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Original Paper

THE EFFECTIVENESS OF *Acanthus Ilicifolius* IN PROTECTING TIGER PRAWN (*Penaeus monodon* F.) FROM *VIBRIO HARVEYI* INFECTION

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ABSTRACT

Sea holly (Achantus ilicifolius) is a mangrove plant that often used by coastal society as a traditional medicine. It is potential to be developed as the sources of pharmaceutical products. This study aims at assessing the effectiveness of sea holly leaves as antibacterial agent and as an agent to enhance the durability of shrimp against Vibrio harveyi. First, dried sea holly leaves were extracted with methanol, after that, it was fractionated with silica gel column method using solvent n-hexane, ethyl acetate, and n-butanol. The treatments given to tiger prawn were crude extract, the fraction of ethyl acetate, and n-butanol, and it was given by immersion. Next, the challenge test was conducted toward Vibrio harveyi. As the result, the extract and the leaves of A. ilicifolius possess activities of inhibiting the growth of V. harveyi in vivo, reducing the prevalence of attacks and improving survival of prawn. In general, based on clinical symptoms and pathological anatomy, n-butanol fraction of the A. ilicifolius leaves possess the best protection, along with ethyl acetate fraction and the crude.

Key words: *Acanthus ilicifolius*; extraction; fractionation; *Vibrio harveyi.*

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INTRODUCTION

Mangrove plants are often seen around the grow-out ponds environment, yet the utilization of its bioactive material has not been developed. "Jeruju" (Sea Holly/*Acanthus ilicifolius*) is a mangrove plant often found in aquaculture coastal area of East Kalimantan, Delta Mahakam in particular. The plant, normally, lives in the area which is rather low in its salinity (± 15-20 ‰), forming bush around mangrove palms. *Acanthus ilicifolius*, mangrove, possesses bioactive compounds that is potential to be used as an antibacterial agent (Manilal, *et al.*, 2009). According to Saptiani, *et al*. (2011), the extract and the fraction of *Acanthus ilicifolius* leaves are potential to inhibit the growth of *V. harveyi in vitro*. The plant contains chemical compound of glucose, alkaloid, flavonoid, fatty acids, steroids, lignans, and component of phenol and terpenoid (Kanchanapoom, *et al*. 2001: Wostmann and Liebezeid 2008). The exploration of bioactive components from natural resources is an alternative in tackling disease in prawn. To be able to control and to prevent disease is one success in prawn cultivation. Disease remains the major problem for prawn cultivator and hatchery business in East Kalimantan. Bacterial disease that attacks the prawns mostly is *Vibriosis*. Generally, the prawns attacked by *Vibrio* show clinically symptoms of reddish black, and some of the outer organ seem red, particularly the gills and limbs. When the prawn looks lit at night, it will be followed by the occurrence of prawns' mass death (Saptiani and Hartini 2008). *Vibrosis* can cause lost in prawn cultivation, starting from larvae stadia to

post larvae, when cultivated grow-out pond. (Kumaravel, *et al.,* 2010; Velmurugan and Citarasu 2010).

In East Kalimantan, antibiotics and chemicals are still the mainstay of grow-out ponds employers and hatchery to overcome the disease. The issue is, the usage of chemicals and antibiotics are often uncontrollable and it leads to a new problem, namely, the resistance. Usually, the larvae coming from hatcheries that use a lot of chemicals and antibiotics often cause many prawn dead when sown in grow-out pond (Saptiani and Hartini 2008; Saptiani, *et al*., 2011). This condition, assuredly, must be very detrimental to aquaculture business. Therefore, an alternative is needed to prevent and overcome the disease, namely; finding natural bioactive compounds originating from surrounding environment which can be utilized as a source to overcome the disease.

The purpose of this study is to assess the effectiveness of the leaves extracts and fractions of *A. ilicifolius* as antibacterial agent and as agent that can enhance the durability of shrimp against *Vibrio harveyi* by examining its inhabitation activity toward the *V. harvei* growth *in vivo*, to examin the ability of its protection potency, and also to find out its effectiveness concentration to inhibit the *V. harvei* growth. The result of this study is expected to be used as a basis for further research; moreover, it is also an alternative as an effort to prevent and control *Vibriosis* disease naturally by utilizing the vegetation around the grow-out pond.

MATERIALS AND METHODS

Sampling of Acanthus ilicifolius

Acanthus ilicifolius taken from the shrimpculture area on Muara Badak District, Kutai Kartanegara Regency of East Kalimantan. Simples were taken and selected in the location of mangrove tighty populatied and ponds area. Leaves of *A. ilicifolius* taken from the leaf stalks are located around the central stem.

Acanthus ilicifolius Extraction

Acanthus ilicifolius leaves were cleaned and washed with fresh water, drained, dried, then finely chopped. The chopped leaves were dried in a room that was not exposed to direct sunlight. The leaves were extracted by storing them in a jar and macerated in a methanol. Then, the macerated result was extracted with evaporation method according to Aknin, *et al.*, (1999) and Manilal, *et al*., (2009) by taking back a solvent that bind the active material with a Rotavapor to per form thick liquid. The extraction result was steamed using water bath, thus extract pellet (crude) was obtained. The extraction result was taken partly to do fractionation using silica gel with column method. The solvents used were n-hexane, ethyl acetate, and n-butanol. Each of the solvents from fractionation was evaporated until pellet was obtained. As the result, 1 extract substance and 3 fractions, crude extract (crude), fraction of n-hexane, fraction of ethyl acetate and fraction of n-butanol, were obtained.

The Pathogenicity Test

The bacteria used in examination were taken from Brackish Water Research Institute, Maros of South Sulawesi. Before used, its pathogenicity was examined by injecting 0,1ml dose (10^3 cfu/ml) to tiger prawns intra-muscularly. After 4-5 days, the prawns showed a clinical symptom of redness. Then, the isolation of bacteria from its hepatopancreas was conducted and infected again to the prawns for 3 times. Next, *V. harveyi* bacteria were isolated and cultured on media of *Thiosulfate Citrate Bile Salt Sucrose Agar* (TCBSA) and incubated for 24 hours at 33^o C, then the bacteria were characterized and fertilized on media of *Triptic Soy Agar* (TSA).

Water Treatment for Media

This study was carried out in the water with good quality and free from any viruses and bacteria since the water treatment had been conducted initially. Salty water was passed into tank, added 30 ppm of chlorine and left in place for about 6 hours, and aerated for 24 hours. After that, the tank was added 10 ppm of sodium thiosulfate ($Na^2S^2O^3$), aerated again, added Ca (limestone) until its pH reached 8.00 and then precipitated. The tub of filtration was filled with water, added with EDTA 5 ppm, tested its pH and salinity, and aerated. The

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following process was to conduct the test whether or not there was *Vibrio sp* on TCBSA culture. The fresh water, used as examination media, was from PDAM that had been precipitated for 48 hours, then removed and aerated continuously for 48 hours, and on TCBSA culture the *Vibrio* bacteria test was performed. Water salinity used as a media research was adapted to the conditions of intermolt-premolt isosmotic. Below is the mixing of seawater and fresh water:

$$S = \frac{[(S_1 x V_1) + (S_2 x V_2)]}{(V_1 + V_2)}$$

Description:
S = Intended/proposed salinity (‰)
= seawater salinity
S_2 = fresh water salinity
V_1 = seawater volume
V_2 = fresh water volume

Prawns Preparation

The prawns used in the study were 2-3 gram in weight and 1,5 months old and came from CV Doddy, Hatchery in Muara Badak District, Kutai Kartanegara Regency of East Kalimantan. Before bred, the healthy prawn was not given antibiotics, chemical substances and other medicine. Next, the healthiest and motile larvae in PL 8 stadia from the breeding were chosen and cultivated in grow-out pond until grew around 2-3 gram weight. The healthy prawns were selected, put in a plastic bag, given oxygen and brought to the laboratory. In the laboratory, the prawns were put in the aquarium to be adapted for 24 hours, and screening test was performed by immersing the prawns using 200 ppm formalin for 15 minutes, then and used as tested object of the research .

Examination of Protection Potency of Sea Holly Leaves Extract and Fraction

The treatment contained 3 active materials with 3 different type doses. They were crude extract (200, 450, 700 ppm doses), ethyl acetat fraction (200, 450, 700 ppm doses), n-butanol fraction (100, 200, 300 ppm doses), negative control (PBS 0,85 %) and positive control (oxytetracycline 0.05 mg /ml), with 3 replications of each. The prawns were put in 33 different aquariums (30 prawns for each), and acclimated for 3 days. Extract and fraction of *A. ilicifolius* leaves treatment was performed by dipping method during 30 minutes, and then, on day 7 challenge test with 10^5 cfu/ml dose of *Vibrio harveyi* as much as 0.1 ml was conducted intra-muscularly on dorsal part.

The inhabitation observation or bactericide was performed with bacteria content test (Total Plate Count/TPC) by taking hepatopancreas sample to be isolated, cultivated on TSA and TCBSA media, and incubated at 33^o C for 24 hours. Then, the amount of colony growth was counted. The inhabitation observation and inspection covered clinical symptom, phonological anatomy, prevalence of attacks and viability. The observation and inspection is illustrated on **Fig. 1.**

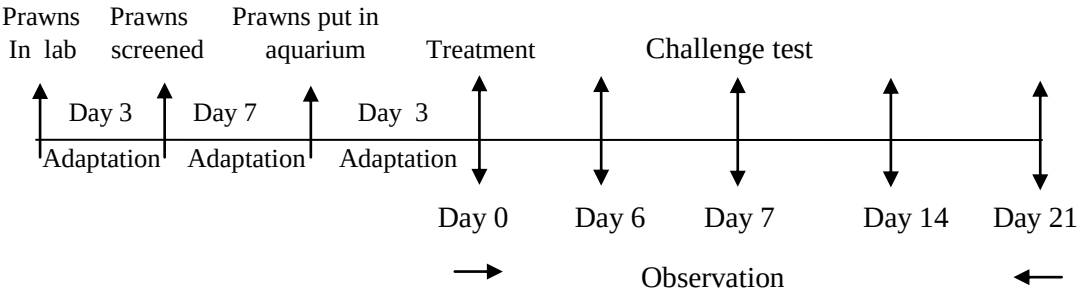

Fig. 1. Research phase of the effectivenes of Sea Holly (*A. ilicifolius*) leaves extract in protecting tiger prawn (*Penaeus monodon F*.) from V. *harveyi* infection

RESULTS AND DISCUSSION

Vibrio harveyi was used because they are the most frequent cause of disease in prawn cultivation in grow-out ponds or in hatchery of East Kalimantan areas (Saptiani and Hartini 2008). Meanwhile, according to Suginta, *et al*., (2010), *V. harveyi* is a family of gram-negative

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bacteria in open seawater that cause luminous *Vibriosis* in prawn, a serious disease in prawn cultivation. The cell wall of the bacteria containing peptidoglycan and lipopolysaccharide functions as a cell protector (Austin and Zhang 2006).

The observation and calculation of bacteria content (TPC) in prawn's hepatopankreas organ, at day 14 after the administration of the leaves extract and fractions of A. *ilicifolius*, showed that the treatment of n-butanol fraction of 300 ppm, and 200ppm, ethyl acetate fraction of 700ppm, and 450 ppm were the lowest in its TPC (2.33 cfu/ml in average). In the meantime, TPC on the other treatment was fraction of n-butanol 100 ppm (2.67 cfu / ml), ethyl acetate fraction of 200 ppm and 700 ppm crude (6.00 cfu / ml), 450 ppm crude (8.67 cfu / ml) and 200 ppm crude (10.33 cfu / ml). Its TPC on the antibiotic treatment was 8.33 cfu/ml, and on the negative control was 14.67 cfu/ml, after day 21, its TPC slowed down except 450 ppm crude, 450 ppm ethyl acetate fraction, and 700 ppm which the content were stable, meanwhile the antibiotic and negative control treatment on crude 200 ppm treatment increased, as showed on **Fig. 2.** The *A.ilicifolius* extract and fraction could inhibit *V. harveyi* on prawns, primarily on the treatment of n-butanol fraction in all concentrations which could inhibit and reduce *V. harveyi*. This result showed that the bioactive content on *A. ilicifolius* extract and fraction was bactericidal toward *V. harveyi* on prawn; therefore it can be utilized as herbal medicine and bio-pharmacy source. Sivaram, *et al*., (2008), stated that the methanol extracts of herbal pathogenic *Vibrio* can control and improve the immunity system of the larvae Grouper (*Epinephelus tauvina*). Some researcher stated that *A. ilicifolius* mangrove contains bioactive compound that is potential to use as antibacterial agent (Manilal *et al*., 2009; Khajure and Rathod 2010; Thirunavukkarasu *et al*., 2011). The extract of *A.ilicifolius* leaves and root can inhibit bacteria growth, but the leaves extract inhabitation is better than the root (Khajure and Rathod 2010). According to Mayer (2011) a material can be categorized as bacteriostatic antibiotic if it can inhibit bacteria growth or bactericidal, or if it can kill bacteria.

The observation of clinical symptom after the conducting of extract and fraction leaves, before challenge test (before day 7), showed that the treatment was safe and did not cause death and specific clinical symptoms. On the beginning of treatment (day 2-4 of the treatment) showed that the body color changed more blue, primarily on the n-butanol fraction treatment. The prawns color changing indicated the occurrence of body reaction toward the extract and fraction of *A.ilicifolius* leaves treatment, and it was caused by the enlargement of prawns' cuticle. The color changing was also a sign that the immunity process occurred in order to fight foreign object entering the body since chromatophore is one of the body's defense system in prawn (Saptiani and Hartini 2008).

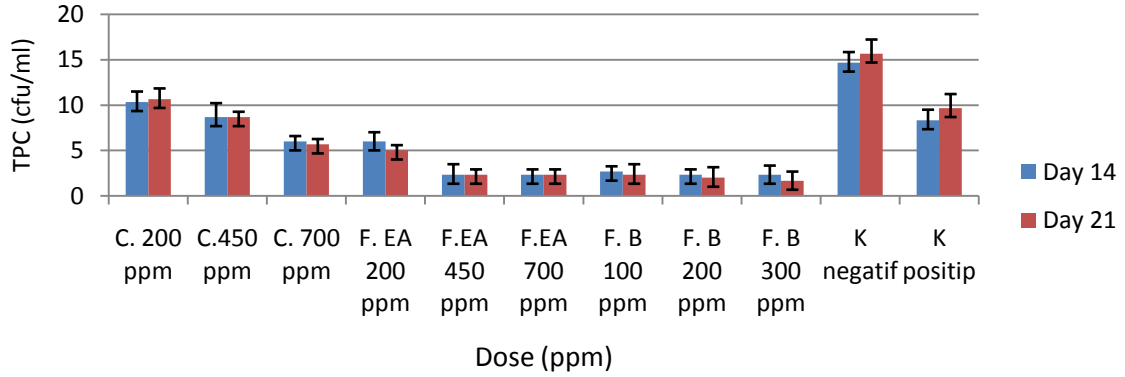

Fig. 2. Bacterial content (TPC) in giant tiger prawn hepatopancreas given the leaves extract and fractions of Sea Holly *(A.ilicifolius)*.
Description : C = crude, F.EA = fraction of Ethyl acetate, , F.B = fraction of n-butanol, K^- = negative control (PBS); K^+ = positive control (antibiotics)

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Generally, the clinical symptom in prawns after given the extract and fraction treatment and after the injection of *V. harveyi* showed better symptom if compared to the symptom on negative control treatment. Similarly on the anatomy pathology changing, the extract and fraction treatment showed that the anatomy pathology changing caused by *V. harveyi* infection could be prevented (**Fig. 3**). The observation of anatomy pathology performed on day 14, after infected by *V. harveyi* on day 7, showed that the prawn hepatopancreas on negative control treatment seemed brownish and mushy, some declined, and its bowel became reddish and harder. The bacteria in prawn digestion and organs functioning as digestive system would disrupt the work of the digestive system because the bacteria were able to parse the various polysaccharides and carbohydrates and took nutrients needed, and finally became the cause of prawns death. According to Austin and Zhang (2006) *V. harveyi* in fish causes *vasculitis*, *gastroenteritis* and *lesi* on its eyes. While in pathogen prawn, frequently, it associates with *vibrioluminous*. The pathogenicity mechanism of these bacteria is hardly understood, but its ability to harm, mostly, associates with its extracellular product, such as *protease, haemolysin* dan *lipopolisaccharide*. On the control negative treatment, the anatomy pathological observation on day 14 or day 7 after *V. harveyi* challenge test, its hepatopancrease changed into brown, on the following day this organ changed into dark brown, shrank and destroyed, and finally did not function normally. The bacteria content on TPC test increased along with the changing of hepatopancreas anatomy pathology. Meanwhile, the *A.ilicifolius* leaves extract and fraction treatment made the changing of anatomy pathology better, even though at the beginning some hepatopancreas turned a little bit brownish.

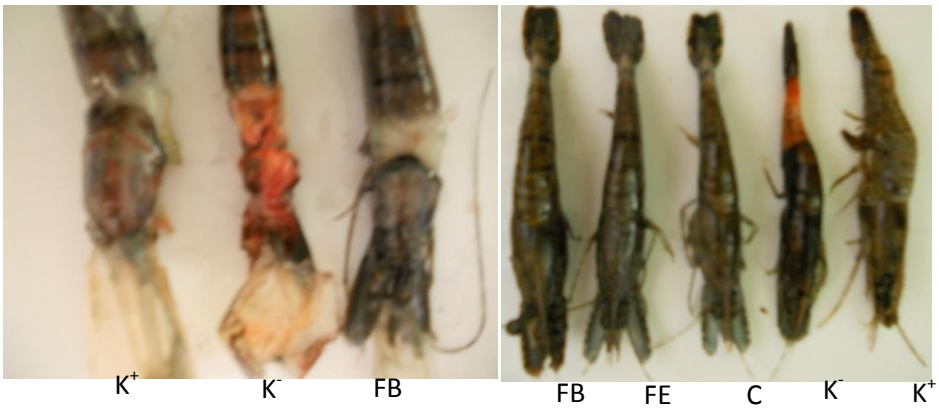

Fig 3. Clinical symptom and anatomy pathology of giant tiger prawn
Description: C = crude, FE = acetate ethyl , FB = n-butanol, K^- = negative control (PBS); K^+ = positive control (antibiotics)

The observation and calculation of the prevalence of the *V. harveyi* infection in prawns, administrated with the extract and fraction of *A.ilicifolius* leaves, showed that bioactive in the leaves could decrease the prevalence of infection or *V. Harveyi* attack (**Fig. 4**). The average prevalence of *V. harveyi* attack on the negative control treatment on day-14 was 65.53%, and after day 21 it decreased becoming 61.065%. The extract leave treatment on day 14 showed the highest attack which is 33.32%, meanwhile the positive control (antibiotics) was 33.7%. The observation and calculation of prevalence on day 21 showed that all the treatments decreased. It occurred because the treatment followed by challenge test or *V. harveyi* injection, precisely, increase the prawn immunity. As reported by Saptiani (2001), test challenge can increase antibody where the antibody is built after the infection, and it will increase more if there is secondary infection. This event will be beneficial to the organism itself, for it will increase resistance to the pathogenic organisms. The result of this research showed the administration of extract and fraction of *A. ilicifolius* can inhibit *V. harveyi* growth and protect the prawns from *V.*

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harveyi attack. Compared with antibiotics, the protection potency and inhabitation of the leaves are still better. According to Kumar, *et al.*, (2008) methanol fraction of the leaves can inhibit peritoneal inflammation in rat.

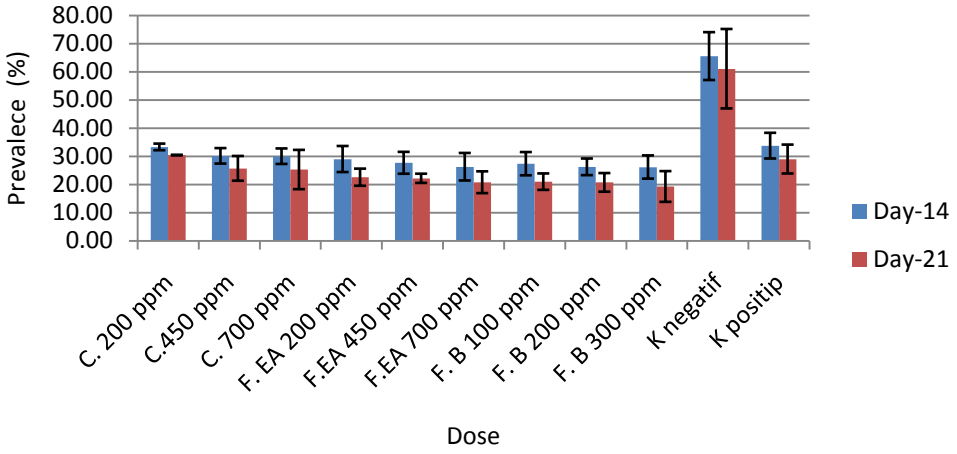

Fig. 4. Occurrence prevalence of *V.harveyi* infection in prawn given leaves extract and fractions of Sea Holly *(A.ilicifolius)*.
Description: C = crude, FE = acetate ethyl , FB = n-butanol, K- = negative control (PBS); K+ = positive control (antibiotics)

The shrimps survival rate on day 14 that was given the extract and fraction of *A.ilicifolius* was around 82.21- 86.77 %, while the survival rate resulted from the positive control treatment (antibiotics) was 83.16% and from the negative control treatment was 53.62%. The complete results can be seen in **Fig. 5.** The administration of *A. ilicifolius* leaves extract and fraction can enhance the survival of tiger prawn better than the negative control treatment can toward *V. harveyi* attack. It showed that the bioactive of *A. ilicifolius* leaves extract and fraction could inhibit *V. harveyi* growth and protect tiger prawn from *V. harveyi* attack.

From the result of chemical compound identification, actually *A. ilicifolius* leaves contain polyphenols, alkaloids and flavonoid. The alkaloids examination result of n-buthanol fraction was more apparent than the ethyl acetate fraction was, while the examination results of polyphenol and flavonoid were relatively similar. Alcaloids and flavonoid class of plants contain a lot of glucoside. As reported by Kanchanapoom, *et al*., (2001), *A. ilicifolius* contains lignan glucoside, phenylpropanoid glycosides and megastigmane glycoside, while according to Huoab, *et al*., (2003) *A. ilicifolius* possesses glucoside component which is 5,11 –epoxymegastigmane of glucoside. N-butanol fraction of *A.ilicifolius* leaves is the best agent to inhibit *V. harveyi* growth in vivo, to increase shrimps survival, and to decrease the prevalence of *V. harveyi* attack on prawn compared to the ethyl acetate fraction and the crude extract. Meanwhile, ethyl acetate fraction in vitro possesses best antibacterial ability compared to crude extract and n-butanol fraction (Saptiani, *et al*., 2011). It is explainable as glucoside or glucan contained in the flavonoid and alkaloid can be bound in the form of ester. Glukoside compounds contained in the ethyl acetate are bound semi-polar, therefore it is difficult to absorb in the shrimps body, whereas n-butanol is more polar in nature, so it is easier to absorb in the target cell. Glycoside is more polar and is primarily metabolite.

The bioactive of *A. ilicifolius* leaves extract and fraction is immunogenic in nature, therefore it can increase the immunity of tiger prawn and protect it from *V. harveyi* attack. *A. ilicifolius* leaves can be utilized as antibacterial agent and immunostimulants in tiger prawn.

According to Mayer (2011), if bacteriostatic antibiotic is used for therapy, it should sufficiently result cellular and humoral immunity of mechanisms to exterminate the bacteria.

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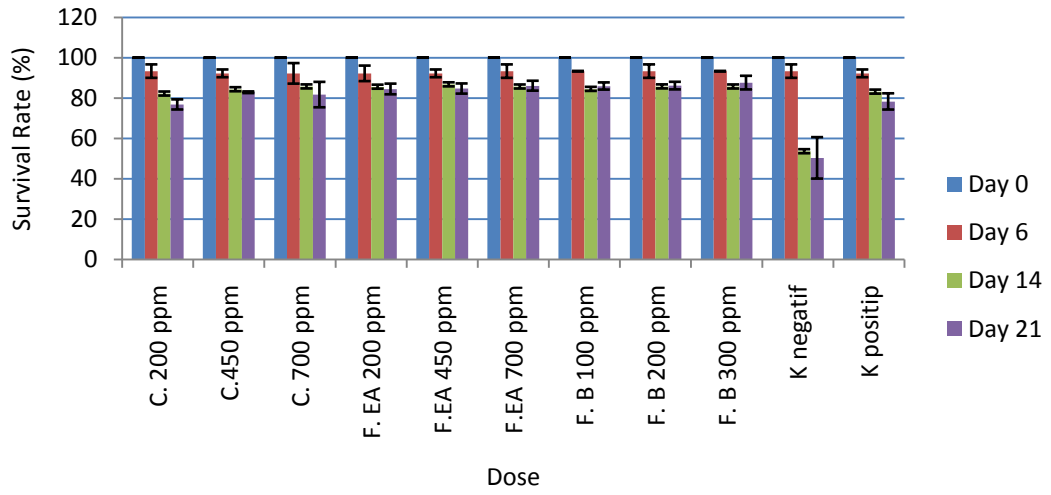

Fig. 5. The survival of giant tiger prawn given the leaves extract and fractions of *A.ilicifolius*
Description: C = crude, FE = acetate ethyl , FB = n-butanol, K- = negative control (PBS); K+ = positive control (antibiotics)

Plant extracts can be used as medicine plant (bio-pharmacy) and as an agent to enhance immunity as well as safe to use in aquaculture systems (Sivaram, *et al*. 2008).

CONCLUSION

The leaves extract and fraction of *A. ilicifolius* effectively inhibit *V. harveyi* growth in tiger prawns, decrease the prevalence of attacks, and increase the tiger prawns survival from *V. harveyi* attack. Fraction of n-butanol of *A. ilicifolius* leaves possesses best protection potency followed by ethyl acetate fraction and the crude.

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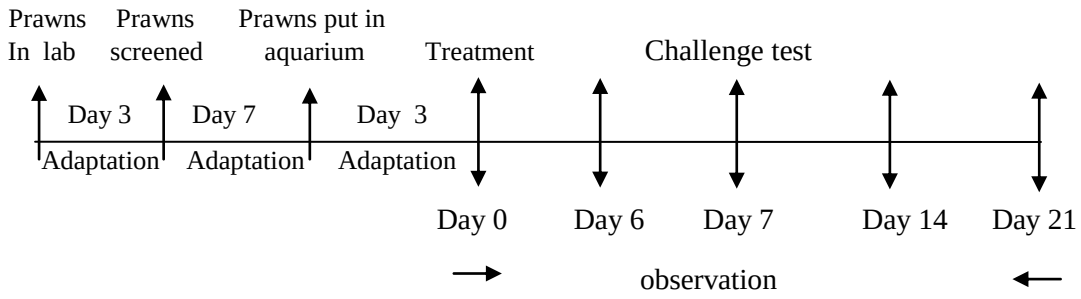

Figure 1. Research phase of the effectivenes of Sea Holly (*A. ilicifolius*) leaves extract in protecting tiger prawn (*Penaeus monodon F.*) from V. *harveyi* infection.

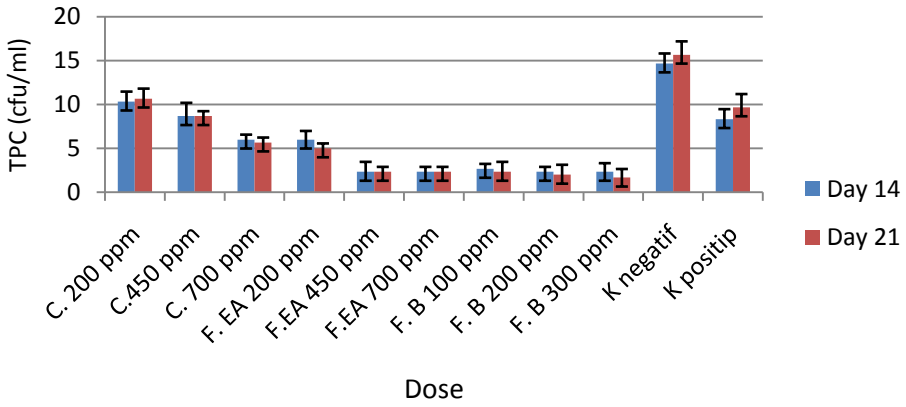

Figure 2. Bacterial content (TPC) in giant tiger prawn hepatopancreas given the leaves extract and fractions of *A.ilicifolius*.
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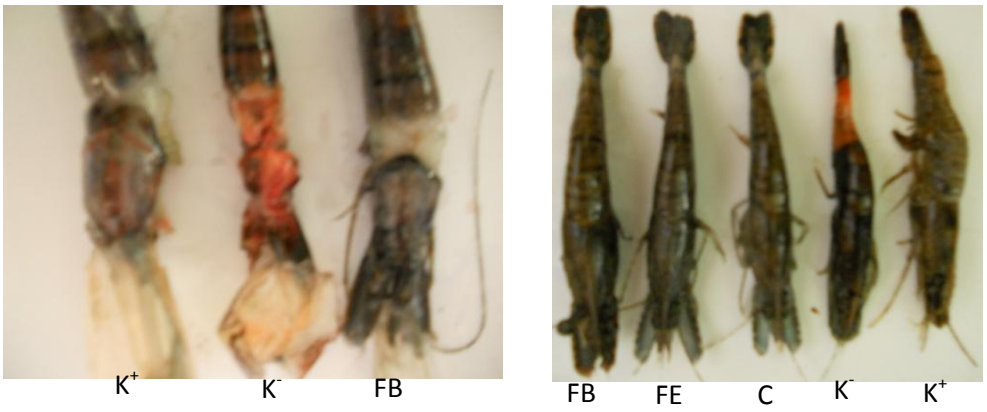

Figure 3. Clinical symptom and anatomy pathology of giant tiger prawn
Description: C = crude, FE = acetate ethyl , FB = n-butanol, K^- = negative control (PBS); K^+ = positive control (antibiotics)

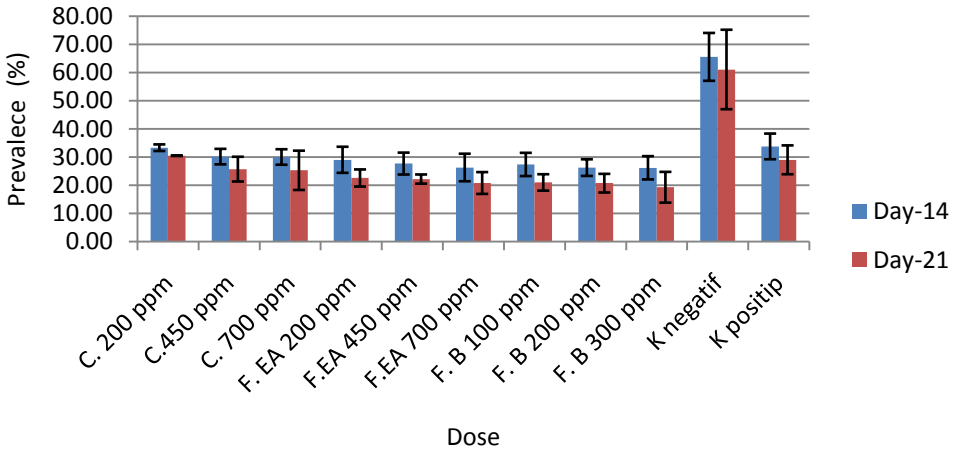

Figure 4. Occurrence prevalence of *V.harveyi* infection in prawn given leaves extract and fractions of A.ilicifolius.
Description: C = crude, FE = acetate ethyl , FB = n-butanol, K- = negative control (PBS); K+ = positive control (antibiotics)

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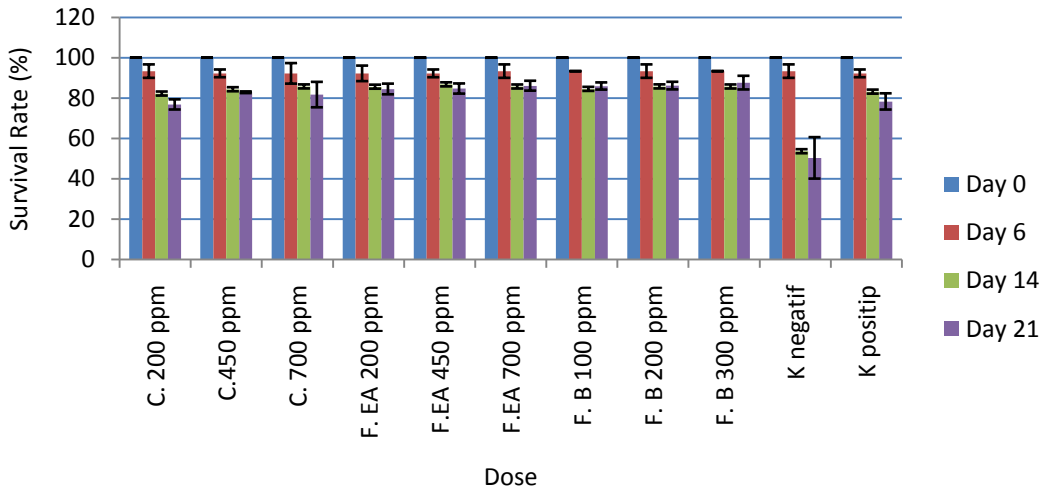

Figure 5. The survival of giant tiger prawn given the leaves extract and fractions of *A.ilicifolius*
Description: C = crude, FE = acetate ethyl , FB = n-butanol, K- = negative control (PBS); K+ = positive control (antibiotics)