FULL LENGTH ARTICLE

Antibacterial effect of *Gracilaria verrucosa* bioactive on fish pathogenic bacteria

Maftuch *, Isma Kurniawati, Awaludin Adam, I’ah Zamzami

Dept. Aquaculture, Fisheries and Marine Science Faculty, Brawijaya University, Malang, East Java, Indonesia

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**KEYWORDS**
Antibacterial; *Gracilaria verrucosa*; Flavonoid; Pathogenic bacteria

**Abstract** *Gracilaria verrucosa* seaweed is a type of seaweed commonly found in water. This study was conducted to investigate the effect of *G. verrucosa* on fish pathogenic bacteria to support fish farming. The method used in this research was the separation of *G. verrucosa* fractions using column chromatography. The active antibacterial fraction of *G. verrucosa* which is obtained from column chromatography indicated fractions containing antibacterial compounds. It was fraction number 3 by using an eluent 16 (ethanol): 4 (ethyl acetate). Furthermore, based on phytochemical screening, ultraviolet spectrophotometer and LC–MS analysis, antibacterial compounds contained in those fraction number 3 are Alkaloid, Flavonoid, Tannin, Phenolic compound. Based on LC–MS and UV–Vis analysis, flavonoid group, Quercetin-7-methyl-ether is a dominant group of the antibacterial compound on fraction no. 3. This fraction had moderate antibacterial activity against *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas putida* and had weak antibacterial activity against *Vibrio harveyi* and *Vibrio algynoliticus* bacteria.

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**Introduction**

Some secondary metabolites derived from marine algae plants have the potency to be the new material for pharmacy (Ely et al., 2004). Chemical compounds contained are the groups of polysaccharides, lipids, proteins, alkaloids and phenolic components (Almeida et al., 2011). The antibacterial characteristic which is obtained from plant products has bioactivity and can be widely studied for potential applications in cultivation systems (Reverter et al., 2014).

Castro et al. (2008) found that 31 species of methanol extracts derived from plants in Brazil had antibacterial activity against *Streptococcus agalactiae*, *Flavobacterium columnare*, and *Aeromonas hydrophila* fish pathogenic bacteria. Several recent studies have been revealed that seaweed and algae are potential sources that can be used as antimicrobial products (Al-Saif et al., 2014; Rabia et al., 2013).

Dubber and Harder (2008) showed that the methanol extract *Ceramium rubrum* (10 mg dry weight/mL) and hexane extract *Laminaria digitata* (31 mg dry weight/mL) gave a strong antibacterial activity against 16 bacterial pathogens. Other study conducted by Genovese et al. (2012) on the ethanol extract *Asparagopsis taxiformis* 100 mg/ml towards 9 types of pathogenic bacteria on fish had the best inhibition towards *V. alginolyticus* (17.0 ± 1.4 mm), *Vibrio vulnificus* (16.8

* Corresponding author.
E-mail address: maftuch@ub.ac.id (Maftuch).
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In red algae, Gracilaria verrucosa is the third largest genus of class Rhodophyta. It is widely known that G. verrucosa contains many bioactive compounds with several bioactivities. Maftuch et al. (2012) and Saraswaty et al. (2015) reported that G. verrucosa enhances innate immune of shrimp and inhibit Salmonella typhimurium. There were not many types of research that examined antibacterial activity using in vitro on red algae called G. verrucosa. So in this study, the researchers would provide information about the compound of antibacterial activity contained in red algae G. verrucosa on various kinds of pathogenic bacteria in the water. Gracilaria is one of the genera with the largest number of species in the family Gracillariaceae (Rhodophyta) in tropical area habitat (Freile-Pelegrin and Murano, 2005). In this study, we investigate the antibacterial activity of G. verrucosa along with their antibacterial compounds which were extracted using specific eluent.

The bacterial species tested challenge is a type of pathogenic bacteria that easily invade the freshwater and brackish water fish. So by testing in vitro results of an extract of Gracilaria verrucosa, we get pertinent information about power extract of G. verrucosa against freshwater and brackish water bacteria.

Materials and research method

G. verrucosa seaweed was obtained from cultivation area in Kraton Pasuruan, East Java, Indonesia. Cultivation of seaweed was conducted at fishpond with an area of about 225 Ha. The cultivation methods used are the spread and longline methods on fishpond, while technology uses a traditional farming system with polyculture shrimps, milkfish, and seaweed. The site is located at a latitude of 7° 40’ 42.64” S and longitude 112° 51’ 13.85” E.

The quality of the seaweed must satisfy the standard. Some of the things that affect the quality itself are the age of the plant, how to harvest and the state of the weather at the time of harvest. Seaweed is harvested when the age of plants has reached 1–1.5 months after being planted. Seaweed harvesting is done in the morning to minimize the moisture content. Harvesting is done in November when the water temperature is relatively cold. High content of agar in seaweed is in summer.

However, the condition of the growth of G. verrucosa is different in each region. In areas with relatively cool conditions, G. verrucosa grow at a temperature of 8–21°C, optimum at a temperature of 12–20°C at the beginning of May until mid-June (Ren-Zhi et al., 1984). Extraction was done using Velmurugan et al. (2012) method which had been modified. Extraction was done using maceration method with ethanol solvent during 2 × 24 h, then it was filtered using Whatman paper and evaporated using rotary evaporator.

G. verrucosa fraction separation using column chromatography

Separation fraction was performed using column chromatography (Velmurugan et al., 2012) which has been modified with a stationary phase of silica gel 70–230 mesh and mobile phase of ethanol: ethyl acetate at the ratio of 20:0, 18:2, 16:4, 14:6, 12:8, 10:10, 8:12, 6:14, 4:16, 2:18 and 0:20. Antibacterial activity against A. hydrophila bacteria was tested based on numbers of obtained fractions. The fraction with the best inhibition zone would be characterized and isolated for antibacterial activity against A. hydrophila, Vibrio harveyi, Vibrio algynoticus, P. aeruginosa, Pseudomonas putida bacteria. Characterization of antibacterial compounds was done using phytochemical screening, ultraviolet spectrophotometer and LC–MS (Liquid Chromatography-Mass Spectrophotometer).

Phytochemical analysis

The phytochemical analysis used Nurdiani et al. (2012). It was aimed to observe the type of active compound contained in the fraction. Alkaloids, flavonoids, tannins, phenolic, steroids and saponins were analyzed. The detail of analysis was described elsewhere.

Spectrophotometer ultraviolet

One use of UV–Vis spectrophotometry i.e. can determine the chemical content of a material by measuring transmit or absorbent of a sample as a function of wavelength. UV rays wavelength ranges between 200 and 400 nm. The Rays seem (UV–Vis) to have a wavelength of 400–750 nm. The magnitude of the radiation absorption is proportional to the number of analyte molecules absorption, thus can be used for quantitative analysis.

Wavelength accuracy is done usually by using samples containing a series of very sharp peaks such as aqueous perchloric and holmium oxide. Alternatively, it could use the measurement of the emission of the lamp. In addition, it can be done by measuring the emission from the lamp (Upstone and Seer Green, 2012).

UV–Vis spectrum measurement was performed at a wavelength of 200–800 nm. A total of 1 mg from the best fraction was dissolved in 100 ml of ethanol then it was measured its wavelength. Spectrophotometer used was Shimadzu UV-1601 PC with a medium scan speed and the sampling interval of 0.5 s (Chatterjee et al., 2011).

LC–MS (liquid chromatography–mass spectrophotometer)

LC–MS analysis was done to the best fraction which had the antibacterial characteristic. Isolates and eluent from LC went into the capillary. Then, isolate and eluent were sprayed through Taylor cone and analyzed using mass spectrophotometer (Pitt, 2009).

Antibacterial test

Antibacterial activity was observed using agar diffusion method following the method of Prihanto et al. (2012) and Genovesse et al. (2012) with slight modification. Pure cultures of A. hydrophila, V. harveyi, V. algynoticus, P. aeruginosa, P. putida bacteria were taken from the Laboratory of Microbiology Laboratory in Faculty of Medicine, University of Brawijaya, Malang. Furthermore, the cultures were grown in liquid media Nutrient broth (NB) and incubated at 35°C for 3 h so that it could form the same turbidity with Mc Farland standard.
solution (10^7 cells/ml), then the suspension was cultured using TSA media.

The fraction was dissolved in DMSO 10%, then 10 µl of the fraction was impregnated onto a paper disc and was placed on TSA media contained bacteria. It was incubated for 24 h at a temperature of 30 °C. Zone of inhibition was measured. Inhibition zone with a diameter of > 15 mm, 9–14 mm, < 8 mm indicated strong, moderate, weak antibacterial activity, respectively.

### Result and discussion

Before fractions were tested for several bacteria, they are preliminary tested against *A. hydrophila* bacteria. The result of each fraction of *G. verrucosa* was depicted in Table 1. This result is corroborated by Varier and Chinnadurai (2013). Marine red seaweed is known to have antibacterial activities. Antibacterial activity of *Gelidium acerosum*, *G. verrucosa* and *Hypnea musciformis* had been investigated. It was revealed that all of the red seaweeds exhibited antibacterial activities against Gram-positive *Salmonella paratyphi*, *Enterococcus aerogenes*, *Staphylococcus epidermidis* and *Gram-negative Salmonella typhi* and *Shigella flexneri* bacteria. Their degree of inhibition is dependent on the eluent of extraction. These antimicrobial properties are affected by several factors: the habitats, seaweed cultivation methods, the age of extracted seaweed, the method used for the extraction of the bioactive components and even the season when seaweed is harvested (Karthikadevi et al., 2009).

Different ratio of ethanol and ethyl acetate was used to extract *G. verrucosa* to obtain the best eluents ratio for extracting antibacterial compounds. The ratio of 16:4 for ethanol and ethyl acetate was the best eluent to extract antibacterial bioactive compounds. It gave the largest inhibition zone. Fraction number 3 was further examined in order to determine its bioactive compounds. It gave the largest inhibition zone. Fraction 3 was impregnated onto a paper disc and was placed on TSA media. The result is corroborated by Varier and Chinnadurai (2013). Marine red seaweed is known to have antibacterial activities. Antibacterial activity of *Gelidium acerosum*, *G. verrucosa* and *Hypnea musciformis* had been investigated. It was revealed that all of the red seaweeds exhibited antibacterial activities against Gram-positive *Salmonella paratyphi*, *Enterococcus aerogenes*, *Staphylococcus epidermidis* and *Gram-negative Salmonella typhi* and *Shigella flexneri* bacteria. Their degree of inhibition is dependent on the eluent of extraction. These antimicrobial properties are affected by several factors: the habitats, seaweed cultivation methods, the age of extracted seaweed, the method used for the extraction of the bioactive components and even the season when seaweed is harvested (Karthikadevi et al., 2009).

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The fraction number 3 contains alkaloids, flavonoids, tannins and a phenolic compound. The extraction of *Moringa peregrina* leaf using the polar eluent, then ethyl acetate showed the same extracted compounds. An alkaloid, a flavonoid, a tannin, and terpenoids are detected (Al-Owaisi et al., 2014). In contrast, by using only ethanol as an eluent, tannin in *G. corticata* was not extracted (Balakrishnan et al., 2013). Here we reported that combination of ethanol and ethyl acetate succeeded to recover tannin in their fraction. It seemed that the use of ethyl acetate is critical to recovering tannin.

To further verify the contained compounds, it was continued with other analysis using a spectrophotometer UV and LC–MS. The spectrophotometer UV and LC–MS results can be seen in Fig. 1.

Interpretation of the data on the UV–Vis results is described in Table 3 below:

On the results of the UV–Vis found that the highest absorption is in pic 4 with a wavelength of 257 nm and an absorbance of 2465. According to Harborne (1984), flavonoids have a wavelength of 250–270 nm and 330–350 nm. So from these results, we can conclude that the compounds were detected mostly from the flavonoids. Then proceed with the analysis of the test of LC–MS to confirm what type of flavonoid compounds was detected. LC–MS results are shown in Fig. 2.

The image above shows the profile, LC–MS on the fraction 16:4 *G. verrucosa*; the first graph is the complete scan results of the fraction, whereas, in the second graph is the result of the spectrum that has been detected. LC–MS result showed that the fraction of *G. verrucosa* had a value of m/z 165.50. According to Gardana et al. (2007), a compound that has a value of m/z 165 and a wavelength of 257 nm and 357 nm is Quercetin-7-methyl-ether. Based on LC–MS result, it could be assumed that the type of flavonoid was Quercetin-7-methyl-ether.

Based on the results of the phytochemical screening test, ultraviolet spectrophotometer and LC–MS, interlinked compounds relations were found. One of the positive compounds in the trial was the group of phenolic phytochemical. The test results from using a UV spectrophotometer and LC–MS also found that antibacterial *G. verrucosa* fraction contained flavonoids. Flavonoids are the largest group of natural phenolic compounds and polar compounds because they have some hydroxyl groups (Arum and Supartono, 2012). Quercetin-7-methyl-ether is a derivative of flavonoids compound (Boros et al., 2010). Quercetin is known to have many beneficial effects for health as cardiovascular protection, anti-cancer, anti-allergic, cataract prevention, anti-virus and anti-inflammatory (Coskun et al., 2004). The test results of antibacterial activity in vitro *G. verrucosa* fraction against some bacteria can be seen in Table 4.

<table>
<thead>
<tr>
<th>Table 1</th>
<th><em>G. verrucosa</em> fraction inhibition zone against <em>A. hydrophila</em>.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
<td>Eluent comparison (ethanol: ethyl acetate)</td>
</tr>
<tr>
<td>1</td>
<td>20:0</td>
</tr>
<tr>
<td>2</td>
<td>18:2</td>
</tr>
<tr>
<td>3</td>
<td>16:4</td>
</tr>
<tr>
<td>4</td>
<td>14:6</td>
</tr>
<tr>
<td>5</td>
<td>12:8</td>
</tr>
<tr>
<td>6</td>
<td>10:10</td>
</tr>
<tr>
<td>7</td>
<td>8:12</td>
</tr>
<tr>
<td>8</td>
<td>6:14</td>
</tr>
<tr>
<td>9</td>
<td>4:16</td>
</tr>
<tr>
<td>10</td>
<td>2:18</td>
</tr>
<tr>
<td>11</td>
<td>0:20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Results of phytochemical screening of <em>G. verrucosa</em> fraction 3.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound</td>
<td>Result</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>Positive (+)</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>Positive (+)</td>
</tr>
<tr>
<td>Tannin</td>
<td>Positive (+)</td>
</tr>
<tr>
<td>Phenolic</td>
<td>Positive (+)</td>
</tr>
<tr>
<td>Steroids</td>
<td>Negative (–)</td>
</tr>
<tr>
<td>Saponins</td>
<td>Negative (–)</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>Negative (–)</td>
</tr>
</tbody>
</table>
Table 3 above shows that *G. verrucosa* fraction inhibition zone against some bacteria was started from the smallest; 4.0 mm, 7.7 mm, 8.0 mm, 8.7 mm and 9.0 mm. Inhibition zone with diameter > 15 mm has strong inhibitory activity, diameter 9–14 mm has moderate inhibitory activity and diameter < 8 mm has weak inhibitory activity (Genovese et al., 2012). Based on the value of inhibition zone, it could be seen that the fraction of *G. verrucosa* had weak antibacterial activity against *V. harveyi* and *V. algynoliticus* (brackish water) bacteria and had moderate antibacterial activity against *A. hydrophila*, *Pseudomonas aeruginosa*, *P. putida* (freshwater) bacteria.

Flavonoids are well known and are synthesized from plants to treat microbial infection and many in vitro researches prove the effectiveness of antimicrobial substances against a variety of microorganisms (Dixon et al., 1983). A flavonoid derived from grub hydroxyl on β-rings is more active against microorganisms as compared to group 2-OH. This shows that the target of these compounds is lipophilic compounds through the bacterial membrane (Chabot et al., 1992).

<table>
<thead>
<tr>
<th>No</th>
<th>Bacteria</th>
<th>Inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Aeromonas hydrophila</em></td>
<td>8.7</td>
</tr>
<tr>
<td>2</td>
<td><em>Vibrio harveyi</em></td>
<td>7.7</td>
</tr>
<tr>
<td>3</td>
<td><em>Vibrio algynoliticus</em></td>
<td>4.0</td>
</tr>
<tr>
<td>4</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>9.0</td>
</tr>
<tr>
<td>5</td>
<td><em>Pseudomonas putida</em></td>
<td>8.0</td>
</tr>
</tbody>
</table>

Figure 1  UV–Vis spectra from fraction 16:4 *G. verrucosa*, the x-axis shows wavelength generated by the extract of *G. verrucosa*, the y-axis shows the resulting absorbance absorption.

Table 4 Antibacterial activity of *G. verrucosa* fraction against some fish bacterial pathogens.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Relative abundance</th>
</tr>
</thead>
</table>

Figure 2  LC–MS profile of fraction 16:4 from *G. verrucosa*, the x-axis shows the time, and the y-axis indicate the abundance of compounds indicated.
This process may be due to the ability of those who are capable of dissolved extracellular protein on bacterial cell walls. In addition to this lipophilic flavonoids can also damage the microbial membrane (Tsuchiya et al., 1996). There are three main mechanisms of flavonoid compounds in inhibiting the growth of bacteria. First is the inhibitory nucleic acid synthesis, inhibitory function of the cytoplasmic membrane and inhibition of metabolic energy (Cushnie and Lamb, 2005).

Conclusions

Based on the characterization test using phytochemical screening, ultraviolet spectrophotometer, and LC–MS showed that G. verrucosa fractions contained flavonoids compound, quercetin-7-methyl-ether as an antibacterial. G. verrucosa fraction had moderate antibacterial activity against A. hydrophila, P. aeruginosa, P. putida bacteria and had weak antibacterial activity against V. harveyi and V. algynoliticus bacteria.

Competing interest

The authors declare that they do not have any significance to compete.

Authors contribution

Maf, IK, and AA participated in the research design and workmanship in the laboratory, IZ involved in the preparation of the manuscript. All authors have read and approved the final manuscript.

Authors detail

Faculty of Fisheries and Marine Sciences, University of Brawijaya, Malang, East Java, Indonesia.

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