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Multidrug-resistant antibacterial activity and active compound analysis several types of seaweed from Karimunjawa, Jepara

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Abstract. Pathogenic bacteria that recently infect humans have been undergone an evolution. The evolution of treatment measures is not in accordance with the dose. Several cases in hospitals have found multi-type bacterial drug resistance. These cases affected much death of the patient. Therefore, this study explores the seaweed from the Karimunjawa Islands, Jepara as the potential sources of new antibacterial active compounds. The study is conducted in the laboratory and used the experimental design of sampling, extraction, resistance test, antibacterial extract test, and analysis of extract active compounds. There are three types of seaweed sampled: *S. crassifolium*, *Padina australis* and *Dictyota dichotoma*. Each extraction is carried out with diethyl ether, methanol, ethanol and chloroform solvents. This study used pathogenic bacterial isolates: *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Escherichia coli* which are tested with 13 antibiotics. The resistance test showed the value of multiple antibiotic resistance index (mar) of 0.84; 0.76; 0.48 and 0.34. Antibiotics with a high percentage resistance value (100%) are from groups of lipopeptides (colistine sulfate) and β -lactams groups (ampicillin). The seaweeds extract tested for antibacterial activity at different concentrations. The results showed that different types and concentrations of extracts had different antibacterial activity values ($p < 0.05$). The extract with high antibacterial activity is an extract from seaweed *S. crassifolium* with methanol solvent. The extract contained phenol 12 mg/g, flavonoid 1.6%, tannin 0.63%, fucoxanthin 0.59 mg/g, carotenoid 165.9 g/g, chlorophyll-a 0.92 mg/g and chlorophyll-b 0.28 mg/g.

1. Introduction

Antibiotics are medicines that are used to treat infectious diseases. The problem of using antibiotics in society is still not under control due to the dosage and rules [1]. This causes bacteria to be resistant to antibiotics/several types of antibiotics (multiple drug resistance).



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Pathogenic bacteria that are resistant could spread rapidly (epidemiology) and the source of its transmission could occur in a hospital environment. This condition makes treatment and infection control were more difficult therefore it is necessary to look for other compounds that have the potential as new antibiotics and one of them comes from brown seaweed [2,3]. Antibacterial compounds in the secondary metabolites of macroalgae such as phlorotannins, fatty acids, polysaccharides, peptides, terpenes, polyacetylenes, sterols, indole alkaloids, aromatic organic acids, shikimic acid, polyketides, hydroquinones, alcohols, aldehydes, ketones, and halogenated furanones, alkanes, and alkenes [4,5]. This macroalga has high natural resources and abundance in the coasts of Jepara - Indonesia. This study aim to screening natural compounds that have prospects in the discovery of new antibiotics in the context of handling the problem of MDR pathogenic bacterial infections.

2. Methodology

2.1. Sampling

The samples obtained were sorted and grouped to get the target samples: *Sargassum crassifolium*, *Padina australis* and *Dictyota dichotoma*. Target samples washed and dried [6,7] which blended into coarse powder and extracted [8].

2.2. Extraction.

Seaweed extraction used macerated process with 4 different solvent: diethyl ether, methanol, ethanol, chloroform [9]. 500 grams of coarse powder was soaked in 1.5L solvents for 24 hours in a dark room condition then filtered [10]. The filtrate with each solvent obtained was evaporated at 40 °C at the pressure of 500 mHg. The extract obtained was dried and stored in a freezer at -4 °C [11].

2.3. Resistance Tests.

The pathogens used was from Dr. Kariadi General Hospital Medical Center. The isolates tested were MDR *P. aeruginosa*, *S. aureus*, *S. epidermidis* and *E. coli*. The incubated test was centrifuged at 1500 rpm for 20 minutes, washed with PBS and measured the OD (Optical Density) between 0.6-0.8 [12] at α 600 nm, 0.1 ml was pipetted and inoculated to the surface of the petri media and incubated for 30 minutes to diffuse. Each antibiotic was dissolved with distilled water at a concentration of 2500 ppm and each sterile paper disc was given an extract solution of 20 μ l (50 μ g / disc) [11]. Furthermore, it is stored in an incubator at 37°C for 24-48 hours and the inhibition zone is observed. The resistance test was carried out on the pathogen with results: *P. aeruginosa* is resistant to chloramphenicol, colistine sulfate, ampicillin, cefriaxone, amoxillin and tetracycline; *S. aureus* is resistant to chloramphenicol, colistine sulfate, ampicillin and aztreonam; *S. epidermidis* is resistant to chloramphenicol, colistine sulfate, ampicillin, cefriaxone, neomycin, streptomycin and ciprofloxacin; and *E. coli* is resistant to colistine sulfate, iminepem, ampicillin, cefriaxone and tetracycline.

2.4. Antibacterial Activity Test.

Antibacterial activity test uses several extracts with different types of seaweed (*S. crassifolium*, *P. crassa* and *D. patens*) and solvents. Extracts tested were 12 types. The incubated test bacterial culture was centrifuged at 1500 rpm for 20 minutes, washed with PBS and measured OD (Optical Density) between 0.6-0.8 [10] at α 600 nm. 0.1 ml was pipetted and inoculated into media surface incubated for 30 minutes

to diffuse. Each extract was dissolved with a DMSO concentration of 5000 ppm and each sterile paper disc was given an extract solution of 20 μ l (100 μ g / disc) [13]. Furthermore, it is stored in an incubator at 37°C for 24-48 hours and the inhibition zone is observed.

2.5. Phytochemical and Pigment Content Analysis.

The sample was extracted by adding 1 ml of 96% ethanol and 5 ml of distilled water. The extract was added 0.5 ml of Folin-Ciocalteu 50%, homogenized and left out for 5 minutes. 1 ml of 5% Na₂CO₃ was added and left out in a dark condition for \pm 60 minutes. The standard used in the analysis of total phenol levels is gallic acid. Uptake was showed at λ 725 nm, with absorbance values and converted to total phenols expressed in mg GAE/g sample weight [13].

The tannin content is condensed [14] with a total of 0.1 mL of 200 mg/L extract solution was put into a test tube and wrapped in aluminum foil, added 3 mL of 4% (w / v) vanillin solution in methanol and vortexed. 1.5 mL of concentrated HCl added and vortexed. The absorbance was read at λ 500 nm after the mixture was incubated for 20 minutes at room temperature. The condensed tannin content is expressed in mg of catechin/kg of extract.

The calculation was done by extracted the methanol. 500 ml of extract was added with 1 ml of 5% NaNO₂ and left out for 6 minutes. Furthermore, 1 ml of 10% AlCl₃ and 10 ml of NaOH 1 M were added and 70% to 25 ml of ethanol were added and left out for 15 minutes then the absorbance was measured with a UV-vis spectrophotometer with a wavelength of 515 nm with 70% ethanol as a control [15].

This analysis uses the Arnon method. A total of 500 mg of sample was sorted with 10 ml of acetone and centrifuged at 3000 rpm for 15 minutes and absorbance was measured at λ 645 nm and 663 nm.

$$\text{Chlorophyll a (mg/g)} = \frac{(12,7 \times A_{663} - 2,69 \times A_{645}) \times V}{1000 \times W}$$

$$\text{Chlorophyll b (mg/g)} = \frac{(22,9 \times A_{645} - 4,68 \times A_{663}) \times V}{1000 \times W}$$

Note = A: absorbance at wavelength spectrophotometry, V: extract volume (ml) and W: wet sample weight.

The amount of carotenoids was calculated by the Kirk and Allen method. Absorbance was measured at α 480 nm and 510 nm.

$$\text{Carotenoid (}\mu\text{g/g)} = \frac{[(7,6 \times A_{480}) - (1,49 \times A_{510})] \times V}{1000 \times W}$$

Note = A: absorbance at wavelength spectrophotometry, V: extract volume (ml) and W: wet sample weight.

Fucoxanthin content were measured at λ 470 nm, 581 nm, 631 nm and 644.

$$\text{Fucoxanthin (mg/g)} = A_{470} - 1,239 (A_{631} + A_{581} - 0,3 \times A_{664}) - 0,0275 \times A_{644} : 141$$

Note = A: absorbance at wavelength spectrophotometry, V: extract volume (ml) and W: wet sample weight.

2.6. Data Analysis

Data extracts, content of phenols, tannins, flafonoids, fukosantin, carotenoids, chlorophyll a and b, are presented using histogram graphs, while resistance ratio data, percentage of resistance and antibacterial

activity of MDR are presented using data tabulation. Data on antibacterial activity, content of phenols, tannins, flavonoids, fucoxanthin, carotenoids, chlorophyll a and b were further tested for homogeneity, normality and additivity with a sig value of 0.050. The data is performed one way ANOVA test with a sig value of 0.050 using the SPSS program version 16.0. In case there is an influence between the treatment of the response then the Tukey test is performed with a sig value of 0.050.

3. Results

Extraction with solvent Diethyl ether, Methanol, Ethanol, Chloroform obtained 12 extracts and extraction results (Figure 1) which showed different types of seaweed have different extract percentage values and different solvents have different extract percentage values.

Figure 1 show *S. crassifolium*, *D. dichotoma* and *P. australis* have the highest percentage of extracts in methanol solvents. The 3 types of samples have the lowest extraction percentage values in diethyl ether solvent. *S. crassifolium* has the highest extraction percentage value in diethyl ether, methanol, ethanol and chloroform.

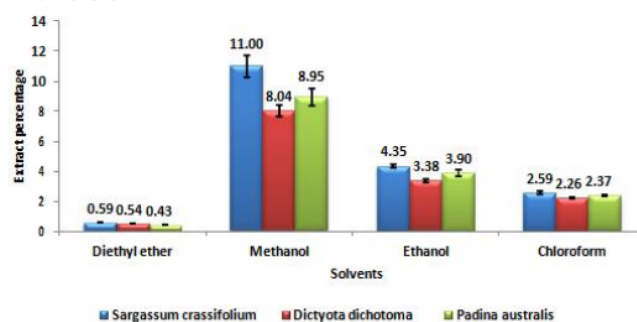


Figure 1. Percentage of brown seaweed extracts from Jepara coasts.

Table 1. Profile of resistance and sensitivity of pathogenic bacterial isolates to antibiotics.

Antibiotic		<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>E. coli</i>
Phenolics	Chloramphenicol	R	R	R	S
Lipopeptides	Colistin sulfate	R	R	R	R
β-Lactams	Imipenem	S	S ₁₂	S	R
	Ampicillin	R	R	R	R
	Ceftriaxone	R	R	S	R
	Amoxicillin	R	R	R	S
	Aztreonam	R	S	R	R
Tetracyclines	Tetracycline	R	R	R	S
Glycopeptides	Vancomycin	R	R	S	R
Aminoglycosides	Neomycin	R	R	S	S
	Gentamicin	R	S	S	S
	Streptomycin	R	R	S	S
Fluoroquinolones	Ciprofloxacin	S	R	S	S
R		11	10	6	5
S		2	3	7	8

R/S	0.85	0.77	0.46	0.38
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Note: R is the resistant response, S is the sensitive response, R / S is the ratio of the resistant response and the sensitive response.

Table 1, shows the isolates of *P. aeruginosa*, *S. epidermidis*, *S. aureus* and *E. coli* have different levels of resistance and sensitivity to antibiotics. *P. aeruginosa* bacterial isolates are resistant to 11 antibiotics : chloramphenicol, colistin sulfate, ampicillin, ceftriaxone, amoxicillin, aztreonam, tetracycline, vancomycin, neomycin, gentamicin and streptomycin. *S. epidermidis* bacterial isolates are resistant to 10 antibiotics: chloramphenicol, colistin sulfate, ampicillin, ceftriaxone, amoxicillin, tetracycline, vancomycin, neomycin, streptomycin and ciprofloxacin. *S. aureus* bacterial isolates are resistant to 6 antibiotics: chloramphenicol, colistin sulfate, ampicillin, amoxicillin, aztreonam and tetracycline. *E. coli* bacterial isolates are resistant to 5 antibiotics: colistin sulfate, imipenem, ampicillin, ceftriaxone, aztreonam, and vancomycin. The bacterial isolates of *P. aeruginosa*, *S. epidermidis*, *S. aureus* and *E. coli* have different sensitivity responses to antibiotics, respectively 2, 3, 7 and 8 types of antibiotics.

Resistant response data of *P. aeruginosa*, *S. epidermidis*, *S. aureus* and *E. coli* were then calculated as the level of resistance in units of percent resistance. Percent resistance shows the level of activity of the type of antibiotic in inhibiting bacterial pathogens (Table 2). Table 2 shows the differences in antibiotics having different antibacterial activity influences on pathogenic bacterial isolates. Percent resistance data consists of 25, 50, 75 and 100% respectively that is resistant to 1, 2, 3 and 4 isolates of pathogenic bacteria. The types of antibiotics that are unable to inhibit the test bacteria are colistin sulfate and ampicillin. Types of antibiotics that can inhibit one type of test bacteria are chloramphenicol, ceftriaxone, amoxicillin, aztreonam, tetracycline and vancomycin. The types of antibiotics that can inhibit two types of test bacteria are neomycin and streptomycin. The types of antibiotics that can inhibit three types of test bacteria are imipenem, gentamicin and ciprofloxacin.

Table 2. Percentage of resistance of pathogenic bacterial isolates to different types of antibiotics.

	Antibiotic	% Resistance
Phenicol	Chloramphenicol	75
Lipopeptides	Colistin sulfate	100
β -Lactams	Imipenem	25
	Ampicillin	100
	Ceftriaxone	75
	Amoxicillin	75
	Aztreonam	75
Tetracyclines	Tetracycline	75
Glycopeptides	Vancomycin	75
Aminoglycosides	Neomycin	50
	Gentamicin	25
	Streptomycin	50
<u>Fluoroquinolones</u>	<u>Ciprofloxacin</u>	<u>25</u>

Table 2 shows the differences in antibiotics with different antibacterial activity influences on pathogenic bacterial isolates. Percent resistance data consists of 25, 50, 75 and 100% respectively which is resistant to 1, 2, 3 and 4 isolates of pathogenic bacteria. The types of antibiotics that are unable to inhibit the bacteria test are Colistin sulfate and Ampicillin. Types of antibiotics that can inhibit one type of bacteria test are Chloramphenicol, Ceftriaxone, Amoxicillin, Aztreonam, Tetracycline and Vancomycin. The types of antibiotics that can inhibit two types of bacteria test are Neomycin and Streptomycin. The types of antibiotics that can inhibit three types of bacteria test are Imipenem, Gentamicin and Ciprofloxacin.

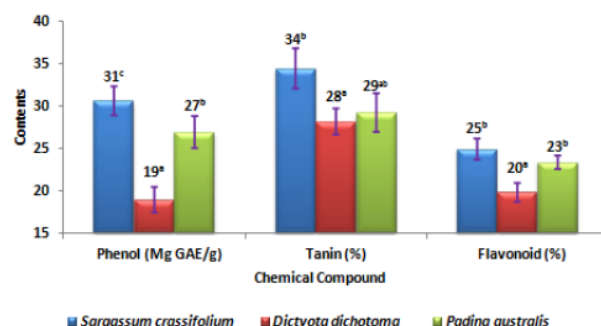


Figure 2. Phytochemical analysis of phenols, tannins and flavonoids in seaweed.

Note: values are averages, super script letters behind different numbers in one group of compounds show significantly different from each other ($p < 0.050$), super script letters from a to z indicate having a greater average value.

In the antibacterial activity test the extract was shown by the size of the inhibition zone formed around the paper disk (Table 3). Table 3 shows the different seaweed have different antibacterial activities, the response zone of pathogenic bacterial inhibition in each type is even different to the extract. Extracts which have the greatest antibacterial activity towards *P. aeruginosa* and *S. aureus* are *S. crassifolium* extracted with diethyl ether solvent. Extracts which have the greatest antibacterial activity towards *S. epidermidis* and *E. coli* are *S. crassifolium* extracted in the methanol solvent. This shows all extracts have MDR antibacterial activity.

The results of the quantitative phytochemical analysis (Figure 2) showed that the different types of seaweed had significantly different levels of phenols, tannins and flavonoids ($p < 0.050$). *D. dichotoma*, *P. australis* and *S. crassifolium* have the lowest to highest levels of phenols, tannins and flavonoids.

In Figure 3 shows the results of quantitative analysis of pigments and different types of seaweed have significantly different contents of fucoxanthin, carotenoids, chlorophyll a and b ($p < 0.050$). Samples which had the lowest to highest levels of fucoxanthin, carotenoids, chlorophyll a and b were *D. dichotoma*, *P. australis* and *S. crassifolium*.

Table 3. Percentage of resistance of pathogenic bacterial isolates to different types of antibiotics (5000 ppm; 24 hours)

Types	Solvents	<i>P. aeruginosa</i>	<i>S. epidermidis</i>	<i>S. aureus</i>	<i>E. coli</i>
<i>Sargassum crassifolium</i>	Diethyl ether	18.23 ± 0.41 ^d	16.73 ± 1.37 ^b	21.56 ± 0.98 ^b	14.97 ± 0.94 ^{ab}
	Methanol	15.89 ± 1.28 ^c	21.54 ± 1.13 ^c	20.24 ± 0.50 ^b	18.44 ± 0.43 ^c
	Ethanol	8.39 ± 0.37 ^a	15.74 ± 0.89 ^b	20.50 ± 0.32 ^b	14.47 ± 1.36 ^b
	Chloroform	9.48 ± 0.52 ^b	13.87 ± 0.79 ^a	16.14 ± 0.85 ^a	13.02 ± 0.80 ^a
<i>Dictyota dichotoma</i>	Diethyl ether	5.95 ± 0.48 ^a	17.13 ± 0.83 ^b	5.34 ± 0.21 ^a	8.31 ± 0.13 ^a
	Methanol	14.81 ± 0.90 ^c	20.67 ± 1.35 ^c	12.92 ± 0.79 ^c	17.46 ± 1.15 ^c
	Ethanol	8.09 ± 0.06 ^b	16.38 ± 0.82 ^b	12.26 ± 0.50 ^c	11.60 ± 0.29 ^b
	Chloroform	14.37 ± 0.25 ^c	8.88 ± 0.46 ^a	7.41 ± 0.08 ^b	8.84 ± 0.23 ^a
<i>Padina australis</i>	Diethyl ether	5.63 ± 0.18 ^a	21.51 ± 0.41 ^d	10.88 ± 0.47 ^a	8.13 ± 0.14 ^a
	Methanol	12.47 ± 0.28 ^c	18.86 ± 0.68 ^c	12.06 ± 0.55 ^{ab}	13.01 ± 0.21 ^d
	Ethanol	10.18 ± 0.08 ^b	14.71 ± 0.39 ^b	12.31 ± 0.16 ^b	11.59 ± 0.26 ^b
	Chloroform	14.32 ± 0.24 ^d	12.09 ± 0.22 ^a	11.95 ± 0.51 ^{ab}	12.01 ± 0.19 ^c

Note: the value is the average ± standard deviation, the super script letters behind different numbers in one column show significantly different from each other ($p < 0.050$), the super script letters from a to z indicate having a greater average value.

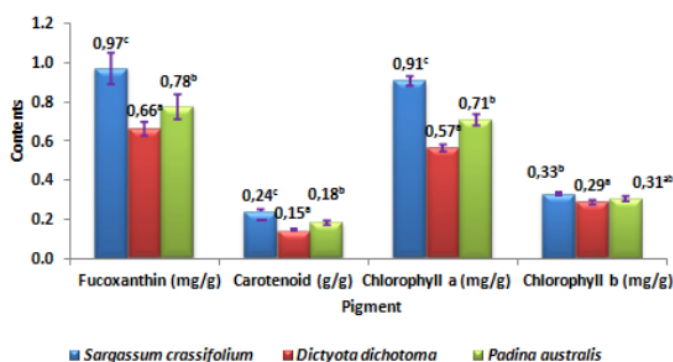


Figure 3. Analysis of fucoxanthin, carotenoids, chlorophyll a and b in seaweed.

4. Discussion

The results of the resistance analysis showed that the antibiotic group which had the greatest antibacterial activity was from the Aminoglycosides group. This class of antibiotics works by inhibiting protein synthesis by interfering with the transfer of peptides to amino acids. Bacterial resistance to Aminoglycosides is the presence of acetylation which makes Aminoglycosides inactive [16]. The majority resistance to Aminoglycosides is due to an enzyme which adds an acetyl group to the antibiotic. Acetylated aminoglycosides are not bound to the bacterial ribosomal 50S subunit therefore they are unable to inhibit the process of protein synthesis of the test organism.

The results of antimicrobial activity tests on extracts showed that the overall extract data which had the lowest antimicrobial activity were extracts of the type *D. dichotoma* and the highest was on *S.*

crassifolium. The difference in zone diameter is assumed that each species has a different morphology, physiology and metabolism. Different types of seaweed have different qualities and quantities of active compounds of secondary metabolites. Factors affecting the size of inhibitory zones are organism sensitivity, compound activity on the extract, culture medium, incubation conditions and agar diffusion rate. The factors which influence the speed of diffusion were the composition of the media, the concentration of microorganisms, incubation time and temperature [17].

Phytochemical analysis shows that *S. crassifolium* extract has the best antibacterial activity. It is suspected that the phytochemical content of *S. crassifolium* is highest when compared to *D. dichotoma* and *P. australis*. [18,19], Sargassum taken from Jepara Beach contains broad spectrum antimicrobial bioactive compounds and antioxidants, such compounds as alkaloids, flavonoids, tannins, triterpenoids, steroids and phenolics. The compounds which play a role in inhibiting bacterial growth are tannin and phenol compounds arranged in polyphenols as well as iodine in *Sargassum* sp. Dried *Sargassum* sp. contained of iodine 0.2-0.5% and 2 grams of dried powder *Sargassum* sp. has a polyphenol content of 4.58% (491.5 mg) [18]. The mechanism of tannin and phenol compounds in inhibiting bacterial cells is by denaturing bacterial cell proteins, inhibiting cell membrane function (transport substances from one cell to another cell) and inhibiting the synthesis of nucleic acids as of bacterial growth can be inhibited [21,22].

Generally flavonoids are in the form of aglycones or bound to sugar as glycosides and are found in plants [23]. Flavonoids are polar because they have a number of sugar groups, therefore flavonoids are generally soluble in polar solvents such as ethanol, methanol, butanol, acetone, dimethyl sulfoxide, dimethyl formamide, water and others. On the contrary less polar aglycons tend to be more soluble in semi-polar solvents such as ethyl acetate, ether and chloroform [24]. Tannin compounds found in plants are bound as salts with plant organic acids [25].

Based on the results of one way anova analysis, the value of chlorophyll a, b, fucoxanthin and carotenoid (mg / g) values was significantly different ($p < 0.05$) between types of seaweed. The highest value of chlorophyll a and b, fucoxanthin and carotenoids ($p < 0.05$) are in *Sargassum crassifolium*. [26] polar fraction of seaweed contains protochlorophyllide as one of the active substances as antibacterial. The structure of protochlorophyllide is a chlorophyll consisting of a porphyrin ring bound by a square structure which is flattened with a magnesium atom in the middle which is bound with nitrogen rings on each side.

5. Conclusions

Brown seaweed *S. crassifolium*, *D. dichotoma* and *P. australis* from Jepara coasts have antibacterial activity MDR of *P. aeruginosa*, *S. epidermidis*, *S. aureus* and *E. coli*. The antibacterial activity is due to the extract contains phytochemical bioactive compounds phenol, flavonoid, tannin, fucoxanthin, carotenoids and chlorophyll a and b. Higher contents of phytochemical and biopigment compounds in the extract give higher antibacterial activity.

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