The effect of several types of mangrove extracrs on tiger shrimp *Penaeus monodon* survival rate challenged with White Spot Syndrome Virus (WSSV)

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**Abstract.** This experiment was aimed to determine the survival rate of tiger shrimp on the use of several types of mangrove extracts challenged with the White Spot Syndrome Virus (WSSV). The experiment was conducted in November 2015 at the Research Institute for brackish water Aquaculture and Fisheries Extension (RIBAFE), Maros. The plastic container of 40 L volume was filled with 30 L of seawater at a salinity of 28 ppt which had been disinfected with chlorine powder of 150 ppm and neutralized with Sodium Thiosulfate of 75 ppm, stocked with 10 ind of tiger shrimps with the size of 5-7 g/pcs. The challenge test of mangrove extract with WSSV was done by mixing 5 μL of WSSV suspension with 10 μL of mangrove extract solution (500 mg/100 mL of NTE buffer). The mixing solution was then incubated at 29 °C for 3 hours and then infected to tiger shrimp by intramuscular injection. The experimental design used was Completely Randomized Design with treatments; A). butanol extract of *Sonneratia alba*; B). butanol extract of *Sonneratia caseolaris*; C). butanol extract of *Sonneratia lanceolata*; D). butanol extract of *Bruguiera gymnorrhiza*; E). diethyl ether extract of *Sonneratia alba*; F). diethyl ether extract of *Bruguiera gymnorrhiza*; G). Control (shrimps injected with WSSV suspension without mangrove extract. Each treatment was repeated 3 (three) times and tiger shrimp were reared for 10 days. Observations of tiger shrimp mortality were performed daily, while Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), ProPO values and WSSV infection were observed at the end of the study. Analysis of variance, which was followed by Least Significant Difference test were conducted on the survival rate of tiger shrimp. The results showed that over 50% of tiger shrimp relative survival was obtained by the treatment that used butanol extract of *S.alba*, butanol extract of *S.caseolaris*, butanol extract of *B. gymnorrhiza*, and diethyl ether extract of *S. alba*. These experiments showed that the four extracts of mangrove effectively increased the survival of tiger shrimp. The highest average survival rate of tiger shrimp was obtained by the treatment that used diethyl ether extract of *S. alba*, while the lowest was found in the positive control, and both treatments were significantly different (*P* <0.05). The result indicated that diethyl ether extract of *S. alba* was found to be the most potential extract to control WSSV disease in tiger shrimp.

1. **Introduction**  
White spot disease in penaeid shrimp is caused by the White Spot Syndrome Virus (WSSV) [1]. This virus is classified as a rod-shaped DNA virus and belongs to the genus *Whispo*ivirus [2,3], family Nimaviridae [4,5]. Several previous reports showed that WSSV also attacked shrimp in the pond [6–8] besides attacking the wild tiger shrimp broodstock [9,10], wild organisms such as speckled shrimp, snap or mysid shrimp, wild fish, crab, and some types of mollusks living in the pond [11,12], as well

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as plankton and insect larvae [13]. According to Raja et al., (2015) *Scylla serrata* was found to be WSSV carrier in shrimp culture [14]. Data showed that WSSV attacked shrimp, both vertically and horizontally [4,15,16].

WSSV was reported to start spreading since 1992 (Escobedo-Bonilla et al., 2008) and until today, the disease continues to be the main cause of shrimp mortality, both in a grow-out pond and hatchery [17–19]. According to Munn (2004), WSSV may cause mortality up to 80% in juvenile (attack for 2-3 days) and adult shrimp (attack for 7-10 days) [20]. Other reports mentioned that WSSV caused 90-100% mortality for 3-10 days since the presence of an attack [4].

Many efforts have been made to overcome the disease attack caused by WSSV, yet this disease is often found to attack the farmed shrimp until now. At the beginning of 2017, WSSV was still found to attack shrimps in the pond, both cultured in super-intensive, intensive, and even traditional systems. This virus did attack not only tiger shrimp but also white leg shrimp in a super-intensive system. Water quality improvement through the use of the reservoir, biofilter, and probiotics is one of the efforts to prevent the existence of disease attack in shrimp farming. Moreover, antibiotic and chemical medicine constantly used to treat disease are no longer suggested and even strictly prohibited by the government for causing accumulation and contamination in the environment. Therefore, alternative approaches that more efficient, effective, and environmentally friendly to replace chemical medicine and antibiotic is necessary to find. The use of probiotics, both international and local products, is expected to improve the water quality and to prevent shrimp stress and stop the spread of the WSSV attack, is one of the efforts conducted by shrimp culturists [21]. However, this effort does not provide much help since the WSSV attack continues to occur.

The use of natural material easily degraded in the environment is one attempt to be done to solve the problem of disease attack in shrimp culture, which still puts the environmental issue into account. Several countries like China, India, Thailand, and Malaysia have studied the use of the medicinal plant, which is commonly used for medical purposes (disease treatment for humans) to be applied as an alternative for disease treatment in aquaculture. One medicinal plant that started being examined and used to treat disease in aquaculture is medicinal mangrove.

The use of medicinal mangrove for disease treatment in the aquaculture includes for WSSV treatment, firstly initiated in the last several years [22–24]. Some types of mangrove were reported to be the potential source of anti-WSSV which includes *Exoecaria agallocha* Acanthus ilicifoilus, *Avicennia* sp, *Rhizophora mucronata*, *Rhizophora apiculata*, *Sonneratia* sp, and *Ceriopstagal* [25]. The potential of mangrove as anti-WSSV agent was also reported by Chakraborty et al. (2014) that MP07X derived from *Rhizophora mucronata* was found to have the potential as an anti-WSSV producer on the use of 1.000 mg/kg shrimp weight/day fed orally, resulted in 85% survival rate in *Vannamei* shrimp [26].

Indonesia is a country rich in natural resources include mangrove forest resources, both quantitatively (area) and qualitatively (diverse type), thus it is possible to assess its use as an alternative to treat disease in shrimp farming including WSSV disease treatment. The Result of a previous study showed that several types of mangrove originated from some pond areas in South Sulawesi are potential as an anti-bacterial producer for treating *Vibriosis* disease and anti-WSSV disease [27].

Based on those statements, the study which aimed to determine the tiger shrimp survival rate on the use of extraction result of several types of mangrove challenged with White Spot Syndrome Virus(WSSV) is required.

2. Materials and Methods

2.1. Preparation of Extraction Result of Mangrove Extract

Approximately 50 mg of each butanol extract of *Sonneratia alba*, *S. caseolaris*, *S. lanceolata*, *Bruguiera gymnorrhiza*, and diethyl ether extract of *S. alba*, and *Bruguiera gymnorrhiza* dissolved
with 10 mL of NTE buffer (0.2 M NaCl, 0.02 M Tris-HCL, and 0.02 M EDTA, pH 7.4), homogenized and stored at a temperature of 4 °C for further use [24].

2.2. Preparation of WSSV Suspension
WSSV suspension isolated from the hemolymph of infected shrimp which signed by the presence of white spot in the carapace and confirmed by the positive result of PCR detection. The shrimp were collected from the traditional pond in Barru Regency, where the outbreak of WSSV occurred at that time. Hemolymph was collected using a 1 ml sterile syringe and put into a 50 mL centrifuge tube. Moreover, hemolymph was centrifuged at the speed of 3000xg for 20 minutes and the temperature of 4°C. The supernatant was transferred to a new centrifuge tube and further was centrifuged again at the speed of 8000xg for 30 minutes at a temperature of 4 °C. The supernatant was then filtered using a 0.4 µL filter paper and stored at temperature -20 °C for further use.

2.3. Experimental Animal and Treatment
The plastic container of 40 L volume was filled with 30 L of seawater at a salinity of 28 ppt which had been disinfected with chlorine powder of 150 ppm and neutralized with Sodium Thiosulfate of 75 ppm, stocked with 10 ind of tiger shrimps with the size of 5-7 g/pcs as an experimental animal. The challenge test of mangrove extract with WSSV was done by mixing 5 μL of WSSV suspension with 10 μL of mangrove extract solution (500 mg/100 mL of NTE buffer). The mixing solution incubated at a temperature of 29 °C for 3 hours [24,28] and then infected to the healthy tiger shrimp as an intramuscular injection. The experimental design used was Completely Randomized Design with treatments; A). butanol extract of Sonneratia alba; B). butanol extract of S. caseolaris; C). butanol extract of S. lanceolata; D). butanol extract of Bruguiera gymnorrhiza; E). diethyl ether extract of S. alba; F). diethyl ether extract of Bruguiera gymnorrhiza; G). Control (shrimp was injected with WSSV suspension without mangrove extract). Each treatment was repeated 3 (three) times, and tiger shrimp were reared for 10 days in a plastic container of 40 L volume filled with seawater at the salinity of 28 ppt as much as 30 L and a density of 10 shrimp/container.

2.4. Relative Percentage Survival (RPS) and Survival Rate of Shrimp
Mortality and morphological symptom of WSSV infection in tiger shrimp were performed daily (Velmurugan et al. 2012), while the survival rate of shrimp was observed at the end of the study. Effectiveness of mangrove extract was done through the calculation of Relative Percentage Survival(RPS) based on the formula developed by Thompson and Adame (2004) in Tampangallo (2012) as follows:

\[ RPS = \frac{\text{Mortality of treatment} \times 100}{\text{Mortality of Control}} \]  

2.5. Observation of Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), and ProPo in Shrimp
Total Hemocyte Count (THC), Differential Hemocyte Count (DHC), and ProPo of tiger shrimp observed at the end of the study. THC was analyzed by collecting 0.1 mL of hemolymph using a 1 ml sterile syringe which was previously filled with 0.3 mL anticoagulant (3.8% sodium citrate). The mixing solution was homogenized and hemocyte cell count was observed using a binocular light microscope at 100x magnification. Total THC was calculated using the formula below:

\[ RPS = \frac{n_1 + n_2 + n_3 + n_4 + n_5}{5} \times 25 \times 10^4 \]  

Differential Hemocyte Count (DHC) observed with the method of Martine & Graves (1985) by taking shrimp hemolymph and making spread on an object-glass which was previously fixed with methanol for 5-10 minutes. The spread of hemolymph was immersed in 10% Giemsa solution for 15-
20 minutes, rinsed under running water, and dried. The preparation was examined under the microscope at a magnification of 400x. Hemocyte was counted until it reached 100 cells and the percentage of each hemocyte cell type was determined based on the formula:

\[
\text{Percentage of hemocyte cell type (\%) = \frac{\text{Amount of each hemocyte cell type}}{\text{Total hemocyte cell}}} \tag{3}
\]

Prophenoloxidase activity (PO) was measured about the procedure by taking 0.1 mL hemolymph and further mixed it with 0.9 mL anticoagulant and the solution was centrifuged at the speed of 700xg and temperature of 4 oC for 20 minutes. The supernatant was removed and the pellet was rinsed using 1mL of cacodylate-citrate buffer (0.01 M sodium cacodylate; 0.45 M sodium chloride; 0.10 M trisodium citrate; pH 7), and later was centrifuged again at the speed of 700xg and temperature of 4 oC for 20 minutes. Supernatant was removed and pellet was diluted into 200 mL of cacodylate buffer (0.01 M sodium cacodylate; 0.45 M sodium chloride; 0.01 M calcium chloride; 0.26 M magnesium chloride, pH 7). The solution was divided into two portions, 100 mL of each. The first solution was added with 50 µL of trypsin solution (trypsin in 1 mL of cacodylate buffer) and the second solution was added with 50 mL of cacodylate buffer to equal the volume and further was incubated at a temperature of 25-26 oC for 10 minutes. Next, each solution was added with 50 µL of L-DOPA solution (3 mg of L-DOPA in 1 mL of cacodylate buffer), let stand for 5 minutes, and added with 800 mL of cacodylate buffer. The activity of the PO was measured using a spectrophotometer at a wavelength of 490nm. Optical density (OD) of PO activity in all experimental conditions was expressed as dopachrome formation in 50µL.

2.6. Data Analysis
The survival rate of tiger shrimp was analyzed for its variance and further continued with the Least Significant Difference test, while the data of THC, Pro-Po, and DHC values were analyzed descriptively and presented in the form of table and figure.

3. Results and Discussion

3.1. Relative Percentage Survival of Tiger Shrimp
Relative percentage survival of tiger shrimp on the use of several types of extraction results of mangrove extract challenged with WSSV is presented in Figure 1. In this figure, the highest relative percentage survival of tiger shrimp was found in the treatment that used diethyl ether extract of \textit{S. alba} (86%), while the lowest was obtained by the treatment that used butanol extract of \textit{S. lanceolata} (20%). Moreover, relative percentage survival of tiger shrimp above 50% was also seen in the treatment that used butanol extract of \textit{S. alba}, butanol extract of \textit{S. caseolaris}, butanol extract of \textit{B. gymnorrhiza}, and diethyl ether extract of \textit{S. alba}. It shows that the four types of extraction results were effective to be used as anti-WSSV, which further will have an impact on the increasing survival rate of tiger shrimp. According to Thompson & Adam (2004) , in Tamfangallo (2012), treatment is effective if the value of RPS >50% [29]. Based on the data, it was also found that each type of extraction result from the same mangrove type led to different effects on WSSV; thus, more strict selection is required concerning the use of mangrove extract to prevent WSSV disease from obtaining the real effective candidate of anti-WSSV. Diethyl ether extract from \textit{S. alba} showed the highest RPS value, as previously mentioned by Muliani et al. (2016) [27]. Besides to be potential as anti-WSSV, \textit{S.alba} has also been reported to contain extremely high antibacterial substance against \textit{Vibrio} with Minimum Inhibition concentration (MIC) value of 1.0 mg/L against \textit{Vibrio harveyi} and 0.1 mg/L against \textit{V. parahaemolyticus} [30].
3.2. Survival Rate of Tiger Shrimp
The survival rate of tiger shrimp at the end of the study is presented in Table 1. As seen in that table, the highest survival rate of tiger shrimp injected with WSSV and mangrove extracts was found in treatment that used diethyl ether extract of *S. alba* (73.33%), while the lowest (0%) was obtained by the positive control (injected with WSSV without mangrove extract). The survival rate of tiger shrimp on the third day for all treatments that used mangrove extracts was still considered high, namely above 80%, while it was 43.33% in positive control which continued to decrease to 6.67% on the fifth day. This finding showed that shrimp in treatment that used mangrove extracts was still able to tolerate WSSV attack until the fifth day, thus the shrimp survival rate was 63-83%, while all shrimp in positive control were dead (100%) on the sixth day. The statistical analysis result indicated that the survival rate of tiger shrimp in treatment injected with WSSV and diethyl ether extract of *S. alba* was significantly different (*P<0.05*) with control (WSSV injected without mangrove extract). This condition showed that diethyl ether extract of *S. alba* is the potential to be used as anti-WSSV and may increase the survival rate of tiger shrimp. This results is the same as with the previous study that survival rate of tiger shrimp with use methanol and diethyl ether extract of *S. alba* was higher than the other treatments [27]. Through immersion method at different doses, Wahyuningrum et al. (2006) reported that a dose of 250 ppm from mangrove *Sonneratia* sp extract was the most optimal dose to decline WSSV pathogenicity thus increased the survival rate of shrimp up to 98.4% [31].

Table 1. The survival rate of tiger shrimp *Penaeus monodon* on the use of extraction result of several types of mangrove challenged with White Spot Syndrome Virus (WSSV)

| Treatments                  | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 | Day 8 | Day 9 | Day 10 |
|-----------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|--------|
| Butanol extract of *S. alba*| 96.67 | 96.67 | 90.00 | 90.00 | 83.33 | 66.67 | 60.00 | 60.00 | 60.00 | 56.67<sup>ab</sup> |
| Butanol extract of *S. caseolaris* | 93.33 | 93.33 | 86.67 | 83.33 | 76.67 | 60.00 | 56.67 | 56.67 | 56.67 | 53.33<sup>ab</sup> |
| Butanol extract of *S. lanceolata* | 93.33 | 86.67 | 83.33 | 80.00 | 63.33 | 36.67 | 23.33 | 23.33 | 20.00 | 20.00<sup>ab</sup> |
| Butanol extract of *B. gymnorrhiza* | 86.67 | 86.62 | 86.67 | 83.33 | 80.00 | 76.67 | 73.33 | 66.67 | 63.33 | 56.67<sup>ab</sup> |
| Diethyl ether extract of *S. alba* | 93.33 | 90.00 | 90.00 | 80.00 | 83.33 | 83.33 | 83.33 | 80.00 | 76.67 | 73.33<sup>a</sup> |
| Diethyl ether extract of *B. gymnorrhiza* | 90.00 | 90.00 | 83.33 | 80.00 | 63.33 | 46.67 | 40.00 | 30.00 | 30.00 | 30.00<sup>ab</sup> |
though the result of PCR detection showed that tiger shrimp was not injected with WSSV, butanol and diethyl ether function as an immunostimulant for tiger shrimp, as seen in the result of treatment that used diethyl ether administration of mangrove extract of diethyl ether. Mangrove can be used as an immunostimulant in shrimp increasing immune response, phagocytic characteristic, and phenoloxidase activity, thus this type of study is expected to be the reason for the high survival rate of shrimp in some treatments in this study, although the result of PRC analysis has already shown severely and moderately positive results. Furthermore, it is said that S. alba and B. gymnorrhiza, besides their function as anti-WSSV, may also function as an immunostimulant. The last function is expected to be the reason for the high survival rate of shrimp in some treatments in this study, although the result of PRC analysis has already shown severely and moderately positive results.

### Table 2. Total Hemocyte Count, Phenoloxidase activity, and WSSV infection in Tiger Shrimp

| Treatment                        | THC \((\times 10^7\) cell/mL) | ProPo (A) | WSSV infection          |
|----------------------------------|-------------------------------|-----------|-------------------------|
| Butanol extract of S. alba       | 1.5667                        | 0.090     | Severely positive       |
| Butanol extract of S. caseolaris | 7.6633                        | 0.020     | Severely positive       |
| Butanol extract of S. lanceolata | 1.1889                        | 0.074     | Severely positive       |
| Butanol extract of B. gymnorrhiza| 1.0333                        | 0.039     | Moderately positive     |
| Diethyl ether extract of S.alba   | 2.8887                        | 0.028     | Moderately positive     |
| Diethyl ether extract of B. gymnorrhiza | 2.1110 | 0.027 | Severely positive |
| Control                          | 3.3330                        | 0.049     | Severely positive       |

Values followed by the same superscripts are not significantly different \((P>0.05)\)

3.4. **Total Hemocyte Count (THC)**

The analysis result of the immune system at the end of the study is presented in Table2. In the table, it is seen that used butanol extract of S. alba, S. caseolaris, S. lanceolata, and diethyl ether extract of B. gymnorrhiza, and control the tiger shrimp were infected with WSSV at a severe category, while that used butanol extract of B. gymnorrhiza and diethyl ether extract of S. alba, tiger shrimp was infected with WSSV at moderate category. According to the data above, although the result of PCR detection showed that shrimp in treatments that used butanol extract of S. alba, butanol extract of S. caseolaris, butanol extract of S. lanceolata, diethyl ether extract of B. gymnorrhiza were infected with WSSV at a severe category, the mortality was not as severe as in control due to the effect of mangrove extract administration. Shrimp still survived until day-10 with quite a high percentage except on the use of butanol extract of S. lanceolata. Whereas, mortality in control, where shrimp was not injected with mangrove extract, occurred since the first day and exceeded 50% on the third day. Tiger shrimp mortality even reached 100% on the sixth day (Table 1). This finding showed that the use of mangrove extracts positively affected shrimp immunity against WSSV attack.
cell/mL and 8.80×10^7 cell/mL. Moreover, an examination of WSSV infection showed a negative result and led to a positive impact on the survival rate of tiger shrimp which reached 100% at the end of the study. Besides its function as an anti-WSSV producer, *S. alba* has also been reported to be able to produce a considerable amount of antibacterial substance against bacteria considered to be the cause of disease in shrimp with MIC value of 1 mg/L for *V. harveyi* and 0.1 mg/L for *V. parahaemolyticus*. Several types of mangrove except for *S. alba* which has been reported to not-activate WSSV included *Rhizophora mucronata*, *Sonneratia* sp, and *Ceriops tagal*. Moreover, it is explained that the water extract of that mangrove was able to not-activate WSSV after collective incubation at room temperature in a ratio of 1:1.

3.5. Phenoloxidase Activity

Phenoloxidase activity of tiger shrimp after 10 days injection with mangrove extract challenged with WSSV is presented in Table 2. In this table, it is seen that the lowest ProPo value was produced by treatment injected with butanol extract of *S. caseolaris*, namely 0.020, and the highest was found in the treatment of butanol extract of *S. alba*, namely 0.090. Nurbaya *et al.*, (2016) reported that the ProPo value of tiger shrimp given methanol extract of *S. alba* was higher than that given methanol extract of *S. lanceolata* and *B. gymnorrhiza*. Prophenoloxidase (ProPO) is one of the main immunity systems for crustacean which has a function to activate phenoloxidase (PO) enzyme, and PO is responsible for melanin synthesis process through the help of proteolytic enzyme which catalyzes hydrocyclic process of monophenol into diphenol and oxidizes diphenol into guinenos and changes it into melanin through the enzymatic process [33,34].

3.6. Differential Hemocyte Count (DHC)

DHC reflects the comparison between hyaline cells, granular cells, and semi-granular cells. Each of the three types of hemocyte cells plays a role in the shrimp immunity system. The hyaline cell is responsible as phagocytic immune, while granular and semi-granular cells have mutual responsibility in performing cytotoxicity activity and the production as well as the release of the prophenoloxidase system [35]. The comparison between the granular, semi-granular, and hyaline cells of tiger shrimp in this study was varied. According to Sung *et al.* (1999), about 50 to 80% of total hemocyte in crustacea is a hyaline cell, 9 to 30 percent is a semi-granular cell, and 4 to 20 percent is a granular cell. However, the proportion between the three types of cells highly depends on species, molting phase, and physiological condition of the organism [36]. In this study, the percentage of the granular cell was far higher than the hyaline and semi-granular cells, while the percentage of the hyaline cell was higher compared to the semi-granular cell (Figure 2). According to Johansson *et al.* (2000), a hyaline cell is responsible for the phagocytic activity, while granular and semi-granular cells are active in the process of protease enzyme, the formation of antibacterial substances, and reactive oxygen such as anion superoxide and hydrogen peroxide [37].
Figure 2. The differential Hemocyte Count of Tiger Shrimp *Penaeus monodon* after 10 days Injection with mangrove extract challenged with WSSV

4. Conclusion

Relative percentage survival of tiger shrimp on the use of butanol extract of *S. alba*, *S. caseolaris*, *B. gymnorrhiza*, and diethyl ether extract of *S. alba* were higher than 50%. Diethyl ether extract of *S. alba* was able to increase the survival rate of tiger shrimp to 70% compared with control (without the use of mangrove extract) and 2-50% compared with other extracts, hence this type of mangrove is potential to be used as an ingredient to control WSSV disease in tiger shrimp.

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