# Screening the antiviral activity of potent "antiviral-immunostimulant herbal extract"* against white spot syndrome virus (WSSV) infection in Penaeid shrimps.

**"Antiviral Herbal Extract" Nomex® contains extract of two different endemic herbs that extracted by patent pending special extraction processes.*

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## Abstract

The sustainability and development of shrimp farming are largely at stake as significant ecological and pathological problems are increasing in the vast majority of the shrimp producing countries. Massive mortalities occur in Penaeid shrimp farms. Control of diseases is the priority for the durability of shrimp industry. The major gross findings of the naturally occurring cases were 2 to 6 mm sized white spots on the inside of the carapace and reddish discoloration. In shrimp market and industry, shrimp immunology is a key element in establishing strategies for the control of diseases in shrimp aquaculture.

Commercial herbal antiviral product Nomex® (developed by Soley Biotechnology Institute against antiviral treatment of marine and freshwater animals) was selected for this study.

Nomex® contains 2 different herbal extract that extracted by special extraction techniques. Nomex® was tested for its activity against WSSV in marine shrimp *P.vannemei*. Its infection prevention and treatment activity were examined by adding 2gr and 10gr to each kg of regular shrimp feed.

Oral administration 2gr/kg concentration Nomex® prevented healthy shrimps in infected tank as prophylactic. Using 10gr/kg concentration cured all infected shrimps. This study also showed that 2gr/kg (adding 2gr Nomex® to each kg of shrimp feed) is continuously preventing WWSV infections, also showed that in this study period, any other infections were not occurred in experiment tank. This study revealed that the application of herbal immunostimulants will be effective against shrimp viral pathogenesis and they can be recommended for shrimp culture.

## INTRODUCTION

The rapid increase in cultured shrimp production was achieved by geographical expansion and technological advancements in reproduction in captivity, larval rearing, artificial diet and intensification in grow-out systems. Shrimp grow-out systems around the world are very various. They are generally divided into extensive, semi-intensive, intensive and ultra-intensive depending on characteristics including production per unit, stocking density, type and amount of feed used, percentage of water exchange, aeration, pond size, pond shape and water depth.

White spot syndrome (WSS) is a severe infection in shrimps, caused by WSSV and causes big losses in commercial shrimp production. The virus is the member of the family *Nimaviridae*, genus *Whispovirus.*

The mortality rate of infected shrimp can reach 100% within 3–10 days after infection. The most important disease affecting shrimp farming worldwide is White Spot Disease (WSD) caused by the widespread, extremely contagious and highly lethal White Spot Syndrome Virus (WSSV). Shrimp showing clinical signs of WSD have an 80-100% mortality rate and outbreaks of disease have destroyed entire populations of shrimp farms throughout the world in just a few days. Natural outbreaks of WSSV are categorized into peracute, acute to subacute and chronic forms, where mortality occurs within 2-3 days, 7-10 days and 15-28 days, respectively. In laboratory challenge tests, WSSV as sole pathogen may cause disease and mortality in SPF *P. vannamei* and other shrimp and

crayfish species. In case of natural infection, several biotic and abiotic factors may influence the course of a WSS outbreak. Co-infections of different viruses including HPV, MBV, IHHNV together with WSSV have been reported. In Europe, WSSV is currently listed as a non-exotic pathogen which prevents the use of uncooked tiger prawns as bait. As example, the losses in India alone have been estimated at several million dollars per year. Diseased shrimp often appear to have a red discoloration and white spots beneath their cuticle. They will stop feeding, become very lethargic and often gather around the edges of their ponds. The virus specifically attacks the cells of the gills, antennal gland, haematopoeitic tissue, nervous tissue, connective tissue, and intestinal epithelial tissue. The infected tissues of these organs eventually die and disintegrate. There were no available treatments for WSD. It is important to recognize that traditional vaccination remedies are not a viable option because vaccines work on the premise that the animal has an adaptive immune system which can produce antibodies to the vaccine. However, shrimp, like all other arthropods, do not have an adaptive immune system and cannot produce antibodies. Antiviral research using plant extracts has gained momentum since 1950. Scores of medicinal herbs have already been tested and used with good results in the control of viral and bacterial diseases in shrimp and fish. WSSV is an enveloped, double stranded DNA virus, ovoid to bacilliform in shape with a tail like extension at one end. Immunostimulants are the substances, which enhance the non-specific defense mechanism and provide resistance against pathogenic organism. Perusal of the literatures indicated that immunostimulants are proven very successfully in treating/preventing microbial diseases in cultured shellfishes. The prevention and control of shrimp diseases appear as an integrated approach in which knowledge about immunity has to be improved, considering other research areas related not only to pathology, shrimp physiology and genetics, but also to nutrition, environment and eco-toxicology. Many herbals have been used for millennia as home remedies and some of these have potent anti-viral properties. Among them, a few have been found to have anti-viral activity against fish viruses in tissue culture and some have been investigated for their ability against shrimp viruses.
Natural plant products have been reported to have various activities like anti-stress, growth promoters, appetizer, tonic, immunostimulants and antimicrobials. Moreover, the substances are obtained from natural sources, besides possessing other interesting properties like non-toxic, biodegradable and biocompatible.
*Penaeus vannamei* is the most important shrimp species in terms of aquaculture production. The other important species are *P. monodon*, *P. chinensis*, *P. merguiensis*, *P. japonicus* and *P. indicus*. *P. vannamei* is naturally present along the pacific coast of Central and South America and Asia.

**Materials and methods:**

**Shrimps:**
400 live healthy *Penaeus vannamei* shrimps were collected from 2 different non-infected shrimp tanks by visually investigation. 400 of them were collected from treated (using probiotics) tanks and 200 of them were Specific Pathogen Free (SPF) according to seed hatchery as seed seller. All shrimps were carried to a new tank (10m3) with the same water conditions with their source tanks. 10000 Liters fiberglass tanks with air-lift biological filters at room temperature (24–28 °C) with salinity 22ppt. The animals were fed with artificial pellet feed. Body weights of shrimps were 14-22gr/each.

**Preparation of viral inoculums:**
WSSV infected shrimps were collected from shrimp farm. The haemolymph samples were drawn from the infected shrimps using sterile syringes followed by centrifugation (3000g for 24 min at 4 °C). The supernatant fluid was then centrifuged again (8000g for 40 min at 4 °C) and the final supernatant fluid was filtered through a 0.4 µm filter. The filtrate was then stored at -20 °C for infectivity study. Inoculums were duplicated in growth media.

**Experiment groups:**
***WSSD Experiment Group (Group A)****:* Total 150 shrimps (75 infected regular + 75 infected SPF shrimps). 2000 Liters fibreglass tanks with air-lift biological filters at room temperature (24–28 °C) with salinity 22ppt.
***WSSD Prevention Group (Group B)****:* Total 150 shrimps (50 infected regular + 50 infected SPF + 25 healthy regular + 25 healthy SPF shrimps). 2000 Liters fiberglass tanks with air-lift biological filters at room temperature (24–28 °C) with salinity 22ppt.
***Control Group (Group C)****:* Total 100 shrimps (25 infected regular + 25 infected SPF shrimps + 25

healthy regular + 25 healthy SPF). 2000 Liters fiberglass tanks with air-lift biological filters at room temperature (24–28 °C) with salinity 22ppt.

**Detection:**
Molecular methods include several protocols of polymerase chain reaction (PCR), *in situ* hybridization and dot blot hybridization.
PCR methods are based on primers designed against a specific part of the genome sequence of WSSV. PCR methods include one step PCR, semi nested PCR, two step PCR, quantitative competitive PCR and real time PCR. One step PCR detects WSSV in shrimp containing a substantial concentration of viral DNA which is usually the case in animals displaying gross signs of disease. In this study, one step PCR was used for detection of WSSV.

**WSSV inoculation procedure:**
Shrimp were inoculated intramuscularly with 50 µl of inoculums containing WSSV.

**Diet:**

Nomex® + Diet Composition (% w/w) for WSS prevention

| | |
|---|---|
| *Spirulina platensis* | 25.0 |
| Fish meal | 22.0 |
| Wheat flour | 12.5 |
| Krill | 12.0 |
| Fish oil | 10.5 |
| Squid meal | 5.0 |
| Mineral mix | 5.0 |
| Liquid binder | 4.0 |
| Vitamin mix | 2.0 |
| Nomex® | 2.0 |

Nomex® + Diet Composition (% w/w) for WSS cure

| | |
|---|---|
| *Spirulina platensis* | 15.0 |
| Fish meal | 22.0 |
| Wheat flour | 12.5 |
| Krill | 12.0 |
| Fish oil | 10.5 |
| Squid meal | 5.0 |
| Mineral mix | 5.0 |
| Liquid binder | 4.0 |
| Vitamin mix | 2.0 |
| Nomex® | 10.0 |

Diet Composition (% w/w) for Control Group

| | |
|---|---|
| *Spirulina platensis* | 25.0 |
| Fish meal | 24.0 |
| Wheat flour | 12.5 |
| Krill | 12.0 |
| Fish oil | 10.5 |
| Squid meal | 5.0 |
| Mineral mix | 5.0 |
| Liquid binder | 4.0 |
| Vitamin mix | 2.0 |

All shrimps were fed by a normal diet (Diet Composition for Control Group) at 5% MBW per day for four days before challenge with WSSV 10000 Liters fiberglass tanks with air-lift biological filters at room temperature (24–28 °C) with salinity 22 ppt.

After WSSV injection and group separation, more four days, they were fed by normal diet at 5% MBW per day too.

For '***WSSD Experiment Group – Group A***' (infected by WSSV and using Nomex® cure), 150 shrimps (75 infected regular + 75 infected SPF shrimps) were fed by Nomex® Diet Composition for WSS cure at 5% MBW per day.

For '***WSSD Prevention Group – Group B***', 150 shrimps (50 infected regular + 50 infected SPF + 25 healthy regular + 25 healthy SPF shrimps) were fed by Nomex® Diet Composition for WSS prevention at 5% MBW per day.

For '***Control Group – Group C***', 100 shrimps (25 infected regular + 25 infected SPF shrimps + 25 healthy regular + 25 healthy SPF) were fed by a normal diet (Diet Composition for Control Group) at 5% MBW per day.

**Experiment Period:**
All shrimps were fed by a normal diet (Diet Composition for Control Group) at 5% MBW per day for four days before challenge with WSSV in 10000 Liters fiberglass tanks with air-lift biological filters at room temperature (24–28 °C) with salinity 22 ppt.
After four days, shrimps were moved to their groups in different 2000 Liters fiberglass tanks.
Total experiment period was 28 days.

**Results:**
Total 300 (150 regular and 150 SPF shrimps) shrimps were infected by intramuscular WSSV inoculation. Total 100 (50 regular and 50 SPF shrimps) shrimps were healthy and used for WSSV contamination risks in infected tanks.

After four days from WSSV injection, WSSV presence was measured by one step PCR.

This research shows that Nomex® exhibits potent antiviral activity on shrimps.

| **WSSV injected shrimps** | **PCR +** | **Visual detection of White Spots** |
|---|---|---|
| 150 regular shrimp | 175 | 172 |
| 150 spf shrimp | 175 | 168 |

At Group A, there were WSSV infected 150 shrimps (75 infected regular + 75 infected SPF shrimps). Within 28 days and by Nomex Diet Composition for WSS cure, experiment showed no mortality and no signs of WSS.

At Group B, there were WSSV infected 100 shrimps (50 infected regular + 50 infected SPF shrimps) and 50 healthy shrimps (25 regular + 25 SPF shrimps). Within 28 days and by Nomex Diet Composition for WSS prevention, experiment showed only 5.3% mortality.

At Group C, there were 100 shrimps (25 healthy regular + 25 healthy SPF shrimps + 25 infected regular + 25 SPF infected shrimps). Within 28 days and by a normal diet (Diet Composition for Control Group), experiment showed only 100% mortality.

| Day | Group A (Mortality) | Group B (Mortality) | Group C (Mortality) |
|---|---|---|---|
| 1 | 0 | 0 | 0 |
| 2 | 0 | 0 | 0 |
| 3 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 |
| 5 | 0 | 0 | 2 |
| 6 | 0 | 1 | 1 |
| 7 | 0 | 0 | 4 |
| 8 | 0 | 0 | 7 |
| 9 | 0 | 1 | 1 |
| 10 | 0 | 2 | 3 |
| 11 | 0 | 0 | 8 |
| 12 | 0 | 1 | 6 |
| 13 | 0 | 0 | 8 |
| 14 | 0 | 0 | 8 |
| 15 | 0 | 1 | 6 |
| 16 | 0 | 0 | 7 |
| 17 | 0 | 0 | 4 |
| 18 | 0 | 0 | 4 |
| 19 | 0 | 0 | 3 |
| 20 | 0 | 0 | 8 |
| 21 | 0 | 1 | 9 |
| 22 | 0 | 1 | 4 |
| 23 | 0 | 0 | 6 |
| 24 | 0 | 0 | 1 |
| 25 | 0 | 0 | - |
| 26 | 0 | 0 | - |
| 27 | 0 | 0 | - |
| 28 | 0 | 0 | - |
| **Total Mortality @28$^{th}$ day.** | **0 (0%)** | **8 (5.3%)** | **100 (100%)** |

Also, this study showed that SPF shrimps can be infected by WSSV.

These results suggest that three possible mechanisms may explain the antiviral activity of Numex®. The first; viral inactivation might occur by the reaction between Numex® and the envelop proteins of the virus, which prevent the entry of the virus into the host, the second; influence of Numex® on the replication of virus, which prevents the multiplication of the virus in the host cell and the third; Numex® might be the immunostimulant which enhances the innate immunity like prophenoloxidase, superoxide anion, nitric oxide of shrimp against WSSV and an antioxidant property of Numex® protects the cells from the free radicals, which raised as the result of WSSV infection.

About $200 was required for the processing of 10 kg of Nomex® to obtained 10% of Nomex® addition, which will effectively increase the shrimp production.

Immunological approaches to prevent fish and shrimp diseases have normally involved antibiotics, chemicals or vaccination against specific pathogens, while the use of immunostimulants is strongly a new and developing area.

At starting of this study, average body weight of shrimps was 18gr. At the end of this study, average body weight of remaining shrimps was 24gr.

Oral administration 2gr/kg concentration Nomex® prevented healthy shrimps in infected tank as prophylactic. Using 10gr/kg concentration cured all infected shrimps. This study also showed that 2gr/kg (adding 2gr Nomex® to each kg of shrimp feed) is continuously preventing WWSV infections, also showed that in this study period, any other infections were not occurred in experiment tank. This study revealed that the application of herbal immunostimulants will be effective against shrimp viral pathogenesis and they can be recommended for shrimp culture.

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**References:**

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