

EFFECTS OF IMMUNOSTIMULANT *ANNONA MURICATA* EXTRACT AGAINST WHITE SPOT SYNDROME VIRUS INFECTION

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Abstract

*Bioactive principles present in the medicinal plants attribute to the therapeutic efficacy and it can be incorporated into modern medicine system for the development of newer drug formulation for therapeutic ailments. Screening of medicinal plants for antimicrobial activities and phytochemicals is important for finding potential new compounds for therapeutic use. In the present study an attempt has been made to congregate the traditional, phytochemical and pharmacological studies done on an important medicinal plant *Annona muricata*. The plant possess the major pharmacological activities includes Anti-viral activity, Anti-oxidant Activity, Larvicidal activity, Anti-inflammatory activity, Antipediculicidal activity, Anti-bacterial activity and wound healing. It also has the anti-carcinogenic and cytotoxic effect. The present study revealed that immunostimulant *A. muricata* extract fed *Fenneropenaeus indicus* exhibited had increased total haemocyte count, oxyhaemocyte count and decreased time of coagulase activity. The biochemical parameters such as protein and carbohydrate and immunological parameters were significantly ($P < 0.01$) differed from the control groups to experimental groups.*

Key words: *Annona muricata*; phytochemical; *Fenneropenaeus indicus*; Immunostimulant

Introduction

The importance of the active ingredients of plants in medicine has stimulated significant scientific interest in the biological activities of these substances. According to the World Health Organization, about 80% of the developing countries (e.g., Brazil, China, India, and Thailand) rely on traditional medicines and of those, 85% use plants or their extracts as the active substance. Numerous studies have been carried out to screen extracts from medicinal plants for the presence of novel compounds and an investigation of their biological

activities. Plants have been known to be a reservoir of secondary metabolites which are being exploited as source of bioactive substance for various pharmacological purposes.

Medicinal plants are considered as the basis for health preservation and care worldwide. One of the medicinal plants is *Annona muricata*. This plant is known as soursop (English) belong to Annonaceae family. This soursop tree is about 5-10 m tall and 15-83 cm in diameter with low branches. This plant is reported to be useful in the treatment of various health ailment such as fever, respiratory illness, malaria (Boyom *et al.*, 2011), liver, heart and kidney infections (Badrie *et al.*, 2009).

Phytochemicals are bioactive chemicals of plant origin. They are regarded as secondary metabolites because the plants that manufacture them may have little need for them. They are naturally synthesized in all parts of the plant; leaves, stem, fruits, seeds, etc. i.e. any part of the plant body may contain active components. The quantity and quality of phytochemicals present in plant parts may differ from one part to another. Moreover, plant secondary metabolites present chemical and pharmaceutical properties interesting for human health (Reddy *et al.*, 2005).

White spot syndrome virus (WSSV) causes up to 100% mortality with in 7 to 10 days in commercial shrimp farms resulting in large economic losses to the shrimp farming industry. Lightner (1996) observed that cultured shrimp are often affected by different pathogen agents, creating a serious economic problem for shrimp farming in many parts of world. In shrimp farming system horizontal transmission of the virus occurs via oral ingestion, the water borne route and cannibalism has been demonstrated to be important in transmission of the virus in conditions (Wu *et al.*, 2001).

Tracing induced changes in first by the use of immunostimulants, vaccines (or) environmental stressors (such as aquatic pollution) is an important part of present day aquaculture (Anderson 1992) Some of the main immunostimulants used are Levamisole, Glucans, Chitins (Cuesta *et al.*, 2003), and many others. It is well known that these agents stimulate the nonspecific immune response (also called innate (or) natural protection) and boost the specific immune response. Therefore, immunostimulants can also be used as improve the specific immune response, these formula cannot be used in food fish because of scarring or tissue injury. As a result, low molecular weight oils are now being mixed and tested with some bacteria for fish bound for the market place. The presence of pollutants and contaminants in the environments can reduce the nonspecific and specific immune responses

in fish. In the field, the final (in *vivo*) results of exposure to these agents of fish are evident by protection against diseases, or morbidities or mortalities.

2. Materials and Methods

2.1. Immunostimulant herb *A.muricata* collection and processing

Leaves of the herb *A.muricata* were collected from Kulasekharam area, Kanyakumari District, India. Collected plant root materials were shade dried with in temperature range of 28-35°C. The drying process was continued to reduce moisture less than 14%. After drying, the plant materials were minced with wooden knife feeding into a grinder, minced materials were made into power using teeth mills and sieved, and then the power was stored in airtight container and kept at room temperature until further use.

2.2. Extraction and Characterization of active principles

Dried powder plant materials were boiled at above 100°C with two hour. After filtered the extracts, the supernatant was collected and the residue were discarded. The supernatant was condensed in the water bath and the condensate was extracted again by methanol. The methanolic extract was concentrated in rotatory evaporator under reduced pressure at the room temperature of 45°C to 50°C in order to avoid the evaporation of plant materials. Aqueous extract was concentrated using Lyophilizer and stored at 4°C. The extract phytochemicals was analysed by standard protocols (Sofowora, 1993; Kumaran & Citarasu, 2015). The aqueous extract was characterized by Thin Layer Chromatography (Wagner, 1988). Chromatographic separation was carried out using solvent system chloroform: methanol: water (6.4: 5:1 v/v) and plates were documented by low range UV and the spots were developed with phosphomolybdic acid.

2.3. Source of infected WSSV

WSSV infected shrimps with prominent white spots were collected from diseases affected shrimp farms. The haemolymph samples were drawn from the infected shrimps using sterile syringes followed by centrifugation (3000 X g for 20 min at 4°C). The supernatant fluid was then re-centrifuged (8000 X g for 30 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 studies.

2.4. Propagation and Purification of WSSV

Normal uninfected adult shrimp *F. indicus* weighing approximately 8.5 ± 3 g were collected from Manakudy estuary and stocked in a fibre glass tank (5000 l capacity) in the laboratory. The shrimps were acclimatized to ambient laboratory condition. Each shrimp was inoculated intramuscularly with the semi purified WSSV (300 μ g of total protein per animal). The shrimp were examined twice daily for signs of morbidity and mortality. When found, moribund shrimp were removed and hemolymph was collected. The pooled hemolymph was centrifuged at 8000X g for 30 min at 4°C for 1 h. The final supernatant fluid was filtered through a 0.4- μ m filter. The filtrate was then stored at -20°C for infectivity studies.

2.5. Preparation of Inactivated WSSV

For preparation of a heat – inactivated WSSV, the viral suspension was diluted 10 fold in Phosphate Buffer saline (PBS) [NaCl 6g, KCl 200mg, Na_2HPO_4 1.44g Potassium phosphate 240mg make one liter and maintain $\text{pH} = 7.4$ and sterilized] and inactivated for 10 min at 60°C water bath. Hot water extract of *A.muricata* (500 mg) were mixed with diluted and inactivated WSSV suspension having 300 μ g of total protein and make up to 1 ml of PBS.

2.6. Immunostimulant coated diet preparation

Ingredients and formulation of the basal ration were followed as described by Boonyaratpalin (1993). The basal diet contained 45% protein, 7.2% Lipid, 14.6% ash, 7.1% moisture and 3% fibre. To prepare the diets, initially the ingredients were mixed thoroughly and 4% gelatin solution containing active principles with appropriate concentration was added along with oil ingredients and sufficient water was added when the pH was adjusted (7 ± 0.1). 1 g/ 100g immunostimulant plant *A.muricata* extract added. After that mixture was cold extruded cut into pellet, air dried and stored at room temperature. They are stored at -20° C deep freezer for further feeding to the culture experiments.

2.7. Cumulative mortality and growth parameters

The percentage of cumulative mortality on 40th days of post-vaccination was assessed. The weight (wet) gain was calculated by deducting the initial weight from the final weight.

2.8. Biochemical parameters

Biochemical parameters such as the total protein and glucose were determined in haemolymph samples of all the experimental groups of vaccinated shrimps. The total protein was determined spectrophotometrically (O.D 595 nm) by (Lowry *et al.*, 1951) and glucose was estimated by the glucose oxidase method (Roe, 1955).

2.9. Haematological parameters

Haemolymph was withdrawn from the pericardial sinus using No.24 hypodermic needle without anticoagulant. The clotting time of the haemolymph was determined by Peters and Long (1973). The Total Haemocyte Count (THC) (cells ml⁻¹) was performed using Burker haemocytometer (Le Moullac *et al.*, 1997). To measure oxyhaemocyanin, 100 ml of haemolymph was immediately diluted with 900 ml distilled water in a 10-mm quartz cuvette and measured the absorbance at 335 nm using a spectrophotometer (Hitachi, Tokyo, Japan). The concentration of oxyhaemocyanin was calculated following the method of Hagerman, 1983.

2.10. Immunological parameter

Phenoloxidase activity in haemolymph samples was determined using L-dihydroxyphenylalanine (L-DOPA) as a substrate (Soderhall, 1983). 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.

3. Results

3.1. Active compound characterization of the immunostimulant *A. muricata*

This study has revealed the presence of phytochemicals considered as active medicinal chemical constituents. Important medicinal phytochemicals including saponin, terpenoids, tannin, flavonoids and steroids were present in the samples. The result of the phytochemical analysis showed that the *A. muricata* had the presence of tannin, saponin, steroids and flavanoids (Table 1). Thin layer chromatographic analysis of the hot water extract of *Asparagus racemosus* revealed that, the spot is confirmed as the of steroidal saponins. There is two types of fractions having the R_f value of 0.92 and 0.43 were detected by the iodine vapor detection (Fig 1).

3.2. Cumulative Mortality after 40 dpv challenging

The cumulative mortality of the Shrimp *F. indicus* fed with and without immunostimulant diets were given in the Fig 2. After 40 dpv, the WSSV challenged *F.indicus* fed succumbed to death the mortality of cent percent with in 9 days. Further the cent percent cumulative mortality was prolonged 15 days in the diet D1 fed *F. indicus* after challenge. The D2 group, the IgY produced with immunostimulant coated diets help to decreased the cumulative mortality. This group significantly increased ($P < 0.05$) the survival of and with stand the resistance after the 40dpv.

3.3. Hamatological Changes after challenging of the immunostimulant treated shrimp *F. indicus*

The Hematological parameters such as Coagulase activity, Total Haemocyte Count (THC) and Oxyhaemocyanin are as follows. The haemolymph was coagulated after 40dpv, within 179 seconds when no anti WSSV antibody in the diet was provided. The time for coagulation is decreased significantly ($P < 0.05$) to 125 and 96 seconds in the D1 and D2 groups. The total Haemocyte count (THC) of 40th day of post vaccination observed 28×10^3 cells ml^{-1} in the control group. The THC was significantly ($P < 0.05$) increased 35 and 45×10^3 cells ml^{-1} in D₁ and D₂ groups respectively. The lowest oxyhaemocyanin level 0.70 (mmol^{-1}) was observed in the control IgY coated diet fed *F. indicus* at 40thdpv. The level of oxyhaemocyanin was significantly ($P < 0.05$) increased to 1.0 and 1.2 (mmol) D₁ and D₂ diets fed *F. indicus* respectively (Table 3).

3.4. Biochemical Changes after challenging of the immunostimulant treated shrimp *F. indicus*

The total protein analysis was performed in the control and experimental groups. Generally the protein values are higher in the infected animals due to higher viral load in the haemolymph. The 30dpv protein level 123 mg ml^{-1} in the haemolymph of control group. This was significantly was ($P < 0.01$) decreased to 106 mg ml^{-1} in the D1 group 40dpv and further it was decreased significantly ($P < 0.01$) to 93 mg ml^{-1} (D2) of the haemolymph respectively (Table 4). The carbohydrate level of 40thdpv challenged *F. indicus* fed on control IgY of 4.54 mg ml^{-1} of haemolymph was recorded. The carbohydrate percentage was significantly ($P < 0.01$) decreased to 2.71, 1.65 mg ml^{-1} of haemolymph respectively in D1 and D₂ groups.

3.5. Immunological Changes after challenging of the immunostimulant treated shrimp

F. indicus

The prophenol oxidase activity (Pro Po) value observed was higher in the immuno adjuvant IgY supplemented diets fed groups than the control group in different days of challenging. All shrimps succumbed to death within 6 days in the control group. After the 5th day, the pro Po value of different anti WSSV IgY supplemented diet D₁ and D₂ fed groups increased significantly. Among them, the maximum Pro Po activity was found in group D₂ on the 15th day. Two way ANOVA revealed that the variation between the Pro Po activity of *F. indicus* fed with control and experimental diets was statistically more significant ($P < 0.05$). The values are raised from control (0.05) group to 0.6 (D₂) in 40dpv (Fig 3).

4. Discussion

Newer vaccines include highly purified subunit antigens that are weakly immunogenic. Vaccine formulations often require adjuvant for increased immunological efficiency and better vaccination schedules (Vogel, 2000). Adjuvant is used as a tool to study immune mechanisms, such as antigen presentation by dendrite cells and modulation of immune responses by cytokines and their receptors. Adjuvant can also be employed in vaccine design research, which could assist in identifying the requirements of protective immunity, since different adjuvant very immune responses to the same experimental antigen. The activities of adjuvant in humans as compared with their effect on small animals should be more fully evaluated. Animal models should be developed that can predict as accurately as possible. *A.muricata* is used as a good adjuvant in human diseases (Chark samhita, 1949) and give a efficient and sustained immunostimulation to improve immunogenicity of weak or low dose of antigens. Experientially, it has been recognized in herbal medicine that the hot water extracts may act on the haematopoietic system, mitogenic and good active adjuvant.

In this study, efficacy of vaccines made of inactivated WSSV with or without immunostimulant used to produce anti WSSV. The selection of a vaccine adjuvant should be based on analysis of the potential benefit of the adjuvant in enhancing the immunogenicity of a vaccine, weighed against its risk to induce adverse local or systemic reactions. A study by Yogeewaran (2007), immunostimulant methanolic extracts of the herbs *Aclypha indica*, *Cynodon dactylon*, *P. Kurooa*, *Withania somnifera* and *Zingiber officinalis* were effectively

suppress the WSSV after the injection with the herbal extracts and WSSV incubated mixture and the extracts have a potent antiviral / immunostimulation activity against the WSSV.

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. Tannins have been shown to form irreversible complex with proline rich proteins, which would result in the inhibition of cell wall protein synthesis. Previous phytochemical analysis of the plant *A. wilkensisiana* has shown the presence of bioactive compounds such as phenols and saponins (Adesina *et al.*, 1980). Most phytochemical analysis showed that the presence of biologically active compounds such as terpenoids and some aliphatic and phenolic compounds with free hydroxyl group showed antimicrobial activity (Rojas *et al.*, 1992). In the present study, the combined effect of herbal extracts and inactivated WSSV vaccination helped to reduce mortality.

Haemocytes are responsible for clotting, exoskeleton hardening and elimination of foreign materials. Mean THC's of healthy penaeid shrimps ranged from 20 to 40 x 10⁶ cells ml⁻¹ (Chang *et al.*, 1999). Molting, development of organs, reproductive status, nutritional condition and disease have been shown to influence haemocyte abundance Le Moullac *et al.*, (1997). The present results of WSSV challenging test in *F. indicus* revealed that the immunostimulants helped to decreased the time of clotting, increased haemocyte count and oxyhaemocyanin level.

For the biochemical aspects of the control and experimental groups, the protein and glucose 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. Lo *et al.*, (1997) reported the high concentrations of protein, amino acids in the haemolymph of crustaceans due to WSSV heavy load, confirmed by PCR detection. The total carbohydrate and glucose levels increased in haemolymph and decreased in muscle and hepatopancreas of WSSV infected shrimp in comparison with healthy shrimp..

Prophenol oxidase, the key enzyme in the synthesis of melanin, occurs in haemolymph as an inactive pro-enzyme prophenoloxidase (proPO). 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 (Söderh al *et al.*,

1986). The *A. racemosus* had highly influenced to enhance the immunological parameters such as PO activity against the WSSV infection. The different concentrations of immunostimulant diets were well influenced and significantly had more survival (74%) and reduction in the viral load (Citarasu *et al.*, 2006). Also the better performance of haematological, biochemical and immunological parameters was found in the immunostimulant incorporated diets fed shrimps.

5. References

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Table 1. Phytochemical analysis of hot water extract of *A. muricata* by standard protocols

Sl.No	Phytochemical constituents	Hot water extract
1	Alkaloid	-
2	Saponin	+
3	Steroids	+
4	Tannin	+
5	Terpenoids	-
6	Flavonoids	+

Table.2. Detailed description of the different types of treatments immunostimulant protocols

Treatments	Details of Immunostimulant and Vaccination	
	Type of treatment	Challenge
Control (C)	No (normal Feed)	Heat Killed WSSV
D-1	Anti WSSV (without extracts)	Heat Killed WSSV
D-2	Anti WSSV (with immunostimulant <i>A. muricata</i>)	Heat Killed WSSV

Table 3. Haematological parameters of the haemolymph of *F. indicus* fed with immunostimulant and challenged with WSSV after 40 day post-vaccination.

Sl.No	Treatments	Haematological parameters		
		Coagulase activity (Seconds)	Total haemocyte (THC) ($\times 10^3$ cell ml^{-1})	Oxy haemocyanin (mmol^{-1})
1	Control	171.66 ^b ± 3.85	28.00 ^b ± 0.81	0.70 ^b ± 0.03
2	(D1)	125.33 ^c ± 3.09	35.66 ^c ± 1.69	0.91 ^a ± 0.01
3	(D2)	96.00 ^d ± 0.81	45.00 ^d ± 2.44	1.08 ^c ± 0.04

Means with the same superscripts (a-d) do not differ from each other ($P < 0.05$).

Table 4. Biochemical Changes of *F. indicus* fed with immunostimulant diets and challenged with WSSV after 40th day post-vaccination in the hamolymph

Sl.No	Treatments	Challenges	
		Protein (mg/ml)	Carbohydrate (mg/ml)
1	Control	123.00 ^b ± 1.41	4.54 ^b ± 0.31
2	D1	106.00 ^c ± 3.74	2.71 ^c ± 0.02
3	D2	93.00 ^d ± 2.16	1.65 ^a ± 0.05

Means with the same superscripts (a-d) do not differ from each other ($P < 0.05$).

Fig.1. Characterization of active elution of the extract of immunostimulant *A.muricata*



Fig 2. Cumulative mortalities of *F. indicus* fed with immunostimulant diets and challenged with WSSV on 40th day post-vaccination

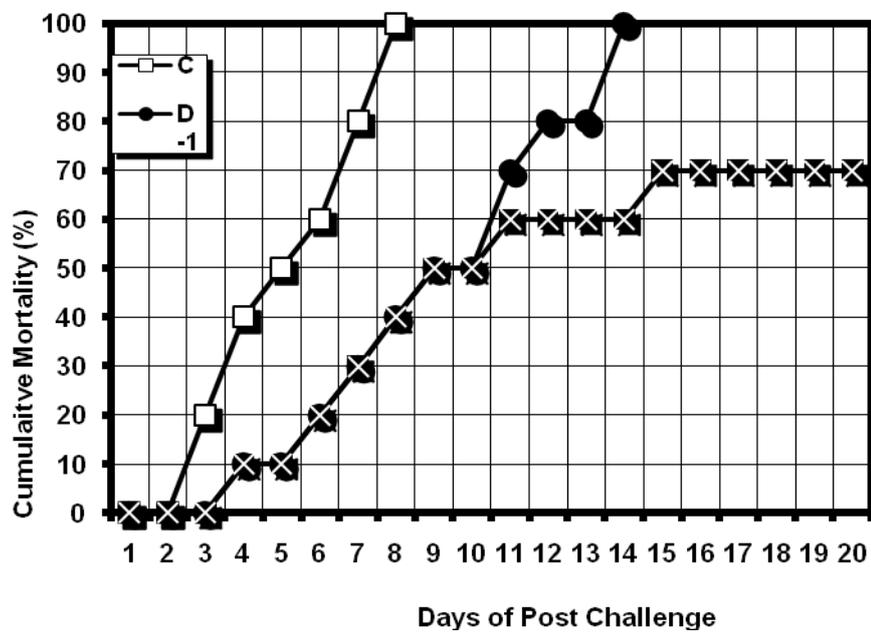


Fig. 3. Phenoloxidase activity (PO) of haemocytes of *F.indicus* fed with immunostimulant diets and challenged with WSSV on 40th day post-vaccination

