Prevention of White Spot Syndrome Virus (WSSV) in tiger shrimp *Penaeus monodon* using boiled mangrove leaf extract *Sonneratia alba* in laboratory scale

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**Abstract.** This study aimed to prevent White Spot Syndrome Virus (WSSV) disease in tiger shrimp *Penaeus monodon* using the boiled extract of *Sonneratia alba* mangrove leaves. The experiment was conducted in the wet laboratory of The Research Institute for Brackish Water Aquaculture And Fisheries Extension using a plastic container of 50 L volume filled with 30 L of seawater at a salinity of 28 ppt. 30 tiger prawns/container in size 1-2.0 g each shrimp used as test animals. Prevention of WSSV disease is done by injecting the extract of *Sonneratia alba* from boiling fresh leaves (1 kg in 5 L of water cooked to 2.5 L) and dry mangrove leaves (300 g in 5 L of water cooked to 2.5 L). The treatments tried were: A) 1% water boiled of fresh leaves; B) 4% water boiled of fresh leaves; C) 7% water boiled of fresh leaves; D) 10% water boiled of fresh leaves; E) 1% water boiled of dry leaves; F) 4% water boiled of dry leaves; G) 7% water boiled of dry leaves; H) 10% water boiled of dry leaves; I) Positive control (shrimp injected with WSSV without mangrove extract), and J) Negative control (without WSSV and mangrove extract) which was repeated three times. Each concentration of mangrove extract was mixed with WSSV antigen solution in a ratio of 2:1 and injected as much as 100 microliters for each shrimp. Water change was carried out every two days at 20%. Feeding is done 2 times a day as much as 10-20% of the total biomass per day. Shrimp reared for 10 days. The parameters observed included the mortality of tiger shrimp which were observed every day. Shrimp immune parameters including THC, DHC, Pro-PO, and WSSV infection were observed on days 1, 3, 5, 7, and 9. The results showed that the mortality rate of tiger shrimp in treatments that did not use mangrove extracts was higher than others. as well as the mortality rate of shrimp in the treatment using boiled fresh leaf extracts was higher than those using boiled dry leaf extract at the same concentration. The WSSV concentration used in this experiment was thought to be high so that there was no visible effect of injury in the incidence of shrimp mortality at the end of the study.

1. **Introduction**

White spot virus (WSV) includes DNA viruses that are rod-shaped and enter the genus Whispovirus, family Nimaviridae. White spot viruses include DNA double strains with sizes ranging from 292.9–307.2 kb [1]. In addition to infecting shrimp in ponds, WSSV has been reported to infect broodstock [2,3], fried prawns, and even wild organisms that live in ponds such as the fire shrimp *Metapenaeus* sp., Jembret shrimp *Acestes* sp., wild fish (*Oreochromis mossambicus*), Crab (*Scylla* sp.), and several types of mollusks as a carrier) [4,5], as well as microalgae and zooplankton [6], plankton and insect larvae.

White spot syndrome virus not only attacks shrimp cultivated in ponds [7–10] but has also been detected to infect tiger prawns *P. monodon* from nature as well as those that have spawned. In addition,
WSSV has also been reported to infect crayfish, Cherax sp. (freshwater shrimp), fry, juvenile shrimp, tiger prawns, *P. monodon*, and even wild organisms that live in ponds [10].

Prevention of white spot disease can be done by using probiotics [11,12], vaccines [13–17], immunostimulants [18–20], and environmental quality management [9], however until now shrimp mortality in ponds and hatcheries due to disease continues to occur. The use of natural materials including mangroves and their associations (plants that live in association with mangroves) for disease prevention in fisheries has begun to be initiated, although it is still limited to a laboratory scale, including antibacterial and antiviral properties.

The use of herbal plants for disease prevention in fisheries has recently begun to be seen by many [21–23]. Wahyuningrum et al., (2007) have used a mixture of sambiloto (*Andrographis paniculata*), guava, and betel leaves for disease prevention in African catfish, while Grandiosa (2010) has used black cumin to treat the bacterial disease *Aeromonas hydrophyla* in carp [24].

Mangroves and associated plants are pure vegetation in aquaculture areas that must be preserved. The biological function of mangrove forests, in this case, mangrove plants and their associations, will run perfectly if their existence is maintained and sustainable. Several types of mangrove leaves have been identified to be bioactive as antiviral and antioxidant producers [25–27], as antibacterial [28–31] as a source of bactericides and fungicides [32], and their effectiveness has been tested against bacterial diseases in a laboratory. One of the mangrove association plants whose existence needs to be preserved is the Kopasanda plant, *Euphorium inolifolium*. This plant is widely distributed in lowland areas to estuarine areas and grows in association with mangroves. Kopasanda leaf extract can function as a bactericide against vibrio bacteria. Muliani et al., (2006), have shown that Kopasanda extract at a concentration of 1000 mg / L is the best [33]. The population of *V. harveyi* in the tiger shrimp larvae rearing container decreased at the lowest point, namely 1.32 x 101 CFU / m, with the highest post-larval survival rate of tiger prawns of 74.37%.

Muliani et al., (2015;2016) reported that several types of mangroves collected from aquaculture areas in South Sulawesi have potential as anti-*V. harveyi*, namely: *S. alba*, *S. caseolaris*, *S. lanceolata*, and *B. gymnorrhiza* [34,35]. In addition, there were also found potential types as antioxidant-producing materials, namely *Xylocarpus granatum* and *Rhizophora lamarkii* [36], and anti-WSSV producers (*S. alba* and *B. gymnorrhiza*) [37]. Furthermore, it was reported that the survival rate of tiger shrimp using boiled mangrove extract was 69% higher and the total *V. harveyi* was about 30% lower than that of methanol extract. While the survival rate of tiger prawns in the use of mangrove extracts through feed was 56% higher than through immersion.

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 [35].

Based on those statements, the study which aimed to prevent White Spot Syndrome Virus (WSSV) disease in tiger shrimp *Penaeus monodon* using the boiled extract of *Sonneratia alba* mangrove leaves is required.

2. Materials and methods

2.1. Boiling extract mangrove preparation

Fresh mangrove leaves *S. alba* were weighted 1 kg and added 5 L of water cooked to 2.5 L and dry mangrove leaves 300 g in 5 L of water cooked to 2.5 L).

2.2. WSSV suspension preparation

WSSV suspension isolated from the hemolymph of infected shrimp which was signed by the presence of a white spot in the carapace and confirmed by the positive result of PCR detection. The Shrimp were collected from a 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 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 [38].

2.3. Location, experimental animal, and treatment
The experiment was conducted in the web Laboratory of Research Institute for Brackishwater Aquaculture and Fisheries extension (RIBAFE). 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 30 ind of tiger shrimps with the size of 0.5-2 g/pcs as an experimental animal. The challenge test of mangrove extract with WSSV was done by mixing WSSV suspension with mangrove extract solution with a ratio of 1:2. The mixing solution was incubated at a temperature of 29°C for 3 hours and then infected to the healthy tiger shrimp as an intramuscular injection at dosage 50-100 µL/pcs [23,39,40]. The experimental design used was a Completely Randomized Design with treatments; A) 1% boiled extract from fresh mangrove leaves; B) 4 % boiled extract from fresh mangrove leaves; D) 10 % boiled extract from fresh mangrove leaves; E) 1% boiled extract from dry mangrove leaves; F) 4% boiled extract from dry mangrove leaves; G) 7% boiled extract from dry mangrove leaves; H) 10% boiled extract from dry mangrove leaves; I) positive control (the shrimp was injected by WSSV without mangrove extract), and J) Negative control (non injected WSSV and mangrove extract). Each treatment was repeated 3 (three) times, and tiger shrimp were reared for 9 days. Water changes are carried out every two days at 20%. Feeding is done 2 times a day as much as 10-20% of the total biomass.

Parameters observed included tiger shrimp mortality which was observed every day. Shrimp immune parameters including THC, Pro-PO, and WSSV infection were observed on days 1, 3, 5, 7, and 9.

2.4. 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. Tiger shrimp mortality
The mortality of tiger shrimp in WSSV disease prevention research using boiled extract of S. alba is presented in Table 1. In the table, it can be seen that tiger shrimp mortality varies based on the type of extract, concentration, and observation time. In the table, it can be seen that on the first day there was no shrimp death in all treatments except in treatment I which was a treatment that was injected with WSSV without mangrove extract, shrimp mortality occurred with an average of 2.22%. Entering the second day, the shrimp mortality rate was seen to be higher, especially in treatment A (using 1% extract from boiling fresh leaves) then followed by treatment D (using 10% extracting from boiling fresh leaves) of 21.11%. On the third day, the average mortality of tiger prawns was above 50% in all treatments except for the negative control which was still 0%. This indicated that the concentration of WSSV used was very virulent and the mangrove extract which was expected to inhibit or suppress WSSV infection was not effective. The mortality of tiger prawns reached ±90% on the fourth day as seen in treatments A (shrimp injected with WSSV + 1% mangrove extract from boiling fresh leaves) and I (shrimp injected with WSSV without mangrove extract).
The mortality of tiger shrimp in treatment I (positive control, namely shrimp injected with WSSV without mangrove extract) reached 100% after entering the fifth day, while in treatment A later after the sixth day. In other treatments, tiger shrimp mortality reached 100% after the sixth, seventh day and some even survived until the eighth day. Muliani and Susianingsih 2018, reported that in the positive control treatment (shrimp injected with WSSV without mangrove extract) tiger shrimp mortality occurred on the third day with total mortality of 100% and statistically significantly different (P<0.05) with other treatments [37].

In Table 1 it can also be seen that there is a tendency for tiger shrimp mortality to be higher in the treatment using boiled extract of fresh leaves compared to that using boiled extract of dry leaves at the same concentration. Likewise, the mortality rate of tiger prawns tends to be higher in the treatment using boiled extract of fresh leaves. In treatment D, for example, where this treatment used 10% boiled extract of fresh leaves, the mortality of tiger prawns on the second day had reached 21.11% while in treatment H which used 10% boiled extract of dry leaves, the mortality of tiger prawns on the same day was 13.33%. Likewise, the shrimp mortality rate was faster in treatment D, where on the seventh day the shrimp mortality had reached 100%, while in treatment H later after the 9th day it only reached 100%. This shows that the extract from boiling dried leaves tends to be better at preventing and protecting shrimp from WSSV infection, although this still needs to be proven by using a lower WSSV concentration so that the mortality rate of shrimp is not too fast so that the effect of mangrove extract is more visible.

Table 1. Average mortality (%) of tiger shrimp during rearing in WSSV disease prevention research using mangrove extract with an injection system

| Treatments | Shrimp Mortality (%) | 1st day | 2nd day | 3rd day | 4th day | 5th day | 6th day | 7th day | 8th day | 9th day |
|------------|---------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| A          | 0±0                 | 25.56±  | 60.0    | 90.0    | 97.78   | 100     | 100     | 100     | 100     | 100±0   |
| B          | 0±0                 | 12.62   | ±6.67   | ±5.77   | ±1.92   | ±0      | ±0      | ±0      | ±0      | ±0      |
| C          | 0±0                 | 8.89    | 53.33   | 87.78   | 95.56   | 98.89±  | 100     | 100     | 100     | 100±0   |
| D          | 0±0                 | ±5.13   | ±10.0   | ±6.94   | ±1.93   | 1.93    | ±0      | ±0      | ±0      | ±0      |
| E          | 0±0                 | 14.44   | 56.67   | 78.89   | 90.00   | 94.44±  | 96.67±  | 98.89±  | 100±0   |
| F          | 0±0                 | ±11.71  | ±8.82   | ±8.39   | ±5.77   | 1.93    | 3.34    | 1.92    | ±0      | ±0      |
| G          | 0±0                 | 21.11   | 62.22   | 82.22   | 94.44   | 98.89±  | 100     | 100     | 100±0   |
| H          | 0±0                 | ±16.44  | ±10.72  | ±1.92   | ±1.93   | 0       | 1.92    | ±0      | ±0      | ±0      |
| I          | 0±0                 | 13.88±  | ±8.82   | ±1.92   | ±1.93   | ±0      | ±0      | ±0      | ±0      | ±0      |
| J          | 0±0                 | ±11.70  | ±1.93   | ±6.94   | ±5.77   | ±0      | ±0      | ±0      | ±0      | ±0      |

Notes: A) 1% water boiled of fresh leaves; B) 4% water boiled of fresh leaves; C) 7% water boiled of fresh leaves; D) 10% water boiled of fresh leaves; E) 1% water boiled of dry leaves; F) 4% water boiled of dry leaves; G) 7% water boiled of dry leaves; H) 10% water boiled of dry leaves; I) positive control (shrimp injected with WSSV without mangrove extract); J) Negative control (without WSSV and mangrove extract)
3.2. Total Hemocyte Count (THC)

The average total hemocytes of tiger prawns in the WSSV disease prevention study using boiled extract of S. alba mangrove is presented in Figure 1. The figure shows that the average total of shrimp hemocytes tends to decrease with time and this is seen in all treatments. This indicates that the shrimp's health condition is getting worse after being injected with WSSV, while the extra mangrove used has not been effective to inhibit WSSV infection of shrimp. It can also be seen by looking at the mortality rate of tiger prawns which is getting higher day by day. The condition of hemocyte cells can indicate the health level of shrimp. In general, in good conditions, shrimp hemocytes consist of granular, semi-granular, and hyaline cells. Each cell has a role in the defense of the shrimp body. One of the body's defense functions performed by hemocytes is phagocytosis. Good hemocyte cells will be able to carry out fogging of foreign objects that enter the hemolymph into dirt/debris.

The lower total hemocytes and the higher mortality rate of tiger prawns from the first day to the ninth day indicated that the WSSV concentration used was very virulent to shrimp and the concentration of mangrove extract used was not effective in preventing WSSV infection in shrimp. A previous report showed that the use of 10% extract from boiled dried leaves gave a better effect on the condition of shrimp hemocytes cells that were challenged with pathogenic vibrio. The condition of shrimp hemocytes cells that were challenged with pathogens both vibrio pathogens and WSSV tended to be better in the treatment using boiled extract of dry leaves compared to wet leaves.

![Figure 1. Total Hemocytes in tiger shrimp injected with WSSV and mangrove extract](image)

**Notes:** A) 1% water boiled of fresh leaves; B) 4 % water boiled of fresh leaves; C) 7% water boiled of fresh leaves; D) 10 % water boiled of fresh leaves; E) 1% water boiled of dry leaves; F) 4% water boiled of dry leaves; G) 7% water boiled of dry leaves; H) 10% water boiled of dry leaves; I) positive control (shrimp injected with WSSV without mangrove extract), J) Negative control (without WSSV and mangrove extract)

3.3. ProPO value

The average ProPO value of tiger prawns in research on WSSV disease prevention using S. alba extract from boiling fresh leaves and dried leaves by injection system is presented in Figure 2. In Figure 2 it can be seen that the ProPO value of tiger prawns varied in all treatments. As with total hemocytes, shrimp ProPO tilapia also tended to decrease with increasing rearing time, but in general, it was seen that ProPo tilapia from the first day to the ninth day was lowest in treatment I (shrimp injected with...
WSSN without mangrove extract. The relatively more stable ProPo value was seen in treatment J (without WSSV and mangrove extract). In the treatment using fresh leaf mangrove extract.

Figure 2. Average ProPO value in shrimp injected with WSSV and mangrove extract

Notes: A) 1% water boiled of fresh leaves; B) 4 % water boiled of fresh leaves; C) 7% water boiled of fresh leaves; D) 10 % water boiled of fresh leaves; E) 1% water boiled of dry leaves; F) 4% water boiled of dry leaves; G) 7% water boiled of dry leaves; H) 10% water boiled of dry leaves; I) positive control (shrimp injected with WSSV without mangrove extract), J) Negative control (without WSSV and mangrove extract).

4. Conclusion

1. The mortality rate of tiger prawns in the treatment using boiled extract of fresh leaves were higher than those using boiled extract of dried leaves at the same concentration
2. The extract from boiling dried leaves tends to be better at preventing and protecting shrimp from WSSV infection
3. Total hemocytes and ProPO values in shrimp tend to decrease with time of rearing

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