2022, Scienceline Publication
World's Veterinary Journal
World Vet J, 12(3): 260-265, September 25, 2022
ISSN 2322-4568
DOI: https://dx.doi.org/10.54203/scil.2022.wvj32

Phytochemical and Antibacterial Effects of Leaf Extract from Mangrove Plant (*Avicennia Marina*) on *Vibrio Parahaemolyticus* in Shrimps

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ORIGINAL ARTICLE
pii: S232456822000032-12
Received: 14 July 2022
Accepted: 08 September 2022

ABSTRACT

Recently, there has been a tremendous increase in the studies addressing the application of bioactive compounds from the natural ecosystem, particularly for medical purposes. Hence, the present study investigated the antibacterial properties of the secondary metabolites possibly contained in the leaves of *Avicennia marina* (*A. marina*) for possible prevention of *Vibrio parahaemolyticus* (*V. parahaemolyticus*), a devastating bacterial pathogen in shrimp aquaculture. In the current study, secondary metabolites were extracted from the leaves of mangrove plant using ethanol extraction method. The ethanolic extracts were then subjected to phytochemical and antibacterial activity tests. The results from the phytochemical analysis demonstrated that the ethanolic extract from the mangrove plant contained varying amounts of flavonoids, tannins, saponins, polyphenols, alkaloids, steroids, and triterpenoids. However, the number of flavonoids and alkaloids seemed to be higher than the other metabolites. The antibacterial activity analysis through the agar diffusion method has shown that different concentrations (50 ppm, 100 ppm, 200 ppm, and 300 ppm) of the ethanolic extract of *A. marina* inhibited the *V. parahaemolyticus.* At 300 ppm, the plant extract exhibited 17.3% antibacterial effectiveness, compared to the antibacterial activity of chloramphenicol. The findings indicated that the secondary metabolites of *A. marina* have the potential that can be developed as an alternative treatment for aquatic animal diseases in the future.

Keywords: Aquaculture, Bioactive compounds, Mangrove ecosystem, Treatment

INTRODUCTION

The application of medicinal plants as phytopharmica products in place of synthetic antibiotics has been increasingly popular in recent years. Many of these products are sold over the counter or online retailing. However, many plant species in tropical countries, including Indonesia, are still not thoroughly investigated for their medicinal properties due to their high diversity. Some of these plants are known to have bioactivity against disease-causing pathogens. For example, the tiwai onion (*Eleutherine Americana*) has been shown to strongly inhibit the growth of *Vibrio parahaemolyticus* (*V. parahaemolyticus*) and *Vibrio harvey* (Azis, 2019; Azis and Cahyadi, 2020).

Similarly, the Dragon Scales Leaf (*Drymoglossum pilosellaoides*) was also reported to contain antibacterial properties against *V. parahaemolyticus* (Azis, 2019). This antibacterial activity is influenced by the presence of various bioactive compounds in phytopharmaceutical extracts that can damage bacterial cell walls causing a modification of the cytoplasmic membrane, the release of core material, changes in protein, and nucleic acid molecules, enzyme inactivity, and preventing protein and nucleic acid synthesis. Interactions include sequential inhibition following common biochemical pathways to enhance antimicrobial diffusion, and inhibition of bacterial protective enzymes (Sintayehu et. al., 2022). Based on toxicity study, antibacterial substances can be classified in three forms, namely bacteriostatic, bactericidal, and bacteriolytic (Parekh and Chanda, 2007). The progress of the bioactivity study of plants is concentrated on plants primarily found in terrestrial ecosystems; however, few studies have been conducted on the plants that are flourishing in the marine ecosystem. Mangrove plants, including *Avicennia marina*, are rich in bioactive substances and have potential as an agent in various biological activities (Al-Mur, 2021). Many of these marine plant species are known

To cite this paper: Azis, Salim G, Indarjo A, Prakoso LY, Hartati R, Daengs AGS, Meiryani, Aslan LOM, Ransangan J, and Rozi (2022). Phytochemical and Antibacterial Effects of Leaf Extract from Mangrove Plant (*Avicennia Marina*) on *Vibrio Parahaemolyticus* in Shrimps. *World Vet. J.,* 12 (3): 260-265. DOI: https://dx.doi.org/10.54203/scil.2022.wvj32

to contain unique bioactive compounds in the leaf extract of a mangrove plant, including *Avicennia alba* leaf extract can prevent the development of cancer cells (Eswariah et. al., 2020).

A total of 81 mangrove species have been reported worldwide, and several biologically active compounds have been isolated from mangroves with various levels of action that have biomedical potential, such as anticancer, antiulcer, antioxidant, antidiabetic, and antimicrobial (Parthiban et al., 2021). One of the most common and easily found mangrove species in Indonesia is the *Avicennia marina*. This species has been extensively used by the indigenous peoples for many purposes, including materials for building the house and traditional medicine for skin diseases, rheumatism, ulcers, and smallpox (Bandaranayak, 2002). Due to its extensive use in traditional medicines, the extracts of this plant are assumed to contain diverse bioactive compounds that could benefit the pharmaceutical industry (Nurjannah et al., 2015).

This *Avicennia* species is predominantly distributed in Tarakan, North Kalimantan (Mahera et. al., 2011). Its leaves, skin, and fruits are highly valuable because of their medicinal properties (Huang et. al., 2016; Oktavianus, 2013). In addition, the bark of the plant can also be further processed to generate many more downstream products, such as alcohol, hydrocarbons, carbohydrates, inorganic salts, minerals, phytoalexins, vitamins, iridoid glucosides, steroids, tannins, triterpenoids, as well as fatty, amino, and carboxylic acids (Ananthavalli and Karpagam, 2017). Biologically active compounds, such as limonoids, terpenoids, alkaloids, glycosides, steroids, flavonoids, esters, quinones, phenols, acids, aliphatic alcohols, amides, lactones, aliphatic ketones, and benzodioxols have been extracted from leaves, stems, bark, fruits, and seeds of Indian mangroves (Parthiban et al., 2021). Overall, this study confirms the antimicrobial and antioxidant activity of mangrove ethyl acetate extract (MEE) and inhibitory and eradicating activity of the *A. marina* biofilm ethyl acetate extract against *Pseudomonas fluorescens* (Ibrahim et al., 2022).

Furthermore, several in-vitro studies have shown the leaf extract of *Avicennia* is highly potent against various pathogenic bacteria *Virgibacillus marismortui* and *Micrococcus iuteus* (Ulmursida et al., 2017). However, most previous studies on the medicinal properties of *A. marina* focused only on human illnesses. A few addressed the use of this plant towards diseases of farmed aquatic animals, such as fish and shrimps. One of the major disease-causing bacteria in shrimps is the V. parahaemolyticus. This halophilic Gram-negative bacterium is ambiguous in the marine environment. This bacterium has been known to cause disease in shrimp aquaculture in North Kalimantan. Mangroves are coastal plants that can adapt and survive in intertidal tropical and subtropical coastal areas, where their presence in marine ecosystems is the second most important after coral reefs (Al-mur, 2021). The bacterium causes acute damage to the digestive system of culture shrimp, particularly the hepatopancreas. *Vibrio parahaemolytics* have also been widely reported to trigger early mortality syndrome (EMS) or the acute hepatopancreatic necrotic disease (APHND) in culture shrimp worldwide. Furthermore, it has also been reported to be responsible for vibriosis in marine crabs (Jithendran et al., 2010).

The widespread use of *A. marina* for traditional medicine might also be helpful for aquatic animal husbandry, particularly for preventing vibriosis in cultured shrimp. Therefore, the present study aimed to examine the antibacterial potential of the ethanolic leaf extract of the *A. marina* towards *V. parahaemolyticus*, a devastating pathogen of culture shrimp.

MATERIALS AND METHODS

The extraction process, bacterial culture, antibacterial activity test, and observation of bacterial growth inhibition zones were all carried out at the Laboratory of Nutrition and Fish Feed, Faculty of Fisheries and Marine Sciences, University of Borneo Tarakan. The leaves of *A. marina* were obtained from the Mangrove and Proboscis Monkey Conservation Area (KKMB) of Tarakan City (Figure 1). Meanwhile, the isolates of *Vibrio parahaemolyticus* were acquired from the Center for Brackish Water Aquaculture Fisheries (BBPBAP) Jepara, Indonesia, with the number of bacterial isolates VpPm 110321-3.

Figure 1. The bioactive compounds of *A. marina* leaf samples were extracted according to Manila et al. (2009) with the modification and antimicrobial activity of extracts of *A. marina* examined against the pathogen.

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Sample preparation and extraction

Crude extract of the leaves of *A. marina* was prepared following the method suggested by Manila et al. (2009). Briefly, the leaves of *A. marina* were cut into small pieces and air dried for 5-7 days. After that, the dried leaves were then finely blended and sifted. The crude preparation began by weighing 200g powder of the finely blended dried leaves of A. marina, macerated three times each for 24 hours with 200 ml of 70% ethanol solution. The solution was then stirred for 1 hour at 45°C using a hot plate with a stirrer. Subsequently, the extracts were filtered using filter paper and concentrated with a rotary evaporator for 2 hours at 40°C.

Finally, the extract solutions were dissolved in 70% ethanol solvent and diluted into different concentrations, and the groups were named A (50 ppm), B (100 ppm), C (200 ppm), and D (300 ppm). Two control solutions were also prepared alongside the plant extract, namely 70% ethanol (C-), and 0.01% chloramphenicol (C+). Each concentration of the solution was prepared in triplicates. The chloramphenicol solution was used as a benchmark or reference (positive control) to determine the effectiveness of the antibacterial activity of the ethanolic leaf extract of *A. marina*. In addition to chloramphenicol, other antibiotics used as positive controls were ampicillin (Amp), ciprofloxacin (Cip), nitrofurantoin (Nit), gentamicin (Gen), oxytetracycline (Otc), tetracycline (Tet), and streptomycin (Str).

Phytochemical tests

The phytochemical assay of *A. marina* leaf extract is briefly described below

Flavonoid Test (Harborne 1998)

To conduct this test, 1 milliliter of *A. marina* leaf extract solution was put into a test tube, and then a little magnesium powder and a few drops of concentrated HCL (Shinoda reagent) were added. If the color of the solution changes to orange, pink, or red, it indicates the presence of flavonoid compounds in the sample.

Saponin Test (Harborne 1998)

Two milliliters of *A. marina* leaf extract solution were put into a test tube and then homogenized for several minutes. The formation of persistent and stable foam for 15 minutes indicates that the sample contains saponins.

Polyphenol (Tannin) Test (Zohra et al., 2012)

One milliliter of *A. marina* leaf extract solution was put into a test tube, followed by the addition of a few drops of 5% ferric chloride ($FeCl_3$) reagent. If a brown precipitate forms, this indicates that the sample contains tannins.

Alkaloid Test (Harborne 1998)

Avicennia marina leaf extract as much as 1 milliliter was put into a test tube, and then 2-3 drops of Dragendorph reagent were added consisting of (Nitrooxy) oxobismuthine (BiNO4xH2O), tartaric acid, and KI. The formation of an orange precipitate indicates the presence of alkaloids in the sample.

Steroids and Triterpenoids Test (Zohra et al., 2012)

One milliliter of *A. marina* leaf extract solution was put into a test tube, followed by the addition of 3-5 drops of chloroform, 3-5 drops of acetic anhydride, and 10 drops of concentrated sulfuric acid. The presence of steroids is seen in the change in the color of the sample from blue to green. The presence of triterpenoids in other parts is seen by changing the color of the sample from brown to reddish brown.

Bacterial culture and antibacterial activity

Culture of *V. parahaemolyticus* was carried out on Thiosulfate citrate bile salts sucrose (TCBS) agar media. Inoculation of the bacteria was done by taking a loopful of bacterial culture using a sterile metal loop and then smeared it onto the surface of the media agar. The inoculated agar media were then incubated for 24 hours at 37°C. Antibacterial activity of the leaf extract of the *A. marina* was examined using the disc diffusion method. Briefly, a loopful of the test bacteria culture was streaked evenly on an agar medium and labelled accordingly. Then, sterile paper discs (zone diameter were soaked in the different concentrations of the plant extract solution, negative control (C-) and positive control (C+), and subsequently incubated for 24 hours at 37°C.

Observation of bacterial inhibition zone

The antibacterial activity of the *A. marina* leaf extract was ascertained by the presence of a clearing zone around the paper discs in each treatment. The diameter of the inhibition zone reflected the strength of the antibacterial activity of the plant extract. The average inhibition zone was obtained through vertical and horizontal measurements of the clearing zones.

Efficacy of antibacterial activity

The effectiveness of the antibacterial activity of the leaf extract against the test bacteria was calculated following the formula suggested by Ghosh et al. (1997). The value of antibacterial effectiveness was obtained from the division of the average diameter of inhibition zone due to plant extract (mm) by the average diameter of inhibition zone due to antibiotic (mm) multiplied by one hundred.

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RESULTS AND DISCUSSION

Based on the results of testing the inhibitory activity of *A. marina* ethanol and chloroform extracts from the Mangrove and Proboscis Monkey Conservation Area (KKMB) of Tarakan City locations, it was shown that the extract had antibacterial potential against *Vibrio parahaemolityus* bacteria. Potency is indicated by the size of the clear zone around the paper disc (Figure 2). Based on the phytochemical tests of the leaf extract, it was evident that the leaves of *A. marina* contained flavonoids, phenols, alkaloids, saponins, tannins, and steroids (Table 1). The results for the antibacterial activity of the ethanolic leaf extract of *A. marina* are presented in Table 2.

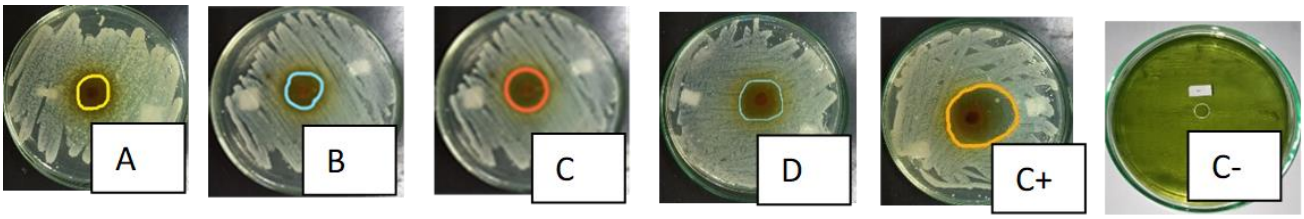

Figure 2. Inhibition zone of the crude extract of *Avicennia marina* against *Vibrio parahaemolyticus.* Treatment of *Avicennia marina* extracts*;* A (50 ppm), B (100 ppm), C (200 ppm), and D (300 ppm). Two control solutions were ethanol 70% (C-), and chloramphenicol (C+) 0.01%.

Table 1. Phytochemical test results of the crude ethanol leaf extract of *Aveciana marina* based on the strength category

Group of compound	Ethanol extracts
Flavonoid	++
Triterpenoids	+
Alkaloids	++
Saponins	+
Tannins	+
Steroids	+

+: Weak; ++: Strong; +++: Very strong based on published data of Marlinda et al. (2012)

Table 2. Inhibition zones of the different concentrations of the crude ethanol leaf extract of *Avicennia marina* against *Vibrio parahaemolyticus*

Treatment	Inhibition zone (mm)			Average (mm)	Inhibitory effectiveness (%)	Growth inhibition response
	1	2	3			
C+	35.5	37.8	37.5	36.9	100	Very strong
C-	0	0	0	0	0	No activity
A	3.4	3.5	3.4	3 .4	9.2	Moderate
B	2.8	3.9	3.7	3.5	9.5	Moderate
C	4.7	4.4	3.8	4.3	11.7	Strong
D	8.3	5.6	5.4	6.4	17.3	Strong

C+: 0.01% Chloramphenicol C-: 70% ethanol, A: 50 ppm, B: 100 ppm, C: 200 ppm, D: 300 ppm. Very strong > 80-100%, Strong > 50-79.9%, Moderate > 10-49.9%, Weak 1-9.9%, No activity 0-0.9% (Marlinda et al., 2012)

The results of this study showed that the treatment of *A. marina* leaf extract 200 and 300 ppm showed strong inhibition of *V. parahaemolyticus*. According to the results of the current study, the content of flavonoids and alkaloids seems to be abundant in *A. marina* leaves. As Edu et al. (2017) mentioned, many alkaloids are found in the roots, leaves, and bark of the *Avicennia* species. They also noted that saponins and tannins were found less in the leaves, which was the case in this study.

The organic leaf extract of *Avicennia* species has inhibitory activity against *V. parahaemolyticus* (Edu et al., 2015; Sachithanandam et al., 2019; Okla et al., 2019). Besides being rich in secondary metabolites, mangrove leaves are also known to contain minerals, vitamins, and amino acids that are important for the nutrition of marine organisms in the mangrove ecosystem (Bandarayake, 2002). Many mangrove plants have been recently used in traditional medicine Extracts of mangrove plants have antibacterial activity against human, animal, and plant pathogens. In addition, the secondary metabolite components of mangrove plants were shown to be positively correlated with the potential of the extract to inhibit bacterial growth. Phytochemical compounds such as flavonoids, phenols, alkaloids, saponins, tannins, and steroids exhibit superior antimicrobial activity against disease-causing pathogens (Sulastrianah et al., 2014). Although these chemical compounds are present in every plant, their distribution may vary according to the species and part of the plant. According to Ningsih et al. (2013), the leaf extract of *Avicennia* species has also been shown to inhibit the growth of other disease-causing bacteria, including *Staphylococcus aureus* and *Escherichia coli*. Since the ethanolic extract of *A. marina* leaves contains high amounts of flavonoids and tannins, the antibacterial activity of the plant can be

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attributed to these compounds. Due to limited tests, the bactericidal or bacteriostatic status of the bioactive compounds in the A. marina leaf extract investigated in this study is difficult to ascertain. Therefore, there is a need to conduct more in-depth research in the future.

The secondary metabolites play an important role in determining the antibacterial properties of the plant extract (Normayunita et al., 2015). Flavonoids and tannins have been reported to inhibit enzyme activities and demonstrated the ability to interact with bacterial DNA, causing damage and increasing the cell walls' permeability (Sachithanandam et al., 2019). Consequently, bacterial cells rupture and lyse (Astriyani et al., 2017). In addition, phenolic compounds are also known to extract cell contents by destroying the lipids in the cell membrane of organisms (Normayunita et al., 2015). Steroid compounds, on the other hand, have been demonstrated to interact with the cell phospholipid membrane, a layer impermeable to lipophilic compounds affecting the cell integrity, changing the cell morphology, and finally making the cells brittle and lysis (Komalasari et al., 2021).

Regarding the diameter of the inhibition zone of the bacterial growth, the effectiveness of the bioactive compounds contained in the leaves of *A. marina* is far lower compared to that of the chloramphenicol (positive control). These could be explained by the extraction methods (Palombo and Semple, 2001; Narasimhudu and Venkata, 2012) and, to some extent, by the climatic condition (Vudhivanich, 2003).

CONCLUSION

The present study demonstrated that the ethanolic leaf extract of *A. marina* contained a high number of flavonoids and tannins, in addition to saponins, steroids, and phenols. The extracts were also shown to inhibit the growth of shrimp pathogen, *V. parahaemolyticus* at the concentration of 300 ppm, which is about 17.3% effective compared to the synthetic antibiotic, chloramphenicol. Such findings can justify further study on the characterization of bioactive compounds in different parts of the *A. marina* tree found in other regions of Indonesia. The results of the present study could also become an alternative treatment for shrimp aquaculture in addition to putting more value on the mangrove ecosystem. Further studies are needed on the efficacy and *in vivo* sub-acute and chronic toxicity of *A. marina* leaf extract in shrimp infected with *V. parahaemolyticus.*

DECLARATIONS

Acknowledgments

This work was supported by the University of Borneo Tarakan grant through the Institute for Research and Community Service of the University of Borneo Tarakan. We would like to thank all those who contributed to our research. This study also collaborated with Research funding from the RKAT Fakultas Perikanan dan Kelautan Universitas Airlangga in 2017, with grant number: 18 /UN3.1.12/KP/2017.

Authors' contribution

Azis, Gazali Salim, Agus Indarjo designed the study, participated in performing the experiments and analyzing the parameter of data, and performing the experiments and writing the manuscript. Lukman Yudho Prakoso, Retno Hartati, Achmad Daengs GS contributed to analyzing the data and writing the manuscript. Meiryani, La Ode Muhammad Aslan, Julian Ransangan participated in performing the experiments and analyzing the data. Rozi checked and confirmed the manuscript's final editing and revision draft before submission to the journal. All authors confirmed the results, and the final version of the manuscript to publish in the present journal.

Competing interests

The authors declare that all authors have no competing interests.

Ethical consideration

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the Turnitin program from the authors.

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To cite this paper: Azis, Salim G, Indarjo A, Prakoso LY, Hartati R, Daengs AGS, Meiryani, Aslan LOM, Ransangan J, and Rozi (2022). Phytochemical and Antibacterial Effects of Leaf Extract from Mangrove Plant (*Avicennia Marina*) on *Vibrio Parahaemolyticus* in Shrimps. *World Vet. J.*, 12 (3): 260-265. DOI: https://dx.doi.org/10.54203/scil.2022.wvj32

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To cite this paper: Azis, Salim G, Indarjo A, Prakoso LY, Hartati R, Daengs AGS, Meiryani, Aslan LOM, Ransangan J, and Rozi (2022). Phytochemical and Antibacterial Effects of Leaf Extract from Mangrove Plant (*Avicennia Marina*) on *Vibrio Parahaemolyticus* in Shrimps. *World Vet. J.*, 12 (3): 260-265. DOI: https://dx.doi.org/10.54203/scil.2022.wvj32