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Effect of dietary *Syzygium cumini* leaf powder on growth and non-specific immunity of *Litopenaeus vannamei* (Boone 1931) and defense against virulent strain of *Vibrio parahaemolyticus*

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ABSTRACT

The study was aimed to assess the growth and immunomodulatory potential of *Syzygium cumini* leaf powder against virulent strain of *Vibrio parahaemolyticus* on the juveniles of *Litopenaeus vannamei*. Two diets such as control diet (without immunostimulant) and immunostimulant diet (containing 1% *S. cumini* leaf powder) were prepared. The juveniles were distributed in four treatments namely; daily feeding of control diet (CD), daily feeding of immunostimulant diet (ID), alternate day feeding of control and immunostimulant diet (CD/ID) and two days control diet and one day immunostimulant diet (2CD/ID) feeding. After feeding trial, shrimps from each treatment group were challenged with 0.1 mL of virulent strain of *V. parahaemolyticus* suspension at a concentration of 10^7 CFU mL^{-1}. The post-challenge sampling was performed after 5 days of injection. The haemolymph prophenol oxidase, superoxide dismutase, catalase and respiratory burst activity of different treatments were significantly different ($P < 0.05$) in both pre- and post-challenge conditions. The haemolymph total protein, globulin, lysozyme and phagocytic activities of *L. vannamei* fed with *Syzygium cumini* leaf powder improved than the control group in pre- as well as post-challenge phase. The survival rate of shrimps fed with immunostimulant diet was significantly ($P < 0.05$) higher as compared to control group. Therefore, *Syzygium cumini* leaf powder added diet protects the host effectively against virulent strains of *V. parahaemolyticus* through both continuous and alternate day feeding.

1. Introduction

Pacific white leg shrimp, *Litopenaeus vannamei* is the potential candidate species for shrimp farming after the down fall of Black tiger shrimp, *Penaeus monodon* in most of the Asian countries (Varadharajan and Pushparajan, 2013). Intensive farming of *L. vannamei* with high stocking density is the most preferred technique in shrimp farming practice. However, intensification of shrimp farming often hampered the shrimp production due to mass mortality caused by infectious bacterial and viral diseases that incurs heavy economic loss in shrimp culture (Walker and Winton, 2010). The Pacific white leg shrimp culture is highly susceptible to the bacterial diseases such as vibriosis and early mortality syndrome caused by *Vibrio* spp. and *Vibrio parahaemolyticus* respectively. The early mortality syndrome also known as acute hepatopancreatic necrosis syndrome (AHPNS) had harassed Pacific white leg shrimp farms in Southeast Asia (López-León et al., 2016). These opportunistic bacterial disease problems were originating from highly endured stress owing to several stressors in general and unlimited stocking of shrimps in particular. So there is an increasing interest in shrimp health, particularly knowledge on the immunological control of disease in shrimp farming is gaining immense importance.

Immunomodulation has been proposed as a potential method to protect the farmed shrimps from infectious pathogens by enhancing their innate immunity. Hence, antibiotics were proposed for the disease prevention. However, the incidence of drug resistant bacteria has become a major problem in shrimp culture due to the irrational use of antibiotics (Aoki, 1992). Regarding the problem of microbial resistance, alternatives to antibiotics were discovered. They are mainly natural compounds, herbs and phytochemicals known as nutraceuticals that are becoming great demand. Besides immunostimulation, these agents also enhance the growth by offering better protection against pathogens and divert the energy exclusively for growth (Abreu et al., 2012). These herbs have little or no side effects which are abundantly available renewable resources and easily degradable (Harikrishnan et al., 2011a;

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Abreu et al., 2012). Several herbs such as *Cynodon dactylon*, *Aegle marmelos*, *Tinospora cordifolia*, *Picrorhiza kurroa* and *Eclipta alba* in black tiger shrimp (Citarasu et al., 2006), *Cardiospermum halicacubum* in *Penaeus monodon* (Rajasekar et al., 2011), *Petalonia binghamiae* in white shrimp (Chen et al., 2014), *Agati grandiflora*, *Justicia tranquebariensis* and *Eclipta erecta* in *P. monodon* (Kumaran et al., 2014), etc. have been studied as immunomodulators and growth promoters in aquafeeds due to their positive effects on innate and adaptive immunity and to prevent and control diseases of crustaceans.

Java plum or Jamun tree, *Syzygium cumini* is one of the most important medicinal plants that cures several infectious diseases in folklore medicine. It is widely available in Southeast Asian countries like India, Bangladesh, Nepal, Sri Lanka, Malaysia, Philippines and Indonesia (Morton, 1987). *Syzygium cumini* leaves possess number of bioactive compounds such as alkaloids, anthocyanins, acylated flavonol glycosides, quercetin, myricetin, myricitin, triterpenoids, tannins, galloyl carboxylase, ellagic acid and kaempferol (Timbola et al., 2002; Ayyanar and Subash-Babu, 2012). Among the active compounds, alkaloids, glycosides and polyphenolic compounds such as gallic acid and ellagic acid are the active antibacterial agents (Chattopadhyay et al., 1998; Pandey and Singh, 2011). Therefore, *Syzygium cumini* leaf powder can be effectively used as an immunostimulant in shrimp farming industry to prevent bacterial disease. The use of *Syzygium cumini* leaves powder (SCLP) in commercial shrimp feed will be a cost effective and an eco-friendlier approach to safeguard the shrimp culture from bacterial diseases through enhancing their non-specific defense mechanism. Hence, in the present study, SCLP was selected as a nutraceutical for *L. vannamei* from the eleven herbs studied due to its higher antibacterial and antioxidant activity. The essential oils particularly sesquiterpenes of carane family present in *S. cumini* leaf powder will also act as a feed attractant and impart attractive fragrance to the finished feed (Kroismay et al., 2006; Machado et al., 2013). This will enhance the feed intake and thus elicit growth of the shrimp. Further, there were scanty reports available at present on the duration as well as frequency of feeding of immunostimulant incorporated diet to the shrimps. Therefore, the present study was aimed to find out the immunomodulatory potential of SCLP and resistance against virulent strains of *V. parahaemolyticus* on *L. vannamei* as well as the required frequencies of immunostimulant feeding i.e. daily, alternate day and once in 2 days immunostimulant incorporated diet feeding.

2. Material and methods

2.1. Collection of herbals

For the selection of best immunostimulatory herbal for the current study, leaves of 11 herbals viz. *Phyllanthus acidus* (L.) Skeels (Otaheite gooseberry), *Tephrosia purpurea* (L.) Pers. (Purple tephrosia), *Murraya koenigii* (L.) Sprengel (Curry leaf), *Moringa oleifera* Lam. (Moringa), *Cassia auriculata* Linn. (Avaram senna), *Sesbania grandiflora* (L.) Poiret (Hummingbird tree), *Syzygium cumini* (L.) Skeels. (Jamun), *Ficus religiosa* Linn. (Peepal tree), *Solanum nigrum* Linn. (Black nightshade), *Cassia angustifolia* M. Vahl. (Senna) and *Acalypha indica* Linn. (Indian nettle) were collected from Tuticorin District of Tamil Nadu, India. The herbs were collected as fresh and washed in running tap water to remove any dirt and other extraneous materials adhering to them. Then dried in shade for three days, ground to fine powder and was stored in clean container for further use.

2.2. Preparation of herbal extract and screening

For the selection of best immunostimulant herbal, methanol extracts were collected from all the 11 herbals. In a beaker 10 g of herbal powder was taken and mixed with 200 mL of methanol to suspend the powder material and kept in shaking incubator for 24 h and centrifuged to collect the supernatant. The supernatant was then evaporated using rotary evaporator and the residue was dissolved in methanol to completely collect from the flask. Then the methanol mixed extract was transferred to pre-weighed petri plate and kept at 50 °C for 2 h to completely evaporate the solvent. Then the dried petri plate was transferred to desiccator to cool the plate and weighed. The yield of each extract was calculated as the weight difference between the plate with dried extract and the empty plate. From the dried extract, 10% standard extract was prepared using 10% DMSO and methanol for antibacterial and antioxidant activity respectively. All the 11 herbals were subjected to screening for antioxidant and antibacterial activity as per standard protocols.

2.3. Antioxidant assays

Total phenolic content from the herbal extracts were estimated by the method of Singleton and Rossi (1965). The Ferric-reducing/antioxidant power (FRAP) assay was carried out according to the procedure of Benzie and Strain (1996). The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of herbal extracts were done by Blios (1958).

2.4. Anti-bacterial assays

The minimum inhibitory concentration (MIC) was determined by Micro Broth Dilution method according to the National Committee for Clinical Laboratory Standard (NCCLS) (NCCLS, 1999) guidelines using a 96 well plate. The wells that showed no growth during MIC determination were selected and a loopful from each well was sub cultured onto herbal extract free Mueller Hinton Agar plates and incubated for further 24 h at 37 °C. The lowest concentration at which there was no growth observed was noted and expressed as MBC.

2.5. Selection of best herbal and feeding trial

After screening of the herbal extracts, Jamun tree leaf (*Syzygium cumini*) powder (SCLP) was selected as the best due to its superior profile of antibacterial and antioxidant activity. An experiment was designed and executed to evaluate the immunostimulant potential of SCLP in the shrimp, *Litopenaeus vannamei*. Java plum tree, *Syzygium cumini* is an evergreen tropical tree widely available in different parts of India. The leaves are available in huge quantity throughout the year for commercial use as immunostimulant. The leaves of *S. cumini* were washed in running tap water to remove any dirt and other extraneous materials adhering to them. Then shade dried for three days and ground finely using mixer grinder. The fine powder was stored in clean and dry container for further use.

2.6. Experimental animals

Juveniles of Pacific white leg shrimp, *L. vannamei* with an average weight of 5.0–6.0 g were used in the experiment. The animals were procured from M/s Sivagnanam Aquafarm, Kattur, Chennai, Tamil Nadu. The shrimps were transported in plastic Sintex™ tanks with sufficient aeration to the wet laboratory. They were carefully transferred to circular tanks of 2000 L capacity and vigorous aeration was provided. The stock was acclimatized under aerated conditions and fed with sinking diet containing 35% crude protein and 6% crude fat for a period of fortnight.

2.7. Experimental design and set-up

The experiment was conducted for a period of 28 days at the wet laboratory of Experimental Research Farm of ICAR-Central Institute of Brackishwater Aquaculture, Muttukadu, Kancheepuram District. There were 4 treatments namely, daily feeding of control diet alone (CD), daily feeding of immunostimulant diet alone (ID), alternate day feeding