JoAS Volume 9 No. 2 October 2024
DOI: https://doi.org/10.20473/joas.v9i2.63662
pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

Journal of Aquaculture Science

In Vitro Evaluation of Brown Algae Extract from *Sargassum aquifolium* in Inhibiting *Aeromonas hydrophila*, the Causative Agent of Motile *Aeromonas Septicemia* (MAS)

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Article info:
Submitted: 27 September 2024
Revised: 20 October 2024
Accepted: 23 October 2024
Publish: 28 October 2024

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ABSTRACT

The widespread use of antibiotics in aquaculture has been associated with the development of multidrug resistance in the environment and in humans. This is also exacerbated by the application of high stocking densities in fish farming systems, including catfish (*Clarias sp)*. This study aims to see the potential of *Sargassum aquifolium* extract, which is a brown macroalgae, as an antibacterial agent for fish disease, *Aeromonas hydrophila*. Previous studies have revealed the phytochemical profile of S. *aquifolium* extract, but its effectiveness as an antibacterial agent has not been studied. The ethanol extract concentrations used were 20%, 40%, 60%, 80%, and 100% with two repetitions. The MIC test method was carried out to determine the lowest concentration of *S. aquifolium* ethanol extract that could inhibit the growth of *Aeromonas hydrophila* bacteria, while the MBC test was carried out to determine the bactericidal properties against *A. hydrophila* bacteria. SEM analysis was carried out to determine the morphological damage to *A. hydrophila* bacterial cells that had been treated with *S. aquifolium* ethanol extract. Based on the results, it was revealed that S. aquifolium extract could inhibit the growth of *A. hydrophila* highest with a very strong category at a concentration of 100%. The MIC value of *S. auifolium* extract was shown at a concentration of 100% but did not cause bacterial cell death based on the MBC value and SEM observations. These findings indicate that *S. aquifolium* extract has the potential to inhibit the growth of *A. hydrophila*. Increasing in vivo parameters such as the immune system, production performance, and survival in catfish is still needed to strengthen these results.

Keywords: *Aeromonas hydrophila*, Bacteriostatic, Clarias sp., Motil Aeromonas Septicemia, *Sargassum aquifolium*.

INTRODUCTION

Indonesia has significant potential for productive freshwater fishery cultivation as a tropical country. Catfish (*Clarias sp)* is among the freshwater fish commodities with great potential for cultivation. According to data from the Ministry of Maritime and Fisheries (KKP), catfish production in Indonesia reached 1.06 million tons in 2022 (Dahliana, Hasmidar & Jumardi, 2023). Indonesian consumers highly favor catfish over other freshwater fish varieties due to the meat's texture and its rich nutritional profile, including protein, fat, carotene, vitamin A, vitamin B1, vitamin B6, vitamin B12, phosphorus, potassium, iron, and beneficial amino acids that contribute to overall health

pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

(Christiand *et al.,* 2022). The increased market demand for catfish has led some farmers to neglect the quality and balance between the cultivation system and fish growth (Nalle *et al.,* 2022). For example, the application of intensive cultivation methods, inadequate water quality management, and the accumulation of large amounts of leftover feed and animal waste are some of the challenges in cultivating catfish (Kamaruddin *et al.,* 2021; Khumaidi & Hidayat, 2018). High stocking densities can reduce water quality due to the buildup of fish metabolism in the form of ammonia and urea as well as feed residues (Sihite *et al.,* 2020; Wiradana *et al.,* 2023).

A. hydrophila is the bacterium that causes one of the diseases that affect catfish as a result of the lower water quality. These bacteria are pathogenic Gram-negative bacteria that frequently affect catfish (Muslikha *et al.,* 2016). Red spot disease, also known as motile *Aeromonas septicaemia* (MAS), is a condition brought on by infection with the *A. hydrophila* bacterium (Agustina *et al.,* 2022). In general, synthetic antibiotics that have activity against aerobic and anaerobic bacteria, such as tetracycline, amprolium, penicillin, streptomycin, tylosin, sulfonamides, and aminoglycosides are used to treat red spot disease in farmed fish (Fauzyyah, 2019). Antibiotic-resistant strains of bacterial have emerged due to the uncontrolled use of synthetic antibiotics, which also have detrimental effects on human health (Felis *et al.,* 2020). Therefore, a natural alternative is needed to control Motile A. septicemia (MAS) in catfish without synthetic antibiotics (Abdul Kari *et al.,* 2022; Semwal *et al.,* 2023; Zhang *et al.,* 2020). One of the natural ingredients that has the potential as an antibiotic is brown algae, *S. aquifolium* (Gazali *et al.,* 2017; Permatasari *et al.,* 2022). *S. aquifolium* is a species of brown algae that is very abundant and spread throughout Indonesian sea waters (Kumalasari *et al.,* 2018). The habitat of *S. aquifolium* is clear waters that have a basic substrate of coral rock, dead coral, volcanic rock, and massive objects located at the bottom of the waters (Lutfiawan *et al.,* 2015).

Based on research conducted by Pakidi and Suwoyo (2017) revealed that *S. aquifolium* contains secondary metabolites such as alkaloids, phenols, terpenoids, and steroids that function as antibacterials. Research conducted by Alamsyah *et al*. (2014), stated that *S. aquifolium* extract given ethyl acetate and methanol solvents has antibacterial activity against *Escherichia coli* and *Staphylococcus epidirmidis*. Furthermore, research conducted by Sidauruk *et al*. (2021), stated that *Sargassum* sp. extract contains bioactive compounds including; alkaloids, steroids, saponins and phenolics which act as antibacterials against gram-positive bacteria (*Listeria monocytogenes*) and gram-negative bacteria *(Pseudomonas aeruginosa*). The purpose of this study is to assess the efficiency of *S. aquifolium* extract in suppressing the growth of *A. hydrophila* bacteria, which cause MAS in catfish. The findings of this study aim to bring more information on the creation of environmentally safe, easy-to-apply, and cost-effective medications for catfish diseases.

MATERIALS AND METHODS

Time and location of research

This study was conducted from February to July 2024. A sampling of *S. aquifolium* was obtained from the waters of Semawang Beach, Denpasar City, Bali Province, at low tide in the intertidal zone. The extraction stage and antibacterial activity test were carried out at the Science Laboratory of Universitas Dhyana Pura, Bali. Pure isolates of *A. hydrophila* were obtained from the collection of the Microbiology Laboratory, Center for Quality

pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

Control and Supervision of Marine and Fishery Products (BPPMHKP), Ministry of Marine Affairs and Fisheries of the Republic of Indonesia (KKP). Observation of morphological damage to bacterial cells due to exposure to *S. aquifolium* extract using a Scanning Electron Microscope (SEM) was carried out at the Marine Education and Research Organisazion (MERO), Karangasem, Bali.

Research materials

The tools used in this study include: beaker glass, Erlenmeyer flask, measuring cup, test tube, petri dish, glass jar, microtube, measuring flask, spray bottle, object glass, dropper pipette, stirring rod, ose needle, spatula, stirrer, tweezers, blue tip, yellow tip, filter paper, ruler, lighter, bunsen, funnel, scissors, cutter, aluminum foil, paper wrap, hand scoop, tissue, sterile cotton, plastic clip, grinder (WILLMAN DE100G), scales (SAYAKI SSA), autoclave (All American 75X), vacuum rotary evaporator (Rotary Evaporator Ika Rv 8 V - Rv 10 Digital V), magnetic stirrer (Hot Plate Stirrer Thermo Scientific Cimarec II SP88857105), vacuum (Rocker 300), vortex (ZX3 Vortex Mixer VELP), oven (UN110 Universal Oven), laminar Air Flow (Robust laminar Air Flow type LAF-140), micropipette (DragonLAB 100-1000 µl), spectrophotometer (Yoke Instrument V1710), and microscope (JSM-IT200 with JEOL brand). The materials used include: *S. aquifolium* obtained from Sanur Beach with a natural brown physical condition without white spots and does not contain dirt, such as sand, gravel, or other organisms. Pure isolates of *A. hydrophila*, Tryptone Soy Agar (TSA), and Tryptic Soy Broth (TSB) with good quality are characterized by a transparent pale-yellow color and good gel formation conditions. Ethanol 70% (technical), distilled water (technical), Chloramphenicol (Apotekku, Denpasar Bali).

Research Design

The design used in this study was a Completely Randomized Design (CRD) with an experimental laboratory exploration approach. The treatment groups in this study were seven (7) treatment groups with 2 repetitions. The treatment groups in this study were as follows:

K_- : Negative Control (aquadest)

K_+ :Positive Control (Chloramphenicol)

ESA_1: Treatment with extract concentration *S. aquifolium* 20%

ESA_2:Treatment with extract concentration *S. aquifolium* 40%

ESA_3: Treatment with extract concentration *S. aquifolium* 60%

ESA_4: Treatment with extract concentration *S. aquifolium* 80%

ESA_5: Treatment with extract concentration *S. aquifolium* 100%

Making ethanol extract *S. aquifolium*

S. aquifolium samples were obtained from the waters of Semawang Beach, Denpasar Bali at the lowest low tide in the intertidal zone. The obtained *S. aquifolium* samples were put into sterile plastic clips and transported to the laboratory. In the laboratory, the samples were washed thoroughly with running water, and then dried until the amount of water in the sample was reduced. After that, a container was prepared that had been lined with aluminum foil for drying using an oven at a temperature of 50℃ for 2×24 hours (Permatasari *et al.*, 2022; Rosiana *et al.*, 2022). The dried *S. aquifolium* sample was blended to obtain small sample pieces and then ground again using a grinder and sieved to produce a finer powder (Hidayah *et al.*, 2017).

pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

S. aquifolium powdered simplisia was weighed as much as 250 grams using a beaker glass, then 2500 ml of 70% ethanol (1:10) was added. The sample was extracted for 3 days and stirred every 24 hours using a stirring rod to obtain a homogeneous extract (Astuti *et al.,* 2024). After that, the sample was filtered using filter paper and vacuum to obtain S. aquifolium macerate. The macerate obtained was then evaporated using a vacuum rotary evaporator at a temperature of 50°C. The thick extract obtained was collected using a dark brownish glass container and stored in a refrigerator for further use.

Determination of extract concentration

The concentration of *S. aquifolium* extract was determined to determine its effectiveness in inhibiting *A. hydrophila* in vitro. The concentration variations used in this study included 20%, 40%, 60%, 80%, and 100%. The positive control used was chloramphenicol at a dose of 250 mg and the negative control was distilled water (Andriyawan *et al.,* 2016).

Rejuvenation of A. *hydrophila* suspension

The *A. hydrophila* bacterial isolates used in this study were obtained from the isolate collection of the Microbiology Laboratory, Center for Quality Control and Supervision of Marine and Fishery Products (BPPMHKP), Ministry of Marine Affairs and Fisheries of the Republic of Indonesia (KKP) which had levels of pathogenicity and virulence as reported in previous studies (Pebriani *et al.,* 2024).

Purification is done by growing bacteria from slant media in liquid media first, namely Tryptic Soy Broth (TSB) and incubated at 300 ^{0}C for 24 hours. Furthermore, the bacterial culture is regrown on solid media, namely Tryptic Soy Agar (TSA) by transferring the bacterial colony using a sterile ose needle from TSB media, then scratched on TSA media and incubated at 300 ^{0}C for 24 hours. Bacterial purification is carried out until a pure isolate is found which is marked by a uniform colony color (Anwar & Tugiyono, 2023).

Inhibitory power test

The inhibition power test of *S. aquifolium* samples against the growth of *A. hydrophila* was carried out using the well diffusion method. In short, the previously purified *A. hydrophila* culture was taken using a sterile ose needle, and then inserted into a test tube containing 9 ml of distilled water until it reached a turbidity level equivalent to the Mc. Farland 0.5 standard. The bacterial suspension was then vortexed and pipetted using a micropipette as much as 200 µL and poured into a sterile petri dish. The petri dish was then added with TSA media, homogenized, and waited until it solidified. The petri dish was then perforated with a diameter of 6 mm as a well. *S. aquifolium* extract was added to each perforated petri dish as much as 20 µl according to the sample concentration. The same method was carried out for positive and negative controls. The petri dish that had been added to the extract was labeled**.** The petri dish was then wrapped using plastic wrap and incubated at 30°C for 24 hours. Observation of inhibition power was carried out by measuring the diameter of the clear zone that appeared in each petri dish around the well. The inhibition zone formed was measured vertically and horizontally and then averaged (Novaryatiin *et al.,* 2018).

Minimum Inhibitory Concentration (MIC) Test

MIC testing was carried out by culturing 250 µl of bacterial suspension and 500 µl of *S. aquifolium* extract of each concentration which was put into a test tube filled with 9 ml of sterile TSB media. The tube was then vortexed for 1 minute, then incubated at 30°C for 24 hours. Observations were made by measuring

pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

the turbidity level of the media using a UV-Vis spectrophotometer with a wavelength of 200-800 nm. The same method was carried out on the positive and negative control groups, repeated twice for each treatment group.

Minimum Bactericidal Concentration (MBC) Test

Minimum Bactericidal Concentration (MBC) is defined as the lowest concentration of antimicrobial capable of bactericidal activity. Briefly, 1 loop of pure culture of *A. hydrophila* was cultured in 9 ml of TSA in a test tube, incubated at 30°C for 24 hours. The bacterial suspension was then diluted to a concentration of 1×10^5 cfu/ml. The S. aquifolium extract used was the lowest concentration capable of inhibiting the growth of *A. hydrophila* at the MIC stage. The bacterial suspension was pipetted as much as 10 µl and spread on a sterile petri dish, then TSA media was added, and homogenized. After homogenization, 10 µl of S. aquifolium extract was pipetted according to the MIC results and spread on a Petri dish filled with *A. hydrophila* suspension. The extract was homogenized with a sterile L rod and incubated at 30°C for 24 hours. If the sample concentration causes growth < 10 cfu/ml it can be considered as the MBC value. The experiment was repeated three times (Hendiani *et al.*, 2020; Mogana *et al.*, 2020).

Observation of the morphology of *A. hydrophila* bacterial cells

A sample of *Aeromonas hydrophila* bacteria that has been cultured in liquid media is inserted into an endorphin tube. The sample is centrifuged, then the centrifugation results are discarded so that only bacterial pellets remain in the tube. Furthermore, the fixation process is carried out to maintain the original structure of the sample so that it does not easily collapse or disintegrate. Fixation is carried out in 2 stages. First, the sample is soaked in a 2% Glutaraldehyde solution, then continued with post-fixation by soaking the bacteria in a 1% Osmium Tetraoxide solution. Furthermore, the dehydration process by soaking in graded alcohol (30%, 50%, 70%, 95% and 100%).

After the dehydration process, the sample is dried at room temperature until dry. The dried sample is then attached to the specimen stub using carbon tape. Finally, the sample is coated using a conductor material. Coating aims to minimize the charging effect due to the scattering of electron energy during scanning. Coating is done with a conductive material in the form of gold (coating thickness: 20 nm). Sample testing using a Scanning Electron Microscope (SEM) was carried out by installing the specimen stub into the specimen holder and then scanned using a Scanning Electron Microscope type JSM-IT200 with the JEOL brand. The scanning process was carried out using the high vacuum method.

Data Analysis

The results of the inhibition and MIC tests were analyzed using SPSS software. The data normality test used Shapiro-Wilk to determine the sample distribution. If the data is normally distributed, it is continued with the One-Way ANOVA test and the DUNCAN test with a significance level stated as $p < 0.05$. The results of the Minimum Bactericidal Concentration (MBC) test and identification of bacterial cell morphology using SEM were analyzed qualitatively.

RESULTS AND DISCUSSIONS

The results of the effectiveness test of *S. aquifolium* ethanol extract using the well method showed inhibitory properties against the growth of *A. hydrophila* bacteria. This is indicated by the formation of a clear zone around the well area that has been treated using *S. aquifolium* ethanol extract (Table 1).

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Available online at https://e-journal.unair.ac.id/JoAS

Ethanol extract of *S. aquifolium* with a concentration of 20% did not have any inhibitory power as indicated by the presence of bacterial growth around the well area. Meanwhile, concentrations of 40%, 60%, 80%, and 100% showed positive inhibitory results as indicated by the formation of an inhibition zone or clear zone around the well area. Furthermore, the positive control (chloramphenicol) showed positive inhibitory results, while the negative control (aquades) did not show any inhibitory activity.

Table 1. Inhibitory power of *S. aquifolium* extract on the growth of *A. hydrophila* based on concentration variation

Treatment	Repetition I (mm)	Repetition II (mm)	Average ± SD	Category
ESA 20%	0	0	0±0,0[a]	No Inhibitor
ESA 40%	18	13. 5	15.75±3. 2[b]	Strong
ESA 60%	14. 3	16	15.15±1. 2[b]	Strong
ESA 80%	16. 5	18. 5	17.5±1. 4[b]	Strong
ESA 100%	32. 5	30. 5	31.5±1. 4[c]	Very Strong
Positive Control	46	47. 5	46. 75±1. 06[d]	Very Strong
Negative Control	0	0	0±0. 0[a]	No Inhibitor

Description: ESA = *Sargassum aquifolium* Extract. Letters with different notations in the same column indicate significant differences between treatment groups ($p < 0.05$) based on the DUNCAN test results.

The diameter of the inhibition zone of *S. aquifolium* extract (ESA) at a concentration of 20% did not form an inhibition zone. Meanwhile, at 40% ESA, an inhibition zone was formed with an average of 15.75 mm with a strong category but not significantly different ($p > 0.05$) with 60% ESA of 15.15 mm with a strong category, and 80% ESA of 17.5 mm with a strong category (Table 1). The 100% ESA treatment showed inhibition of *A. hydrophila* of 31.5 mm with a very strong category or significantly higher when compared to the 20% ESA to 80% ESA treatment.

The positive control (chloramphenicol) had the highest inhibition zone of 46.75 mm with a very strong category. The research conducted by Azzahra dan Trimulyono (2024) reported that *S. aquifolium* extract has antibacterial activity against gram-negative bacteria *Pseudomonas fluorescens*. The research conducted by A'yunin *et al.* (2021) reported that watercress macroalgae extract has inhibitory power against *A. hydrophila* bacteria at a concentration of 0.007%.

The inhibition test in this study was categorized based on the average measurement of the inhibition zone formed in the well area that had been treated with *S. aquifolium* extract. According to Rahayu *et al.* (2019), the diameter of the inhibition zone formed is greater than 20 mm which is a very strong category.

Meanwhile, the diameter of the inhibition zone formed at 11-20 mm is a strong category. Research conducted by Atmaja *et al.* (2017) reported that the greater the concentration given, the greater the inhibitory power produced. Statement from Sa`adah and Nurhasnawati, (2015), revealed that the main factor affecting the results of the inhibition test is the choice of solvent in the extraction process. Ethanol solvent is an organic solvent with a high level of polarity. This is in accordance with the level of polarity of phytochemical compounds in *S. aquifolium* which are suspected of having antibacterial

pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

activity, such as flavonoids, alkaloids, tannins, phenols, terpenoids, and steroids (Magvirah *et al.,* 2019).

Flavonoids are antibacterial compounds that are able to inhibit cell function by forming complex compounds from extracellular proteins to damage cells and cell structures in bacteria (Parubak, 2019). In addition to flavonoids, active compounds that act as antibacterials contained in *S. aquifolium* extract are alkaloids and steroids. The mechanism of action of alkaloids as antibacterials is to disrupt all components of peptidoglycan in bacterial cells, so that the cell wall layer in bacteria is not formed completely and causes death in these cells (Anggraini, Nisa *et al.,* 2019). Based on observations of the MIC test results using a spectrophotometer, it was found that the *S. aquifolium* extract had a concentration of 100% as the MIC against *A. hydrophila.* At a concentration of 20%, there was no inhibition indicated by the difference in the average absorbance value which was not much different from the negative control. The average absorbance value at a concentration of 40% was 2.524 mm, 60% was 2.587 mm, 80% was 1.958 mm, and 100% was 1.263 mm. Meanwhile, the absorbance value in the positive control was 0.89 nm and the absorbance value in the negative control was 3.586 nm (Table 2).

Table 2. Minimum Inhibitory Concentration (MIC) value of *S. aquifolium* extract on the growth of . *A.hydrophila* measured using a UV-Vis spectrophotometer.

Treatment	Wavelenght		Absorbans		Average ± sd
	Repetition I (nm)	Repetition II (nm)	Repetition I	Repetition II	
ESA 20%	220	220	3.412	3.405	3.408±0.004[a]
ESA 40%	215	215	2.610	2.438	2.524±0.121[b]
ESA 60%	215	215	2.564	2.611	2.587±0.033[b]
ESA 80%	215	215	1.973	1.942	1.957±0.021[c]
ESA 100%	215	215	1.222	1.303	1.262±0.057[d]
Positive Control	215	215	0.848	0.928	0.888±0.056[e]
Negative Control	215	215	3.607	3.569	3.588±0.026[a]

Description: ESA = *S. aquifolium* Extract. Letters with different notations in the same column indicate significant differences between treatment groups ($p<0.05$) based on the DUNCAN test results.

The results of the MIC test in this study showed differences in turbidity levels at each concentration, which was indicated by the results of absorbance value measurements using a spectrophotometer with a wavelength of 200-800 nm. This is in accordance with research conducted by Magvirah *et al*. (2019), the number of bacteria can be measured based on the level of turbidity of the culture media, if the media is increasingly turbid, the number of bacterial cells in it will increase. Research conducted by Arivo dan Annissatussholeha, (2017) reported that the higher the absorbance value, the higher the bacterial growth at that concentration. The research conducted by Lingga *et al*. (2016) states that the antibacterial activity of a natural compound can be said to be strong if the minimum inhibitory concentration is small, but has a large inhibitory power. The results of the MBC test of *S. aquifolium* extract at a concentration of 100% against *A. hydrophila* bacteria showed positive results due to bacterial growth in the test medium after incubation for 24 hours. This means that *S. aquifolium* extract with a concentration of 100% is only able to inhibit the growth of *A. hydrophila* bacteria (bacteriostatic), but is not bactericidal. Bacteria that grew after incubation for 24 hours showed cream-colored colonies on TSA media

pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

that had been added with S. aquifolium extract at a concentration of 100%. MBC test of *S. aquifolium* extract at 100% concentration against *A. hydrophila* bacteria showed bacterial growth in the media that had been treated and incubated for 24 hours.

This means that the optimum concentration of *S. aquifolium* extract is only able to inhibit the growth of *A. hydrophila* bacteria, but has not been able to kill them completely. This is by research from Sastrawan *et al.* (2020) that the calculation of the MBC value becomes invalid if there is bacterial growth after treatment.

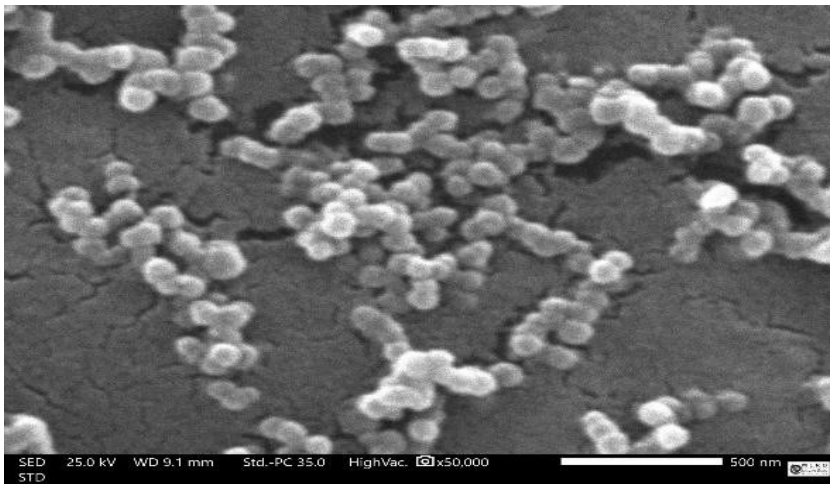

Figure 1. Morphology of *A. hydrophila* bacterial cells exposed to 100% concentration of *S. aquifolium* extract. Observation of bacterial cells using a Scanning Electron Microscope (SEM) at a magnification of 50,000×

Likewise with the research conducted by Yusriyani *et al*. (2023) one of the factors that affect the minimum bacterium concentration is the level of susceptibility of the test bacteria. *A. hydrophila* bacteria are gram-negative bacteria so they are more resistant to antibacterial substances. The cell wall structure of negative bacteria is permeable so that it stops antibiotics from penetrating bacterial cells (Astrini *et al.,* 2014). Scanning Electron Microscopy (SEM) analysis aims to identify the effect of compounds contained in 100% concentration of *S. aquifolium* extract on the morphology of *A. hydrophila* bacterial cells. Based on observations made, the morphology of *A. hydrophila* bacteria with a magnification of 50,000× did not show any damage.

This is by the results of the MBC test, where 100% concentration of *S. aquifolium* extract was unable to kill *A. hydrophila* bacteria (Figure 1). Research conducted by Panjaitan *et al.* (2020) reported that mangrove leaf extract (*Rhizophora mucronate*) caused damage to *A. hydrophila* bacterial cells. The damage was analyzed using SEM with indications of damage, namely the elongation of cell size accompanied by swelling. The mechanism of antibacterial action on phenol compounds is protein inactivation in cells. In addition, several components of phenol compounds that bind to proteins cause protein structures, such as cell walls and cells to be damaged (Kambey *et al.,* 2019).

CONCLUSION

Ethanol extract of *S. aquifolium* with a concentration of 100% is the best concentration with the highest inhibitory activity against *A. hydrophila*. *S. aquifolium* extract did not show bactericidal properties based on the results of the MBC test and was confirmed by the morphology of *A. hydrophila* cells using SEM. Further research is still

pISSN 2550-0910; eISSN 2579-4817
Available online at https://e-journal.unair.ac.id/JoAS

needed to analyze the use of other organic solvents to extract active compounds from *S. aquifolium* so that it can increase its effectiveness in controlling the growth of *A. hydrophila* on a field scale.

ACKNOWLEDGEMENTS

The authors would like to thank the Microbiology Laboratory, Center for Quality Control and Supervision of Marine and Fishery Products (BPPMHKP), Ministry of Marine and Fisheries of the Republic of Indonesia (KKP) for supporting the implementation of this research through permits for the use of bacterial isolates and materials related to the research.

AUTHORS' CONTRIBUTIONS

The contribution of each author is as follows, EW, AJ, and PAW; collected the data conducted the study, data analysis, drafted the manuscript, and designed the tables and figures. AAAPP, IMWAP, PES, and MKJK; devised the main conceptual ideas, reviews the study, conducted a critical revision of the manuscript. All authors discussed the result and contributed to the final manuscript.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

FUNDING INFORMATION

This research did not receive any specific grant from any funding agency in the university, public, commercial, or not-for-profit sectors.

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