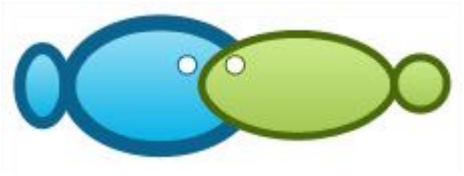

Inhibiting *Vibrio harveyi* infection in *Penaeus monodon* using enriched *Artemia salina* with mangrove fruit *Sonneratia alba* extract

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Abstract. The high level of attack of both infectious and non-infectious diseases results in low production in giant tiger prawn (*Penaeus monodon*) in North Kalimantan's traditional coastal aquaculture ponds. One of the most severe pathogens of concern is *Vibrio harveyi*. The present study investigates whether *Sonneratia alba* fruit extract inhibits *V. harveyi* infection challenging giant tiger prawn postlarvae through a feed of *Artemia salina*. Three different doses of *S. alba* extract (15 ppm, 20 ppm, and 25 ppm), and one control (0 ppm) were used in this study. The extract was used to enrich *A. salina* as feed for giant tiger prawn postlarvae, which were then challenged with *V. harveyi*. The experimental methods involved a completely randomised design (CRD) with three replications. Among the four groups, the highest survival rate (78.33%) was observed in the treatment using 20 ppm of *S. alba* extract, and this group exhibited significantly (P>0.05) better survival and inhibition of *V. harveyi* infection in giant tiger prawn.

Key Words: mangrove fruit, bio enrichment, phytochemicals, immunostimulantsi.

Introduction. Efforts to achieve high production in giant tiger prawn *Penaeus monodon* (Fabricius, 1798) cultivation continue to encounter various problems. One of the most significant problems is the high prevalence of infectious and non-infectious diseases. Various types of diseases have been observed in giant tiger prawn farming that are often caused by *Vibrio harveyi* bacterial infection (Trianto et al 2004). *Vibrio* species are ubiquitous in marine and brackish water environments; however, some species are considered opportunistic pathogens of immune-compromised fish and shrimp (Alonzo et al 2017). Diseases caused by *Vibrio* bacteria often referred to as vibriosis.

Vibrio disease often occurs in the nauplii, zoea, and mysis stages, and sometimes occurs in the postlarvae stage in aquaculture (Saptiani et al 2012). *V. harveyi* is an opportunistic pathogen because it changes from saprophytic to pathogenic if environmental and host conditions deteriorate (Diggles et al 2000).

Several studies of herbal ingredients or phytochemicals have been conducted on aquatic biota using petal extract and fruit of the mangrove *Sonneratia caseolaris* (L) Engler as an antibacterial against the infection of *V. harveyi* in giant tiger prawn (Maryani et al 2002). Moreover, ethanol extract of *S. alba* contains active compounds in the form of alkaloids, flavonoids, triterpenoids, carbohydrates, carotenoids, tannins, and coumarin (Satriani et al 2017). Phytochemical analysis of *S. alba* ethanol extraction samples has also revealed that active ingredients contained in the extract are potential antibacterial candidates. Notably, several studies have reported that mangroves can inhibit the growth of *V. harveyi*. Naiborhu (2002) stated that leaf extracts, petals, fruit and seeds of *S. caseolaris* were able to kill and inhibit the growth of *V. harveyi*. This plant contains bioactive compounds such as flavonoids, steroids, phenol hydroquinone and powerful tannins as antimicrobial agents.

Generally, saponin compounds are contained in plants that have a bitter taste - such as papaya leaves and bitter melon fruit - while *S. alba* has an acidic taste that is similar to that of tamarind. Inhibitory effect of *S. alba* fruit ethanol extract on *V. harveyi* bacteria in thiosulfate citrate bile salts sucrose agar media using the paper disc diffusion technique demonstrated that the extract was capable of producing marked inhibitory power with the formation of clear zones (2.50±0.31 cm diameter) at a concentration of 2% (Cahyadi et al 2017).

The present study aims to examine the effect of mangrove fruit *S. alba* extract as an immunostimulant on *V. harveyi* bacteria when applied *in vivo* to giant tiger prawn postlarvae.

Material and Method. The study was performed at the Laboratory of Fish Nutrition and Feed, Water Quality Laboratory, and Mini hatchery of the Faculty of Fisheries and Marine Sciences, University Borneo Tarakan (UBT), Tarakan City, Indonesia, from January to August 2018.

A quantity of 35 kg of young *S. alba* fruit were taken, with light green criteria from the coast of Tarakan Island, Indonesia. The fruit was then washed under running water, cut into small pieces and dried in an hot air oven at 40°C for three days. The dried fruit was mashed into simplisia through a maceration process. The maceration process was performed by soaking the simplisia in 96% ethanol solution with a comparison of simplisia and ethanol of 1:4. Using a magnetic stirrer, the solution was kept in a closed glass beaker for 72 hours to ensure that parts of the small simplisia tangent were optimised with a solvent to facilitate the active ingredients contained in the simplisia to be extracted and to expand the surface of the tangent between the material and the solution. The filtrate solution from the maceration process separated the active ingredient and 96% ethanol solvent to create concentrated extract in the form of a dilute paste containing phytochemical material using a rotary evaporator at 40°C. The aqueous paste was formed and then evaporated in an oven at 40°C continuously for 24 hours until a thick *S. alba* paste was created (Puspitasari & Proyogo 2016).

A total of 0.5 gram of *A. salina* cyst was weighed and then incubated in 1 liter of 32 mg L^{-1} salinity water, strong aeration for 24 hours, and then harvested. *S. alba* extract test on *A. salina* had a 50% lethal concentration (LC_{50}) at a concentration of 20 ppm. The utilisation of ethanol extract bioenrichment of *S. alba* in the essential feed of *A. salina* was divided into four different aquaria (glass tanks) with a volume of 100 mL, and each was filled with immersion ethanol extract of *S. alba* fruit with a concentration of P1 (without extract), P2 (25 ppm), P3 (20 ppm), P4 (15 ppm). *A. salina* culture bioenrichment was performed every three days according to each respective treatment. This was designed to refresh *A. salina* bioenrichment so that giant tiger prawn larvae obtained optimal feed during maintenance. The study used approximately 300 giant tiger prawn postlarvae (12 days old) in this investigation. The postlarvae were collected from a local hatchery in Tarakan City and were acclimatised for 1-3 days. Healthy larvae advanced against water current in their container and actively consumed the feed.

The purified isolate of *V. harveyi* bacteria collected from Balai Penelitian Budidaya Air Payau Jepara, Indonesia was rejuvenated in Thiosulfate Citrate Bile Salts Sucrose Agar (TCBSA) selective media on Petri dishes, scratched on sterilised TCBSA selective media and then incubated at 30°C for 24 hours. *V. harveyi* that were successfully rejuvenated were used for serial dilution, which involved bacteria produced from a culture in TCBSA media being taken and placed into a test tube containing Alkali Pepton Water and then homogenised with vortex until well blended and incubated for 24 hours. Thereafter, the optical density (OD) value was tested using a spectrophotometer with a wavelength of 600 nm to reach a value of > 0.600. Then, 0.1 mL of culture was taken and placed into a test tube containing 0.9 mL APW (dilution 10^{-1} to 10^{-7}) (Hadioetomo 1990).

The present study used 24 aquaria of a 5-litre volume containing 2 litres of seawater. The establishment of each container included washing with soap and rinsing with water until it was clean and dry. All sides of each container were covered with colored plastic to avoid stressing on the larvae. Aeration systems were installed that used

blowers, aeration tubing, and aeration stones. The amount of aeration stones were used is 24 pieces. Water inserted into the container was left and actively aerated for 24 hours before the 20 *A. salina* were placed inside.

This study used 300 postlarvae 10 ($P.L._{10}$). Samples were obtained from a local hatchery in Tarakan City and acclimatized for 1-3 days. In the first period, samples were fed *A. salina* (± 20 individuals per shrimp larvae) based on Joni et al (2007) for 10 days, and then on the second period, the challenge test used *V. harveyi* bacteria to infect the sample for another 2 days. The analysis consisted of observing clinical symptoms of giant tiger shrimp larvae, survival rates, and applying bacterial biochemical tests based on the procedures of Bergey & Holt (1994) and Cahyadi & Gusman (2009).

The present study used an experimental method performed with a completely randomised design (CRD) consisting of four treatments of *S. alba* extract and three replications, which included Group 1: *A. salina* with 0 ppm of extract (control); Group 2: *A. salina* added with 25 ppm of extract; Group 3: *A. salina* with 20 ppm extract; and Group 4: *A. salina* with 15 ppm extract. The survival rate of giant tiger prawn postlarvae was measured using the method developed by Effendie (2002):

$$SR = \frac{N_t}{N_0} \times 100$$

where: SR = survival rate (%);
Nt = the number of larvae at the end of the study;
No = number of larvae at the beginning of the study.

Data collected from the results of this study were analysed descriptively and statistically using SPSS 18 Software. To determine the effect of treatment on the survival of giant tiger prawns, analysis of variance (ANOVA) was used with the significance set to 95% confidence level (α 0.05). If significant differences existed, further least significance difference (LSD) was performed.

Results and Discussion. *A. salina* enriched with *S. alba* fruit extract treatment showed positive results, as they contained alkaloid compounds, flavonoids, saponin, steroids, and phenols with zero tannins or colour detection at the time of testing. The results of the qualitative phytochemical examination indicate that *S. alba* extract can be absorbed by *A. salina* (Table 1).

Table 1

Sonneratia alba extract absorption by *A. salina*

Parameters	*S. alba extract*	*Bioenrichment*	*Non-bioenrichment*	*Indicators*
Alkaloids	+++	+++	+++	Produce deposits of brown compounds.
Phenol hydroquinone	+++	+++	+	Produce deposits of brown compounds.
Flavonoids	+++	+++	+++	Produce foam.
Saponins	+++	+++	+++	Produce orange compounds.
Steroids	+++	++	-	Produce blue compounds.
Tannins	+++	-	-	Produce black blue, blue and green compounds.

+++ (very strong); ++ (strong); + (less strong); - (not contained).

A. salina has nonselective filter properties in the consumption of feed (Sivaji 2016; Rizk et al 2018). *A. salina* will eat anything that can be consumed around it, including *S. alba* extract. According to Nedi et al (2006), the safety concentration of organisms from toxicity is 10% of the LC_{50} value. However tannins can be toxic compounds, because the

tannin content can bind proteins to form a bond of tannin-protein complexes, while *A. salina* has a high protein content and cannot absorb *S. alba* extract (Zamsari et al 2012). In the control group, some of the same positive compounds carried in *S. alba* fruit extract were observed, but this group was negative for steroid and tannin compounds. Some compounds can inhibit bacterial growth, such as alkaloid compounds, which can produce an inhibitory mechanism by disrupting the fundamental components of peptidoglycan in bacterial cells, where the cell wall layer is not formed intact and causes cell death (Juliantina et al 2009).

The mechanism of phenols as antibacterial compounds at low concentrations can damage the cytoplasmic membrane and cause leakage from the cell nucleus. At high concentrations, phenol compounds cope with cellular proteins. These activities are very active when bacteria are in the cleavage stage (when the phospholipid layer around the cell is in a delicate condition), and phenol compounds can easily damage the cell contents (Volk & Wheeler 1993). Puupponen-Pimiä et al (2001) suggests that different bacterial species exhibit different sensitivities towards phenolics, since fruits extracts in their study inhibited Gram-negative but not Gram-positive bacteria. These variations may reflect differences in cell surface structures between Gram-negative and gram-positive bacteria. Moreover, a study by Mitani et al (2018) demonstrated that phenolic compounds - rather than citric acid - contribute to antimicrobial activity against enterobacteria in the digestive tract of *Penaeus monodon* larvae.

As an antibacterial, flavonoids have a mechanism of action that can damage microbial cell wall permeability and inhibit microbial growth through binding toward the functional cell protein and DNA (Sabir 2005). Our phytochemical testing of *S. alba* extract show that saponin compounds were not contained in the extract of *S. alba*.

Steroid compounds are widely found in nature as lipid fractions from plants or animals. This substance is an essential regulator of biological activity in living organisms. Steroids are compounds found in the waxy layer of leaves and fruit that serve as protection against insects and microbial attacks (Harborne 1988).

The highest optical density (OD) test value in *V. harveyi* culture used APW liquid media with dilution between 10^{-1} and 10^{-7} with a wavelength of 600 nm was 0.531. A bacterial absorbance value of 0.788 was derived from a bacterial dilution between 10^{-4} to 10^{-5} with the number of bacterial colonies from the calculation of TPC media test being approximately 6.4 x106 CFU mL^{-1}. It was able to kill 41.67% of black tiger prawn larvae in the control group within 12 days. Mustika et al (2014) explains, the spectrophotometer method which is done by detecting the level of turbidity of bacteria, will affect the amount of light transmitted (wavelength) through the cuvette media and then convert it to OD values.

In vivo observation of clinical symptoms shows that the control group experienced a decrease in motion activity on days 11 and 12 (Table 2). This symptom was characterized by reduced activity of prawn movements, staying at the bottom of the container, swimming pattern tilted with body position to the right, appetite reduced (measured by the amount of *A. salina* that has not been eaten). A decrease in prawn activity (i.e., swimming patterns) tends to be normal behaviour; however, reflex movements tend to be aggressive due to the presence of natural body defences in the prawn's body. The results indicate that enrichment using *A. salina* extract can cause greater survival among larval giant tiger prawns infected by *V. harveyi*. The characteristics of attacked giant tiger prawn include individuals that appear weak and tend not to swim, as well as body parts exhibiting visible red spots (Ulna et al 2016). The survival rate of giant tiger prawn fry was calculated based on the average survival rate at the end of the study after being given bio enrichment for 10 days and a challenge test for 2 days (Figure 1).

The survival of giant tiger prawn larvae in the control treatment experienced the greatest decrease compared to the 20-ppm treatment. This is due to the immune system of giant tiger prawn larvae being weakened, thereby resulting in the death of fry during bacterial infection (Table 2).

Table 2

Clinical symptoms of giant tiger prawn postlarvae

Dose (ppm)	*Days*	*Motion activity*		*Swimming pattern*		*Reflex motion*		*Survival rate (%)*	*Mortality (%)*
		Active	*Passive*	*Normal*	*Unbalanced*	*Normal*	*Aggressive*		
0	0-4	√		√		√		58.33	41.67
	5-8		√	√		√			
	9-10	√		√			√		
	11-12		√		√	√			
25	0-4	√		√		√		68.33	31.67
	5-8		√		√	√			
	9-10		√	√			√		
	11-12	√		√			√		
20	0-4	√		√		√		78.33	21.67
	5-8		√	√		√			
	9-10		√		√	√			
	11-12	√		√		√			
15	0-4	√		√		√		70	30
	5-8		√		√	√			
	9-10	√			√		√		
	11-12	√		√			√		

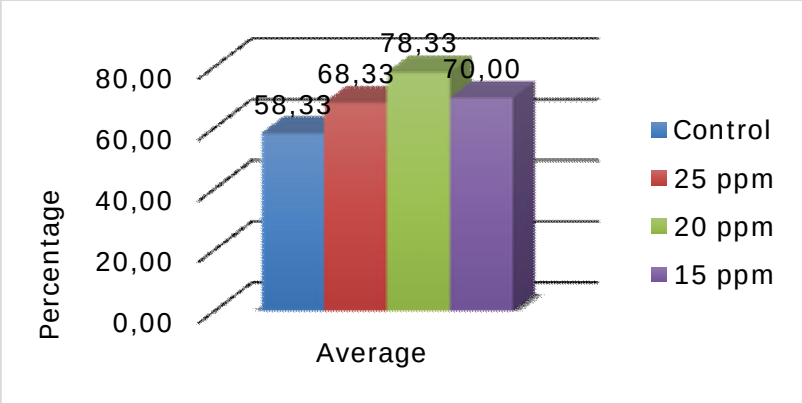

Figure 1. Survival rate of giant tiger prawn larvae.

The highest survival rate (78.33%) was observed in the group with 20 ppm of extract, which indicated that the extract was capable of inhibiting the growth and infection of *V. harveyi* bacteria. Arun et al (2010) reported that the flavonoid, alkaloid, and tannin compounds contained in *S. alba* extract function as antibacterial compounds that can inhibit bacterial growth. The control group exhibited the lowest survival rate (58.33%). ANOVA analysis indicates that the use of *S. alba* fruit extract at a predetermined concentration provides a significant effect ($p > 0.05$), and LSD tests for the 15 ppm and 20 ppm groups exhibited a considerable difference when compared with the control and 25 ppm groups (p-value < 0.05 is higher than the LSD value).

Bacterial biochemical tests on giant tiger prawn larvae using Gram staining ensure that the *V. harveyi* species attach to the giant tiger prawn larvae (Table 3). The test indicates a Gram-negative (-) result, which is characterised by the formation of concentrated suspensions (such as mucus) and stickiness. Beyhan & Yildiz (2007) reported that *Vibrio* are Gram-negative, rod-shaped bacteria that are usually short (0.5 x 1.3-3 mm) but can also be curved or comma shaped. *Vibrio* are non-sporulating, non-capsulated, facultative anaerobes that are catalase-positive and motile using a single flagellum pole. In liquid media, all *Vibrio* species exhibit rapid motility. Most species are positively oxidase and reduce nitrate to nitrite. When bred in agar media, *Vibrio* species form two very different types of colonies, namely rugose and smooth. The formation of these two colony variants is related to population diversity and adaptation to environmental conditions.

Table 3

Biochemical test results for *Vibrio harveyi* bacteria

Test media	*Test result*	*Control isolate*
Gram test	-	-
Motility test	+	+
Catalase test	+	+
Oxidase test	+	+
Glucose test	+	+
Oksidatif/Fermentatif test	F	F
Genus	*Vibrio* sp.	*Vibrio* sp.
Ornithin	+	
Indole	+	
TSIA is'nt upright	A	
Upright TSIA	K	
Gas on TSIA	-	
H_2S on TSIA	-	
TCBS Test	+ (yellow)	+

Conclusions. Based on research that has been done, it can be concluded that the administration of bioenrichment in *Artemia salina* showed significantly different results on survival rate of giant tiger prawns (*Penaeus monodon*) infected by *Vibrio harveyi* at 78.33% with an extra concentration of 20 ppm *Sonneratia alba*. *Artemia salina* was given bioenrichment proven to absorb *Sonneratia alba* fruit extracts from phytochemical tests that showed positive results of alkaloids, flavonoids, saponins, steroids and phenols.

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