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Potency of Local *Gracilaria* sp. Extract as an Antibacterial against Skin Disease Pathogen

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Abstract **Original Research Article**

Skin disease is one of the most common diseases in tropical countries such as Indonesia, which might be caused by infection of pathogens like *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Red algae *Gracilaria* sp. originated from Wediombo Beach, Yogyakarta, Indonesia has not been widely used for medicinal purpose, especially as an antimicrobial for skin diseases in human. Therefore, this research aimed to study about the potency of local *Gracilaria* sp. extract as an antibacterial against skin disease pathogen. Red algae extraction was carried out using maceration method in ethanol solvent. Identification of phytochemical groups was determined using basic biochemistry analysis and Thin Layer Chromatography (TLC), while active phytochemical compounds were identified using Gas Chromatography-Mass Spectrophotometry (GC-MS). Antibacterial tests were performed by minimum inhibition concentration (MIC) using MTT indicator and inhibition assay using paper disc method. Alkaloids and saponins were identified in phytochemical based on biochemical tests. Compound separation by TLC using chloroform and methanol solvents with a ratio of 90:10 resulted in seven color spots with ninhydrin spray and six color spots with anisaldehyde spray. The GC-MS results showed that there were 38 compounds identified in the crude extract, which 8 of these 38 compounds are known to have anti-bacterial activity. Anti-bacterial assay of *Gracilaria* sp. extract on concentration of 100% showed medium strength to inhibit *Staphylococcus aureus* and strong strength to inhibit *Pseudomonas aeruginosa*. Crude extract of local *Gracilaria* sp. is potential to be used as antimicrobials against *Staphylococcus aureus* and *Pseudomonas aeruginosa* common agents for skin disease.

Keywords: *Gracilaria* sp., anti-bacterial, skin diseases pathogen, active compound

INTRODUCTION

Wediombo Beach, which is located in Girisubo District, Gunungkidul Regency, Special Region of Yogyakarta Province, is known to have a variety of aquative biodiversity such as fish, mollusks, crustaceans, sea urchins, sea cucumbers, and seaweeds. Seaweed of Macroalgae that are often utilized on Wediombo Beach includes *Gelidium* sp., *Sargassum* sp., *Ulva* sp., and *Gracilaria* sp. these macroalgaes are dried or processed to be sold as industrial raw materials. Macroalgae in general have various potentials that can be used as a source of food, fuel, cosmetics and even medicine, because of their secondary bioactive metabolites [1]. Of three major macroalgae (rhodophyta, phaeophyta and chlorophyta), rhodophyta or red algae is an important source of various bioactive compounds and has most species member compared to green and brown algae [2].

Skin disease is one of the most common diseases contracted by humans. The prevalence of skin disease in developing countries is around 20-80% [3] and this disease is often found in tropical countries, such as Indonesia. Skin diseases can be caused by bacteria and fungi. The bacteria that frequently infects humans is Staphylococcus aureus which causes ulcers [4] and *Pseudomonas aeruginosa*, which may cause systemic skin infections including subcutaneous nodules, ectima gangernosum, and gangrenous cellulitis [5].

Gracilaria sp. has many useful phytochemical compounds that act as antibiotic such as flavonoids, alkaloids, saponins, tannins, and producing polyunsaturated fatty acids, some even contain PGE-E2 [6]. Phycoerithrine, phycocyanin, prostaglandin, and hemagglutinin extracted from *Gracilaria* sp. have also been shown to have pharmaceutical activity [7]. *Gracilaria* sp. also contains chlorophyll which has a positive effect on inflammation, oxidation and wound healing by acting directly as a free radical scavenger and has the potential to protect lymphocytes against oxidative DNA damage by free radicals [8-10].

Antibiotics are treatments that aim to kill infectious agents, but the irrational use of antibiotics can lead to bacteria-resistant which happen quite often nowadays.

MATERIALS AND METHODS

Sample Collection

Gracilaria sp. was collected from Wediombo Beach, Gunungkidul, Indonesia. Epiphytes, sand particles and other debris were removed from samples by washing thoroughly with fresh water. *Gracilaria* sp. was shade dried at 40ºC, and to be grinded into fine powder using blender.

Sample Extraction

Extraction was carried out by maceration method using 96% ethanol solvent. The extraction process was carried out by mixing 500 grams of *Gracilaria* sp. with 1500 ml of 96% ethanol, and then soaked for 24 hours. Maceration extraction was repeated 3 times until all the active compounds contained in the sample were extracted. The extracts were filtrated using Whatman No.1 filter paper. All of filtrate obtained was evaporated using a vacuum rotary evaporator at a temperature of 40ºC until it became a paste.

Determination of Yield

The percentage of yield was calculated using the following equation:

$$\text{Yield (\%)} = \frac{\text{Massofdriedextract}}{\text{Massofextractpowder}} \times 100\%$$

Qualitative Biochemical Analysis

a. Alkaloid

Zero point three gram (0,3 g) extract was put in a test tube, added with 0,5 mL of 2%HCl and the solution was divided into two tubes. Tube I was added with 0.5 mL Dragendroff reagent, while tube II was added 2-3 drops of Mayer's reagent. Presence of orange sediment in tube I and yellowish precipitate in tube II indicates the presence of alkaloids.

b. Terpenoid and steroid

Zero point three gram (0,3 g) extract was put in a test tube with 0,5 mL of chloroform added, then 0,5 mL of acetic acetic acid anhydride and 1-2 drops of concentrated H_2SO_4 through the tube wall. If a brownish ring is formed, the sample extract contains terpenoids, whereas if it is formed a bluish green color indicates the presence of steroids.

c. Saponin

Zero point three gram (0,3 g) extract of *Gracilaria* sp. was put into a test tube, added 10 mL of hot water, cooled, then shaken vigorously for 10 seconds, if a steady foam is formed for not less than 10 minutes, it means that the sample contains saponins.

The second test is the Libermann-Burchard test. Extract of *Gracilaria* sp. was put into a test tube, then heated with 1 mL of acetic acid anhydra, cooled, then dripped with 2 drops of concentrated sulfuric acid. If it contains steroid compounds, it will form a blue green color, or will form pink to red colors if it contains triterpenoid compounds [11].

d. Flavonoid

Zero point three gram (0,3 g) was shaken with 3 mL n-hexane until the n-hexane extract is colorless, then the residue was dissolved in 20 mL 80% ethanol and divided into three parts (A, B, and C). Solution A was used as a blank, B was added with 0.5 mL of concentrated HCl and the color change was observed, if the color changes to bright red it contains leuko-antocyanin compounds. Solution C was added with 0.5 mL of concentrated HCl and 4 pieces of Mg, and then the color change was observed, if the color changes to orange indicate the presence of flavones, pale red indicates the presence of flavonols, dark red indicates the presence of flavanones.

e. Tannin

Zero point three gram (0,3 g) extract was put in a test tube, added with 2-3 drops of 1% $FeCl_3$ solution. If the solution produces a dark green or dark blue color, the extract contains tannins.

Thin Layer Chromatography

The crude extract of *Gracilaria* sp. was put into a TLC plate using a capillary tube. The TLC plate was put into a closed glass which already contained mobile phase that were consisted of chloroform and methanol with a ratio 90:10. TLC plate would be then sprayed using two reagents (ninhydrin and nisaldehyde) to identify the type of compound. To determine the presence of active compounds, UV light was employed and Rf value were calculated to determine the group of active compound identified.

Gas Chromatography-Mass Spectrophotometry (GC-MS)

The GC-MS analysis of bioactive compounds from *Gracilaria* sp. was done using screening method equipped with HP-5MS UI column (30 m in length × 250 mm in diameter × 0.25 µm in thickness of film). Pure helium gas was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50-240°C. One gram of the prepared crude extract diluted with 96% ethanol was used. Relative quantity of the chemical compounds present in each of the extracts of *Gracilaria* sp. was expressed as percentage based on peak area produced in the chromatogram.

Minimum Inhibitory Concentration (MIC)

Staphylococcus aureus and *Pseudomonas aeruginosa* was prepared by taking several inoculation needles from a 24 hours old bacterial culture that had been grown in slant Nutrient Agar. The 100% extract

was prepared using 1 gram of crude extract diluted with 1 mL of 10% DMSO. Then the first well was filled with 100 µg / ml, the second well was filled with 50 µg / ml distilled water. Subsequently, 50 µg / ml was taken from the first well into the second well and so on that a series of dilutions was obtained from 50 µg / ml to 25 µg / ml, and 12.5 µg / ml. All wells were added 30 µl of Nutrient Broth and 20 µl of bacterial suspension and incubated at 37ºC for 24 hours and 10 µl of MTT solution was added to each well and the color change to purple was observed. The lowest concentration that did not change color indicates the absence of growth from microbes.

Determination of Inhibition Zone

Nutrient Agar, Nutrient Broth, and Mueller-Hinton Agar media were used in this study. All media, distilled water, and DMSO were sterilized using autoclave at a temperature of 121ºC for 15 minutes. Petridish sterilized in an oven at a temperature of 150ºC for 2 hours. The bacteria used in this study were Staphylococcus aureus and *Pseudomonas aeruginosa*. Ciprofloxacin was used as positive control. One ose culture from slant Nutrient agar (NA) was suspended into Nutrient Broth (NB) medium then incubated at 37ºC for 24 hours. *Gracilaria* sp. extract, positive control (ciprofloxacin), and negative control (DMSO 10%) were made in concentrations of 100, 50, 25, and 12,5%, this concentration series was taken from 1 gram crude extract dissolved in 1 ml of DMSO 10%. Bacteria were streaked into petridish containing MHA media, and then inserted 6 mm paper discs that had been immersed in the extract sample, positive control and negative control using tweezers. Incubated at 37ºC for 24 hours, and the inhibition zone was measured standards from [12].

STATISTICAL ANALYSIS

Obtained data were analyzed using Microsoft Excel to calculate the standard deviation.

RESULT

Yield Determination

Dried red algae have 1000 g of weight. The crude extract produced from maceration method was 15, 78%. The resulting yield from this research is 1, 57%.

Qualitative Biochemical Analysis

Based on the result of the phytochemical screening of *Gracilaria* sp. extract, which is shown on Table 1 informed that *Gracilaria* sp. only contains saponin shown by the formation of foam during testing and alkaloids shown by the presence of orange sediment and yellowish precipitate.

Table-1: Results of preliminary photochemical screening of 96% ethanol extract of *Gracilaria* sp.

Active compounds	Test	Result
Alkaloid	Wagner	+
	Mayer	+
Terpenoid	Terpenoid	-
Saponin	Foam	+
	Liebermann-Burchard	-
Flavonoid	Bate-Smith Metcalf	-
	Wilstater	-
Tannin	Gelatin	-
	Polyphenol	-
Steroid	Steroid	-

Note: The sign (+) indicates the presence of a compound contained in the sample, the sign (-) indicates the absence of the compound contained in the sample.

Thin Layer Chromatography Analysis

The result of Thin Layer Chromatography analysis with ninhydrin as spray shows seven spots with Rf value for each spot are 0,87; 0,75; 0,63; 0,52; 0,43; 0,27; and 0,13. Meanwhile, Thin Layer Chromatography with anisaldehyde as spray shows six spots with Rf value for each spot are 7,5; 6,5; 5,5; 4,5; 3 and 2,5.

Table-2: Semi-qualitative compounds identification using TLC

Spray	Pictures	Color spot	Rf value	Compound
Ninhydrin		Red	0,87	Alkaloid [13]
		Pink	0,75	Unknown
		Pink	0,63	Unknown
		Peach	0,52	Unknown
		Pink	0,43	Unknown
		Pink	0,27	Unknown
		Orange	0,13	Unknown
Anisaldehyde		Red	7,5	Amines, aldehyde, ketone, carbohydrate, ester [14]
		Brown	6,5	Amines, aldehyde, ketone, carbohydrate, ester [14]
		Purple	5,5	Amines, aldehyde, ketone, carbohydrate, ester [14]
		Purple	4,5	Amines, aldehyde, ketone, carbohydrate, ester [14]
		Yellow	3	Unknown
		Brown	2,5	Diterpenes

Characterization of Active Compounds by Gas Chromatography-Mass Spectrophotometry

The principle of GC-MS is evaporation. Volatile compounds are compounds that have a low molecular weight. The results of GC-MS crude extract of *Gracilaria* sp. showed that there are eight compounds that have the potential as antibacterial. Pentadecane is a compound that has the highest percentage of all compounds (14.1%). Here is the result of the GC-MS test:

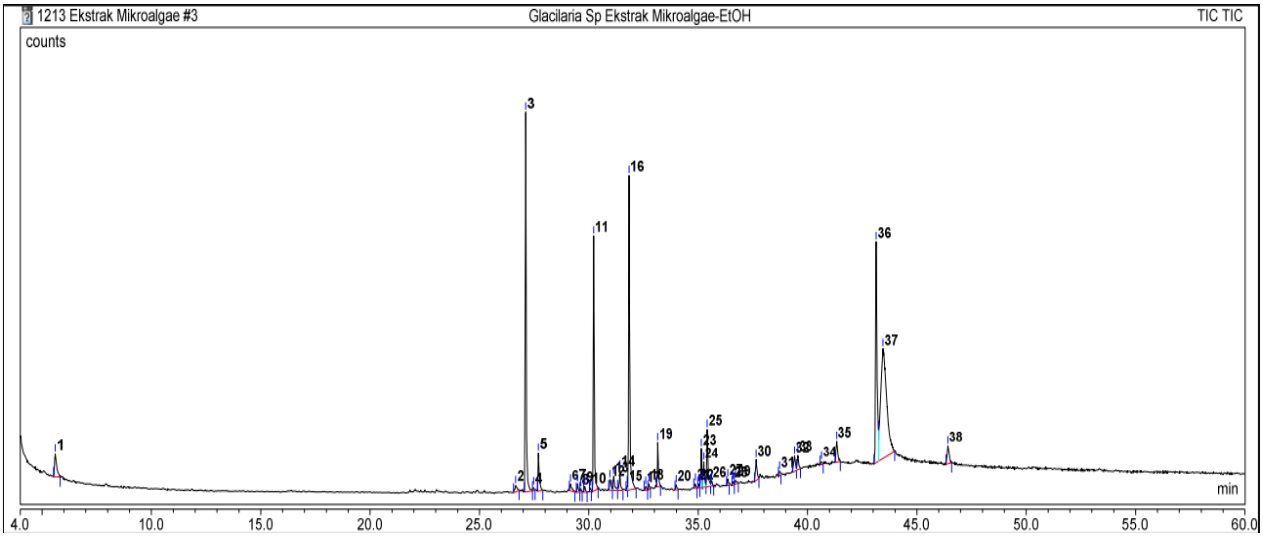

Fig-1: GC-MS chromatogram of *Gracilaria* sp. Ethanol extract

Table-3: Phytoconstituents of ethanolic extract of *Gracilaria* sp

Compounds	Nature of compounds	Percentage (%)	Chemical formula
Pentadecane	Hidrocarbon	14,1	$C_{15}H_{32}$
Imidazole, 2-amino-5-[(2-carboxy)vinyl]-	-	0,14	$C_6H_7N_3O_2$
Hexadecanoic acid, 15-methyl-, methyl ester	Carboxyl acid	1,87	$C_{18}H_{36}O_2$
E-11-Hexadecenoic acid, ethyl ester	Caboxyl acid	0,44	$C_{18}H_{34}O_2$
2-Pentadecanone, 6,10,14-trimethyl-	Terpene ketone	9,88	$C_{18}H_{36}O$
11-Octadecenoic acid, methyl ester	Fatty acid	1,50	$C_{19}H_{36}O_2$
3,7,11,15-Tetramethyl-2-hexadecen-1-o	Terpenol	2,79	$C_{20}H_{40}O$
Ethyl iso-allocholate	Steroid derivative	1,34	$C_{26}H_{44}O_5$

Minimum Inhibitory Concentration (MIC)

MIC was performed to determine the potential of the extract as an antibacterial. Of the four concentrations tested, all of them showed positive results, which means that the growth of Staphylococcus aureus and *Pseudomonas aeruginosa* could be inhibited by *Gracilaria* sp. crude extract.

Table-4: MIC result of *Gracilaria* sp. against Staphylococcus aureus and *Pseudomonas aeruginosa* using MTT indicator

Concentration of *Gracilaria* sp. crude extract	Result	
	Staphylococcus aureus	*Pseudomonas aeruginosa*
12,5%	+	+
25%	+	+
50%	+	+
100%	+	+
Positive control	+	+
Negative control	-	-

Determination of Inhibition Zone

The inhibition zone determination test was carried out with four concentrations. The results showed that the greater the concentration, the wider the zone inhibition. The results also showed that almost all of the test concentrations were able to inhibit the tested bacteria at a medium level.

Table-5: Determination of zone inhibition

Bacteria	Sample	Concentration	Diameter ± SD	Strength
Staphylococcus aureus	Crude extract	100%	8,3 ± 1,15	Medium
		75%	8 ± 1	Medium
		50%	7,6 ± 0,57	Medium
		25%	7 ± 0	Medium
	Positive control	100%	27 ± 1	Very strong
	Negative control	100%	-	-
Pseudomonas aeruginosa	Crude extract	100%	10,3 ± 1,15	Strong
		75%	9,6 ± 1,15	Medium
		50%	8,3 ± 0,57	Medium
		25%	7,3 ± 0,57	Medium
	Positive control	100%	35,3 ± 0,57	Very strong
	Negative control	100%	-	-

DISCUSSION

Yield Determination

Wediombo beach is an open beach with lots of sun exposure. Sunlight is definitely needed for photosynthesis process and greatly determines the speed of seaweed to meet nutrient needs such as carbon, nitrogen and phosphorus for its growth rate, feedback control, enzyme inactivation, and enzyme induction. Nutritional limitations and decreased growth rate will produce signals that have regulatory effects resulting in secondary metabolites and differentiation of morphology or morphogenesis [15].

The existence of secondary metabolites is important for the life of *Gracilaria* sp. and human as its consumer because of its benefits, such as antibacterial function. The obtained yield of *Gracilaria* sp. from extraction process was 1, 57%. The yield was higher than the study of [16] with a yield of 0,9%. Yield results can vary due to the different sizes of simplicia used. The smoother the sample used the more sample's surface contacts with the solvent, so that the solvent can extract sample's compound more optimally and the more compounds that are extracted, the higher the yield value of the sample [17].

Qualitative Biochemical Analysis

In contrast to the research of Manalu et al. [18] which can detect flavonoids, saponins, and steroids/triterpenoids. In this study, only two compounds were detected, alkaloid and saponin. This can occur due to the low concentration of the content contained in the *Gracilaria* sp. extract or the low amount of extract used so that it is difficult to detect using qualitative testing with reagents, besides the environmental conditions in which *Gracilaria* sp. lives also greatly affect the content of secondary metabolites.

The presence of these compounds in the sample depends on environmental factors and how *Gracilaria* sp. lives because the presence of secondary metabolites depends on organism-environment interactions and stress resistance [16, 19]. From the data obtained, the secondary metabolite of *Gracilaria* sp. should be quite high when viewed from the ecological characteristics of the coast [1]. The small number of secondary metabolites contained in *Gracilaria* sp. can also occur because *Gracilaria* sp. lives in a good environment, such as not too many predators and clean water conditions such as high dissolved oxygen. The high oxygen in the coastal waters of Wediombo is an indicator that the waters are still in natural conditions and there is no pollution.

Thin Layer Chromatography Analysis

The TLC results are based on the spot color formed and the Rf value corresponding to the polarity of the compound contained. The higher the Rf value, the less polar the compound is. More compounds were identified in TLC test than in the qualitative phytochemical test because TLC testing is able to detect a variety of more specific compounds [20].

The results of the TLC on Table 2 were carried out by spraying the ninhydrin reagent which functions to detect amino acids, amines, and as a general spray on alkaloids, and also sprayed by anisaldehyde which functions to detect many compounds including secondary metabolites. Spraying TLC plate with ninhydrin spray obtained seven color spots: red, pink, peach and orange. In his book, Cannell [13] stated that alkaloid compounds appear red with the use of ninhydrin reagents. It can be seen that the TLC with ninhydrin spray showed a red color with an Rf of 0.87 which is thought to be an alkaloid, while the other colors are still unknown. On the other hand, spraying TLC plate with anisaldehyde spray obtained six colors: red, brown, purple, and yellow which are thought to be amines, aldehyde, carbohydrate, or ester.

Characterization of Active Compounds by Gas Chromatography-Mass Spectrophotometry

GC-MS tests showed that there were more specific and many detectable compounds than TLC or phytochemical tests. There were thirty eight compounds detected. Table 3 shows the detected compounds that have antibacterial abilities [21-28]. Apart from being antibacterial, these compounds also have other roles. Pentadecane; 11-Octadecenoic acid, methyl ester; and 3, 7, 11, 15-Tetramethyl-2-hexadecen-1-o has a role as an

antioxidant [22, 27, 28]; Imidazole, 2-amino-5-[(2-carboxy)vinyl]- as an anti-inflammatory [22]; E-11-Hexadecenoic acid, ethyl ester as anti-tumour and antifungal [24]; 2-Pentadecanone, 6, 10, 14-trimethyl- as an antiostheoporotic [25].

Minimum Inhibitory Concentration (MIC)

Of the many compounds detected, some have an antibacterial effect. This is evidenced by screening test using MTT indicator (Table 4). Through the screening data, it is known that the crude extract of *Gracilaria* sp. was able to inhibit the growth of Staphylococcus aureus and *Pseudomonas aeruginosa*. The inability of the extract to kill the tested bacteria can be influenced by several factors such as the low concentration used or the low content of the sample.

Determination of Inhibition Zone

The inhibition test was carried out to determine the potency of *Gracilaria* sp. crude extract in inhibiting tested bacteria. The criteria for antibacterial strength are: (1) weak category if the resulting inhibition zone is ≤ 5 mm, (2) medium category if the resulting inhibition zone is 5-10 mm, (3) strong category if The resulting zone of inhibition is 10-20 mm, and (4) is very strong if the resulting zone of inhibition is ≥ 20 mm [12]. Most of the results of the inhibition test showed medium criteria (Table 5). *Gracilaria* sp. appears to be more able to inhibit *Pseudomonas aeruginosa* (gram negative bacteria) than Staphylococcus aureus (gram positive bacteria). The different results are due to the ability of each bacterium to resist antibacterial activity of this red algae extract. Bacterial resistance is helped by their cell wall thickness and composition. There are differences in the composition and structure of the cell walls in each bacterium [29]. *Pseudomonas aeruginosa* contain a higher percentage of lipid, fat or fat-like substances than Staphylococcus aureus. *Pseudomonas aeruginosa* cell wall is thinner than Staphylococcus aureus cell wall because the structure of *Pseudomonas aeruginosa* has an outer membrane covering a thin layer of peptidoglycan which is a bilayer containing phospholipids, proteins, and lipopolysaccharides. Meanwhile, Staphylococcus aureus have a cell wall consisting of a thick peptidoglycan layer containing teichoic and lipoteichoic compounds [30]. The sensitivity level of bacteria is quite diverse and really depends not only on the type of active compound but also on the bacterial strain.

Taken together, the practical benefit of this research is to provide new information about the phytochemical content of local red algae *Gracilaria* sp. taken from Wediombo Beach which shown to have antibacterial potency.

CONCLUSION

Local red algae species *Gracilaria* sp. from Wediombo Beach, Yogyakarta are potential to be utilized as sources of antibacterial compounds against Staphylococcus aureus and *Pseudomonas aeruginosa* as common skin infection agents.

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