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## Performance of natural oil blend formulation (NOBF) against white spot syndrome virus (WSSV) agent in *Penaeus vannamei* boone, 1931

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### Abstract

The white spot syndrome virus (WSSV) is lethal in penaeid shrimp. Successful efforts made to develop a natural oil blend formulation (NOBF) by blending *Eucalyptus globulus*, *Pinus sylvestris*, and *Lavandula latifolia* in different compositions with anti-WSSV properties. A bioassay challenge trial conducted using 1 g of specific pathogen-free *Penaeus vannamei* Boone, 1931 shrimp in 4 replicates of aquaria for each group. The NOBF dose of 0.4 ppm applied throughout the trial period by mixing in the aquarium water daily, starting seven days before the challenge. The efficacy of NOBF against WSSV was measured using the per os method of a challenge. The cumulative mortality in the positive control group reached 100 % after ten days of the challenge. The NOBF was applied in the commercial shrimp ponds to study its efficacy and palatability. A study in 8 ponds in a WSSV prone area in Indonesia, demonstrated no significant difference in crucial water quality parameters, especially on algae as natural food. The NOBF applied ponds had higher productivity (1.28 Kg of shrimp per m<sup>3</sup>) as compared to control (1.18 Kg of shrimp per m<sup>3</sup>). The FCR of NOBF group was lower (1.41) than control (1.53). The survival rate of the NOBF group was higher (86.96%) than control (80.36). The trial outcomes show that NOBF is safe and user-friendly, with properties to reduce pathogen load.

**Keywords:** White spot syndrome viral disease, natural oil, *per os* challenge, shrimp pond culture, shrimp productivity, natural food

### Introduction

The white spot syndrome virus (WSSV) is the most lethal pathogen of culture shrimp worldwide <sup>[1]</sup>. It is an enveloped non-occluded DNA virus of the family Nimaviridae under the new genus Whispovirus and is also the most devastating shrimp pathogen ever isolated and studied. This virus is extremely virulent and causes up to 100 % mortality in 3-7 days in all the cultured species of penaeid shrimp <sup>[2, 3, 4]</sup>.

Some successful efforts made previously using natural herbs against shrimp viruses <sup>[5, 6, 7, 8]</sup>.

The plants, such as *Eucalyptus globulus*, *Pinus sylvestris*, and *Lavandula latifolia* are well documented for their anti-viral properties. *Eucalyptus* extract shows strong anti-viral and anti-bacterial properties <sup>[9, 10, 11]</sup>. *Pinus* species have potent anti-viral, anti-bacterial, and immunomodulating properties <sup>[12, 13]</sup>. The *Lavandula* have shown strong anti-viral <sup>[14]</sup> as well as immunomodulatory <sup>[15]</sup> effects.

A successful effort made to blend the oils of similar properties to fight against WSSV more vehemently. The efforts made to test the palatability and its impact on critical water quality parameters in commercial shrimp ponds.

### Materials and Methods

The materials and methods divided into two sections, section 1 as laboratory level trials and section 2 as field trials.

#### Section 1: Laboratory trials

##### NOBF preparation and composition

*Eucalyptus globulus*, *Pinus sylvestris* and *Lavandula latifolia* oils obtained from vendors who comply with the strictest industry practices:

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Demeter Agro Research and Improvements Pty., Ltd., New Directions Australia Pty., Ltd., and Australian Botanical Products Pty., Ltd. Each essential oil is obtained through the steam distillation process and should undergo thorough checking for the quality and chemical compositions based on European Pharmacopeia. After the essential oils are declared

to pass the quality checking, the mixture of the NOBF created with the following sequence and percentage: *Eucalyptus globulus*, *Pinus sylvestris*, and *Lavandula latifolia* are added in equal quantities to form the oil mixture then mixed with potable water. Hence, a 1% concentration of the final product obtained.

**Table 1:** Dose of natural oil blend formulation (NOBF), its procedure, and frequency of application in the shrimp tanks and ponds. NOBF application in the aquarium, NOBF dose of application and schedule in the culture pond

Dose of NOBF	Dose (ppm)	Frequency of application	Procedure
Aquarium	0.4 ppm (4 L.ha <sup>-1</sup> )	Daily (starting from 7 days prior to trial to end of trial)	Required amount of NOBF poured in the aquarium
Pond: before Post larvae stocking	0.4 ppm (4 L.ha <sup>-1</sup> )	Two times, 7 days and 3 days before stocking	Required amount of NOBF diluted in 10 L of pond water and sprayed over the pond in high current area
Pond: during culture	0.4 ppm (4 L.ha <sup>-1</sup> )	Weekly application, total 15 times in one crop	

The predetermined doses of NOBF were applied in the trials conducted in the lab and in the ponds. The 0.4 ppm of NOBF was applied in the tank as a water supplement throughout the experiment starting from 7 days before challenge. A prophylactic dose of 0.4 ppm (40 L.ha<sup>-1</sup>) was applied weekly as a preventive dose in the shrimp ponds.

#### Animal preparation for lab trial

The specific pathogen-free (SPF), juvenile shrimp, *Penaeus vannamei* Boone, 1931, was selected for the trial. The post larvae (PL) of shrimp procured from a bio secure hatchery of PT. Central Proteina Prima, Indonesia. The PLs produced using SPF broodstock procured from Shrimp Improvement System, Hawaii. The PLs were tested and screened free from known pathogens, such as WSSV, infectious myonecrosis virus (IMNV), *Enterocytozoon hepatopenaei* (EHP), and early mortality syndrome (EMS). The PL 10 raised to 1 g of average weight in the bio secure facility of a lab for the bioassay trial.

#### Disease challenge laboratory

The bioassay trial conducted at the Disease Research Center of PT. Central Proteina Prima, Indonesia. A trial was set-up using 12 glass aquaria of 80-litre water capacity. The glass aquaria cleaned, sun-dried, and finally disinfected with 70 % alcohol. Each aquarium filled with 50 L of seawater provided with the required dissolved oxygen (DO) supply. The 12 juvenile shrimps, each with an average weight of 1 g, were evenly distributed in 12 aquaria. The shrimp acclimatized for three days before the experiment.

#### Water quality parameters

The seawater utilized during the experiment underwent the process of sedimentation, filtration, and disinfection with 30 ppm of active chlorine (sodium hypochlorite) and finally treated with ultraviolet radiation (SS-L75W, Matala Water Technology, Taiwan). The water quality parameters maintained during the trial were as followed, DO (> 5 ppm), temperature (27 ± 1 °C), salinity (20-25 ppt), pH (7.5-8.15), and alkalinity (80-120 ppm).

#### Shrimp food

The shrimp feed was produced in Feed Mill, Surabaya of PT. Central Proteina Prima, Indonesia. Shrimp were fed at 5 % of mean body weight per day on pelleted feed in the aquaria trial. The standard feeding table was followed for the commercial pond trial.

#### Challenge preparation (WSSV)

The WSSV-infected tissue was used for the challenge. The fresh challenge was prepared following the methods of [3, 16]. The significant steps described below:

1. The lethal dose of pure WSSV was injected to 10 SPF shrimp of 15 g each. The moribund shrimp with clear symptoms of WSSV were collected from day 3 of the challenge. The tissue was screened for other potential contaminants before use for WSSV extraction. The tissue was cut into fine slices and stored at -20 °C.
2. The viral number was quantified in the challenged tissue using the quantitative polymerase chain reaction (qPCR) method. The average number of viral solutions was log 9.

#### WSSV challenge procedure

The applied *per os* challenge method was a combination of those developed by [3, 17]. The challenge was shrimp tissue carrying WSSV fed at same quantity as feed i.e., 5 % of mean body weight. No other feeding was done on the day of challenge. No water was exchanged in the tanks for 3 days after challenge.

#### Observation

The daily feed consumption, shrimp activeness, and mortality were observed and recorded at least twice a day.

#### Lab confirmation using PCR and histopathology

PCR tested the shrimp of all the groups for the presence of WSSV at day 8 of challenge.

#### NOBF formulation evaluation

The relative per cent survival was used to evaluate the efficacy of the anti-pathogen formulation. The relative per cent survival value was measured using the following formula:

$$\text{RPS value} = 1 - (\% \text{ mortality in NOBF} / \% \text{ mortality in Positive control}) \times 100.$$

#### Section 2: Field trials

##### Animal preparation for pond stocking

The specific pathogen-free (SPF), juvenile shrimp, *Penaeus vannamei* Boone, 1931, was selected for the trial. The postlarvae (PL) of shrimp procured from a biosecure hatchery of PT. Central Proteina Prima, Indonesia. The PLs produced using SPF broodstock procured from Shrimp Improvement System, Hawaii. The PLs were tested and screened free from known pathogens, such as WSSV, infectious myonecrosis

virus (IMNV), *Enterocytozoon hepatopenaei* (EHP), and early mortality syndrome (EMS). The shrimp at stage post-larvae 10 (PL 10) stocked in the experimental ponds.

**Field trial in shrimp culture pond**

A total of 8 commercial ponds, i.e., four each from treatment and control of each 1,000 m<sup>2</sup> area was selected for the trial. The 5-month trial was conducted in a WSSV prone area in Lampung, Indonesia. The standard operating procedure of PT. CP Prima was followed. The shrimp PL were stocked at 70 pieces m<sup>-2</sup>. The culture period was 70 days. Two partial harvests were performed to maintain the carrying capacity of the ponds.

**NOBF dose and application in culture pond**

The NOBF was considered novel. Its application, acceptance to shrimp, was required to study. A full pond level trial was designed and conducted for product optimization and recommendation. The pond trial was conducted by keeping the following observations in mind:

1. Impact of NOBF on critical water quality parameters: The critical water quality parameters, such as DO, pH, salinity, total ammonia, temperature, and water transparency, were observed weekly.
2. Palatability and growth performance: The shrimp feeding rate, growth performance and the survival rate were measured and compared.

**Shrimp health condition**

The shrimp health condition in the ponds was checked by pathogen presence, including PCR screening of WSSV, IMNV, EHP, EMS-*Vibrio parahaemolyticus*, and other *Vibrio* levels at different time intervals. The hematological analysis was also performed after 3 weeks of NOBF application.

**Statistical analysis**

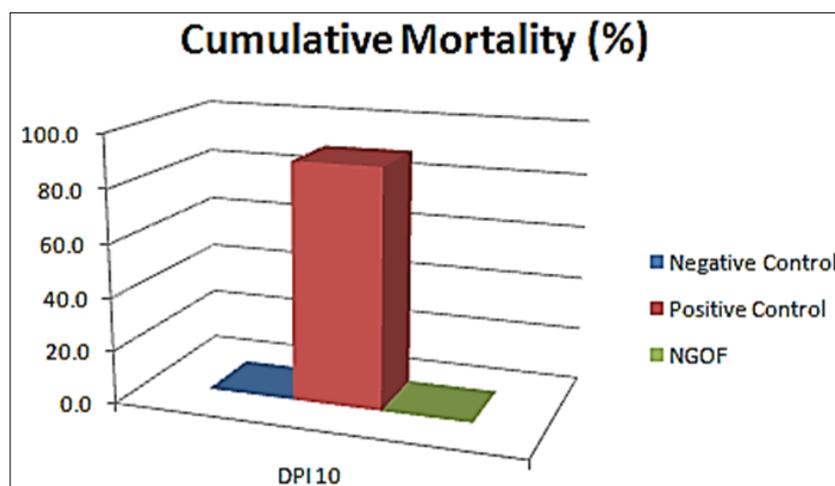
The statistical analysis, ANOVA (analysis of variance), at 0.05 significance level was performed using software SPSS Statistics, Version 23 from IBM for the challenge test. The statistical analysis was performed using the general linear model of Statistical version 10 (Stat Soft) for the pond trial data.

**Results**

**Part 1: Laboratory trials**

**Cumulative mortality observation**

The cumulative mortality was recorded two to three times a day. The moribund shrimp or those about to die were immediately removed from the aquaria to avoid the cannibalism and accelerated horizontal transmission. The mortality started in positive control tanks from the third day post-infection (dpi 3). There was no mortality observed in the negative control and NOBF groups (Figure 1). The NOBF group was significantly different from the positive control group (Figure 1 and Table 2).



**Fig 1:** Cumulative mortality of WSSV challenged shrimp in three replicated each. Mortality is presented in percentage and on daily cumulative basis. No mortality reported in negative control and NOBF groups whereas mortality started day post infection (DPI) 3 in positive control

**Table 2:** Statistical analysis using ANOVA, between challenged groups on day post infection 10. The positive control significantly different from NOBF and negative control groups

Group	Rep.	No. of shrimps	Cumulative mortality (%) at DPI 10
Negative Control	1	12	0.0
	2	12	0.0
	3	12	0.0
	4	12	0.0
	Average		
Positive Control	1	12	83.3
	2	12	75.0
	3	12	100.0
	4	12	100.0
	Average		
NGOF	1	12	0.0
	2	12	0.0
	3	12	0.0
	4	12	0.0
	Average		

**Table 3:** Statistical analysis using ANOVA, between challenged groups on day post infection 10. The positive control significantly different from NOBF and negative control groups

ANOVA					
Cumulative mortality at DPI 10					
	Sum of squares	df	Mean square	F	Sig.
Between groups	21396.482	2	10698.241	205.223	0
Within groups	469.167	9	52.13		
Total	21865.649	11			

**Feed consumption observation (Gut content)**

The food consumption was monitored at least twice a day. It was recorded in terms of gut content, i.e., empty gut and full gut after 30 minutes of feeding. The food consumption was lowest in the positive control group, whereas negative control and NOBF-treated shrimp had normal diet. The feed consumption determines the stress level of shrimp. The full gut of negative control and NOBF shrimp determines no stress in those shrimps due to WSSV challenge.

**WSSV clinical signs observation (Shrimp activeness and clinical signs)**

The typical clinical sign of WSSV infection are feed

consumption, lethargy, red color appearance, and finally white spot appearance on the carapace. All the typical clinical signs were observed clearly in positive control shrimps.

**Lab confirmation using PCR and histopathology nested PCR observation**

The surviving shrimp were collected at the end of the trial and checked using a commercially available PCR kit from Genreach, Taiwan. The positive control shrimp were found to be positive for WSSV, whereas the negative control and NOBF groups were negative for WSSV.

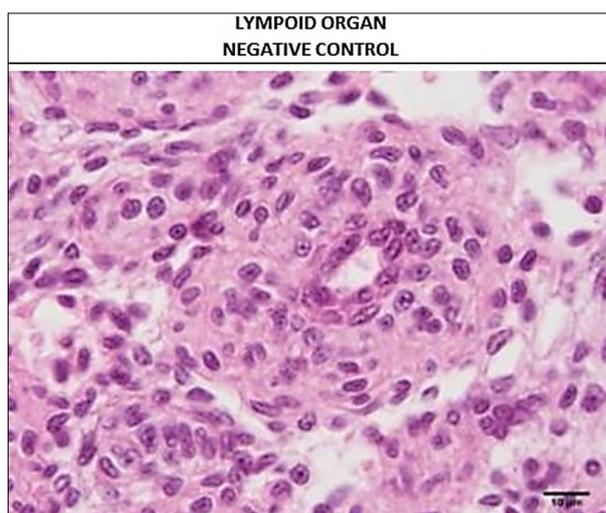
**Table 4:** Nested PCR report of positive control, negative control, and NOBF groups. The positive control shrimp samples were found to be positive for WSSV, whereas the NOBF and negative control groups were negative. The samples collected at the end of the trial

Group	Sample number	Nested PCR results (WSSV)
Negative control	1	Negative
	2	Negative
	3	Negative
Positive control	1	Positive
	2	Positive
	3	Positive
NOBF	1	Negative
	2	Negative
	3	Negative

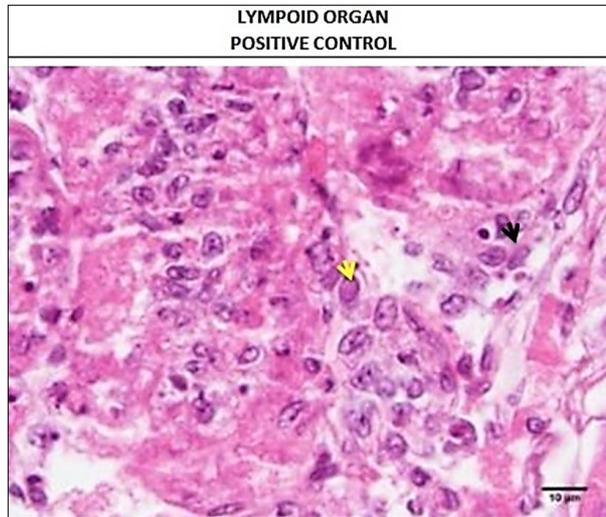
**Histopathological observation**

The shrimp were collected on the day 8 of challenge and preserved for histopathological analysis of lymphoid organs of negative control (4A), positive control (4B) and NOBF

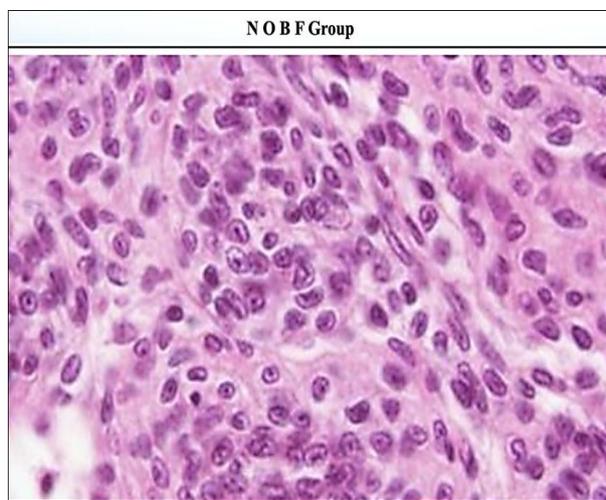
group (4C) are shown. The positive control slide has shown typical symptoms like presence of picnotic nuclei and intranuclear inclusion bodies in the later stage.



**Fig 2:** Histopathological analysis of lymphoid organ of negative control. The absence of picnotic nuclei and intranuclear inclusion bodies (Yellow arrow) in negative control



**Fig 3:** Histopathological analysis of lymphoid organ of positive control group. The presence of picnotic nuclei and intranuclear inclusion bodies (Yellow arrow) in positive control at the later stage shows the severe WSSV infection



**Fig 4:** Histopathological analysis of lymphoid organ of NOBF group. The absence of picnotic nuclei and intranuclear inclusion bodies (Yellow arrow) in NOBF

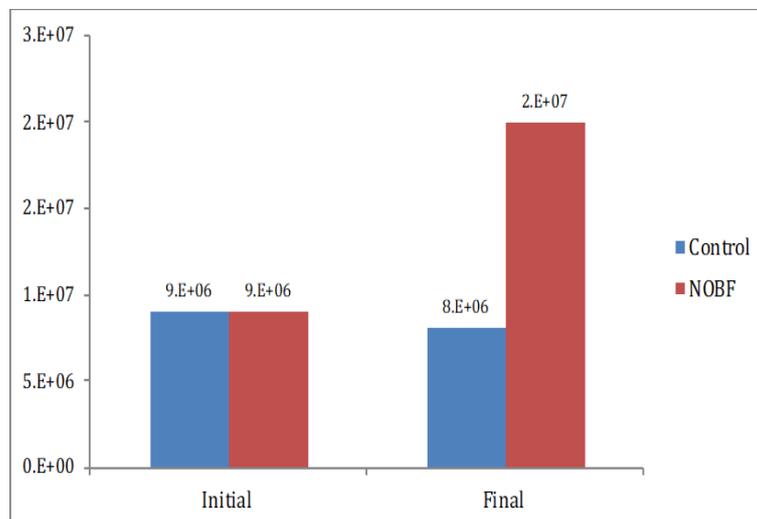
**NOBF formulation evaluation**

The relative percent survival was used to evaluate the efficacy of anti-pathogen formulation. The relative percent survival value was 100 % for the NOBF.

**Field observation**

**Immunity testing of experimental animals**

The hemocyte count of NOBF applied animals increased from  $9 \times 10^6$  to  $2 \times 10^7$  in 3 weeks whereas the control remains almost the same i.e.  $9 \times 10^6$  (Figure 5).



**Fig 5:** Hemocyte count of NOBF applied shrimp and control shrimp after 3 weeks

**NOBF application in culture ponds**

The NOBF was evaluated in the shrimp culture pond by observing impact on crucial water quality parameters, palatability, shrimp health condition, and finally the productivity. The obtained results are as follows,

control were in the optimum range. There was no significant difference among the crucial water quality parameters. The impact on natural food i.e. algae is shown in the figures 6A, 6 B and 6C. The data shows no negative impact on the growth of natural food due to NOBF application.

**Crucial water quality parameters**

All the crucial water quality parameters of both NOBF and

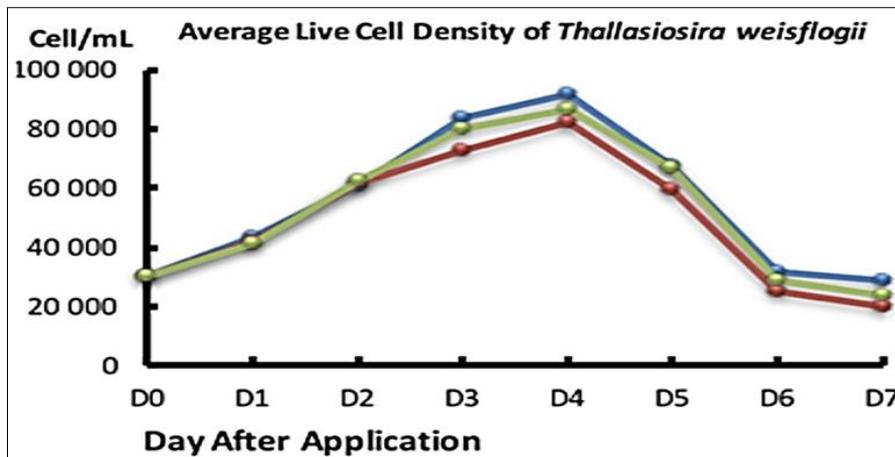


Fig 6A: *Thalassiosira weissflogii*

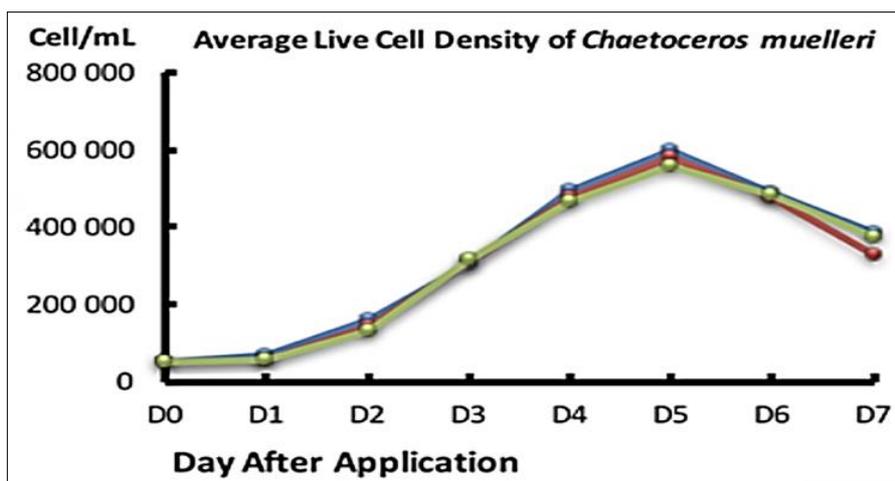


Fig 6B: *Chaetoceros muelleri*

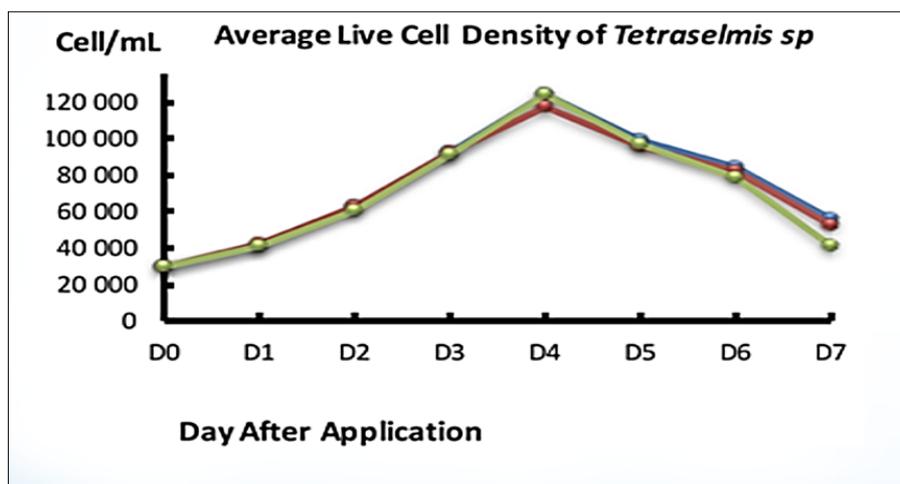


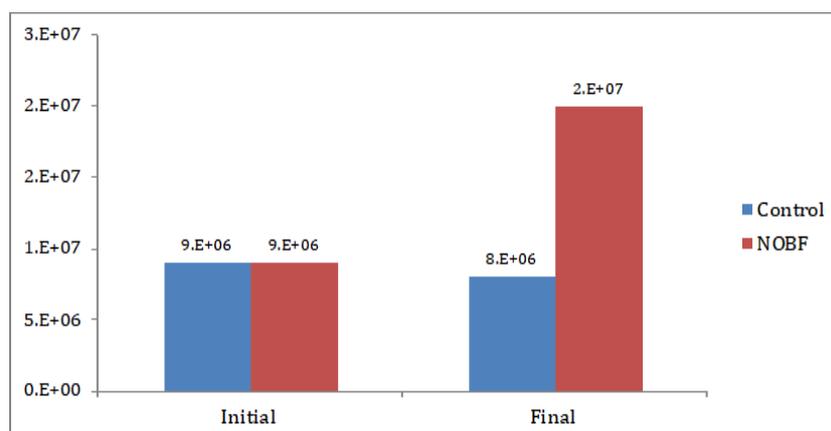
Fig 6C: *Tetraselmis spp*

Fig 6A, 6B and 6C: Study on growth of algae, *Thalassiosira weissflogii*, *Chaetoceros muelleri* and *Tetraselmis spp* in NOBF applied ponds and control ponds. No difference found between control and NOBF ponds. NOBF doesn't have any negative impact on the crucial water quality parameters

### Shrimp health condition

The shrimp health condition was checked by pathogen presence and hemocyte count. The hemocyte count of shrimp, which was higher in NOBF applied ponds and at the optimum level [18], was one of the major signs of good health (Figure

7). The total bacterial count and total *Vibrio* count were in the acceptable range [19, 20] with no significant difference between NOBF and control ponds. All the trial ponds were detected negative for pathogens WSSV, IMNV, EHP, and EMS-*Vibrio parahaemolyticus* throughout the trial period.



**Fig 7:** Total hemocyte count of shrimp at initial stage (20 days of culture) and at advanced stage (70 days of culture) in control and NOBF ponds. The final data showed that hemocyte count of NOBF shrimp increased to log 7 at optimum level

### Shrimp production performance

The obtained results show that the NOBF application was well accepted by shrimp, which resulted in higher growth rate

than the control. The overall shrimp production in NOBF applied ponds were better with no significant difference as shown in Figure 8.

Treatment	Description	Number of Tank	St. Density (pc/m <sup>3</sup> )	DOC	Harvest MBW (g)	Acc. ADG (g/day)	Biomass (kg/tank)	Productivity (kg/m <sup>3</sup> )	SR (%)	FCR (%)
CONTROL	NO NOBF application	4	70	90	21.01±0.355a	0.232 ±0.004a	2.363±0.062a	1.18±0.31a	80.36±1.071a	1.53 ±0.040a
NOBF	NOBF application	4	70	90	21.04 ±0.339a	0.233 ±0.004a	2.562±0.073a	1.28±0.37a	86.96±3.600a	1.41 ±0.041a
P value					0.4384	0.4384	0.4640	0.4640	0.1290	0.4487

Values are means ± SEM  
Means in the same row with different superscripts are significantly different at p<0.05

**Fig 8:** Overall, shrimp production performance and comparison between control and NOBF applied ponds. There is statistically no significant difference in performance between control and NOBF ponds. Overall, NOBF applied ponds have better productivity and performance

The NOBF group has higher productivity (1.28 Kg of shrimp per m<sup>3</sup>) as compared to control (1.18 Kg of shrimp per m<sup>3</sup>). The FCR of NOBF group was lower (1.41) than control (1.53). The survival rate of NOBF group was higher (86.96%) than control (80.36 %).

### Discussion

In the present study, attempts have been made to blend the natural oil with anti-viral properties in required compositions potent enough to be an anti-WSSV component and its acceptability in the shrimp culture ponds as a water supplement. Previously, [21], tried the blend of *C. dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurooa*, and *Eclipta alba*, and successfully tested it against WSSV. Previously [22], successfully tested *Pongamia pinnata* against WSSV in *P. monodon*. Likewise, several researchers have successfully proven the anti-WSSV properties of terrestrial plant extracts [23, 24, 25, 21, 26, 27, 28]. Most of the developed herbal-based formulations either had unproven efficacy in the field

or were not compatible with the cost of shrimp production. Our previous studies [29, 30, 31, 32] on the efficacy of NOBF against shrimp pathogens encouraged us to proceed further. Use of oil instead of the herbal extract was a better choice to maintain the consistency and accuracy of the formulation. The obtained PCR and histopathological analysis showed that NOBF has been able to deactivate WSSV. The obtained results are in alignment with the previous results obtained by [29] using the per os and immersion methods of challenge. The immediate next step was to evaluate the performance of NOBF in the shrimp ponds. Efforts were made to study the palatability and impact of NOBF on crucial water quality parameters in the commercial shrimp ponds. No negative impact was recorded on the water quality parameters and the natural food in the presence of NOBF. The impact of NOBF was studied on the natural food, i.e. algae. The obtained data showed that NOBF does not have any negative impact on the growth and performance of natural food. The crucial water quality parameters refer to the quality

of water that enables the successful growth and production of the desired organisms. The higher shrimp productivity in NOBF applied ponds by keeping lower FCR, and higher survival rate proved that NOBF could provide a healthy environment to the shrimp to grow in its optimum level.

### Conclusion

The bioassay lab trial data showed that the mode of action of NOBF is viricidal. We found that the NOBF is a natural agent to apply in the shrimp culture ponds to maintain shrimp health and enhance productivity.

### Conflict of interest

We declare that we have no conflict of interest.

### Acknowledgments

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