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Influence of Selected Antiviral Herbal Active Principles against Shrimp White Spot Syndrome Virus (WSSV)

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Abstract

Antiviral herbals such as *Agati grandiflora, Justicia tranquebariensis* and *Eclipta erecta* were selected and extracted using polar, mid polar and non polar extracts of hexane, ethyl acetate and methanol. Among the *in vitro* antiviral assay against WSSV, the methanolic herbal extracts effectively suppressed the WSSV replication. Further the combination (1:1:1) of the three methanolic herbal extracts were incorporated in artificial feed and made the diet such as 100, 200, 400 and 800 mg. kg^{-1}. These diets were fed to the shrimp *Penaeus monodon* weighed of 8 ± 0.5 g for 60 days. After a 30 days interval, they were challenged with WSSV and studied the hematological, biochemical and immunological changes. Among the different diets fed, *P. monodon* the 400 and 800 mg. kg^{-1} diet were highly resistance against the WSSV infection, improved hematological and immunological parameters. The alternative herbal treatments were highly applicable against the WSSV infection in the Shrimp aquaculture industry.

Keywords: WSSV; Herbals; Alternative medicines; Antiviral

INTRODUCTION

White spot syndrome virus (WSSV) is an economically significant shrimp disease, which causes high mortalities and severe damages to shrimp culture. In cultured shrimp WSSV infection can cause a cumulative mortality of up to 100% with in 3 to 10 days. The major targets for WSSV infection are tissues of ecto and mesodermal origin, such as the gills, lymphoid organ and cuticular epithelium. Replication initiates in the nucleus, where virions are assembled. The virions are released from infected

cell by cell rupture. Infected animals show gross signs of lethargy, such as lack of appetite and slow movement. Characteristic for infected shrimps are the white spots on the exoskeleton (Vlak *et al*., 2005).

Several products have been experimentally tested for the control of viral diseases on shrimp due to their potential to stimulate the invertebrate non specific immune system. Strategies for prophylaxis and control of WSSV theoretically include improvement of environmental conditions, stocking of specific pathogen free shrimp post larvae and enhancement of disease resistance by using immunostimulants. Immunostimulants are the substances, which enhances the non specific defense mechanism and provide resistance against pathogenic organism. Perusal of the literatures indicated the immunostimulants are proven very successfully in treating /preventing microbial diseases in culture shrimp fishes. (Citarasu *et al*., 2006)

Application of antibiotics and other chemicals in aquaculture has is own intricate problems. For example, regular use of antibiotics in fish and shrimp hatcheries or grow out system may lead to development not only antibiotic resistant fish/shrimp bacteria, but also human disease causing bacteria.Information is still lacking on the absorption and distribution of antibiotics in fish and shrimp persistence of residues or effect of them in the environment.Hence, promoting a holistic systems approach to fish /shell fish health management needs specific attention. Citarasu *et al*. (2002) described the positive effect of the herbal active principles such as antibacterial, antiviral, immunostimulant and antistress effect in shrimp aquaculture.

Historically plants have provided a source of inspiration for novel drug compounds as plant derived medicines have made large contribution to human health and well being. Their role is two fold in the development of new drugs. (1) they may became the base for the development of a medicine, a natural blue print for the development of new drugs or (2) a phytomedicines to be used for the treatment of disease (Iwu *et al.*, 1999). Many herbs have been used for millennia as home remedies and some of these have potent anti-viral properties. A few have been found to have anti-viral activity against fish viruses in tissue in culture (Direkbusarakom, 1996). Most of these plants and plant extracts do not act by non-specifically stimulating specifically the immune system of the shrimp (Direkbusarakom, 1996).The present focus on the influence of herbal extract having antiviral and immunostimulant properties against WSSV in *Penaeus monodon.*

MATERIALS AND METHODS

Three herbs having the antiviral and immunostimulant characteristics such as *Agati grandiflora, Justicia tranquebariensis* and *Eclipta erecta*, were selected following Nadkarni. The details of the antiviral herbs are given in the Table 1. The dried powders were extracted with the above mentioned solvents by percolation extraction. The extracts were filtered, centrifuged and concentrated in rotatory evaporator under reduced pressure at the temperature of 45^0C to 50^0C. Aqueous extract was concentrated using lyophilizer and stored 4^0C. 500 mg of condensed plant extracts were dissolved in 100 ml of NTE buffer (0.2 N NaC1, 0.02 M Tris-HCL and 0.02 M EDTA, pH 7.4) as stock for bioassay studies. 5 µ1 of viral suspension (300 µg of total

protein) were mixed with 10 µ1 of plant extract and incubated at 29^0C for 3 h. After 3 h, the mixture was injected intramuscularly into *P. monodon*, weighed 8.0 ± 0.5 g. Mortalities were recorded for each day and the experiment was carried out up to 10 days. Control and experiment all groups were also maintained as for the mixture of 25 µ1 NTE buffer and 5 µ1 viral suspensions. Based on the initial antiviral screening, herbal extracts were purified through silica column chromatography (60-120µm mesh size). Approximately 2 gm of Plant extract was loaded as dried slurry of the top of silica gel column and eluted with the different combinations of non-polar and polar solvents. Elution was collected, condensed and concentrated and stored 4^0C.

Table 1. Detailed description of the herbs having Antiviral and immunostimulant properties

Sl. No	Botanical name	Family	Distribution	Useful parts	Active principles	Biological Effect
1	*Agati grandiflora*	Lagumin asae	South or west India in the Ganges vally and in Bengal	Bark leaves, flowers, gum, root, bark & fruits	Tannin	Antiviral, Antidiarrhoea
2	*Justica tranquebariensis*	Acanthaceae	India	Leaves	Legnin	Antiseptic, anitiviral, antidiabetic
3	*Eclipta erecta*	Astraceae	Throughout India mainly in Himalya	Leaves, Flower	Ecliptine	Antiparasitics, Antiviral

The fractions were incubated with WSSV again and the mixture was injected intramuscularly into *P. monodon* and mortalities were recorded until 7 days. Herbal extract supplemented diets were prepared using equal concentrations of the active fractions of the all herbal methanolic extracts. Ingredients and formulation of the basal ration were done followed by Boonyaratpalin. The basal diet contained 45.1% protein; 7.2% lipid; 14.6% ash; 7.1% moisture and 3% fibre. Four test diets were prepared at the concentration of 100, 200, 400 and 800 mg/kg. A control diet, devoid of herbal active principle was also prepared.

Healthy shrimps, *P. monodon* weighing approximately 8.0 ± 0.5 g were purchased from a local shrimp farm at Manakudy, Tamilnadu, India. They were stocked in a fibre glass tank (5000 l capacity) in the laboratory. The shrimps were acclimatized to ambient laboratory condition. The culture water was first chlorinated with 25 ppm of sodium hypochlorite and de chlorinated by vigorous aeration. Before starting the experiment, the shrimps were randomly selected and screened by PCR for WSSV infection using the primer having the base pair of 643. Uniform size of *P. monodon* were selected from the stock and transferred in individual experimental fibre glass

tanks (1000 l capacity) of four experimental groups and a control group in triplicate (n = 50 X 3 = 150) with continuous flow-through water and constant aeration system. The shrimps were fed thrice a day at 8.00, 13.00 and 18.00 h at 10% of the body weight. Uneaten food and waste matters were removed before feeding. The water quality parameters such as temperature (27 ± 1.0 ^{0}C), salinity (28 ± 1.5‰), and pH (8.2± 0.1) were maintained every day.

After termination of the experiment, 50 shrimps from each dietary group were injected intramuscularly with WSSV filtrate which is prepared from infected shrimps (300 µmg of total protein per animal) in the second abdominal segment. The blank control was injected with 0.1 ml of Phosphate buffered saline. Survival was monitored until 7 days and DNA was extracted and PCR amplification was carried out followed the method described by Chang et al.. Haemolymph samples of experimental and control shrimps were tested by first step PCR. The negative samples detected in the first step were further subjected for second step PCR analysis.

The haematological parameters such as coagulation time of the haemolymph, total haemocyte count (THC) and oxyhaemocyanin were calculated after challenging the experiment. The coagulation time of the haemolymph was determined by capillary method. Total haemocyte count (cells ml^{-1}) was performed using Burker haemocytometer. The concentration of oxyhaemocyanin was calculated following the method of Hagerman. Phenol oxidase activity in haemolymh samples was determined using L-dihydroxyphenylanine (L-DOPA) as a substrate (Soderhall, 1983).

All data obtained from experiments were analysed using one way ANOVA ($P < 0.05$ as significant level) in Statistica 6.0 computer package (Statsoft, UK). Means were also compared using SNK test

Results

The percentage survival of the *P. monodon* was given in the Table 2, after the viral suspension incubated herbal extracts injection. Among the different solvent extractions, methanol extract was very effectively suppressed the WSSV and replication. There is no mortality was observed in this treatment after 7 days. There are 50 to 60 % of the survivals observed in the hexane and ethyl acetate extract treatments respectively. The cumulative mortality of the control groups and different concentrations of the herbal extracts incorporated diets fed shrimp; *P. monodon* is given in Figure 1. Only 20 % CMI was observed in the blank control with in 20 days. This was significantly increased ($P < 0.05$) to 100 % in the control groups. The survival of shrimps was increased significantly ($P < 0.05$) when they fed on increasing concentrations of immunostimulants. Within 10 days of challenging experiment with WSSV, the control group of shrimp fed on diet devoid of immunostimulant succumbed to death (100%) within 5 days. One way ANOVA revealed that variation in the survival of *P. monodon* fed with control and immunostimulant supplemented diets was statistically significant ($P < 0.05$).

First and second step PCR diagnosis for WSSV in control all herbal diets fed *P. monodon* were given in Figure 2. The results revealed that, there is cent percent PCR positive in the shrimps fed without herbal extracts incorporated diets. The signal was

significantly decreased (P< 0.05) when increasing concentrations of herbal extracts. The herbal extracts having the antiviral and immunostimulant properties suppressed the WSSV replication. The overall percentage of PCR detections are 80, 55, 20 and 25 % in the 100, 200, 400 and 800 mg/ Kg diets fed *P. monodon* respectively.

The haemolymph coagulated in 164 seconds when no immunostimlants was given in the diet. The time for coagulation decreased significantly (P<0.01) to 120, 114, 104, and 91 seconds in the 100 to 800 mg/Kg diets fed groups respectively. The decreased time for coagulation is responsible for the decreased viral load in the haemolymph. The Total Haemocyte Count (THC) of 23.5X 10^6 cells/ml were observed in the control group. The THC was significantly (P<0.01) increased to 30, 37, 42 and 45 X 10^6 cells/ml in 100 to 800 mg/Kg diets fed groups respectively. The lowest oxyhaemocyanin level, (0.82 (mmol l^{-1})) was observed in the control diets fed *P. monodon*. The level of oxyhaemocyanin significantly (P<0.01) increased to 0.9, 0.91, 1.11 and 1.0 (mmol l^{-1}) in the 100 to 800 mg/Kg diets fed groups respectively (Table 3).

The prophenol oxidase activity (pro PO) value observed was higher in the herbal immunostimulants incorporated diets fed groups than the control group in different days of challenging. The less value was observed (0.157) in the control group within 5 days. The value was significantly (P<0.01) increased to 0.28, 0.712, 0.83 and 1.12 in 100, 200, 400 and 800 mg/Kg diets fed groups respectively after 10 days (Figure 3).

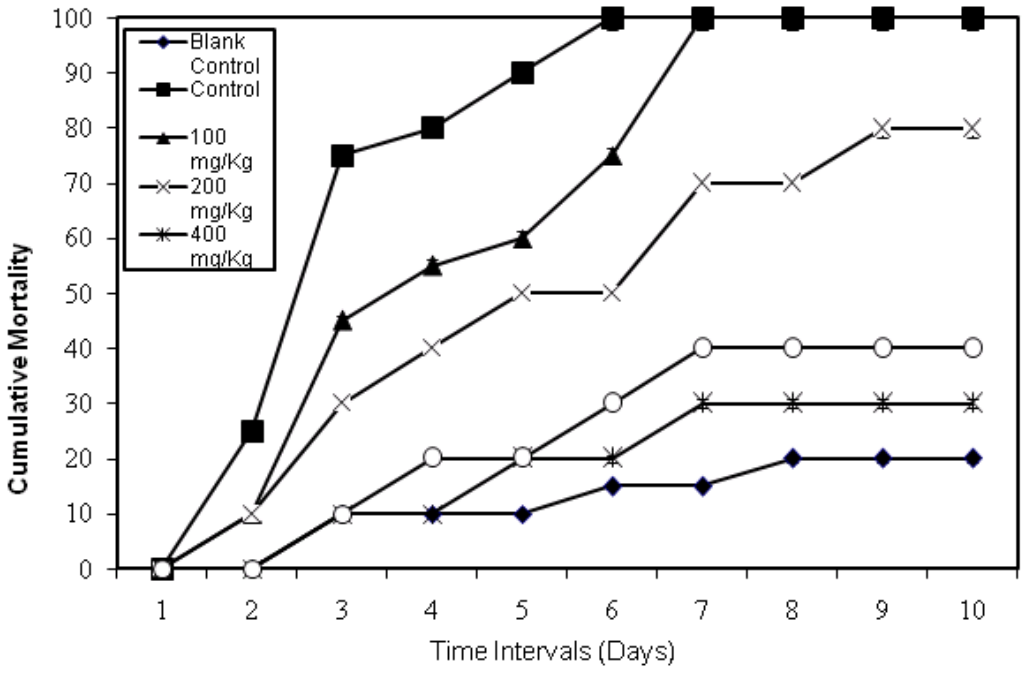
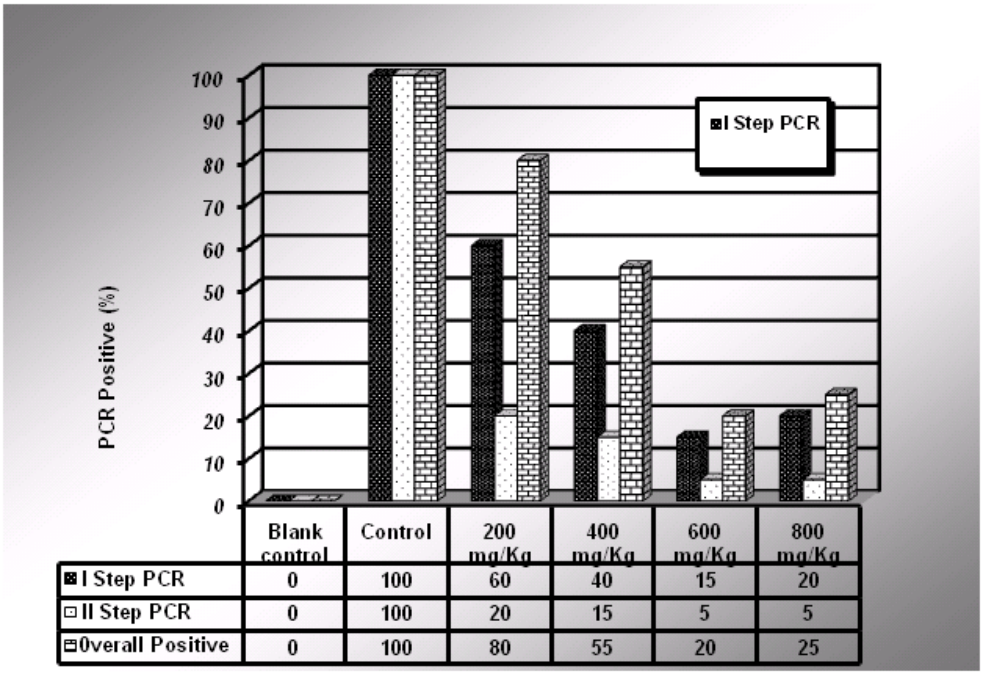


Fig. 1. Cumulative mortaliy of P. monodon fed on herbal immunostimulant diets after challenged with WSSV



	Blank control	Control	200 mg/Kg	400 mg/Kg	600 mg/Kg	800 mg/Kg
I Step PCR	0	100	60	40	15	20
II Step PCR	0	100	20	15	5	5
0verall Positive	0	100	80	55	20	25

Figure 2. Percentage PCR detection of haemolymph samples of *Penaeus monodon* fed herbal immunostimulant diet after challenged with WSSV

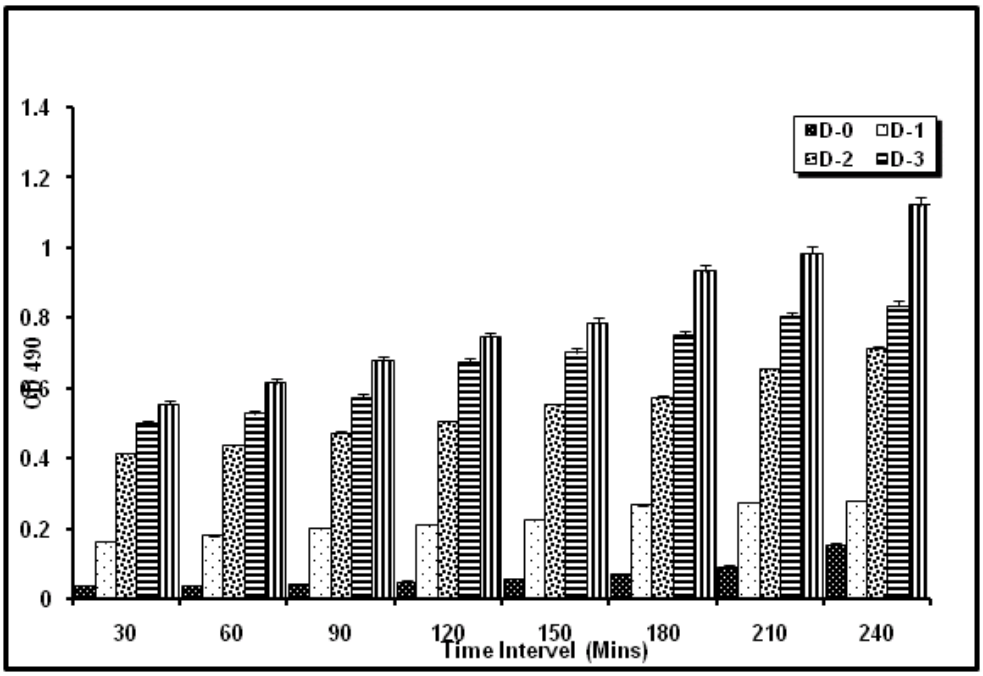


Fig. 3. Pro Phenoloxidase activity (ProPO) of haemocytes of *P. monodon* fed on herbal immunostimulants incorporated diets and control diet after challenged with WSSV

Table 2. Percentage survival of *P. monodon* treated with WSSV incubated with herbal extracts after 7 days

Sl. No	Antiviral Herbs	Survival (%) of different organic solvent Extraction		
		Hexane	Ethyl acetate	Methanol
1	*Agati Grandiflora*	60	75	100
2	*Justica tranquebariensis*	75	75	100
3	*Ecliptaerecta*	50	75	100

Table 3. Haematological changes in the haemolymph of *P. monodon* fed on herbal antiviral/ immunostimulants diets and control diet after challenge with WSSV

Treatments	Haematological Changes		
	Coagulase activity (Sec)	Total Haemocyte Count ($X10^6$ cells ml^{-1})	Oxyhaemocyanin (mmol l^{-1})
D-0	164^a ± 6. 01	$23. 33^a$ ± 1. 24	$0. 82^a$ ± 0. 01
D-1	$120. 66^b$ ± 0. 94	$30. 66^b$ ± 0. 94	$0. 9^a$ ± 0. 002
D-2	$114. 66^b$ ± 3. 68	$37. 66^c$ ± 1. 24	$0. 91^a$ ± 0. 02
D-3	$104. 66^c$ ± 7. 31	$42. 00^d$ ± 0. 81	$1. 11^b$ ± 0. 09
D-4	$91. 00^d$ ± 5. 88	$45. 33^d$ ± 1. 24	$1. 00^b$ ± 0. 47

Means with the same superscripts (a-d) do not differ from each other ($P < 0. 01$).

DISCUSSION

The development of viral resistance towards anti viral agents enhances the need for new effective compounds against viral infections. Medicinal plants have a variety of chemical constituents, which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses. Plants have been rich sources of medicine because they produce a variety of bio active molecules most which are probably evolved a chemical defense against prediction or infection. Medicinal plants contain active constituent like terpenes, alkaloids, steroids, saponins, tannins, phenols, quinines and flavonoids (Leven et al., 1979 ; Harborne, 1982; Bever, 1986).

The present study, the extracts such as *Justicia transquebariensis*, *Eclipta erecta* and *Agathi grandioflora* were effectively suppress the WSSV in the *in vivo* systems of the tiger shrimp, *Penaeus monodon* the viral suspension incubated *E. erecta* herbal extract cent percent suppress the WSSV and no mortality observed. The methanol extract of *Justicia transquebariensis* leaves was tested for antiviral activity against various fish pathogenic viruses namely, Infectious Haematopoietic Necrosis Virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV) and *Oncorhynchus masou* virus (OMV) (Direkbusarakom *et al*., 1993).

The present study different concentrations of the herbal extracts incorporated diets were highly influence on the *P. monodon* against WSSV infection. The diets helps to decreased cumulative mortality, i.e. increase resistance against, improved haematological parameters such as coagulase activity, Total haemocyte count and oxyhaemocyanin level. PCR molecular diagnosis of different herbal extracts incorporated diets fed groups had less signal. The immunological parameters such as PO and intracellular super oxide anion activity are highly improved in the herbal diet fed groups. Citarasu et al (2002) fed with the antiviral and immunostimulant herbal extracts incorporated diets to the WSSV infected Shrimp, *P. monodon* juvenile and the impact of the herbals were accessed with PCR diagnosis. The plants like *Cyanodon dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa* and *Eclipta alba* were effectively controlled the WSSV, *in vivo* system. Also Yogeeswaran(2007), antiviral and immunostimulant characteristics such as *Acalypha indica, Cynodon dactylon, P. Kurooa, Withania somnifera* and *Zingiber officinalis* were extracted with polar and non-polar solvents and screened against WSSV by incubating with WSSV infected haemolymph of shrimp and injected to the shrimp. The screening results revealed that, the methanolic extracts of all herbs were very effective against the WSSV.

Due to the active principle nature such as alkaloids, flavanoids, pigments, phenolics, terpenoids, starch, steroids and essential oils of the herbs, they act as good antimicrobiological agents. The herbal active compounds may inhibit or block the transcription of the virus to reduce the replication in the host cell. This practice will reduce the side effects of applying the synthetic compounds, less cost and eco-friendly. Hence, the alternative herbal biomedicines prove to be very effective in the aquaculture operations.

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