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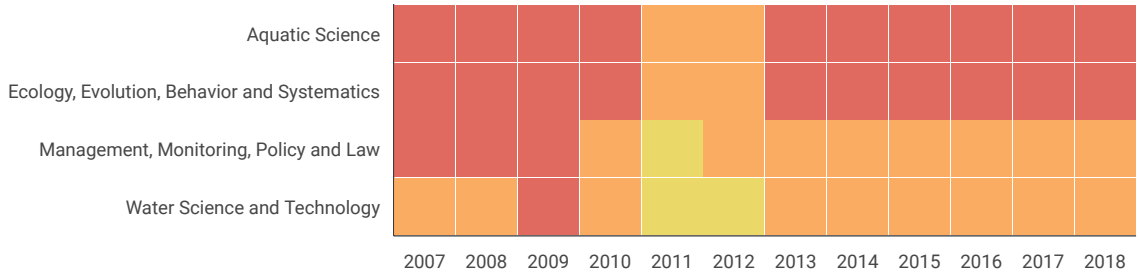

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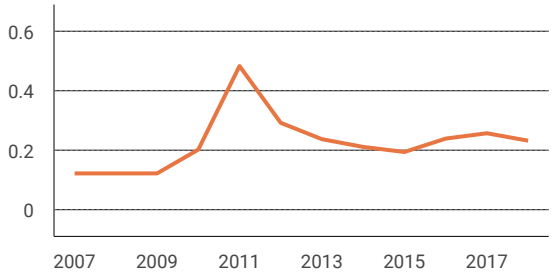

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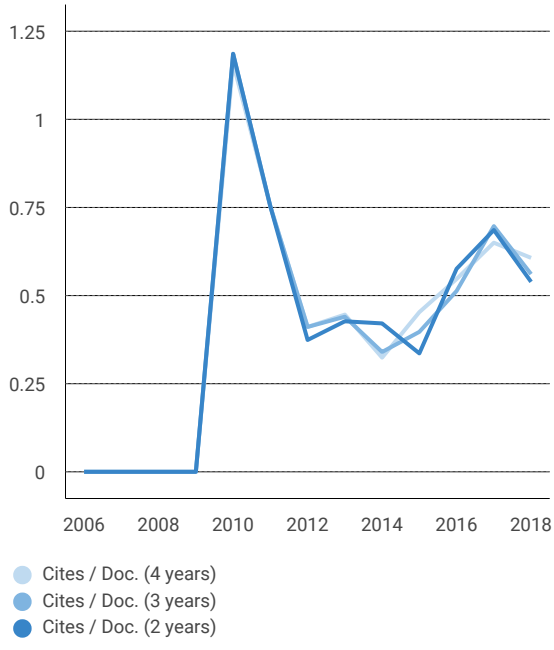

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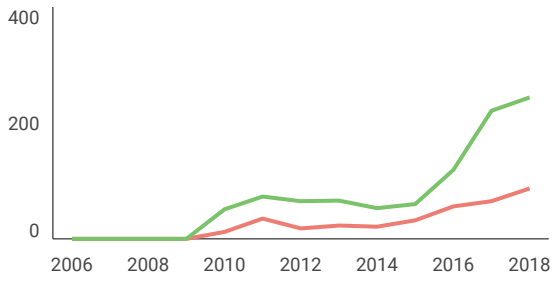

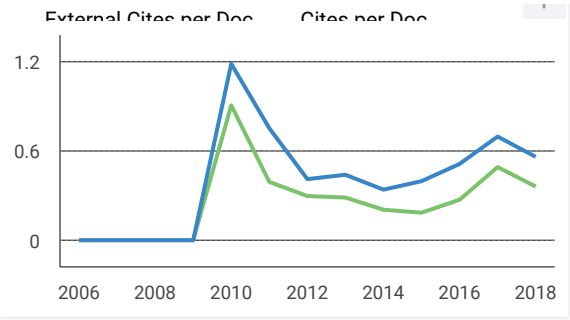

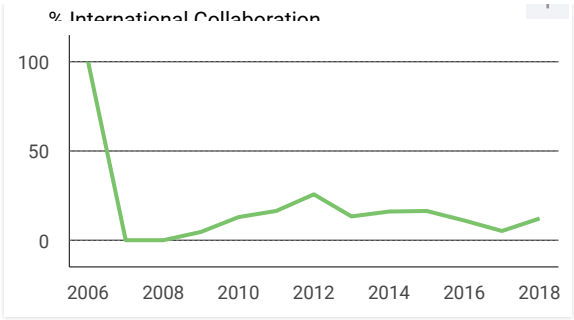

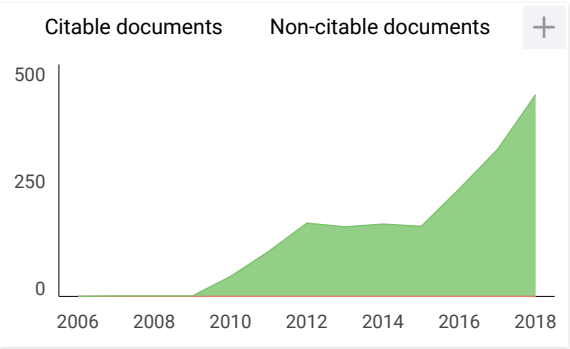

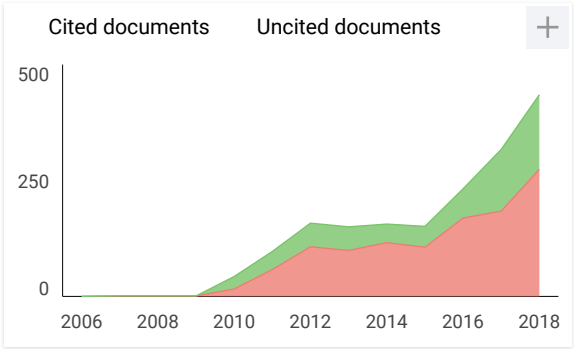

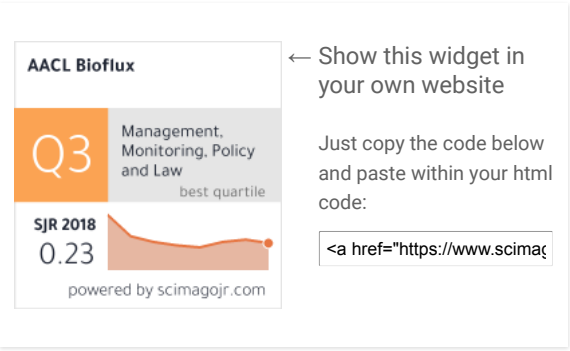

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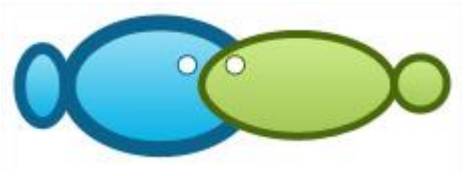

The inhibition of *Vibrio alginolyticus* by the flavonoid extract of *Sonneratia alba* fruit

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Abstract. This study aimed at investigating the antibacterial activity of flavonoids extracted from the fruit of *Sonneratia alba* towards *Vibrio alginolyticus.* Positive control (chloramphenicol) and negative control (distilled water) were applied as comparator parameters. This research applied complete randomized design through 2 repetitions. Treatments for the determination of the minimum inhibitory concentration of flavonoid extracts had concentrations of 62.5, 125, 250 and 500 ppm. Treatments concentration for the determination of the flavonoid inhibition zone diameter had 250, 375, and 500 ppm. Data were analyzed using the Anova and the Duncan test. Findings revealed that the minimum inhibitory concentration value is at 250 ppm, as shown by the clean appearance of the *V. alginolyticus* inoculums suspension. The Duncan test revealed that 250, 375, and 500 ppm concentrations are able to develop significantly different inhibition activities towards *V. alginolyticus*. The inhibition zone for each concentration are 8.07, 9.23, and 10.33 mm, respectively. It was found that a concentration level of 500 ppm has an antibacterial potential against *V. alginolyticus*, which is one of the pathogens causing the ice-ice disease on *Kappaphycus alvarezii*.
Key Words: antibacterial activity, minimum inhibitory concentration, inhibition zone, ice-ice disease.

Introduction. *Sonneratia alba* is one of the mangrove species found in Gorontalo and well-known as "Tamindao" by the people of Gorontalo Utara. This species is mostly found in the village of Katialada, sub-district of Kwandang, District of Gorontalo Utara. Based on data from the Gorontalo Statistics Center, in 2016 the mangrove area in the District of North Gorontalo was 2,886 ha (GSC 2016). Normally, the fruit of *S. alba* is consumed as food, such as *rujak*, and is processed into flour, used as a basic ingredient of other processed food products. Besides, *S. alba* has also the potential to be a natural antibacterial. According to Lakoro (2017), the *S. alba* fruits contain antibacterial compounds (flavonoids) in a greater concentration than in its leaves and stem bark: 6.86 µg/0.5 g in the fruit, 6.20 µg/0.5 g in the leave, and 3.91 µg/0.5 g in the stem bark. The amount of flavonoids in the *S. alba* fruit is due to its secretion as secondary metabolite, by the self-defense mechanism of the fruit, face to aggressions from predators such as insects, cave bats, birds, and caterpillars.

The use of antibacterial compounds, such as flavonoid extracted from herbal plants, helps preventing diseases caused by humans, animals, and plants bacterial contamination, while avoiding or decreasing the use of synthetic antibiotic compounds, which creates bacterial resistance. The continuously increasing use of synthetic antibiotic and the lack of knowledge about the safe dosage will cause new issues, such as the resistance to bacterial. In the current study, the inhibition ability of flavonoid compounds, extracted from *S. alba,* was tested on *Vibrio alginolyticus* colonies growing on ice-ice disease contaminated seaweeds. The ice-ice disease epidemic has been found in cultivation locations of the *Kappaphycus alvarezii* seaweed, in the village of Tihengo, sub-district of Ponelo, district of Gorontalo Utara, in September 2016. The pathogens identification revealed that the *V. alginolyticus* bacterium was at the origin of seaweed contamination with the ice-ice disease.

V. alginolyticus is a bar form gram-negative marine bacterium moving with flagellum (Austin & Austin 1999). It is categorized as an opportunistic bacterium living in sea or brackish water, in particular in stationary waters, enriched with organic material. This bacterial can grow up optimally temperatures ranging between 30-35°C (Reskika 2011). *V. alginolyticus* acted as death cause on fish and other marine biota in up to 80-90% contamination cases, causing zoonosis in fishery products (Achmad et al 2016). Up to date, information has not yet been provided on *S. alba* extracted flavonoids' inhibition potential on the *V. alginolyticus* causing ice-ice disease. This study aims to provide information and scientifically prove the possibility to develop new alternative antibiotics from *S. alba* mangrove.

Material and Method

Description of the study sites. This research was conducted from March to April 2017 in the Biology Laboratory of the Faculty of Mathematics and Natural Science, State University of Gorontalo. The tools used during the research activities were: analytical balance, autoclave, incubator, hotplate stirrer, rotary shaker, spectrophotometer, UV-Vis, laminar flow, Bunsen, Erlenmeyer, test tube, measuring cup, beaker, Petri dish, stirring rod, micropipette, forceps, inoculating loop, and digital caliper. The materials were: concentrated extract of *S. alba*, tested bacteria (*V. alginolyticus*), nutrient broth (NB) media, nutrient agar (NA) media, Mueller Hinton agar (MHA) media, distilled water, antibiotic (Chloramphenicol), alcohol 70%, disc paper, label paper, aluminum foil and tissue.

This research was conducted in three steps, namely: the preparation of flavonoid extract, the preparation of *V. alginolyticus* stock and the antibacterial activity test through two methods: dilution method for minimum inhibitory concentration (MIC) and diffusion method for inhibition zone diameter (IZD).

Preparation for flavonoid extract. This research used an amount of 3 g of flavonoid compounds from the fruit of *S. alba* with a flavonoid content of 6.68 µg/0.5 g. The stock of flavonoid extract was obtained as in Sulistijowati (2017), in the form of concentrated extract with 1 month storage duration at room temperature. The stock of flavonoid extract was used in minimum inhibitory concentration and inhibition zone diameter tests.

Preparation for stock of *Vibrio alginolyticus*

Sterilization of tools and materials. The glassware was sterilized using an oven so the equipment would not to be contaminated by undesirable microbes or bacteria (Cappucino & Sherman 2001). The following tools were wrapped by aluminum foil: beaker, stirring rod, micropipette, forceps, knife, spatula, and inoculating loop. Erlenmeyer and measuring cup were closed down by cotton, and then wrapped by aluminum foil. Further, a Petri dish was wrapped by paper. Finally, after all the tools were ready, they were introduced into an oven with a temperature of 100°C. However, NA, NB, and MHA media were sterilized in autoclave at a temperature of 121°C, 1 atm for 15-30 minutes.

The preparation of media and rejuvenation of Vibrio alginolyticus. 1.26 g nutrient agar (NA) was inserted in an Erlenmeyer and there were added 45 mL of distilled water. Then, it was placed on hot plate stirrer at a temperature of 100°C and stirred until it boiled. Afterwards the media was cooled down, 9 mL were extracted and distributed in each of the five beakers and sterilized in autoclave at a temperature of 121°C for around 15-30 minutes. The sterilized media were cooled down first in laminar flow in inclined position till it became compact. Finally, the cultured *V. alginolyticus* was inoculated in 2 continuous inoculations in inclined surface, then incubated for 24 hours at a temperature of about 37°C.

The preparation of media and suspension of Vibrio alginolyticus. 0.4 g nutrient broth (NB) liquid was inserted in an Erlenmeyer and there were added 50 mL of distilled

water. Then, it was placed on hot plate stirrer at a temperature of 100°C and stirred till it boiled. After that, the medium was sterilized in the autoclave at a temperature of 121°C for around 15-30 minutes. The sterilized medium was first cooled down in a laminar flow. Finally, the rejuvenated *V. alginolyticus* was inoculated in the media in 1-2 inoculations, then it was cultured using a rotary shaker during 48 hours (2 days) at a temperature of about 37°C with an agitation speed of 160 rpm (Suduri 2017).

After being cultured, the optical density value on bacterial suspension was measured using an UV-Vis spectrophotometer by pouring 0.5 mL bacterial suspension in a cuvette, followed by the addition of 1.5 mL distilled water. Then, the cuvette filled with the bacterial suspension was placed in the UV-Vis spectrophotometer and its optical density was measured at a wave length of 580 nm before treatment and at 0.600 after treatment. The value was used as standard optical density of the tested bacterial suspension.

Antibacterial activity of the flavonoid extract. The antibacterial activity test can be done using two methods, dilution and diffusion. The dilution method is used to determine the minimum inhibitory concentration (MIC) of antibacterial substance. According to Sulistijowati et al (2015), the minimum inhibitory concentration test is conducted to investigate the minimum concentration of an antibacterial substance that still has inhibition activity against the tested bacteria, while the diffusion method through agar diffusion is applied to understand antibacterial activity by observing the inhibition zone diameter (IZD) formed around the disk plate.

Minimum inhibitory concentration (MIC) of the flavonoid extract. MIC test in this research was conducted by preparing variable concentrations of flavonoid extract solutions from the fruit of *S. alba,* starting by a 5,000 ppm stock solution of flavonoid extract, where 0.25 g of flavonoid extract was dissolved with 50 mL distilled water, then it was vortexed. The 5,000 ppm stock solution of flavonoid extract was diluted successively at a concentration of 500, 250, 125, and 62.5 ppm.

The flavonoid extract at the above mentioned concentrations was applied according to the indications of Rahmadani (2015), stating that an extract has an antibacterial potential if at a concentration ≤1,000 µg/mL (ppm) it is able to inhibit bacteria's growth (Rahmadani 2015). Minimum inhibitory concentration of the flavonoid extract from the fruit of *S. alba* was determined for each extract concentration, by pouring 2 mL of each in a beaker already containing 2.5 mL nutrient broth media and by adding 0.5 mL *V. alginolyticus* suspension with optical density standard value of 0.600. The mixture was vortexed till it was homogeneous, and then incubated at a temperature of about 37°C during 24 hours. After incubation, for each concentration, the solution's turbidity was observed visually.

The MIC was determined by observing the lowest concentration of flavonoid extract solution that can inhibit the *V. alginolyticus,* marked by visual limpidity. However, to further prove the MIC value, the bacterial suspension's optical density value was measured for each concentration of the tested extract, by using a UV-Vis spectrophotometer. It was referred to what Rahmadani (2015) stated, namely that the MIC value can be also determined by measuring the optical density value with the UV-Vis spectrophotometer.

Inhibition zone diameter (IZD) of the flavonoid extract. The antibacterial activity test was undertaken using the agar diffusion method referred by Rahmadani (2015). The first step was preparing a Mueller Hinton agar media and a flavonoid extract at a concentration according to the MIC test results. The minimum concentration obtained from the MIC result is 250 ppm. Therefore, the applied concentrations of the flavonoid extract from the fruit of *S. alba* for the inhibition zone diameter determination were successively 250, 375 and 500 ppm. After that, paper disk was submerged for 30 minutes and placed on the agar media's surface containing bacterial suspension. Subsequently, it was incubated during 24 hours at a temperature of about 37^0C and observed. Finally, the inhibition zone diameter was measured using a digital caliper.

Experimental design and data analysis. The main research topic, which is the antibacterial activity test of flavonoid extract through the MIC dilution method, was analyzed descriptively. The inhibition zone diameter test through the agar diffusion method was analyzed using a complete randomized design with 2 repetitions. The applied treatment consisted of different concentrations of flavonoid extract from the MIC test.

The data obtained from the inhibition zone diameter test was analyzed by applying one-way ANOVA. Then, in order to find out the significance of the differences for each treatment concentration in inhibiting *V. alginolyticus*, the Duncan test was applied.

Discussion

Minimum inhibitory concentration of flavonoid extract of Sonneratia alba fruit. Based on Table 1, after a 24 hours incubation, at a temperature of about 37^0C and at concentrations of 62.5 and 125 ppm, the visually turbid suspensions showed that the two concentrations did not have any inhibition activity on *V. alginolyticus.* At the contrary, at concentrations of 250 and 500 ppm the flavonoid extract had an inhibition activity on *V. alginolyticus*.

Table 1

Result of MIC test on flavonoid extract of fruit of *Sonneratia alba* against *Vibrio alginolyticus*

Treatment of flavonoid extract concentration	*MIC test result*
500 ppm	-
250 ppm	-
125 ppm	+
62.5 ppm	+

-: clean (it gives inhibition activity); +: turbid (it does not give any inhibition activity).

The result of the MIC test in Table 1 showed that inhibition increases with the concentration, as suggested by the turbidity decrease with the concentration increase. Pelczar et al (1986) previously stated that the antibacterial capacity increases with the antibacterial concentration increase.

After the MIC value was visually investigated, the optical density value of each concentration was measured using a UV-Vis spectrophotometer at a wave length of 580 nm and compared with inoculum solutions before treatment.

Table 2

Optical density value measurement result of each flavonoid extract concentration of fruit of *Sonneratia alba*

Treatment of flavonoid extract solution concentration (ppm)	*Result of optical density value measurement of each flavonoid extract concentration*	
	Inoculums solution before treatment	*Inoculums solution after treatment*
62.5	0.600	0.553
125	0.600	0.508
250	0.600	0.453
500	0.600	0.384

Based on Table 2, the measured optical density for each suspension's concentration after treatment's minimum inhibitory concentration which starts preventing the growth of *V. alginolyticus* is 250 ppm, fact confirmed by an optical density value lower than for concentrations of 125 ppm and 62.5 ppm.

As shown in Table 2, the optical density for a 62.5 ppm concentration is 0.553 and for a 125 ppm concentration it is 0.508, values close to the optical density of inoculums

solution before treatment (0.600), suggesting a lack of inhibition activity on the *V. alginolyticus* in the extract with 62.5 ppm and 125 ppm concentrations. Conversely, optical density for a 250 ppm concentration (0.453) is quite different from the optical density of inoculums solution before treatment (0.600). It indicates that a 250 ppm concentration has an inhibition activity on the growth of *V. alginolyticus*.

Inhibition zone diameter of fruit of Sonneratia alba flavonoid extract. The test result of the inhibition zone diameter for each concentration of flavonoid extract towards *V. alginolyticus* is presented in Table 3.

Table 3

Test result of inhibition zone diameter (mm) for concentration of fruit of *Sonneratia alba* flavonoid extract towards *Vibrio alginolyticus*

Concentration of flavonoid extract solution (ppm)	*Average inhibition zone diameter (mm)*
250	8.07
375	9.23
500	10.33

Based on the inhibition zone diameter presented in Table 3, it has been shown that each concentration of fruit of *S. alba* flavonoid extract has antibacterial activity against *V. alginolyticus*, a fact spotted by the existence of a transparent, clear zone around the paper disk. Findings presented in Table 3 revealed that the average inhibition zone diameters of flavonoid extract are: 8.07 mm for the 250 ppm concentration, 9.23 mm for the 375 ppm concentration and 10.33 mm for the 500 ppm concentration.

The Table 3 reveals that 250 ppm, 375 ppm, and 500 ppm concentrations have different inhibition zone diameters, with a peak of the inhibition zone diameter at the concentration of 500 ppm, which suggests a strong antibacterial activity. 375 ppm and 250 ppm concentrations are in the moderate category of antibacterial inhibitory activity, producing inhibition zones of 9.23 mm and 8.07 mm, respectively.

Results are in line with the statements of Rastina et al (2015), according to whom, if the inhibition zone formed at agar diffusion test is less than 5, the inhibition activity is absent, while a 5-10 mm inhibition zone is categorized as moderate, a 10-19 mm inhibition zone is categorized as strong, and a 20 mm inhibition zone is categorized as very strong. According to Saikhia & Upadhaya (2011), flavonoid compounds have the potential to be antibiotic and antibacterial, due to their antioxidant activity. The antibacterial flavonoid from the fruit of *S. alba* is an active compound disrupting the synthesis of bacteria's cell wall and eventually destroying the bacterial plasma by cell lysis. Besides, flavonoids inhibit DNA gyrase, an ATPase enzyme involved in the bacterial energy mechanism (Chusnie 2005).

According to Kurniaji (2019), the antibacterial flavonoid compounds', work mechanism in inhibiting the growth of bacteria is performed by destroying their cell wall, causing lysis or the inhibition of cell wall synthesis and the change of permeability of cytoplasm membrane, with loss of nutrients. The protein denaturation in cell and the alteration of the metabolic system in cell, due to the presence of the antibacterial compound in the work system of intracellular enzyme, influence the bacteria DNA replication process.

Based on statistical analysis, it was found that the flavonoid extract of fruit of *S. alba* influences the growth of *V. alginolyticus.* Results analysis using ANOVA, aiming at testing the hypothesis of the influence of the flavonoid extracted from the fruit of *S. alba* on the culture of *V. alginolyticus,* revealed a statistical significance for p=0.004. After investigating the influence of the flavonoid extract of the fruit of *S. alba* on the culture of *V. alginolyticus*, a Duncan test was processed to determine the significant difference between the concentrations of the flavonoid extract.

Table 4

The Duncan test result on each flavonoid extract concentration of fruit of *Sonneratia alba* towards the growth of *Vibrio alginolyticus*

Concentration of flavonoid extract solution (ppm)	*Average inhibition zone diameter (mm)*
250	8.07[a]
375	9.23[b]
500	10.33[c]

The analysis results, showed in Table 4, revealed that each concentration of flavonoid treatment had a significantly different inhibition effect. Treatment A (250 ppm) had a significant difference compared with treatment B (375 ppm) and C (500 ppm). Treatment B (375 ppm) had a significant difference compared with treatment C (500 ppm). Treatment C (500 ppm) had the most significant difference among others. These results are in line with the statement of Ncube et al (2008), according to whom the antimicrobial activity of a chemical compound is determined by the concentrations and characteristics of the material used.

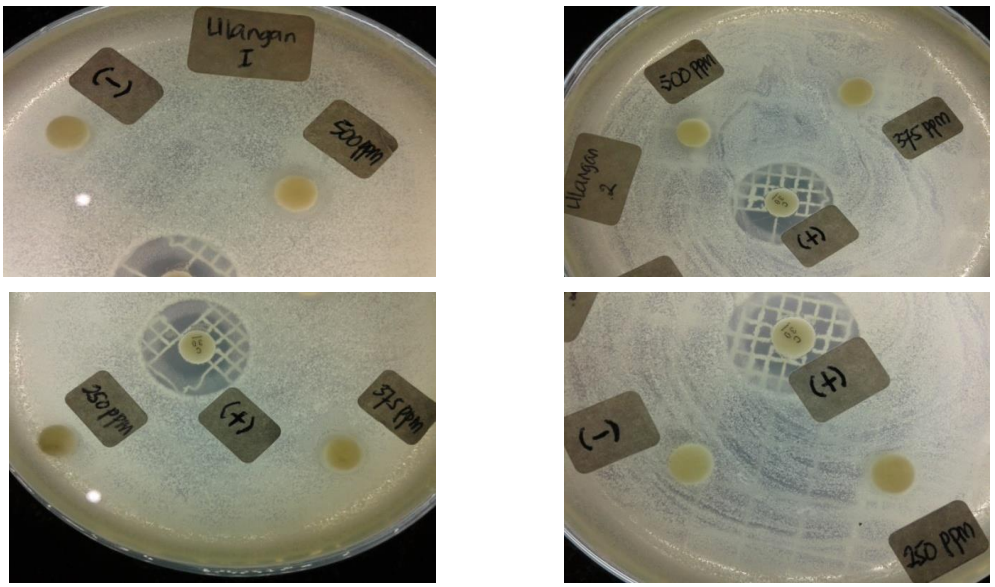

Figure 1. Inhibition zone of *Vibrio alginolyticus* (original).

The difference of the inhibition zone diameters for each treatment revealed a variability in the bacterium sensitivity to the flavonoid extract concentrations. This can be explained as the inhibition activity for concentrations between 375 pm and 250 ppm is categorized as moderate, the *V. alginolyticus* is less sensitive to the antibacterial.

V. alginolyticus, as gram-negative marine bacterium, has a complex multilayered structure, consisting of a cytoplasm membrane, a single layer of peptidoglycan, and an outer membrane, containing lipoprotein and lipopolysaccharide, which also consists of a protein molecule called porin. Porin from the outer membrane of gram-negative bacteria's cell wall is hydrophilic (Candrasari et al 2012). The difference of structures and components of the cell wall causes the gram-negatives developing more resistance (Brooks et al 2007).

Based on the determinations result for the inhibition zone diameter and statistical analysis test, the most effective flavonoid extract in inhibiting *V. alginolyticus* had a concentration of 500 ppm, producing an average inhibition zone diameter of 10.33 mm, which placed the concentration in the strong inhibition category, as antibacterial for *V. alginolyticus* bacterium, identified as the cause of the ice-ice disease in seaweed (*K. alvarezii*).

Based on the findings of Rahmadani (2015), 500 µg/mL (ppm) of 96% ethanol extract of java wood's stem bark containing flavonoids have a moderate inhibition category on *E. coli*, *Helicobacterium pylori* and *Pseudomonas aeruginosa*, with inhibition zones of 8.5 mm, 8.2 mm and 8.5 mm, respectively.

Conclusions. Based on the current study's findings, it can be concluded that the flavonoid extract of *S. alba* is able to inhibit the growth of *V. alginolyticus.* The inhibition activity of a flavonoid extract at a concentration of 500 ppm is categorized as having the strongest inhibition, which is showed by an inhibition zone diameter of 10.33 mm. Consequently, the flavonoid extract from the fruit of *S. alba* has a good antibacterial potential.

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