. The yield of all fractions was calculated and then observed organoleptically include shape, color, odor, and taste.

Identification of Test Bacteria

1. Gram Staining

Gram staining done by making a bacterial test suspension on the object glass and then the object glass is fixed on the fire. Rubbing on the glass of the object is inundated with carbolic gentian violet for 1 minute. Excess dye is removed and rinsed with distilled water. Then smeared with Lugol for 2 minutes. Lugol, which is overgrown, is then discarded and rinsed with distilled water. After that, smear washed with 96% alcohol drop by drop until the dye dissolves then rinse with distilled water. The last coloring stage smeared with methylene blue for 30 seconds and excess dye is removed and rinsed with distilled water. Then the object glass is dried with filter paper and added immersion oil and then observed under a microscope. Gram-positive bacteria will be purple and Gram-negative bacteria will be blue.

2. Test of Bacterial Assertion

The bacterial assertion test is done by streaking bacterial suspensions on specific media. For Staphylococcus aureus bacteria MSA media (Mannitol Salt Agar) is used. After bacteria scratched on the media, then incubated for 18 hours at 37ºC and observed the color changes that occur.

Especially for MRSA resistance tests are carried out to find out whether the bacteria are reactive or not. Resistance tests are carried out by making bacterial suspensions in physiological NaCl whose turbidity is equal to 0.5 McFarland. Then as many as 20 µL of bacterial suspension was put into a sterile petri dish, and 20 mL of sterile MHA were put in, then homogenized. Antibiotic discs placed on the agar, then incubated for 20 hours at 37 ° C. The caliper then measure the inhibition zone that is formed. The absence of an inhibitory zone indicates that the bacteria has been resistant to antibiotics.

3. Biochemical Test

Biochemical tests were carried out including sugar fermentation test, TSIA media, urea agar medium, citrate agar medium, indole, methyl red, Voges-Proskauer, and motility. Metabolites produced by bacteria can be detected by the reaction that occurs with changes in the color of the reagent.

Antibacterial Activity Test

Red algae (*E. cottonii*) extract and all of LLE fractions were tested by agar diffusion method through the following stages:

1. Sterilization of Tools and Media using autoclave at 121^{o}C for ± 15 minutes

3.4 g of *Mueller Hinton Agar* (MHA) dissolved in aquadest100 mL, then heated until completely dissolved and a clear solution formed.

2. Preparation of Test Bacterial Suspension and samples

Bacterial culture suspended in 2 ml of physiological NaCl, then its turbidity was adjusted with McFarland 0.5 (bacterial suspension where every 1 ml contained 1.5 x 108 bacteria).

Samples of red algae extract was dissolved in dimethyl sulfoxide (DMSO) to obtain extract solution at 60%, 50%, 40%, 30%, 20%, and 10% (w /v) of concentrations. While fractions was tested at 50% (w /v) of concentration.

3. Agar plate assay

20 µL of bacterial suspension put into a sterile petri dish, then 20 ml of MHA media added which was still liquid at a temperature of ± 40^{o}C. The mixture is beating slowly until homogeneous and allowed to solidify. Furthermore, the agar plate was divided into four zones and perforated using a 6.1 mm perforator.

50 µL of extract or fractions at various concentration put into the wells. Agar plates were incubated in an incubator at 37 ° C for 18-24 hours for bacteria or at 25-27^{o} C for

3x24 hours for fungi. The diameter of the inhibitory zone is measured using a caliper run.

4. Microdilution assay

The most active fraction further tested by microdilution method using 96 wells microplate in order to determine minimum inhibitory concentration (MIC) and minimum kill concentration (MKC).

The first well as a negative control filled with 100 µL *Mueller Hinton Broth* (MHB) media. The second weel as a positive control consisted of 10 µL of bacterial suspension in 100 µL MHB. The 3rd well as the fraction control consisted of 10 µL of the most active fraction in 100 µL MHB. All 4th-8th wells were filled with 100µL MHB respectively. In the 4th well added by 100 µL the most active fraction at 50% (w/v) of concentration. Proceed dilution by pipetting 100µL from the 4th well to 5th well until the 8th well, then 100 µL of the last dilution results are removed. In this way, the solution in the 4^{th} well is the highest concentration of the most active fraction and in the 8th column is the lowest concentration of the most active fraction or at 50%, 25%, 12.5%, 6.25%, and 3.125% (w/v) of concentration respectively. All 4th-8th wells filled with 10 µL of bacterial suspension. The microplate incubated at 37° C for 18-24 hours for bacteria or at 25-27^{o} C for 3x24 hours for fungi. The growth of bacteria indicated by turbidity or sediment forming at each well.

MIC and MKC determination done by streaking the solution from several wells that look clear to agar plate of MHA. The plates incubated for incubated at 37° C for 18-24 hours for bacteria or at 25-27^{o} C for 3x24 hours for fungi. MIC is determined at agar plate with the smallest concentration of fraction which did not show the growth of bacteria (no growth of bacterial colonies).

RESULTS AND DISCUSSION

Extraction and Fractionation Results

The crude extract has been measured with several parameters to determine the quality. The results showed in Table 1. The 50 g of extract fractionated by LLE methods obtained 43.39 g water fraction (86.78% (w/w) of yield), 4.18 g n-hexane fraction (8.36%) and 2.43 ethyl acetate fraction (4.86%). Organoleptic observations on the three fractions showed that water fraction in the form of white-brown crystals, typical smelled, and salty. Ethyl acetate fraction in the form of a brownish black sticky mass and a distinctive smell. While the n-hexane fraction in the form of thick brown black and smells typical.

Bacterial Identification Test Results

1. Test of bacterial assertion

This research did preliminary identification of *S. aureus* and MRSA clinical isolates was done by them on MSA media. The MSA media contains mannitol and uses methyl red as a pH indicator. Staphylococcus aureus will ferment mannitol which makes acid production, thus indicating a change in color to yellowish [8]. The culture results obtained were a single culture of MRSA and Staphylococcus aureus clinical isolates on each MSA medium with a rounded and yellowish white colony shape. The further test is resistance tests were carried out to prove whether the Staphylococcus aureus bacteria that would be used in the study were indeed resistant to methicillin antibiotics and other beta-lactam antibiotics (Table 2)

2. Gram staining

The results of Gram staining showed that the test bacteria were rod-shaped and purple. The test bacteria are included in the Gram-positive bacteria because Gram-positive bacteria will retain the blue-violet crystal so that under the microscope they appear purple [9].

3. Biochemical Test

S. aureus can ferment glucose so that it can be identified that the bacterium is Staphylococcus aureus (Kelly, 1995). *S. aureus* bacteria were positive mean able to ferment of citrate, urea, lactose fermentation, sucrose, glucose [10].

Antibacterial Activity Test Results

1. Agar diffusion assay results

The results showed that *E. cottonii* extract was active against *S. aureus*, *B. subtilis* and *S. epidermidis* at a concentration of 30-60 % (w/v). Based on the size of a diameter of zone inhibition looks like *E. cottonii* extract more active to *B. subtilis* than other bacteria (Table 4). Table 4 also showed that extract has no potency as antifungal.

Table 1. Standard Extract Parameters

Parameters	Results
Organoleptic	Amorphous, salty, typical smells, dark-brown
% Yield	7,25% (w/w)
Water content	10% (v/w)
Water Soluble content	47,5% (w/w)
Ethanol Soluble content	23,5% (w/w)

Table 2. MRSA Bacterial Resistance Test

Antibiotic	**Concentration (µg/mL)**	**Inhibition zones (mm)**	**Breakpoint (R<)**	**interpretation**
Ampicillin	10	0	26	Resisten
Oxacillin	5	0	26	Resisten
Amoxicillin	25	0	26	Resisten
Methicillin	5	0	26	Resisten

Table 3. Bacterial Biochemical Test

Test	*S. aureus*	MRSA
Glucose	+	+
Sucrose	+	+
Lactose	+	+
Maltose	+	+
Urea	-	+
TSIA	+	+
Citric	-	-
metil red	+	+
Manose	+	+
VogesProskauer	-	-
Motil	-	-

Table 4. The results of An Antimicrobial assay of *E. cottonii* extract

Bacteria and Fungi	**Diameter of inhibition zone (mm) at a Concentration (% w/v)**				
	30	**40**	**50**	**60**	**80**
S. aureus	11.93	12.43	13,28	14,31	NT
MRSA	-	-	-	-	NT
P. aeroginosa	-	-	-	-	6.07
PAMR	-	-	-	-	NT
S. epidermidis	11,93	12,96	14.01	14,85	NT
B. subtilis	12.86	13.45	14.19	14.40	NT
C. albicans	-	-	-	-	NT
A. niger	-	-	-	-	NT

Note: (-): no inhibition zone mean not active; NT: not tested

The fractions of LLE at concentration 50 % (w/v) showed active against some bacteria. n-hexane fraction only active against *B. subtilis*. Ethyl acetate fraction was active against bacteria *S.aureus*, *S. epidermidis*, *P.aeruginosa* and *B. subtilis*. Water fractions was active against *S.aureus* and *B. subtilis*. However, all fractions were not active against fungi (Table 5). Based on these results, ethyl acetate fraction is the most active fraction that will be tested again by microdilution method.

2. Microdilution Assay result

The ethyl acetate fraction tested by microdilution method using 96 wells microplate against *S.aureus*, *S. epidermidis* and *B. subtilis*. The result showed that the concentration range of MIC and MKC value lies at 25-50% (w/v) of concentrations to all bacteria tested. MKC is the lowest concentration capable of killing 99.9% of bacterial growth or 0.01% of the colonies allowed from the initial number of inoculums.

Table 5. The results of Antimicrobial assay of *E. cottonii* fractions

Bacteria and Fungi	**Fractions**	**The Diameter of inhibition zone (mm)**
S. aureus	n-hexane fraction	-
	ethyl acetate fraction	7.14
	water fraction	6.77
MRSA	n-hexane fraction	-
	ethyl acetate fraction	-
	water fraction	-
S. epidermidis	n-hexane fraction	-
	ethyl acetate fraction	19.7
	water fraction	-
P. aeroginosa	n-hexane fraction	-
	ethyl acetate fraction	0.73
	water fraction	-
PAMR	n-hexane fraction	-
	ethyl acetate fraction	-
	water fraction	-
B. subtilis	n-hexane fraction	17.9
	ethyl acetate fraction	18.38
	water fraction	16.54
C.albicans	n-hexane fraction	-
	ethyl acetate fraction	-
	water fraction	-
A. niger	n-hexane fraction	-
	ethyl acetate fraction	-
	water fraction	-

Note: (-): no inhibition zone mean not active

CONCLUSIONS

Extract and fraction of red algae (*E.cottonii*) has antibacterial activity against *S.aureus*, *S. epidermidis*, *P.aeruginosa* and *B. subtilis*. Ethyl acetate fraction is the most active fraction to those bacteria with MIC and MKC value at range 25% -50% (w / v) of concentration. So, red algae (*E.cottonii*) has the potential activity and can develop as a natural antimicrobial.

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