Application of gallic acid, rutin and quercetin from marine macroalgae \textit{Sargassum} sp. to control \textit{Vibrio parahaemolyticus}

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\textbf{Abstract.} \textit{Sargassum} sp is a type of brown marine macroalgae with bioactive or phytochemical compounds that function as antibacterial. This study aims to detect and isolate bioactive compounds and determine antibacterial activity of flavonoid compounds (rutin and quercetin) and phenols (gallic acid) from \textit{Sargassum} sp to control \textit{Vibrio parahaemolyticus}. The methods were optimizing extraction and fractionation methods of \textit{Sargassum} sp extract, as well as testing efficacy of antibacterial activity of flavonoid and phenol compounds from \textit{Sargassum} sp extract against \textit{V. parahaemolyticus} bacteria by observing the inhibition zone around the paper disc. The experimental design used the ONE-WAY ANOVA test. The results showed that the yield of \textit{Sargassum} sp extract could be obtained optimally by the multi-staged maceration method around 82.56\%, 89.78\%, and 87.78\% with n-hexane, ethanol, and ethyl-acetate solvents, respectively. Fractionation of \textit{Sargassum} sp extract, which was dissolved with ethyl-acetate and ethanol separately to produce isolates of flavonoids (rutin and quercetin), and phenol (gallic acid). Antibacterial activity of the rutin compound from \textit{Sargassum} sp extract has the highest inhibition and can inhibit the growth of \textit{V. parahaemolyticus} bacteria with an inhibition diameter of > 25 mm. \textit{Sargassum} sp extract can be used to control bacteria disease \textit{V. parahaemolyticus} in-vitro.

\section{1 Introduction}

\textit{Sargassum} sp is a type of brown algae that can grow in cold waters and on rocky beaches and widely spread in tropical and subtropical waters [1], including in Indonesia. The genus \textit{Sargassum} also has a high diversity. Its distribution is in the intertidal area, the upper littoral zone to the sublittoral zone, usually in the coastal areas [2]. Live attached to rocks or other hard materials, and some are attached to the seabed using a holdfast [3] and can grow at 0.5-10 m depth [4]. The highest biomass of \textit{Sargassum} sp in May and June was during the dry season with suitable water temperatures of 25-35°C and salinity of 32-33.5‰ [5,6]. The most important parameters affecting the growth of this algae are nutrients, substrate, light quantity, pH, salinity, temperature, and hydrodynamics [7].

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Sargassum sp is a type of brown macroalgae that contains bioactive compounds that function as antibacterial, antioxidant, antiviral, and antifungal. Based on existing research results, it showed that the bioactive compounds from Sargassum sp contain several active compounds such as alginates, steroids, alkaloids, flavonoids, phenols, fucoidans, phlorotannins, fucoxanthins, and triterpenoids. The highest antibacterial activity in seaweed is from the brown algae class Phaeophyta [8]. Several scientific studies on the utilization of Sargassum sp extract and other types as antibacterial agents, among others, for the antibacterial properties of E. coli and S. epidermidis [9], antibacterial of V. parahaemolyticus and V. harveyi [10], antibacterial of E. coli, P. aeruginosa, S. aureus, and B. cereus [11], antibacterial of S. aureus and B. cereus [12]. Exploration usage of Sargassum sp is more in the food and cosmetics sector, while in the fisheries sector, it is generally used to enhance the immune response in fish and shrimp. Therefore, this research aims to screen, isolate bioactive compounds, and determine the antibacterial activity of flavonoid compounds (rutin and quercetin) and phenols (gallic acids) from marine macroalgae Sargassum sp to control bacterial disease of Vibrio parahaemolyticus in vitro.

2 Materials and Method

The materials used were Sargassum sp, ethanol, n-hexane, ethyl-acetate, distilled water, silica gel, Thin-Layer Chromatography (TLC) silica plates, mobile phase solutions (toluene, formic acid, acetic-glacial acid), qualitative reagents (Dragendorff, Wagner, Meyer), filter paper, V. parahaemolyticus bacterial isolates, TCBS, and Mueller-Hinton agar.

Sargassum sp was collected from Anyer beach, Banten Province, rinsed thrice with freshwater, then dried with an oven at 60°C. Dried Sargassum sp was grinded with a grinder until it became a crude powder called simplicia.

The equipment used chromatographic columns (Pyrex, USA), TLC instruments (Camag® Linomat 5, Swiss), immersion chamber (Camag® Twin Trough Chamber, Swiss), and UV-Chamber (Camag® UV Cabinet 4, Swiss).

2.1 Optimization method of extraction of Sargassum sp extract

The experimental design was carried out to obtain the yield value of Sargassum sp extract using a three-replication Completely Randomized Design (CRD). The treatment used the multi-staged maceration method (MSM) and the non-staged maceration method (NSM).

Extraction using the Multi-staged Maceration Method (MSM) was carried out by means of 10 g of Sargassum sp seaweed simplicia powder, dissolved in 100 ml of n-hexane solvent and allowed to stand for 24 hours with occasional stirring (every 6 hours). After 24 hours the residue was separated from the filtrate, and the simplicia residue was macerated again with the same solvent until the extract was clear. The residue and filtrate were again separated, then the simplicia residues were dried in an oven at 50°C. The dried simplicia were macerated again with 100 ml of ethyl acetate for 24 hours. The residue and filtrate were separated, and the simplicia residue was macerated again with ethyl-acetate solvent until they looked clear. The simplicia residues were dried in an oven and then macerated in the final stage using 100 ml of ethanol for 24 hours. The residue and filtrate are separated, and the simplicial residue is macerated with the same solvent until the extract looks clear. In the extraction using the Non-staged Maceration Method (NSM), 10 g of Sargassum sp simplicia was dissolved separately, dissolved in n-hexane, ethyl-acetate, and ethanol, and carried out 3 times replication. The residue and filtrate are separated using filter paper. The extraction results are stored in a Duran bottle. The yield of the extraction results is calculated based on the resulting extraction volume.
The simplicia residues were dried in an oven and then macerated in the final stage using 100 g of \textit{Sargassum sp} extract and other types as antibacterial agents, among others, for the exploration usage of \textit{Sargassum sp} extract. The \textit{Sargassum sp} extract was grinded with a grinder until it became a crude powder called simplicia. The fractionated sample for each \textit{Sargassum sp} extract was in the form of extract solution and dissolved in 100 ml of n-hexane solvent to control bacterial disease of \textit{Vibrio parahemolyticus}.

The bioactive compounds contained in the \textit{Sargassum sp} extract were analyzed qualitatively following the Harborne [14] qualitative test method. The bioactive compounds to be analyzed were alkaloids, flavonoids, saponins, and tannins. Furthermore, the bioactive compounds extracted from \textit{Sargassum sp} were fractionated using the following column chromatography method.

Prepare the chromatography column with a mixture of 50 grams of dry silica and the mobile phase. Then, cover the top of the column with aluminum foil, and let stand for 24 hours.

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Analysis of the yield data used one-way ANOVA, which showed significant differences or not between treatments.

2.2 Optimization method of fractionation of \textit{Sargassum sp} extract

The bioactive compounds contained in the \textit{Sargassum sp} extract were analyzed qualitatively following the Harborne [14] qualitative test method. The bioactive compounds to be analyzed were alkaloids, flavonoids, saponins, and tannins. Furthermore, the bioactive compounds extracted from \textit{Sargassum sp} were fractionated using the following column chromatography method.

\begin{equation}
\text{yield} = \frac{\text{final volume of extract}}{\text{initial volume of extract}} \times 100\%
\end{equation}

2.3 Screening of bioactive compounds using TLC

The fractionated sample for each \textit{Sargassum sp} extract was in the form of extract solution isolates that were taken about 1 ml and placed in a ceramic cup. The isolate was then dried in a fume cupboard. Then each isolate was added with its original solvent, such as ethanol, n-hexane, and ethyl-acetate, each as much as 1 ml. Isolate that has been dissolved and then homogenized evenly.

Silica plate paper with a size of 10 x 20 cm was prepared and given dots with a 10 mm distance. Each isolate that has been dissolved was then taken as much as 1 drop using a pipette to the spotting point that has been made on silica plate paper. After all the isolates were dripped, the silica plate paper was immersed in the mobile phase solution prepared in the immersion chamber. The mobile phase used as the immersion solution is adjusted to the fractionation activity for each type of bioactive compound to be analyzed. Then, after being immersed in the mobile phase solution, the silica plate paper was dried for about 5-10 minutes. Then, the silica plate paper was observed in UV-Camag to see changes in the movement of the spot from the isolate that was dropped at an adjusted wavelength, namely at 254 nm or 366 nm.

Silica plate paper that had been eluted was then analyzed by Thin Layer Chromatography to determine or identify the type of active compound present in \textit{Sargassum sp} isolate based on the standard used. Compound standards used include alkaloids (piperine standards), flavonoids (rutin and quercetin standards), and phenols (gallic acid standards).
2.4 The in-vitro testing of Sargassum sp extract against Vibrio parahaemolyticus pathogenic bacteria

The efficacy of the Sargassum sp extract product, which had been fractionated into several eluates, was tested in-vitro for V. parahaemolyticus pathogenic bacteria that causes white feces disease by observing and measuring the diameter of the inhibition zone formed around the paper disk of the herbal extract.

The tested bacterial isolate, V. parahaemolyticus which had been cultured in TCBS media, was taken as much as 100 µL, and then spread evenly onto Mueller-Hinton Agar media. 5 mm paper disks were prepared by soaking them in fractionated Sargassum sp extract isolates. Leave it for 1 x 24 hours.

Next, tweezers placed the paper disk containing the Sargassum sp extract on the surface of the inoculating medium. Bacteria were incubated for 24 hours at 37°C. The diameter of the inhibition zone formed was measured using a ruler. All processes are carried out aseptically.

3 Results and Discussion

3.1 Optimizing method of extraction of Sargassum sp extract

Table 1 shows that the highest average yield of Sargassum sp extract was obtained by the multi-staged maceration method with ethyl acetate solvent. This was presumably due to the different polarity of each solvent so that it could attract the compounds contained in an extract based on their polarity.

If seen from the results of the ANOVA test on the Sargassum sp extract, the p-value <0.05 was significantly different, meaning that the type of solvent affected the yield value of the Sargassum sp extract. On average, the best solvent in the Sargassum sp extract was ethyl acetate with the multi-stage maceration method. In the Sargassum sp extract, where the p-value <0.05 was significantly different, it means that the type of solvent used affected the yield data of the dissolved Sargassum sp extract.

Table 1. Average yield data of Sargassum sp extract (%) with different extraction methods and solvents.

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>Solvents type</th>
<th>n-hexane</th>
<th>Ethyl-acetate</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Stage Maceration</td>
<td></td>
<td>55.33 ± 7.02</td>
<td>72.33 ± 5.69</td>
<td>78.33 ± 0.58</td>
</tr>
<tr>
<td>Multi-Stage Maceration</td>
<td></td>
<td>82.56 ± 0.96</td>
<td>89.78 ± 0.39</td>
<td>87.78 ± 2.17</td>
</tr>
</tbody>
</table>

Solvent polarity was the most essential factor in producing extract yield data. In addition, the solvent's boiling point can also affect the yield of the extract. According to Maulida & Zulkarnaen [15], non-polar solvents, such as n-hexane, can dissolve non-polar substances. Ethyl-acetate is a semi-polar solvent that dissolves semi-polar molecules on cell walls, whereas ethanol is a polar solvent that dissolves polar chemicals like phenol groups [16,17]. Therefore, ethanol can optimally attract polar chemicals found in the extract of Sargassum sp.

3.2 Optimizing method of fractionation of Sargassum sp extract

Based on Table 2, the results of the fractionation of the bioactive compounds of the Sargassum sp extract produced several isolates of the active compounds either by non-staged
maceration or multi-staged maceration methods. In addition, the optimal mobile phase formulation for each bioactive compound was also obtained.

**Table 2.** The number of isolates of the active compound of *Sargassum* sp herbal extract from a result of column chromatography fractionation

| Compounds | Non-staged Maceration | | Multi-staged Maceration | |
|---|---|---|---|
| | n-hexane | ethyl acetate | ethanol | n-hexane | ethyl acetate | ethanol |
| Alkaloid | | | | | | |
| Piperin | x | x | 4 isolates | x | x | 5 isolates |
| Flavonoid | | | | | | |
| Rutin | x | 6 isolates | 2 isolates | x | 6 isolates | 1 isolate |
| Quercetin | x | 3 isolates | x | x | 3 isolates | x |
| Fenol | | | | | | |
| Gallic acid | x | x | 1 isolate | x | x | 3 isolates |

Qualitatively, the extract of *Sargassum* sp produces active compounds (phytochemicals), alkaloids, flavonoids, and phenols (gallic acid). In general, *Sargassum* sp was known to have bioactive compounds such as alkaloids, flavonoids, phenols, and tannins, but in this activity, tannins were not detected. The method was not suitable during the extraction process, so the tannin compounds evaporated quickly or were not bound, as it was known that this tannin compound was a polyphenolic compound that evaporated very quickly.

The production of a white precipitate indicates the presence of an alkaloid in the Mayer test. It was assumed that the precipitate was a potassium-alkaloid combination. A solution of mercury (II) chloride and potassium iodide reacts to generate a crimson precipitate of mercury (II) iodide during the production of Mayer's reagent. If too much potassium iodide is added, potassium tetraiodomercurate (II) is produced [18]. Alkaloids, composed of nitrogen atoms with lone pairs of electrons, can form coordinate covalent bonds with metal ions [19]. The nitrogen in the alkaloids was expected to react with the K+ metal ion from potassium tetraiodomercurate (II) to generate a precipitated potassium-alkaloid complex in the alkaloid test using Mayer's reagent.

The production of a light brown to yellow precipitate in the Wagner test showed a positive alkaloid result. These precipitates were thought to be potassium-alkaloids. Iodine combines with I- ions from potassium iodide to form brown I3+ ions in the manufacture of Wagner's reagent. K+ metal ions will coordinate covalent connections with nitrogen in the alkaloids to generate a precipitated potassium-alkaloid complex in the Wagner test.

The production of a light brown to yellow precipitate suggested that the alkaloids performed well in the Dragendorff test. A potassium alkaloid precipitated. Bismuth nitrate was dissolved in HCl during the production of Dragendorff reagent to avoid hydrolysis reactions from occurring because bismuth salts were easily hydrolyzed to create bismuthyl ion (BiO3-). Additionally, Bi3+ ions from bismuth nitrate react with potassium iodide to generate a dark precipitate of Bismuth (III) iodide, which dissolves in excess potassium iodide to form potassium tetraiodobismutat [18]. Nitrogen was utilized in the alkaloid test with Dragendorff reagent to establish coordinate covalent bonds with K+, which was a metal ion.

In the flavonoid test, an orange color was obtained from the reaction with the betel leaf and *Sargassum* sp liquid extract. Flavone compounds provide red to orange colors, flavonols
or flavanones produce dark red hues, and aglycones or glycosides produce green to blue colors. Furthermore, the ensuing orange color might be generated by the production of flavylum salts [20].

The tannin test yielded positive results for betel leaf extract but negative results for Sargassum sp extract, and tannins would precipitate protein in the gelatin. Tannins combine with gelatin to generate a stable copolymer that is not water soluble. [14]. This reaction becomes more sensitive when NaCl is added to increase tannin-gelatin salting.

The test was continued by fractionating Sargassum sp using column chromatography. The results of the fractionation of the Sargassum sp extract were collected into several isolates, which were separated by a gradient according to the color of the solution. Bioactive compounds isolated from Sargassum sp extract were identified by reading silica gel plates on Thin Layer Chromatography. The phytochemical standards analyzed included piperine standards (alkaloids), rutin standards (flavonoids), quercetin standards (flavonoids), and gallic acid standards (phenols). These isolates were tested regarding existing standards. Observations of the elution results of herbal extract isolates were observed in TLC with a wavelength of 254 nm and 366 nm.

The identification results of the bioactive compounds extracted from Sargassum sp, which were fractionated in isolated solutions and confirmed with the available standard compounds, are shown in Table 3. It was found that 9 isolates confirmed the piperine compound (alkaloid) contained in the Sargassum sp extract. For routine compounds (flavonoids), 15 isolates of Sargassum sp extract. For quercetin compounds (flavonoids), 6 isolates of Sargassum sp extract. For the gallic acid (phenol) compound, Sargassum sp extract obtained as many as 6 confirmed isolates.

As for obtaining the optimal mobile phase for each Sargassum sp extract bioactive compound, it was necessary to have a type of developer solvent according to the standard characteristics used. After carrying out several stages of TLC testing, it was obtained that the developer solvent in the mobile phase was optimal for isolates from Sargassum sp extract so that the TLC readings could be seen that the isolate had a rf value equivalent to the rf of the standard used. The following is the developer solvent used in the analysis of bioactive compounds in the isolated extract of Sargassum sp.

1. Quercetin (flavonoid) = Toluene : Ethyl-Acetate : Formic acid (5 : 4 : 0.2)
2. Rutin (flavonoid) = Ethyl-Acetate : Acetate Glacial Acid : Formic Acid : Distilled water (10 : 1,1 : 1,1 : 2,8)

Using the right developer solvent in the elution process can produce good separation of bioactive compounds. The polarity level of the developer solvent must match the polarity level of the bioactive compounds being measured so that when elution was carried out on the silica plate, good separation could be seen between the standard bioactive compounds and the isolated bioactive compounds.

### 3.3 Antibacterial activity of flavonoid compounds (rutin and quercetin) and phenol from Sargassum sp extract against Vibrio parahaemolyticus bacteria in vitro

The average inhibition diameter obtained was moderate to very strong, from 7.25 to 26.75 mm (Table 3). The diameter of the inhibition zone < 5 mm is in the weak category, the diameter of 5-10 mm is in the medium category, the diameter of 10-20 mm is in the strong category and the diameter > 20 mm is in the very strong category [21]. Based on the results in Table 3, the active compound rutin obtained from Sargassum sp extract produced the best inhibition diameter against V. parahaemolyticus bacteria, which was 26.75 mm. The
compound rutin (polar) was sensitive to the *V. parahaemolyticus* so that it produced a large enough inhibition zone. The flavonoid (rutin) extract of *Sargassum* sp could diffuse into the media to produce the largest inhibition zone.

**Table 3. Diameter of inhibition (mm) of *Sargassum* sp extract against *V. parahaemolyticus* bacteria**

<table>
<thead>
<tr>
<th>Code</th>
<th>Sample</th>
<th>Inhibition Diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Piperin (<em>Sargassum</em> sp, ET1, MB)</td>
<td>9.75 ± 0.96</td>
</tr>
<tr>
<td>A2</td>
<td>Piperin (<em>Sargassum</em> sp, ET5, MT)</td>
<td>10.75 ± 0.96</td>
</tr>
<tr>
<td>B1</td>
<td>Rutin (<em>Sargassum</em> sp, EA4, MB)</td>
<td>26.25 ± 2.63</td>
</tr>
<tr>
<td>B2</td>
<td>Rutin (<em>Sargassum</em> sp, EA6, MT)</td>
<td>26.75 ± 2.22</td>
</tr>
<tr>
<td>C1</td>
<td>Quercetin (<em>Sargassum</em> sp, EA2, MB)</td>
<td>12.25 ± 1.26</td>
</tr>
<tr>
<td>C2</td>
<td>Quercetin (<em>Sargassum</em> sp, EA3, MT)</td>
<td>14.00 ± 0.82</td>
</tr>
<tr>
<td>D1</td>
<td>Gallic acid (<em>Sargassum</em> sp, ET2, MB)</td>
<td>15.00 ± 0.82</td>
</tr>
<tr>
<td>D2</td>
<td>Gallic acid (<em>Sargassum</em> sp, ET3, MT)</td>
<td>14.00 ± 0.82</td>
</tr>
</tbody>
</table>

Rutin compounds are a class of flavonoid compounds. The content of flavonoids in *Sargassum* sp extract can act as an antibacterial. Flavonoids, as antibacterials, can form complex compounds with extracellular proteins. This causes the layer on the cell wall not to form intact. Damage to the bacterial cell wall causes changes in the permeability of the cell membrane, thereby inhibiting the work of intracellular enzymes and water entering the cell in an uncontrolled manner [22]. This causes the cell wall to lysis finally, and the bacteria die.

The quercetin compound has good antibacterial activity due to the presence of a phenol group with a working mechanism of coagulating proteins by deactivating enzymes and disrupting cell walls. It has good bactericidal properties [23]. Meanwhile, quercetin has antibacterial activity because it can denature cell proteins and bacterial cell membranes [23]. This mechanism was related to the formation of complex bonds with proteins in the membrane (phenol-proteins), causing the permeability to decrease. The complex bonds that have been formed then decompose and penetrate the cell, resulting in protein coagulation and causing bacterial enzymes to be inactive. As a result, the bacterial cell wall is not formed properly, resulting in cell leakage, and the bacteria die.

Bioactive compounds from *Sargassum* sp extract can inhibit the growth of Gram negative bacteria such as *V. parahaemolyticus*. Because of the cell wall of Gram-negative bacteria contains a thin peptidoglycan layer, an outer membrane consisting of proteins, lipoproteins, phospholipids, lipopolysaccharides, and an inner membrane. In addition, the cell walls of Gram-negative bacteria contain polysaccharides and are more susceptible to mechanical and chemical damage [24].

Several factors determine a chemical’s capacity to be antibacterial and antioxidant, including the quantity of phenol groups, the position of the -OH group in the compound, and the presence of other functional groups [25]. In addition, the presence of polyphenols in *Sargassum* sp extract also affects antioxidant activity. Polyphenols were compounds produced by seaweed that were used to protect themselves from sunlight [26]. Phenol is a molecule with a hydroxy group that can transfer its hydrogen to be stabilized by resonance in the phenolic structure, allowing it to operate as an antibacterial and antioxidant [27].
4 Conclusion

The yield of Sargassum sp extract can be obtained optimally by the multi-staged maceration method at around 82.56%, 89.78%, and 87.78% with n-hexane, ethanol, and ethyl-acetate solvents, respectively. Fractionation of Sargassum sp extract which was dissolved with ethyl-acetate and ethanol separately to produce isolates of flavonoids (rutin and quercetin), and phenol (gallic acid). The antibacterial activity of the rutin compound from Sargassum sp extract has the highest inhibition and can inhibit the growth of V. parahaemolyticus bacteria with an inhibition diameter of > 25 mm.

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