The potential of *Rhizophora mucronata* extracts to protect tiger prawn from pathogenic infections

G Saptiani1*, A N Asikin1, F Ardhani2 and E H Hardi1

1Laboratory of Aquatic Microbiology, Faculty of Fisheries and Marine Sciences, Universitas Mulawarman. Jl. Gunung Tabur Kampus Gunung Kelua, Samarinda 75124, Indonesia

2Department of Animal Science, Agriculture Faculty, Universitas Mulawarman. Jl. Paser Balengkong, Kampus Gunung Kelua, Samarinda 75124, Indonesia

*E-mail: ginaoesman@gmail.com

**Abstract.** The research aims to study *Rhizophora mucronata* leaves extract to inhibit and protects tiger prawn from pathogens infection. The leaves of *R. mucronata* were chopped, dried, macerated and extracted in three different solvents, namely ethanol, water and sea water. In vitro inhibitory tests were performed using disc diffusion and minimal inhibitory concentration methods. The in vivo test on tiger prawn was given by submersion, which then tested with *Vibrio harveyi* and *Saprolegnia* sp. The observation and inspection covered clinical symptom, pathological anatomy, total vibrio count, survival rate, and Relative Percentage of Survival. The result showed that mangrove leaves extract can inhibit pathogens on tiger prawn, improve survival rate and relative percentage of survival. The best *R. mucronata* extract that can inhibit and protect the tiger prawn from pathogen infection is ethanol extract 1,250-1,500 ppm, followed by water extract 1,500 ppm, and sea water extract 1,500 ppm.

1. Introduction

The disturbance of disease in tiger prawn culture in East Kalimantan province, Indonesia until now can not be solved. Tiger prawn deaths can occur from larval stadia in seeding to post larva stadia that have been cultured in ponds, and even before harvest. The common cause of this death is *Vibrio harveyi*, and in larval stadia can also be caused by fungi. The main disadvantage in aquaculture is the sudden outbreak of the disease, mainly due to *Vibrio* spp, which is considered a significant problem for the development of this sector, with severe economic losses worldwide [1]. Disease prevention solutions to date are still not resolved properly. Sometimes farmers still use chemicals and medicines. The use of these chemicals and drugs is often uncontrolled and leads to resistance and toxicity [2]. Several studies have shown that some plant extracts can against fungi and bacteria, both in vitro and in vivo, so as to produce healthy tiger prawn and fish larvae [2, 3, 4, 5, 6].

Mangrove crops grown in coastal areas of East Kalimantan, Indonesia. Mangrove is widely used for household needs, as well as food, beverages and traditional medicine. Mangrove is a source of several bioactive components. Mangrove is used as a medicine by the community and its extract has pathogenic inhibitory activity in humans, animals and plants [7, 8]. Mangrove is a plant rich in bioactive compounds. Mangrove research as a medicine has been widely practiced, as well as mangrove screening as an antibacterial to against disease in shrimp [3, 9]. Mangroves can be used as a new bioactive source of potential natural products used to control microbial disorders [10]. The mangrove phytochemical analysis has revealed important chemicals such as saponins, alkaloids, glycosides, tannins, steroids, flavonoids, gums, phytosterols, and reducing sugars [11].

The mangrove plants are found in many ponds, but the utilization of bioactive materials has not been developed yet [12]. This study aims to study *Rhizophora mucronata* leaf extract to inhibit the growth of pathogens in vitro and in vivo, and to improve the survival of tiger prawn.
2. Materials and Method

2.1. R. mucronata extraction
Leaf of R. mucronata comes from the coast in Muara Badak District, Kutai Kartanegara Regency, East Kalimantan. The leaves are cleaned, washed and drained, after dried chopped and dried in a room not exposed to the sun directly, about 30 days to dry. The leaves are macerated with three different solvents, i.e. 80% ethanol, water and sea water salinity 22 ‰ for 24 hours. The ratio between leaf and solvent is 1:7. The result of maceration is extracted by evaporation method with rotary evaporator. When the concentrated extract becomes about 25%, the salt content that exists in the mangrove plant is removed by liquid method, until the salt is exhausted. The extraction was evaporated on top of the bath until the ethanol solvent evaporated, so extract pellets were obtained, while in the water extract and seawater the process was stopped if the liquid stayed 10% from the original.

2.2. Pathogens preparation.
The pathogens used as the challenge test were V. harveyi and Saprolegnia sp. which come from the Laboratory of Aquatic Microbiology Faculty of Fisheries and Marine Sciences Mulawarman University. V. harveyi tested its pathogenicity by injecting five tails of 2.5 gram tiger prawn size intramuscularly by 0.05 mL with a dose of 10^5 CFu / mL, after five days and the tiger prawn showed a reddish clinical symptom, V. harveyi was isolated from hepatopancreas and infected back to the tiger prawn three times. V. harveyi was then isolated and cultured on Thiosulfate Citrate Bile Salt Sucrose agar (TCBSA) medium a nd incubated for 24 hours at 33 ℃ and observed colonies. If bacterial colonies glow, then V. harveyi is malignant again. Before use the bacteria are fertilized again on the medium of Triptic Soy Broth (TSB) plus 2% NaCl. The Saprolegnia sp fungus is rejuvenated, by culturing on Potato Dekstro Agar (PDA) medium incubated at 33° C for 24 hours.

2.3. Post larva tiger prawn
The tiger prawn used is Post larva tiger prawn (PL) 20, which originated from the hatchery of the people in Muara Badak of East Kalimantan. Post larvae come from the mother and the controlled hatchery, which does not use chemicals or drugs. Tiger prawns are certainly free of Vibrio by sampling for bacterial isolation and culture to TCBSA media. After that the tiger prawn is acclimatized for 1 day in the aquarium, and each aquarium filled 25 tiger prawn.

2.4. Water and aquarium
Sea water used is certainly free from pathogens. Shelter is disinfected with disinfectant, rinsed, immersed in water and dried. Water is deposited on the tub for three days, then flowed into a reservoir and aerated. The water used for salinity research is 22 %, then the isolation and test of V. harveyi, and Saprolegnia sp., should be negative. The aquarium is arranged in such a way that it is hoped that there will be no difference in place, temperature and light. Aquarium filled with water as much as six liters and aerated.

2.5. In vitro test
In vitro tests were performed using disc diffusion (ADD) and Minimum Inhibitory Concentration (MIC) methods. The ADD test of ethanol extract of R. mucronata leaves, water extract and sea water of R. mucronata was carried out on V. harveyi and Saprolegnia sp. The bacteria were cultured on TSB medium plus NaCl 2%, then incubated for 24 hours with temperature 33 ℃. After incubation, the bacteria were diluted to 10^4 CFU/mL and cultured on TSA media plus 2% NaCl in petridish. Mushroom Saprolegnia sp. cultured on Potato Dekstro Broth medium (PDB), then incubated at 33 ℃, for 24 hours. After incubation, it was subsequently diluted to 10^6 CFU/mL and cultured on PDA media in petridish. Treatment of concentration of each extract was 200, 400, 600, 800, and 1,000 ppm and negative control using 0.85% phosphat buffer saline (PBS) and positive control using 0.1 mL/100 mL oxytetrachlin antibiotic solution. Each treatment performed three replications. The treatment was
administered by extracting an extract solution on whatman filter paper disc which is 6 mm in diameter, then placed and arranged in such a way as to bacterial culture and fungus culture. After incubation, observations and measurements of the inhibitory zone diameters were established, starting at 24, 36, 48, and 60 after incubation.

The MIC test was performed on a sterile microplate having 96 holes. all holes are filled with 0.5 mL TSB plus 2% NaCl. In the first row hole, each treated ethanol extract, water and sea water of R. mucronata leaf, as well as negative control of PBS and positive antibiotic control of 0.5 mL each, with 3 replications. Next dilution dilution, until the 8th hole. Then all the holes were filled with V. harveyi 0.1 mL with concentration 10^6 CFU/mL and microplate incubated for 24 hours with temperature 33 °C. Similarly, the MIC test of the fungus Saprolegnia sp., But the media used is PDB, and incubation 33 °C. Bacterial growth was observed from media turbidity, while the clear showed extract capable of inhibiting bacteria or fungi. To corroborate the MIC test results, in the dilution hole which results are dubious, culture is done on TSA or PDA media with spread method.

2.6. In vivo test
In vivo tests were performed on post larvae of tiger prawn. The experimental treatments were ethanol extract of R.mucronata leaf, water extract R.mucronata, and R.mucronata sea water extract, with each concentration 500, 750, 1,000, 1,250 and 1,500 ppm, positive control of 1mL/3 liter oxitetracycline antibiotic and negative control PBS 0.85%. The treatment was administered by immersion and each treatment consisted of 25 Tiger prawn of PL 20, and repeated 3 times. V. harveyi and Saprolegnia sp. were performed after 24 hours of treatment, which was administered by immersion of 1 mL/liters of water medium, with a concentration of 10^6 CFU/mL. The prawn are kept for 28 days.

2.7. Data analysis
The results of the ADD test were observed and measured by the diameter of the clear zone formed around Whatman filter paper disc. MIC was analyzed by observing bacterial growth in each treatment. The determination of MIC is based on the highest dilution yield (or lowest extract concentration) which is still capable of inhibiting bacteria.

Clinical symptoms were observed twice daily during the study. Clinical symptoms observed were changes in behavior, swimming patterns, reflex motion, appetite, and the completeness of frying bodies. Anatomical pathology (PA) observation was performed on dead tiger prawn and at the end of the study. Observation of PA based on the change of color and body shape fry. The survival of tiger prawn fries was observed daily and the Survival Rate (SR), which was obtained from the percentage of larvae that lived until the end of the study. In addition, at the end of the study, the total bacterial content of V. harveyi was calculated by using total count count or total vibrio count (TPC/TVC) and also the effectiveness of the extract to protect the fry from V. harveyi infection by Relative Percentage of Survival (RPS), by the formula, RPS=1-[(j(prawn treatment mortality) x (control prawn mortality))^-1] x 100%.

This study used a complete randomized design and the in vitro and in vivo test results were analyzed using anova test, when there were significant differences followed by Duncans test.

3. Results and Discussion

3.1. In vitro test
Overall, The result of in vitro test of ethanol extract of R. mucronata leaf, R. mucronata water, and R. mucronata sea water using ADD method showed that R. mucronata leaf extract could inhibit the growth of V. harveyi and Saprolegnia sp. with an inhibitory zone of 8.33-12.33 mm and 8.33-12.00 mm, as shown in Figure 1. Treatment of ethanol extract of R. mucronata 1,000 ppm was highest inhibition zone to V. harveyi, but not significantly different from ethanol extract 800 ppm, 800-1,000 ppm water extract and seawater leaves of R. mucronata. The highest inhibit zone against Saprolegnia sp. was found in ethanol extract treatment of R. mucronata 1,000 ppm leaf, which is not different from
600-800 ppm ethanol extract and 600-1,000 ppm sea water extract and 800-1,000 ppm water extract of *R. mucronata* leaf.

![Figure 1](image_url)

**Figure 1.** Inhibition zone of *R. mucronata* leaf extract against *V. harveyi* and *Saprolegnia*

The results of in vitro test on microbial using MIC method showed mangrove extract significantly different with negative control but not different with positive control on ethanol extract treatment and *R. mucronata* leaf sea water. The ethanol extract of *R. mucronata* leaves was able to inhibit *V. harveyi* and *Saprolegnia* sp. with the lowest concentration of 13.02 μg/mL, then sea water extract 20.84 μg/mL, while water extract *R. mucronata* was 26.04 and 31.25 μg/mL as in Figure 2.

Several studies on antimicrobials in mangrove extracts show different inhibitory zones. The ethyl acetate leaf fraction of *A. ilicifolius* has the fastest and best inhibitory, ie 10.67-12 mm to *V. harveyi* [13]. The extract of methanol bark of *S. alba* and *A. marina* fruit showed a considerable drag zone, ie 15 mm to *S. typhi*. The acetic acid extract of *S. alba* showed an inhibitory zone of 14 mm, when tested against *L. monocytogenes* [14]. The ethanol extract of *R. stylosa* showed an inhibitory zone of 12.33 and 11.67 mm, water extract 11.00 and 10.67 mm, and sea water extract 11.67 and 11.33 mm, when tested against *A. harveyi*, and *Saprolegnia* sp. [15]. In this research, ethanol extract of *R. mucronata* leaves able to produce 12.33 mm inhibition zone against *V. harveyi* and 12.00 mm to *Saprolegnia* sp. This shows the *R. mucronata* leaf extract deserves to be used as an antimicrobial material.

![Figure 2](image_url)

**Figure 2.** MIC leaf extract of *R. mucronata* against *V. harveyi* dan *Saprolegnia**
MIC of mangrove plants against pathogenic bacteria ranged from 20 mg/mL to 640 mg/mL [16]. MIC ethanol extract from *Avicenna marina* leaves to *Aspergillus flavus* and *Penicillium italicum* respectively 16 and 8 mg/mL [17]. MIC ethanol extract from *R. stylosa* leaves to *A. harveyi*, and *Saprolegnia* sp. respectively 5.21-15.63 and 6.51-20.84 μg/mL [15]. MIC ethanol extract of *R. mucronata* leaves against *V. harveyi* and *Saprolegnia* sp. is 13.02-31.25 μg/mL. The results of this study showed that *R. mucronata* leaf extract had low MIC value, so it was effectively used as an antimicrobial agent.

3.2. In Vivo Test
In vivo test results showed ethanol extract of *R. mucronata* leaves, water *R. mucronata*, and *R. mucronata* sea water can inhibit *V. harveyi* and *Saprolegnia* sp. on tiger prawn post larvae. Survival on tiger prawn given ethanol extract of *R. mucronata* leaf tested against *V. harveyi* and *Saprolegnia* sp. ranged from 58.67-82.67% and 56.00-81.33%. Water extract of leaves *R. mucronata* ranged from 53.33-72.00% and 45.33-69.33%, while sea water extract is 56.00-76.00% and 53.33-74.67%, as shown in Figure 3. Extract of ethanol leaves *R. mucronata* 1,500 ppm most high survival against *V. harveyi*, which is no different from the antibiotic treatment, but significantly different from other treatments. Next followed by ethanol extract 1,250 ppm and sea water extract 1,500 ppm and subsequent ethanol extract 1,000 ppm and water extract 1,500 ppm. In tiger prawn tested challenge with *Saprolegnia* sp. the highest survival was also in the treatment of ethanol extract of *R. mucronata* 1,500 leaves which was not different from the antibiotic treatment, but significantly different from other treatments. The second best treatment is sea water extract 1,500 ppm, ethanol 1,250 ppm, 1,000 ppm and water extract 1,500 ppm.

Relative Percentage of Survival on tiger prawn given ethanol extract of *R. mucronata* leaf tested against *V. harveyi* and *Saprolegnia* sp. ranged from 35.38-72.59% and 31.21-57.50%, *R. mucronata* leaf water extract is 27.03-56.40% and 14.49-50.97%, while sea water extract leaves *R. mucronata* 31.33-62.40% and 26.89-59.58%, as shown in Figure 4. The ability of mangrove extracts to protect post larvae of tiger prawn against the highest *V. harveyi* attack is ethanol extract of *R. mucronata* 1500 ppm leaf which is not different from antibiotic treatment but significantly different from other treatment. The second best treatment is ethanol extract 1,250 ppm and sea water extract 1,500 ppm, followed by water extract 1,500 ppm. The ability of mangrove extracts protects against *Saprolegnia* sp. the best is also on ethanol extract treatment of *R. mucronata* 1,500 ppm leaf which is not different from antibiotic treatment but different from other treatment. The second best is sea water extract 1,500 ppm, followed by ethanol extract 1,250 ppm.

![Figure 3](image_url)
Figure 4. The Relative Procentage Survival ability of _R. mucronata_ leaf extract given to Post larva of tiger prawn to protect from infection of microbial

TVC measurements showed the lowest antibiotic treatment, not unlike the ethanol extract treatment of _R. mucronata_ leaves 1,500 ppm. The second best is ethanol extract 1,250 ppm followed by treatment of sea water extract 1,500 ppm and ethanol 1,000 ppm.

In vivo test, to prove the effectiveness of _R. mucronata_ leaf extract inhibited microbial infection in tiger prawn, the challenge test was done with _V. harveyi_ and _Saprolegnia_ sp. The results showed that generally tiger prawn treated with clinical symptoms extract normal, there are only about 10% appear reddish on tail and carapace that occur in the treatment of water extract 500 ppm. Similarly, the condition of the pathology of tiger prawn anatomy appears normal. Clinical symptoms of microbial disease appear in negative control treatments, such as redness and damaged tail, legs redness and fracture, reddish rostrum and broken, reddish black body. Generally, tiger prawn that are attacked by _Vibrio_ sp show clinical symptoms of redness, and some external organs appear red, especially gills and limbs [3]. _Vibrio_ sp. causing dirty gills, yellow or pale red, dark and red colour changes in the carapace with abdominal discharge, and tail, dark hepatopancreas with slight redness [18].

_Rhizophora mucronata_ leaf extract is able to inhibit microbial infection and improve post larvae of tiger prawn larvae. The ability to inhibit these microbes can be seen from the results of TVC test, which shows the content of TVC on tiger prawn treated with extracts of low value, especially on ethanol extract and sea water extract _R. mucronata_ leaves. Besides being able to inhibit microbial infections and increase tiger prawn survival, _R. mucronata_ leaf extract is able to protect against microbial attacks. The ability of _R. mucronata_ leaf extract indicates that this plant contains antimicrobial ingredients. The mangrove plant is considered an important source for chemical components of medicinal materials [17]. Bioactive mangrove materials can be used as antioxidants, anti-cancer and component drugs [19, 20]. Mangrove plants are a good source of steroids, triterpenes, saponins, flavonoids, alkaloids and tannins [21]. _Rhizophora mucronata_, a tropical red mangrove, has been widely used in traditional medicine as an antiseptic, antibacterial, anti-ulcusogenic and anti-inflammatory agent [22]. _Rhizophora mucronata_ leaf components have the potential to be used in the treatment of gastrointestinal motility such as diarrhea [23]. _Avicennia marina_ and _Rhizophora mucronata_ leaf ethanol extracts have antifungal activity on _P. pupurogenome_, _P. chrysogenum_, _P. notatum_, _A. niger_, _A. alternata_ and _Penicillium italicum_ [24].

4. Conclusion
_Rhizophora mucronata_ leaves extract can inhibit _Vibrio haveyi_ and _Saprolegnia_ sp. on tiger prawn, improve survival rate and relative percentage of survival. The best extract that can inhibit and protect
the tiger prawn from pathogen infection is ethanol extract of *Rhizophora mucronata* 1,250-1,500 ppm, followed by water extract 1,500 ppm, and sea water extract 1,500 ppm.

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