

Effectiveness of Natural Herbal Oil Formulation against White Spot Syndrome Virus in *Penaeus vannamei*

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Abstract

The Natural Herbal Oil Formulation (NHOF) have been designed to work against White Spot Syndrome Virus (WSSV) in Shrimp. The essential oil blend extracted from the following plants, *Lavandula latifolia*, *Pinus sylvestris*, *Jasminum officinale*, *Citrus limon*, *Prunus avium*, *Viola odorata*, *Gardenia jasminoides*, *Cocos nucifera*, *Rosa damascene* and *Eucalyptus globulus*, mixed together to develop as anti-WSSV product. The product were added in the feed, as feed additive. Another product developed which was applied in tank water as water supplement named as "Pondguard" (Reg no. KKP RI no. D16060285-HBC). The Pondguard is composed of three essential oils i.e., *Eucalyptus globulus*, *Pinus sylvestris* and *Lavandula latifolia*. A bioassay trial was conducted to determine the efficacy of developed formulation as an anti-WSSV candidate. The experimental shrimp was fed on NHOF-mixed feed and "Pondguard" applied in the tank water whereas control shrimp were fed on the regular feed. The shrimp of both experimental and control were challenged by WSSV on 15th day of feeding. Two methods of WSSV challenge were applied, per-os challenge and immersion challenge. The cumulative mortality in positive control reached up to 100% on DPI 10 whereas no mortality appeared in the experimental group and in negative control. The trial results show that the developed natural herbal formulation has significant effect against WSSV in a controlled condition.

Introduction

White Spot Syndrome Virus is the most deadly pathogen of cultured shrimp [1,2]. The cumulative mortality in infected shrimp reaches up to 100% in 4-7 days in lab and 10-14 days in the ponds [3-6]. The intensity of infection and severity depends on the viral load. The stress factors, like, fluctuation in critical water quality and environmental parameters, like temperature, pH and Dissolved Oxygen parameters stimulates the process of infection.

Researchers and technicians have been continuously trying to control the spread of White Spot Viral Disease [7]. The limited knowledge on the mode of action and method of application of various agents restricts the success [8]. Various plants in the form of phenolic, polyphenolic, alkaloid, quinone, terpenoid, lectine, and polypeptide compounds etc. have shown anti-viral properties. Various efforts have been made to identify the anti-WSSV properties in the plants, such as, *Cynodon dactylon* [9,10], *Durio zibethinus* Murr (Durian) [11] and *Ceriops tagal* [12] and *Eucalyptus* spp. and *Plelargonium roseum* [13].

A successful effort has been made to develop a formulation by adding essential oil blend extracted from ten plants together and mixed it with the feed as feed additive. The feed was supported by a liquid formulation, named as Pondguard, containing three essential oil extract. A trial was conducted to determine the efficacy of feed with Natural Herbal Oil formulation and Pondguard against White Spot Syndrome Virus (WSSV) in *Penaeus vannamei* [14].

Materials and Methods

Bioassay lab and glass aquaria

A bioassay trial was set-up using 10 aquaria each for experimental groups as well as for control groups. The Specific Pathogen Free (SPF)-juvenile shrimp of average weight 0.25 g, 10 in number were maintained in each aquarium. The glass aquaria were cleaned, sun dried and disinfected with 70% alcohol. Each aquarium was filled with 5 L of seawater provided with required Dissolved Oxygen (DO) supply.

Water quality parameters

The seawater utilized during the experiment was gone through the process of sedimentation, filtration and disinfected with 30 ppm active chlorine and finally treated with Ultra Violet radiation (SS-L75W, Matala Water Technology, Taiwan). The water quality parameters maintained during the trial were as followed, Dissolved Oxygen (DO) (>5 ppm), Temperature ($27 \pm 1^\circ\text{C}$), Salinity (20 ppt), pH (7.5-8.15) and alkalinity (80-120 ppm).

Experiment start

The duration of trial was of 30 days i.e., 5 days acclimatization, 14 days feeding, day 15 challenge and from day 16 onwards continues feeding and observation for 10 days. Shrimp of treatment group were fed on experimental feed whereas regular feed was fed to the control. There was no change in feed quality and the rate of feeding throughout the experiment. The liquid supplement, Pondguard was applied on daily basis in experimental tanks throughout the experiment. The shrimp were observed intensively for their behavior and feeding rate and cumulative mortality during the trial.

Shrimp food

The shrimp feed was produced in Feed Mill of PT. Central Proteina Prima, Indonesia. Treatment group of shrimp was fed on experimental

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feed i.e., CPP regular feed mixed with Natural Herbal Oil Formulation (NHOF) and control group on regular feed. The anti-WSSV NHOF were developed by combining the essential oil blend extracted from the following ten plants, *Lavandula latifolia*, *Pinus sylvestris*, *Jasminum officinale*, *Citrus limon*, *Prunus avium*, *Viola odorata*, *Gardenia jasminoides*, *Cocos nucifera*, *Rosa damascene* and *Eucalyptus globulus*. Using expeller-pressing method (Anderson International Corporation) performed the oil extractions from the selected plants. The essential oil blends were mixed with the feed in required amount. The basic formulation of both the feed were same except essential oil mixed in the experimental feed.

Pondguard application

A liquid product called Pondguard (KKP RI no. D 16060285–HBC) was developed as a water supplement. The Pondguard is composed of three essential oils i.e., *Eucalyptus globulus*, *Pinus sylvestris* and *Lavandula latifolia*. It was applied in the treatment tank at the rate of 1.5 ppm on daily basis after tank cleaning and water exchange (approximately 20% per day).

WSSV disease challenge procedure

Two kinds of challenge methods i.e., per-os and immersion were applied in the trial [15]. The treatment tanks were divided into two group (5 tanks each), one group challenged by per-os method whereas another one by immersion method. The method in detail were as followed,

Challenge method 1: Per-os method of challenge: The muscle to use in per-os challenge was prepared from the artificially WSSV challenged SPF shrimp. The method applied, in short, was pure WSSV viral isolate was injected to the SPF juvenile shrimp. The moribund shrimp with gross sign of WSSV were collected, peeled and de-veined. The muscle were blend and homogenized. The viral load was checked by Real-Time PCR. The collected tissue containing viable WSSV were stored at -80°C by adding sterile glyserol till further use.

On the day of challenge, the shrimp were not fed on artificial feed. The muscle containing WSSV were fed to shrimp at the rate of 5% MBW as morning meal.

Challenge method 2: Immersion method of challenge: The WSSV-contaminated water was utilized for the immersion challenge. The method applied, in short was, pure WSSV viral isolate was injected to the SPF juvenile shrimp. The dead shrimp with gross sign of WSSV were collected from the tanks after 24 h of 100% cumulative mortality. The tank water was filtered. The viral load was checked by Real-Time PCR (copy number per microliter of water). The 5 L of WSSV-contaminated water were poured into each experimental and positive control tanks (Table 1).

Post challenge observation: The challenged shrimp were under intensive observation. The feeding rate and tank bottom siphoning schedule and maintained the same for both control and treatment group throughout the experiment. The water exchange was only done in per-os challenged tanks. No water exchange was done in immersion challenged tanks.

Lab confirmation: The WSSV was confirmed by nested PCR analysis.

Statistical analysis: Statistical analysis will be done by ANOVA with $P < 0.05$ confidence level (Table 2).

Result and Discussion

A successful effort was made to develop anti-WSSV product. The product is combination of blend of essential oils with anti-viral properties. The artificial feed as a carrier of anti-viral product is the best way to provide the protection to the shrimp. The extra protection was provided by applying the liquid product in the tank water. It was done with the assumption that the rate of feed consumption will be reduced during the stress condition including at the time of the viral attack. The pondguard was applied on daily to maintain the optimum protection level. The shrimp were fed for first fourteen days to develop enough protection. The feed consumption data in Figure 1 show after the challenge. There was feed drop on DPI 2 and 3 in all the groups which indicates that the challenged animals were under stress. The feed consumption started getting normal from DPI 4 in treatment groups.

Two types of viral challenge methods, per-os and immersion, were applied to test the product at the maximum. The pure WSSV was first used to infect the SPF shrimp and then the tissue of diseased shrimp were utilized for per-os challenge. The tank water containing WSSV were collected from the injection challenged tanks to conduct the immersion challenge. Higher rate of cumulative mortality recorded in immersion challenged positive control group as virus could enter to the shrimp body through the digestive system as well as via osmoregulation. There was no mortality occurred in the experimental feed fed groups which showed the anti-viral activities of the product. The mortality started from day 2 of challenge in per-os challenge whereas day 3 in immersion challenge. This could happen due to the variation in viral load as the rate of feeding would vary in the individual shrimp of per-os challenge group (Figure 2).

There was significant difference between experimental group and control group.

The developed product is user friendly as it's mixed and incorporated into the shrimp feed. The developed blended oil extract formulation have shown anti-WSSV properties in the controlled condition.

Group	Challenge		Rep.	MBW (g); Density/5 L	Feed	Pond guard	Water exchange
	Method	Dose					
Treatment	Challenge 1 Immersion	141.66 copy/uL	10	0.25 g; 10 pcs	Regular feed+NHOF	Daily; 1.5 ppm	-Pre-challenge 20% per day -Post-challenge; immersion challenge no water exchange; per-os challenge 20% water exchange from DPI 3
	Challenge 2 Per-os	WSSV infected tissue at the rate of 5% of biomass for 1 day		0.25g; 10 pcs			
Positive control	Challenge 1 Immersion	141.66 copy/uL		0.25; 10 pcs	Regular	-	
	Challenge 2 Per-os	WSSV infected tissue at the rate of 5% of biomass for 1 day		0.25 g; 10 pcs			
Negative control	-	-		0.25 g; 10 pcs			

Table 1: Experimental design of anti-WSSV trial.

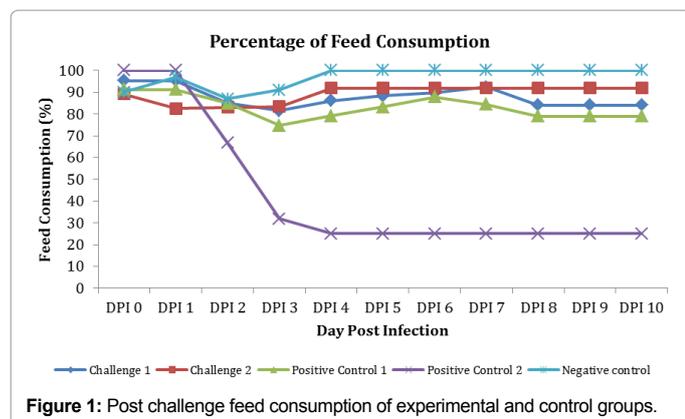


Figure 1: Post challenge feed consumption of experimental and control groups.

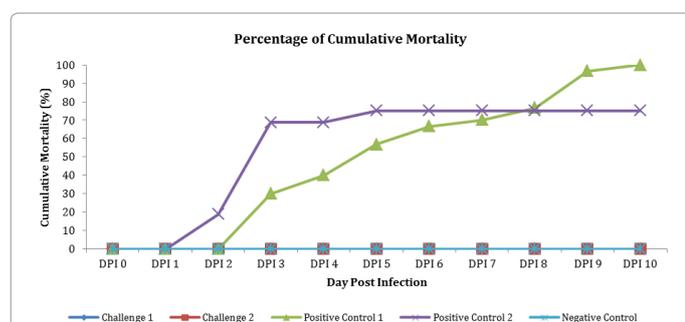


Figure 2: Cumulative mortality percentage of experimental and control shrimp.

Mortality	Sum of Squares	df	Mean Square	F	Sig.
Between groups	4000.000	1	4000.000	2.667	0.141
Within groups	12000.000	8	1500.000	-	-
Total	16000.000	9	-	-	-

Table 2: ANOVA test result.

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