

Combination of *Nigella sativa* and *Phyllanthus niruri* as An Immunostimulant for The Prevention of White Spot Disease in *Litopenaeus vannamei*

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Abstract

Pacific whiteleg shrimp (*Litopenaeus vannamei*) is a very important commodity, which accounts for almost 70% of the total world shrimp production. However, this production is still threatened by disease attacks, one of which is caused by white spot disease (WSD). Herbs are widely used as immunostimulants in an effort to prevent diseases. Black cumin (*Nigella sativa*) and stone breaker (*Phyllanthus niruri*) have long been known as excellent immunostimulants in humans. This study aimed to evaluate the potential of *N. sativa* and its combination with *P. niruri* as an immunostimulant in *L. vannamei* for preventing WSD. Tested shrimp (± 3.0 g) were treated with *N. sativa* (N), *P. niruri* (P) and the combination of both (1N:1P, 2/3N:1/3P, 3/4N:1/4P, 1/3N:2/3, 3/5N:2/5P, 1/4N: 3/4P, 2/5N:3/5P) through the feed for 28 days. They were then infected by the white spot syndrome virus (WSSV) and observed for 7 days. The parameters observed included immune response and production performance. *N. sativa* was able to reduce the mortality of test shrimp infected with WSSV, with an RPS value of 71%. *N. sativa* can be combined with *P. niruri* in the right composition (1/2N:1/2P and 2/3N:1/3P), while the other combinations are antagonistic. The administration of the best treatments in this study relatively did not affect the value of total hemocyte count, phenoloxidase activity, respiratory burst activity, hemolymph clotting time and production performance of tested shrimp.

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INTRODUCTION

Pacific whiteleg shrimp is the most important commodity in the world and accounts for 63% of the total world shrimp production. The rest is made up of red swamp crawfish *Procambarus clarkia*

(22%), giant tiger prawn *Penaeus monodon* (9.5%), giant river prawn *Macrobrachium rosenbergii* (3%) and other shrimp species (FAO, 2020). However, disease outbreaks remain a major issue in

shrimp farming. The outbreaks such as ones caused by white spot disease (WSD), yellow head disease (YHD), and infectious myonecrosis (IMN) still pose the most serious threat to *L. vannamei* culture. White spot disease is still a serious threat to pacific whiteleg shrimp production in the world (Thitamadee *et al.*, 2016). This virus attack can even result in 100% of shrimp mortality within 7-10 days after being infected (Wiyoto *et al.*, 2017).

The use of immunostimulants is one of the most common preventive measures to fight diseases in shrimp that is deemed to be effective. This is due to the fact that shrimp is dominated by a non-specific immune system. According to Apines-Amar and Amar (2015), various types and sources of immunostimulants used in shrimp farming include those herbs with various active substances. Herbs have various properties, such as antimicrobial, antistress, growth stimulation, appetite stimulation, and improve the immune system in fish and shellfish because of their active components, such as alkaloids, flavonoids, phenolics, terpenoids, steroids, and essential oils (Citarasu, 2010). The interest in herbs is getting higher throughout the world due to the prohibition of antibiotics and other chemical administration. Moreover, these plants are considered easy to prepare, inexpensive, and possess fewer side effects on animals and the environment (Hai, 2015).

Among different kinds of herbs that have been studied and proven to increase body immunity in humans and terrestrial animals are black cumin *N. sativa* and stonebreaker *P. niruri*. Various benefits of *N. sativa* have been reported, including immunomodulator, antioxidant, anti-inflammatory, antibacterial, antifungal, antiparasitic, antiviral, analgesic, neuroprotective, gastroprotective, cardioprotective, hepatoprotective, and nephroprotective (Ijaz *et al.*, 2017; Islam *et al.*, 2017). *P. niruri* is known as an immunomodulatory, antioxidant, anti-inflammatory, analgesic, wound healing and antiulcer, antiviral, antibacterial,

cardioprotective and hepatoprotective (Lee *et al.*, 2016).

Several studies of *N. sativa* on aquatic commodities have shown that its administration can increase the immunity of rainbow trout (*Oncorhynchus mykiss*) (Awad *et al.*, 2013; Bektaş *et al.*, 2019), tilapia (*Oreochromis niloticus*) (Elkamel and Mosaad, 2012; Dey *et al.*, 2020), carp (*Cyprinus carpio*) (Khondoker *et al.*, 2016; Yousefi *et al.*, 2021), climbing perch (*Anabas testudineus*) (Khatun *et al.*, 2015), rohu (*Labeo rohita*) (Ali *et al.*, 2020) and pacific whiteleg shrimp (Lei and Xiao-En, 2019; Nur *et al.*, 2020). *P. niruri* can improve the immunity of catfish *Clarias gariepinus* (Setiaji *et al.*, 2013), carp (Sunitha *et al.*, 2017), tiger shrimp *P. monodon* (Direkbusarakom *et al.*, 1995; Vibin *et al.*, 2020), and Pacific whiteleg shrimp (Sukenda *et al.*, 2011). *P. niruri* has also been reported to improve the growth performance of catfish (Johri *et al.*, 2011) and carp (Sunitha *et al.*, 2017), and also reduce stress in carp (Ibrahim *et al.*, 2015).

The combination of herbs that are synergistic has several advantages over a single application, including : (1) having a wider effect, (2) reducing the side effects of giving only a single herb, (3) having a stronger effect as one herb can cover the other's weaknesses (Yang, 2010). Both *N. sativa* and *P. niruri* have been known for their effectiveness in increasing the immune response in humans, but their application to control viral diseases in shrimp, especially WSD, is still very limited. The combination of *N. sativa* and *P. niruri* as an immunostimulant also needs to be evaluated. Therefore, this study is conducted to examine the potential of *N. sativa*, *P. niruri* and their combination as immunostimulants to prevent WSD, as well as its effect on the production performance of *L. vannamei*.

METHODOLOGY

Place and Time

This research was carried out in January-March 2020 at the field

laboratory of Technology and Management of Applied Aquaculture Hatchery, College of Vocational Studies, IPB University. The measurement of blood parameters was carried out at the Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University.

Research Materials

The shrimp used in this study came from a hatchery in Anyer, Banten, which was certified as specific pathogen-free (SPF). Black cumin oil extract was purchased commercially, while the meniran used was in the form of powdered simplicia, obtained from Biofarmaka, IPB University. The feed used was a commercial shrimp feed measuring 1.2 mm x 1.5 mm, with a protein content of 40%. The equipment used included

aquarium (40 cm x 30 cm x 40 cm) with aeration, analytical balance (NEWTECH NT-A, China), micropipette (Dragon Lab), syringe (Terumo 1 cc), microscope (Olympus, Japan), hemocytometer (Neubauer Improved Assistant, Germany), glass preparations and test tubes.

Research Design

The experiment used a completely randomized design with three replications. The treatments were *N. sativa* (N), *P. niruri* (P) and the combination of both as described in Table 1. The treatment combinations were given to the test shrimp through the feed for 28 days. In the end, the parameters for production performance were measured, followed by a challenge test with white spot syndrome virus (WSSV) infection to measure the parameters of the immune response.

Table 1. The combination treatments of *N. sativa* and *P. niruri*.

Treatment combinations	<i>P. niruri</i> (P) composition (1%)			
	0	1	2	3
<i>N. sativa</i> (N)	0	1P		
composition	1	1/2N:1/2P	1/3N:2/3P	1/4N:3/4P
(0,1%)	2	2/3N:1/3P		2/5N:3/5P
	3	3/4N:1/4P	3/5N:2/5P	

Work Procedure

Feed Treatment Preparation

P. niruri was extracted through brewing and filtration. Simplicia meniran as much as 5 g was put into 50 mL of boiling water then stirred and cooled. The herbal water extract was separated from its pulp by using two layers of fine cloth filter as a filter. *P. niruri* extract (1%) and *N. sativa* oil extract (0.1%) were mixed according to the treatment combinations as described in Table 1. It was then given a binder (1% of gelatin) and an attractant (0.5% of squid oil). This was mixed with commercial feed containing 40% protein content (coating method). After that, the treatment feed was dried in an oven at 40 °C for 60 minutes (until dry). It was made every three days to maintain the quality of the feed.

Feeding Treatment

Shrimp at an average body weight of ± 3.00 g were transferred to the aquarium (72 L), whose population was 0.2 ind/L. The treatment feed was given four times a day at 07.00 a.m., 11.00 a.m., 03.00 p.m., and 07.00 p.m. with a feeding rate (FR) of 3-6%, adjusted to the shrimp's biomass. During this process, no water change was conducted. The water quality was maintained at optimal conditions with temperatures ranging from 29-31 °C, the salinity was at 25 g/L and DO > 4 mg/L.

The treatment feed was given to the test shrimp for a duration of 28 days. At the end of the rearing, the final length and weight of the shrimp were measured to calculate the production performance. After that, the test shrimp were infected with WSSV. The infection of WSSV was performed in accordance with the method by Escobedo-Bonilla *et al.* (2005). Ten

shrimp in each aquarium were infected with WSSV through intramuscular injection on the shrimp's abdominal segment between the second and the third. Virus extract was diluted with phosphate-buffered saline (PBS) to up to 10^{-3} . Then, 200 μ L of the solution was injected. Immune response parameters were observed for 7 days after infection.

Observation Parameters

Production parameters measured in this research were average body weight (ABW), growth rate (GR), specific growth rate (SGR), feed conversion ratio (FCR), and production. These parameters were observed, based on the calculations performed by Ponce-palafox *et al.* (2019). Immune response parameters that were observed included mortality, relative percent survival (RPS), and blood parameters such as total hemocytes count (THC), phenoloxidase (PO) activity, respiratory burst (RB) activity, and hemolymph clotting time. The mortality of the test shrimp was measured every day.

The parameters for THC were observed one day before infection (D-1), day 1 post-infection (D1), day 4 post-infection (D4), and day 7 post-infection (D7). The parameters for PO activity, as well as RB activity, were measured on D-1 and D4, while parameters for hemolymph clotting time were measured on D1. The methods used to measure THC value, phenoloxidase activity, respiratory burst activity and hemolymph clotting time are based on Blaxhall and Daisley (1973), Song and Hsieh (1994), Liu and Chen (2004), and Jussila *et al.*'s (2001) studies, respectively.

Data Analysis

Analyses of variance (ANOVA) were used to determine the differences between treatments. The normality and homogeneity of the variance were determined using Kolmogorov-Smirnov and Levene test, respectively. Statistical analyses were conducted using SPSS statistics version 22 for windows (SPSS

Inc.) at a significance level of 0.05. Significant differences between treatments were determined using a Tukey test.

RESULTS AND DISCUSSION

Immune Response

The administration of *N. sativa* and *P. niruri*, along with seven different combinations of both showed varied resistance to WSSV infection. *N. sativa* (1N), 1/2N:1/2P, and 2/3N:1/3P treatments were able to significantly reduce the mortality of the tested shrimp infected with WSSV. The RPS value of the three treatments reached 71%, which demonstrated a significant difference ($p > 0.05$) compared to the control (+). While *P. niruri* (1P) and other combination treatments showed a tendency of better immunity than shrimp that were not given the herbal treatment, except for the 1/3N:2/3P treatment (Table 2). Similar results were also reported by Sukenda *et al.* (2011) that the survival value of *L. vannamei* treated with *P. niruri* (20 mg/kg of feed for seven days) tended to be better, at 86.7%, although not significantly different from the control at 66.7%.

This positive result is thought to be due to the function of *N. sativa* and *P. niruri* as immunomodulatory. *N. sativa* is able to increase the immune response in humans, terrestrial animals, and aquatic animals (Abd El-Hack *et al.*, 2016; Gholamnezhad *et al.*, 2016; Yousefi *et al.*, 2021), with the main active component named Thymoquinone (2-Isopropyl-5-methylbenzo[1,4-quinone) (Islam *et al.*, 2017). Similar to *N. sativa*, *P. niruri* is able to increase the immune response in aquatic animals (Sukenda *et al.*, 2011; Setiaji *et al.*, 2013; Sunitha *et al.*, 2017; Vibin *et al.*, 2020), and modulate the immune system of both innate and adaptive immune components (Eze *et al.*, 2014). Flavonoid group, which are the main compounds of *P. niruri*, optimizes the cells' work by sending intracellular signals to cell receptors. The other compounds are phyllanthin and

hyphophyllanthin, which possess anti-inflammatory activity so that they can strengthen immunity (Rosidah *et al.*, 2021).

N. sativa and *P. niruri* have also antiviral properties. *N. sativa* as an antiviral agent in humans has been discussed by Abd El-Hack *et al.* (2016) and Islam *et al.* (2017). *N. sativa* therapy can reduce viral load, improve oxidative stress, and clinical conditions in patients infected with the Hepatitis C virus (Barakat *et al.*, 2013). *P. niruri* extract can also reduce viral antigen levels in chronic hepatitis B patients, due to the ability of *P. niruri* to inhibit hepatitis B virus replication. Direkbusarakom *et al.* (1995;1996) and Direkbusarakom (2004) also reported the antiviral efficacy of several species of *Phyllanthus* sp. against several types of viral diseases in fish and shrimp, such as infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), Oncorhynchus masou virus (OMV), WSSV, and yellow-head baculovirus (YHV). *Phyllanthus* sp. can

directly inactivate the virus, block the absorption of the virus into the host cell and inhibit viral replication. However, the effectiveness of these antivirals differs depending on the species of *Phyllanthus* sp. and the type of virus used. Lee *et al.* (2016) stated that phenolic compounds, flavonoids, and alkaloids contained in many herbs have potential as antiviral agents.

The hemolymph of treated shrimp 1N and 2/3N:1/3P also showed a tendency for faster hemolymph clotting time that reached 36-37 seconds or 20% faster than the control (46 seconds) (Table 2). The speed of hemolymph clotting is very important in the immune system. In general, the clotting system of humoral immune response is the first defense line and an integral part of the immune system of invertebrates. This system is also crucial in preventing blood loss during injury or wound recovery. The clotting system also relates to the activation of AMPs that have anti-bacterial properties (Maningas *et al.*, 2013).

Table 2. The value of mortality rate, relative percent survival (RPS) and agglutination of *L. vannamei* that were given different *N. sativa* and *P. niruri* combination treatments.

Treatments	Mortality rate (%)	RPS (%)	hemolymph clotting time (second)
C (-)	7.0 ± 6.0 ^a	71.0 ± 25.0 ^c	46.0 ± 5.0 ^a
C (+)	23.0 ± 6.0 ^{bc}	0.0 ± 25.0 ^{ab}	46.0 ± 5.0 ^a
N	7.0 ± 12.0 ^a	71.0 ± 49.0 ^c	36.0 ± 2.0 ^a
P	13.0 ± 6.0 ^{ab}	43.0 ± 25.0 ^{bc}	43.0 ± 2.0 ^a
1N : 1P	7.0 ± 6.0 ^a	71.0 ± 25.0 ^c	44.0 ± 8.0 ^a
1/3N : 2/3P	30.0 ± 0.0 ^c	-29.0 ± 0.0 ^a	47.0 ± 6.0 ^a
1/4N : 3/4P	20.0 ± 0.0 ^{abc}	14.0 ± 0.0 ^{abc}	46.0 ± 4.0 ^a
2/3N : 1/3P	7.0 ± 6.0 ^a	71.0 ± 25.0 ^c	37.0 ± 18.0 ^a
2/5N : 3/5P	23.0 ± 15.0 ^{bc}	0.0 ± 65.0 ^{ab}	37.0 ± 2.0 ^a
3/4N : 1/4P	17.0 ± 6.0 ^{abc}	29.0 ± 25.0 ^{abc}	43.0 ± 1.0 ^a
3/5N : 2/5P	17.0 ± 6.0 ^{abc}	29.0 ± 25.0 ^{abc}	46.0 ± 7.0 ^a

Tested shrimp that were given treatments of 1N and 2/3N:1/3P (the best treatments), did not show any difference in THC value compared to K (+) at D-1 before infection, except for the 1/2N:1/2P treatment (Figure 1). This result is different from Nur *et al.* (2020) who reported an increase in the THC value in Pacific whiteleg shrimp given a 0.75%

dose of *N. sativa* extract and Vibin *et al.* (2020) who reported an increase in the THC value in Pacific whiteleg shrimp given a 3% dose of *P. niruri* extract. The increase of immune response is not always marked by the increase of hemocytes, but also by the hemocyte cells' ability to cope with any pathogens that entered the body, including those that were achieved by

phagocytosis mechanism, encapsulation and nodule formation, apoptosis, antioxidant system, PO system, melanization, cytokine production, protein clotting (coagulation), etc.

The other combination of *N. sativa* and *P. niruri* has greatly increased the value of THC of the tested shrimp, where the tested shrimp treated with 2/5N:3/5P reached more than 200% compared to K (+) (Figure 1). Interestingly, the high value of THC was not in line with the value of its RPS. The high value of THC is thought to have a backfire effect and become harmful as it responded exaggeratedly when infection occurred. On the other hand, WSSV infection caused a drastic decrease in the value of THC in the shrimp with high THC values. These THC values fluctuated greatly at around 9-11 million cells/mL. In this case, the immunomodulatory activity of *N. sativa* combined with *P. niruri* in tested shrimp was not seen. It is suspected that the combination of *N. sativa* and *P. niruri* in the wrong combination is actually antagonistic, although many studies have also shown the strong immunomodulatory activities in *N. sativa* or *P. niruri* in a single administration. This result is different from that of Dirjomuljono *et al.* (2008), who combined 360 mg *N. sativa* and 50 mg *P. niruri* extracts and proved

significant benefits for the treatment of acute tonsillopharyngitis. The immunomodulatory of these herbals is beneficial to recover from this viral disease.

Phenoloxidase activity and respiratory burst activity are humoral responses that are commonly used in shrimp immune response studies. Activation of the proPO system is the dominant part of a crustacean defense system that plays a role in cell behavior, important functional molecule releases and/or activation, and neutralization of infectious agents (Smith *et al.*, 2003). Respiratory burst activity is the activity of releasing free radicals, that play an important role in the phagocytosis process (Yang *et al.*, 2016). Giving *P. niruri* to shrimp decreased the value of PO activity and RB activity ($p > 0.05$) of the tested shrimp at D-1. In contrast, *N. sativa* treatments did not show any significant difference compared to the control (+). The value of RB after WSSV infection showed similar patterns in all treatments, where it decreased on D+4, with the exception of 3/4N:1/4P treatment (Figure 2). The treatments of 1N, 1/2N:1/2P, and 2/3N:1/3P which resulted in the highest RPS value, showed that PO activity and RB activity values were not significantly different from K (+).

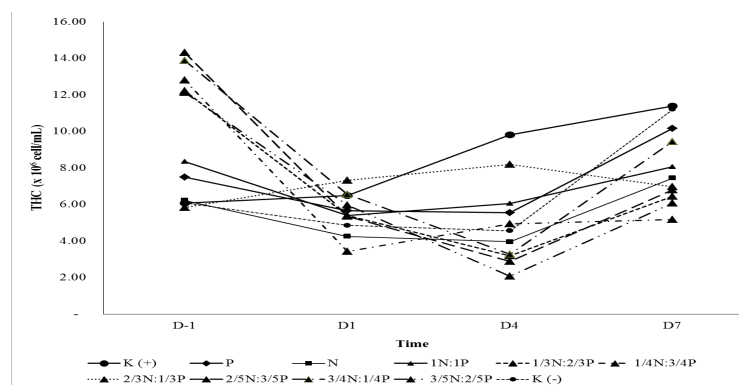


Figure 1. Total hemocyte count of *L. vannamei* that were given different *N. sativa* and *P. niruri* combination treatments on feed for 28 days and then infected by white spot syndrome virus.

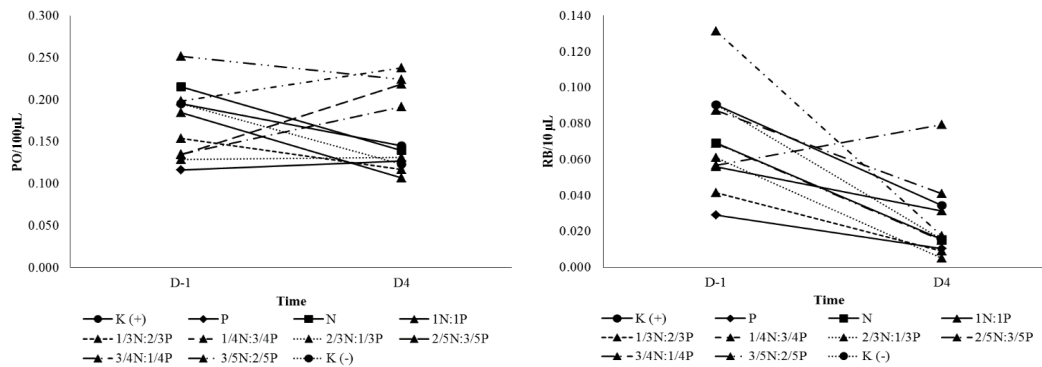


Figure 2. The value of phenoloxidase activity (PO) and respiratory burst activity (RB) of *L. vannamei* that were given different *N. sativa* and *P. niruri* combination treatments on feed for 28 days and then infected by white spot syndrome virus.

Production Performance

The use of *P. niruri*, *N. sativa*, and their combination did not show any significant differences ($p > 0.05$), compared to the control in all growth parameters (Table 3). It means that these treatments do not have a negative impact on the production performance of *L. vannamei*. These results are in line with the research of Niroomand *et al.* (2020) and Sukenda *et al.* (2011) which showed that giving *N. sativa* up to a dose of 3% or giving *P. niruri* to a dose of 2%, did not affect the growth of *L. vannamei*. On the

other hand, improved growth performance of fish fed with *N. sativa* and *P. niruri* was reported by Johri *et al.* (2011), Sunitha *et al.* (2017) and Yousefi *et al.* (2021). Administration of *N. sativa* at 0.5% and 1.0% for 60 days showed an increase in the activity of digestive enzymes in carp such as proteases, lipases, and amylase resulting in better SGR and FCR (Yousefi *et al.* 2021). Giving *P. niruri* to catfish (5%) and to carp (2%) for 60 days, can increase SGR and decrease FCR value (Johri *et al.*, 2011; Sunitha *et al.*, 2017).

Table 3. The value of survival, average body weight (ABW), growth rate (GR), specific growth rate (SGR), feed conversion ratio (FCR), and production of *L. vannamei* that were given different *N. sativa* and *P. niruri* combination treatments on feed for 28 days.

Treatment	Survival (%)	ABW (g)	GR (g/day)	SGR (%/day)	FCR	Production (g/m ²)
C	91.67 ± 6.38 ^a	8.15 ± 0.49 ^a	0.15 ± 0.02 ^a	4.52 ± 0.38 ^a	0.96 ± 0.06 ^a	466 ± 30 ^a
P	93.33 ± 9.42 ^a	8.23 ± 0.81 ^a	0.15 ± 0.03 ^a	4.33 ± 0.47 ^a	1.00 ± 0.08 ^a	479 ± 60 ^a
N	96.67 ± 3.85 ^a	8.43 ± 0.54 ^a	0.16 ± 0.02 ^a	4.63 ± 0.30 ^a	0.88 ± 0.02 ^a	508 ± 15 ^a
1N:1P	91.67 ± 6.38 ^a	8.15 ± 0.58 ^a	0.15 ± 0.02 ^a	4.53 ± 0.46 ^a	0.97 ± 0.05 ^a	465 ± 12 ^a
1/3N:2/3P	88.33 ± 3.33 ^a	8.81 ± 0.32 ^a	0.17 ± 0.01 ^a	4.81 ± 0.14 ^a	0.97 ± 0.05 ^a	486 ± 23 ^a
1/4N:3/4P	96.67 ± 3.85 ^a	8.13 ± 0.47 ^a	0.15 ± 0.02 ^a	4.44 ± 0.48 ^a	0.91 ± 0.05 ^a	491 ± 27 ^a
2/3N:1/3P	93.33 ± 0.00 ^a	8.43 ± 0.42 ^a	0.16 ± 0.01 ^a	4.63 ± 0.26 ^a	0.92 ± 0.01 ^a	492 ± 27 ^a
2/3N:3/5P	93.33 ± 7.70 ^a	8.49 ± 0.18 ^a	0.16 ± 0.01 ^a	4.68 ± 0.22 ^a	0.92 ± 0.10 ^a	495 ± 44 ^a
3/4N:1/4P	88.33 ± 6.38 ^a	8.89 ± 0.21 ^a	0.18 ± 0.01 ^a	4.90 ± 0.30 ^a	0.94 ± 0.08 ^a	491 ± 39 ^a
3/5N:2/5P	88.33 ± 3.33 ^a	8.76 ± 0.46 ^a	0.17 ± 0.02 ^a	4.80 ± 0.37 ^a	0.96 ± 0.05 ^a	483 ± 12 ^a

CONCLUSION

The application of *N. sativa* through the feed for 28 days, was able to increase the body resistance and reduce the mortality of *L. vannamei* infected with WSSV, with an RPS value of 71%. *N. sativa*

can be combined with *P. niruri* in the right composition (1/2N:1/2P and 2/3N:1/3P), although its effectiveness is not better than the application of *N. sativa* alone, while the other combinations are antagonistic. The administration of *N. sativa* alone (1N)

or in combination with *P. niruri* (1/2N:1/2P and 2/3N:1/3P), which are the best treatments in this study, relatively did not affect the value of total hemocyte count, phenoloxidase activity, respiratory burst activity, hemolymph clotting time and production performance of test shrimp.

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