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PHYTOCHEMICAL SCREENING AND IMMUNOSTIMULANT EFFICIENCY OF SELECTED MEDICINAL PLANT EXTRACTS AGAINST WHITE SPOT SYNDROME VIRUS

D. Beula Shiny*, J. Vijila Jasmin, S. Sujithra, M. Jenifer Tamizharasi and T. Kumaran

Muslim Arts College, Affiliated to Manonmaniam Sundaranar University, Thiruvithancode, Kanyakumari 629174 (Tamilnadu), India.

Abstract

Diseases are a crucial factor which inhibits the expansion of aquaculture. Various chemotherapeutants have been used for treatment or prevention of disease. White spot syndrome virus (WSSV) is one of the most important shrimp viruses. It caused massive production losses of cultured shrimp in world-wide. Current treatment protocols are inefficient and cause environmental hazards. To develop alternative practices, attention should be diverted to find novel drugs, especially from plant sources. Many herbal compounds have been found to have non-specific immune stimulating properties in fish and shrimp specifically against WSSV. The present work investigates the screening of immunostimulant plants having the immunostimulant activity against the WSSV and *in vivo* delivery of the plant active extracts in the Indian white shrimp *Fennerropenaeus indicus* against WSSV infection.

Key words : Plants, Phytochemical, *Fenerropenaeus indicus,* Immunostimulant, WSSV.

Introduction

Aquaculture has become a popular food producing sub-sector complement to agriculture (Castaudella and Crusethi, 1993) and has become the fastest growing food producing industry and grown more than five fold as faster than global population (Csavas, 1994). Aquaculture industry, especially in mariculture, shrimp farming is one of the most outstanding commercial success stories in the history of Asian aquaculture, which currently produces more than 80% of the global cultured shrimp. Diseases problems in aquaculture are currently an important constraint to the growth of aquaculture, which has impacted both socio-economic development and rural livelihoods in some countries (FAO, 2000). Viruses are the most economically significant pathogens of cultured shrimp worldwide. The WSSV has a wide host range and it has been observed not only in shrimps but also in crabs but also in crops and other arthropods such as copepods, insects and pest prawns (Lo *et al.,* 1996).

***Author for correspondence :** E-mail: beulashiny7@gmail.com

WSSV has had greatest impact on shrimp on culture and at present still causes the major disease problem (Rosenberry, 2004). In cultured shrimp WSSV infection can cause a cumulative mortality of up to 100% within 3-10 days (Lightner, 1996). The active phase of the disease is characterized by the presence of white spots on the inner surface of the exoskeleton from which the name is derived (Lo *et al.,* 1996). Other clinical sings include anorexia, lethargy and reddish discoloration of the body (Wang *et al.,* 1999). Application of antibiotics and other chemicals in aquaculture has its own intricate or grow out system may lead to development not only antibiotic resistant fish/shrimp bacteria, but also human disease causing bacteria. Information is still locking on the absorption and distribution of antibiotics in fish and shrimp persistence of residues or effects of them in the environment.

Use of immunostimulants, in addition to chemotherapeutic agents and vaccines, has been widely accepted by fish farmers, in many questions about the

efficacy of immunostimulants from users still reaming. Citarasu *et al.,* (2006) describe antibacterial, antiviral, immunostimulant and anti-stress effect of herbal product the significantly influenced in shrimp aquaculture. Direkbusarakom *et al.,* (1993) reported that *Phyllanthus amarus* and *P. urinaria* contained an antiviral substance which was active against yellow head virus. Many kinds of Thai traditional herbs showed antiviral and antibacterial activity against fish and shrimp pathogenic agents. The phytotherapetic approaches will be eco-friendly, economic and reduce the side effects in the aquaculture.

The natural plant products have been reported to have various activities like anti-stress, growth promoting, appetizing, tonic, immunostimulation, aphrodisiac and antimicrobials in the finfish and shrimp larviculture due to the active principle natures such as alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils (Sivaram *et al.,* 2004) Herbs have been widely used in veterinary and human medicine. They are natural products that are not only safe for consumers but also widely available throughout Asia. Nowadays herbs or herbal products also have a significant role in aquaculture.

Materials and Methods

Collection and Processing of immunostimulant herbs

The herbs such as *Pisidium guajava, Annona muricata* and *Agathi grandiflora* were collected from rural area of Kulasekharam, Kanyakumari District. Collected plant materials were shade dried with in temperature range of 28-35^0 C the drying process was continued to reduce moisture level less than 14%. After drying, the plant materials were minced with wooden knife before feeding into a grinder minced materials were made into powder using teeth mills and sieved then the powder was stored in airtight container and kept at room temperature until further use.

Extraction

The powdered plant materials were extracted with hexane three times. Then ethyl acetate was added to the residue and extracted three times, and finally methanol was added and extracted three times. Soaking the material for overnight carried out each extraction. Each of these solvent extracts was concentrated in rotatory evaporator under reduced pressure at the temperature of 45^0 C to 50^0 C in order to avoid the evaporation of plant materials. Aqueous extract was concentrated using lyophilized and stored at 4^0 C.

Phytochemical analysis

Phytochemical screening of the methanol and ethyl acetate extract was carried using standard methods (Evans, 2002) to screen for the presence of various chemical constituents.

Experiment set-up and feeding

Healthy shrimp of *F. indicus* juvenile weighed 9 ± 1 g were purchased from local extensive shrimp farm, Thamaraikulam, Tamil Nadu, India and acclimatized in the laboratory condition. They were stocked in a FRP tank (5000 l capacity) in the laboratory. Uniform size of *F. indicus* were selected from the stock culture and transferred in individual experimental fiber glass tank (100 1capacity) for three experimental groups, D-1, D-2, D-3 and Control. Triplicate culture (n = 10 x 3 = 30) were maintained in each groups with continuous flow-through water and constant aeration system. The water quality parameters such as temperature (27±1.0^0 C) salinity (25±1.5%) and pH 969971, were maintained every day. The shrimp were fed thrice a day at 8.00. 13.00 and 18.00 at 10% of the body weight. Uneaten food and waste matters were remove before feeding. After 30 days of the feeding experiment, the shrimps from each experimental and control groups were injected intramuscularly with WSSV. Haemolymph and abdominal muscles were collected from the individual groups for further biochemical, haematological and immunological studies.

Cumulative mortality and Growth parameters

The percentage of cumulative mortality after 30 and 60th day post vaccination were assessed. The weight (wet) gain was calculated by deducting the initial weight from final weight.

Biochemical analysis

Biochemical parameters such as total protein (Lowry *et al.,* 1951) and total carbohydrate (Roe, 1955) were determined in the haemolymph samples of control and experimental groups challenged shrimps.

Heamatological parameters

Haemolymph was collected from the pericardial sinus using No. 24 hypodermic needle without anticoagulant. The clotting time was determined following the procedure described by Peters and Long (1973). To measure oxyhaemocyanin, 100 µl of haemolymph was immediately diluted with 900µl distilled water in a 10 mm quartz cuvette and the absorbance was measured at 335 nm using a UV spectro photometer. The concentration of oxyhaemocyanin was calculated based on the method of Nickerson and Vanholde (1971).

Prophenol oxidase activity

Phenol oxidase activity in haemolymph samples was

determined using L-dihydroxyphenylanine (L-DOPA) as a substrate (Söderhäll, 1983). TBS (30 µl) was added to the experimental cuvette containing 30 µl of haemolymph sample. Then 60 µl L-DOPA solutions (1.6 mg/ml in TBS) were added followed by immediate mixing. Next 200 µl of TBS was added as a diluent and enzyme activity was determined by measuring the absorbance of dopachrome at 490 against a blank containing 260 µl of TBS and 60 µl of L- DOPA. The absorbance value at 1 and 3 minutes after the addition of 200 µl of TBS was recorded. Enzyme activity was expressed as units, defined as the amount of enzyme giving an increase in absorbance at 490 nm of 0.001 per min/mg/protein.

Results

Phytochemical analysis

Immunostimulant of the extract of the plants such as *Annona muricata, Agathi grandiflora* and *Pisidium guajava* were carried out against WSSV. The phytochemical screening of the plants revealed that, flavonoids, terpenoids, saponins, tannins and other phytochemicals were observed. *A. grandiflora* and *A. muricata* was showed the absence of carboxylic acids. *A. grandiflora* and *P. guajava* tested negative results for the presence of terpenoids and *A. muricata* negative results for the presence of tannins (Table 1).

Cumulative mortality after WSSV Challenging

After the culture period, the *F. indicus* were challenged, intramuscularly injected with WSSV. There is cent percent cumulative mortality observed within 5 days when no immunostimulant and antiviral characteristics herbal extract given in the diets. The D2 and D3 had decreased percentage of cumulative mortality. That means the groups survived 60 and 50 % 0f survival in the above groups respectively. The herbals having the immunostimulant characteristics were highly influenced to resist the WSSV (Fig. 1).

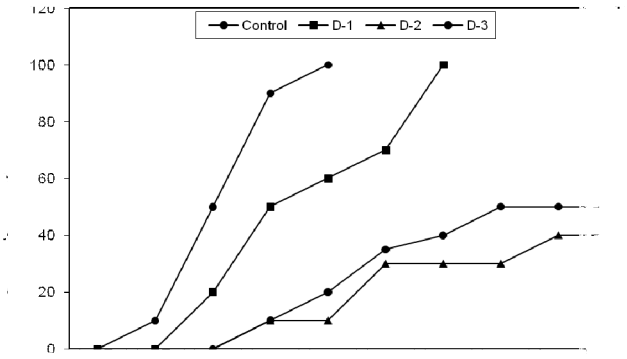

Fig. 1: Cumulative mortality of herbal diets fed with *F. indicus.*

Survival & Growth parameters

The survival, weight gain and specific growth rates were given in the table 2. The *F. indicus* survived 65, 75, 85, and 80% in the control, D-1, D-2, and D-3 diet fed groups. The minimal weight gain of 1 mg/d was observed the control diet. The gain was significantly increased to 2.5, 2.2 and 2.6 mg/day in the D-1, D-2 and D-3 diet fed groups. The minimum specific growth of 0.17 % and the maximum of 0.35 % were observed in the control and D-3 group respectively.

Heamatological Studies

The Haematological parameters such as coagulase activity and Oxyhaemocyanin results were given in the table 3. The haemolymph is coagulated 167 seconds when no anti viral and immunostimulant diets were given. The time for coagulation is decreased to 120, 111 and 96 seconds in the D-1 to D-3 groups respectively. The decreased time for coagulation is responsible for the decreased viral load in the haemolymph. The lowest oxyhaemocyanin level, 0.88 (mmol l^{-1}) was observed in the control diets fed *F. indicus*. The level of oxyhaemocyanin was increased to 0.9, 0.96 and 1.77 (mmol l^{-1}) D-1 to D-3 diets fed *F. indicus* groups respectively.

Table 1: Phytochemical constituent for the anti viral and immunostimulant plants.

S.No.	Tests	*Agathi grandifolia*	*Pisdium guajava*	*Annona muricata*
1.	Steroids	+	+	+
2.	Saponins	+	+	-
3.	Flavanoids	-	-	-
4.	Coumarin	+	+	-
5.	Carboxylic acids	-	+	-
6.	Cardiac glycosides	+	-	-
7.	Phenols	+	+	+
8.	Quinone	-	+	-
9.	Resins	+	+	+
10.	Terpenoids	-	+	+
11.	Tannins	+	+	-

Biochemical Changes of immunostimulant treated *F. indicus*

The total protein analysis was performed in the control and experimental groups. The protein values are higher in the infected animals due to higher viral load in the haemolymph. After WSSV challenge, the protein level is 115 mg ml^{-1} in the haemolymph of control group. The value was decreased to 110, 99 and 95 mg ml^{-1} in the D1, D2

Table 2: Growth characteristics of herbal immunostimulant active principle incorporated diets fed shrimp *F. indicus*.

Treatment	Length(mm)		Wet weight(mg)		Weight gain (mg/day)	Specific growth rate (%)	Survival (%)
	Initial	Final	Initial	Final			
Control	8.6±0.16	11.2±0.16	10.6±0.12	12.1±0.35	1.3^{a}±0.12	0.17^{a}±0.16	65
D-1	8.4±0.29	11.3±0.12	10.4±0.24	12.7±0.16	2.5^{b}±0.20	0.28^{b}±0. 08	75
D-2	7.9±0.12	11.6±0.16	10±0.18	12.4±0.18	2.2^{b} ±0.16	0.31^{c}±0.12	85
D-3	8.2±0.29	11.6±0.16	9.6±0.14	12.2±0.24	2.6^{b}±0.18	0.33^{c}±0.18	80

Note: Means with the same superscripts (a-c) do not differ from each other (P < 0.01).

Table 3: Haematological changes in the haemolymph of *F. indicus* fed on herbal immunostimulant diets and control diet after challenge with WSSV.

Treatments	Haematological Changes		
	Coagulase activity (Sec)	Total Haemocyte Count ($X10^5$ cells ml^{-1})	Oxyhaemocyanin (mmol l^{-1})
Control	167^{a}±2.16	27.33^{a}±1.63	0.88^{a}±0.01
D-1	120.66^{b}±0.94	36.50^{b}±0.94	0.9^{a}±0.002
D-2	111.33^{b}±2.94	42.22^{c}±2.16	$0. 96^{a}$±0.01
D-3	96.33^{c}±2.86	46.43^{d}±1.24	1.77^{b}±0.23

Note: Means with the same superscripts (a-e) do not differ from each other (P < 0.01).

and D 3 groups of the haemolymph respectively (Table 4). The carbohydrate level of WSSV challenged *F. indicus* fed on control 117 mg/100ml of haemolymph was recorded. The carbohydrate percentage was significantly (P< 0.01) increased to 120 and 140 mg/100ml of haemolymph respectively in D_2 and D_3 groups. The results were shown in table 4.

Table 4: Biochemical changes in the haemolymph of *F. indicus* fed different herbal immunostimulant diets after WSSV challenge.

Treatments	Biochemical changes	
	Protein (mg/ml)	Carbohydrate (mg/100ml)
Control	115.4 ± 0.32^{a}	117.7 ± 1.24^{a}
D-1	110.2 ± 0.43^{b}	98.3 ± 1.24^{b}
D-2	99.6 ± 0.47^{c}	120.4 ± 0.32^{c}
D-3	95.5 ± 0.37^{d}	140.4 ± 0.32^{d}

Note: Means with the same superscripts (a-e) do not differ from each other (P < 0.01).

Immunological Changes of immunostimulant treated *F. indicus*

The prophenol oxidase activity (proPO) value observed was higher in the herbal anti viral and immunostimulants diets fed groups than the control group in different days of challenging. The less value of 0.163 was observed in the control group. The value was significantly (P<0.01) increased to 0.29, 0.68, 0.83 and 1.12 for D-1 to D-2 groups respectively after 240 mins incubation of the reaction mixture (Fig. 2).

Discussion

In recent years, natural products from the plant kingdom have been investigated for their immunemodulating potential against infectious and neoplastic diseases. Herbal therapy, or "phytomedicine," the therapeutic use of plants, plant parts, or plant-derived substances, is generally considered a form of complementary medicine (Jones, 1997). Herbal compounds such as volatile oils, tannins, phenolics, saponins, alkaloids, polysaccharides and polypeptides were shown effective alternatives to that of antibiotics. The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents (Press, 1996) as well as serve in drug discovery from natural products for primary lead compounds.

The present study revealed that, steroids, tannins, titerpenoids, flavonoids, coumarin and many other phytochemical compounds were analyzed. Hangerman and Bulter (1981) in their studies stated that phytochemical analysis showed the presence of biologically active compounds such as phenols and tannins in the extracts. These organic compounds have been known to possess antimicrobial action. The herbal immunostimulants were improved the immunue system and reduced the microbial infection in the gold fish *Carassius auratus* (Minomol, 2005) and similar work was carried out by Magdelin (2005) on the ornamental fish *Poecilia sphenops* using herbal immunostimunants.

Haemocytes are responsible for clotting, exoskeleton hardening and elimination of foreign materials (Song and Hsieh, 1994). Mean THCs of healthy penaeid shrimps ranged from 20 to 40 x 10^6 cells ml^{-1} (Chang *et al.,* 1999). The present results of WSSV challenging test in *F. indicus* revealed that the the immunostimulants helped to decreased the time of clotting and oxyhaemocyanin level. Stewart *et al.,* (1969) have observed increase in hemolymph clotting time in *G. homari*-infected lobsters as observed in our study. The WSSV might be responsible

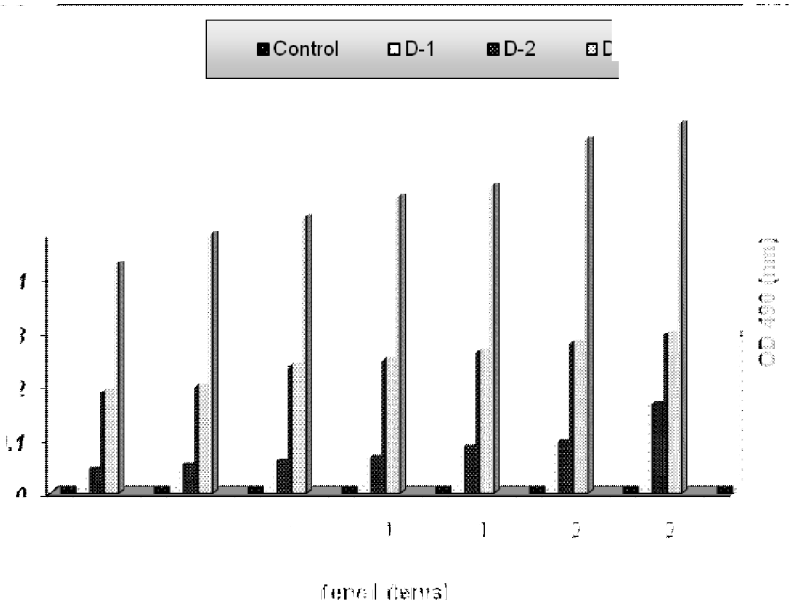

Fig. 2: Prophenol Oxidase activity of *F. indicus* fed on herbal immunostimulants diets and control diets.

for failure in coagulation of haemolymph in infected shrimp.

For the biochemical aspects of the control and experimental groups, the protein values are higher in the control groups. It is mainly due to the heavy viral load in the infected *F. indicus*. The D1 and D2 group have less viral load due to the less infection. Beckage (1996) reported the possibility for the increase of protein content in haemolymph and decrease in muscle as well as hepatopancreas of infected shrimps is that baculoviruses encode a variety of proteases and other enzymes that 'melt' the tissues. The total carbohydrate and glucose levels increased in haemolymph and decreased in muscle and hepatopancreas of WSSV infected shrimp in comparison with healthy shrimp. Generally, the glucose level increases in infected or stressed animals to ward off the infection or stress (Kumaran *et al.,* 2018). The possibility of high levels of glucose and total carbohydrate in haemolymph might be due to the transport of glucose and carbohydrate from hepatopancreas and muscle to haemolymph.

Prophenol oxidase, the key enzyme in the synthesis of melanin, occurs in haemolymph as an inactive pro-enzyme prophenoloxidase (proPO). ProPO is activated to form PO when it reacts with zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ions, trypsin, or heat. Results from several experiments have implied that apart from their role in melanisation, components of the putative proPO activating system stimulate several cellular defence reactions, including phagocytosis, nodule formation, encapsulation, and haemocyte locomotion (Söderhäl *et al.,* 1986). From this study, concluded that, there is some resistance found against the WSSV infections. The hematological and immunological results revealed that, a clear cut idea of resistance. The herbals having the immunostimulating characteristics had better growth promoting ability, immunostimulation and act as enhanced performance during the oral administration against the WSSV infection.

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