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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

Table 1
Feed formula and proximate composition of feeds.

Ingredients	Immunostimulant diet (g kg^{-1})	Control (g kg^{-1})
Soybean meal[1]	280.00	280.00
Fish meal[2]	160.00	160.00
Acetes meal[3]	100.00	100.00
Wheat gluten[4]	30.00	30.00
Ground nut oil cake[5]	30.00	30.00
Sunflower oil cake[6]	16.00	16.00
Wheat flour[7]	170.00	170.00
Rice flour[8]	110.00	110.00
DORB[9]	20.00	30.00
Fish oil[10]	10.00	10.00
Soy lecithin[11]	10.00	10.00
BHT[12]	1.00	1.00
Chitin[13]	2.00	2.00
Cholesterol[14]	1.50	1.50
Choline chloride[15]	2.50	2.50
Vitamin C[16]	2.00	2.00
Binder[17]	10.00	10.00
Vitamin and mineral[1]	25.0	25.0
Jamun leaf powder[19]	10.00	0.00
Proximate composition (as is basis)		
Crude protein (%)	35.18 ± 0.67	35.09 ± 0.43
Crude fat (%)	7.74 ± 0.22	7.61 ± 0.27
Crude fibre (%)	4.40 ± 0.19	4.53 ± 0.18
Total ash (%)	9.06 ± 0.21	9.11 ± 0.17
Moisture (%)	10.64 ± 0.51	10.78 ± 0.40
NFE (%)	32.98 ± 0.73	32.88 ± 0.66
DE (kcal g^{-1})	3.423 ± 0.16	3.403 ± 0.13

NFE-nitrogen free extract; DE-digestible energy.
[1]Growel Feeds Pvt. Ltd., Gudivada, Andhra Pradesh.
[2&10]Raj Fishmeal and Oil Co., Malpe, Mangalore.
[3]KhajaMohideen, Wall Tax Road, Chennai.
[4]Viveka Essence Mart, Wall Tax Road, Chennai.
[5–9]From the local market, Chennai.
[11–15]Hi-Media, Mumbai.
[16]Stay – C from DSM Nutritional Technologies, Mumbai.
[17]Bentoli AgriNutrition India Pvt. Ltd., Chennai.
[18]Agrimin from Vibrac Healthcare India, Pvt. Ltd., Mumbai.
[19]Collected in CIBA campus, Chennai.

of control and immunostimulant diet (CD/ID) and two days control diet and one day immunostimulant diet feeding (2CD/ID). Each treatment was with 3 replicates following completely randomized design. Total experiment was conducted on 12 numbers of circular FRP tanks with 1 m diameter and 500 L water holding capacity. Twenty shrimps with initial weight ranging from 5.0–6.0 g were stocked in each tank in sea water with 30‰ salinity. The light and dark regimen was maintained at 11 and 13 h respectively (11L:13D) during the experiment. The total volume of the water in each tank was maintained uniformly throughout the experimental period. Round the clock aeration was provided. The uneaten feed pellets if any, and faecal matter were removed by siphoning twice in a day. Water quality parameters viz. temperature, pH, dissolved oxygen, free carbon dioxide, total hardness, ammonia, nitrite and nitrate were recorded during the experimental period.

2.8. Formulation and preparation of experimental diets

There were 2 practical diets prepared with the crude protein and lipid levels at 35% and 6% respectively. One diet contained 1% SCLP as an immunostimulant (ID) and the other diet without immunostimulant (i.e.) control diet (CD) (Table 1). The experimental diets were prepared using the following ingredients such as soy bean meal, fish meal, acetes meal, wheat gluten, groundnut oil cake, sunflower oil cake, wheat flour, de-oiled rice bran, fish oil, soy lecithin, binder and other additives. All the ingredients were weighed properly as per the requirement and were kept in a big plastic container. Water was added to make the dough of ingredients and the dough was transferred to an aluminum container, which was then placed in an autoclave for cooking/steaming. The steaming was done for 20 min. The cooked dough was then removed from the autoclave and kept aside for cooling. When the steamed dough was completely cooled, vitamin C and butylated hydroxytoluene were added so as to avert their loss during steaming. After incorporation of these elements, the dough was mixed properly and was pressed through the die of a motor driven pelletizer to get uniform sized pellets, which were spread on trays of oven and kept in hot air oven overnight for complete drying at 45–50 °C. After drying, the pellets were packed in polythene bags and were sealed airtight and labeled according to the treatments. Proximate analysis of feed and feed ingredients was performed as per the prescribed method of AOAC (1995) in the Nutrition Laboratory of ICAR-CIBA.

2.9. Growth and survival

The Pacific white leg shrimp juveniles were fed to apparent satiation. The daily ration was offered in three frequencies at 09:00, 13:00 and 17:00 h. The body weight of the shrimps from each replicate was measured at an interval of fifteen days to assess the growth. The experimental shrimps were starved overnight before taking the body weight and weighed at a precision of 0.1 g using electronic balance. At the end of the experiment, the shrimps from each treatment were collected and the numbers were counted. The survival rate (%) was calculated.

2.10. Haemolymph collection and tissue homogenate preparation

After completion of the feeding experiment, the shrimps from each treatment were sampled and sacrificed for biochemical and immunological analysis. The haemolymph was collected from experimental shrimps using 1 mL Tuberculin syringe with 26-gauge needle by inserting it into the ventral sinus located at the base of the first abdominal segment. Haemolymph was collected in 1:1 ratio of anticoagulant (30 mM trisodium citrate, 338 mM sodium chloride, 115 mM glucose, 10 mM EDTA, pH 7.0) in the syringe (Le Moullac et al., 1997). After haemolymph collection the shrimps were used for tissue collection for the enzymological assays. The tissues such as gill, muscle and hepatopancreas were collected from the shrimps of each treatment and were weighed and homogenized with 0.25 M chilled sucrose solution in a glass tube using teflon coated mechanical tissue homogenizer. The tube was kept in ice to avoid heating during homogenization. The homogenate was centrifuged at 10000 rpm for 10 min at 4 °C in a refrigerated centrifuge. The supernatant was stored at −20 °C until use. A 5% homogenate was prepared for the tissues of interest for the assay of enzymes.

2.11. Estimation of vitamin C and E

Vitamin C was estimated by the colorimetric method of Roe and Kuether (1943). Vitamin C level was expressed as µmol mg protein^{-1}. Vitamin E was estimated based on the method of Baker et al. (1980). The vitamin E content from the tissue sample was expressed as µmol mg protein^{-1}.

2.12. Superoxide dismutase (SOD) and catalase (CAT)

Superoxide dismutase was assayed according to the method described by Misra and Fridovich (1972) based on the oxidation of epinephrine–adrenochrome transition by the enzyme. One unit of SOD activity was the amount of protein required to give 50% inhibition of epinephrine auto oxidation. Catalase was assayed according to the method described by Takahara et al. (1960). Enzyme activity was expressed as nmol H_2O_2 decomposed min^{-1} mg protein^{-1}.

Table 2
Antioxidant assays of different herbal extracts.

Herb spp.	DPPH (inhibition %)	FRAP (μmol Fe(II)/g dry wt)	Total phenolic (mg phenols/100 g)
Phyllanthus acidus	$25.39^{c} \pm 2.83$	$89.04^{ab} \pm 4.45$	$38.41^{a} \pm 3.04$
Tephrosia purpurea	$52.24^{d} \pm 2.74$	$144.46^{d} \pm 12.15$	$139.01^{b} \pm 11.00$
Murraya koenigii	$53.12^{d} \pm 4.25$	$181.62^{e} \pm 10.16$	$171.42^{c} \pm 14.43$
Moringa oleifera	$10.74^{ab} \pm 0.79$	$93.79^{c} \pm 7.45$	$75.66^{ab} \pm 6.80$
Cassia auriculata	$82.10^{e} \pm 4.98$	$303.75^{f} \pm 23.19$	$316.42^{d} \pm 27.29$
Sesbania grandiflora	$7.35^{ab} \pm 0.71$	$67.62^{abc} \pm 4.57$	$31.08^{a} \pm 2.71$
Syzygium cumini	$86.31^{e} \pm 5.25$	$369.29^{g} \pm 23.60$	$429.50^{e} \pm 35.34$
Ficus religiosa	$7.69^{ab} \pm 0.57$	$70.20^{abc} \pm 6.48$	$37.25^{a} \pm 3.06$
Solanum nigrum	$6.19^{ab} \pm 0.35$	$58.45^{ab} \pm 3.39$	$19.50^{a} \pm 0.78$
Cassia angustifolia	$12.53^{b} \pm 1.21$	$82.20^{bc} \pm 5.40$	$51.58^{ab} \pm 2.95$
Acalypha indica	$2.99^{a} \pm 0.31$	$40.29^{a} \pm 1.31$	$8.66^{a} \pm 0.73$

Mean values in the same column with different superscript (a, b, c, d) differ significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.
Concentration of herbal extract is 50 μg mL^{-1}.

2.13. Aspartate aminotransferase (AST) and alanine amino transferase (ALT)

The AST activity was assayed in different tissue homogenates as described by Wootton (1964). The procedure adopted for ALT activity was same as for AST activity except the substrate, 0.2 M D,L-alanine instead of aspartic acid.

2.14. Prophenol oxidase assay (ProPO), respiratory burst, lysozyme activity and phagocytic activity

ProPO assay was carried out in the haemolymph drawn from the shrimps by the modified method of LeMoullac et al. (1997). The ProPO activity was expressed as the changes in absorbance min^{-1} mg $protein^{-1}$. The respiratory burst activity of haemolymph (1:1 anticoagulant) was estimated by Nitroblue tetrazolium assay which was done by the method of Stasiack and Bauman (1996). Haemolymph lysozyme activity was carried out by the method of Parry et al. (1965). The activity was expressed as U min^{-1} mg $protein^{-1}$. Phagocytic cells were detected by using *Staphylococcus aureus* as described by Anderson and Siwicki (1995). Phagocytic activity was expressed as the number of phagocytising cells divided by the total number of phagocytes counted.

2.15. Total haemocyte count and granular haemocyte count

The haemolymph from unchallenged and challenged shrimp was stored in Eppendorf tubes with 100 μL anticoagulant at 4 °C. Fixed haemolymph was diluted 2, 4, 8, 16, and 32 times with ice-cold PBS (20 mM, pH 7.2) and total haemocyte counts (THC mL^{-1}) were done using a haemocytometer (Le Moullac et al., 1997) in the di1utions with light microscope. The granular haemocyte count was performed according to the method of Sritunyalucksana et al. (2005). The proportion of granular haemocyte in 200 haemocytes was recorded and this portion was calculated as granular haemocyte count.

2.16. Haemolymph biochemical profile

The total protein of haemolymph was determined by the Biuret method (Reinhold, 1953) using diagnostic kit (Nice chemical Pvt. Ltd., Cochin, India). Bovine serum albumin was used as the standard protein and the absorbance was measured at 530 nm using a spectrophotometer. Albumin was estimated by Bromocresol green (BCG) binding method (Doumas et al., 1971) using diagnostic kit. Albumin in a buffered medium binds BCG and produces a green color whose absorbance is proportional to the albumin concentration. The absorbance of standard (S) and test (T) were measured immediately against blank (B) in a spectrophotometer at 620 nm. Globulin was calculated by subtracting albumin values from total plasma protein. A/G ratio was calculated by dividing albumin values by globulin values.

2.17. Challenge study and percentage of survival

After 28 days of feeding experiment, shrimps from each experimental group were challenged with virulent strain of *V. parahaemolyticus* isolated from the hepatopancreas of the *L. vannamei* obtained from Aquatic Animal Health and Environment Division, CIBA, Chennai, India. The virulent pathogenic isolates of *V. parahaemolyticus* were grown in nutrient broth for 24 h at 37 °C in a BOD incubator and harvested by centrifuging the culture broth at 4000 rpm for 10 min at 4 °C. The cells were then washed thrice in sterile PBS (pH 7.2) and finally maintained in PBS at a base line dosage of 10^{7} CFU mL^{-1}. The shrimp in each experimental group were injected 0.1 mL of bacterial suspension. The challenge experiment was conducted in accord with previous studies (Phuoc et al., 2008; Phuoc et al., 2009; Vieira et al., 2010; Xia et al., 2015). In the present study experimental shrimps were challenged through injection rather immersion because injection is the better route to evoke infection (Xia et al., 2015). Mortality was observed for 5 days. The percentage of survival in different treatment groups was calculated that was the routine method for the estimation of infection (Phuoc et al., 2009; Xia et al., 2015).

2.18. Statistical analysis

The data were statistically analyzed by statistical package SPSS version 16 in which data were subjected to one-way ANOVA and Duncan's multiple range tests was used to determine the significant differences between the means. The mean values for pre- and post-challenge parameters were compared by Student's *t*-test. Comparisons were made at 5% probability level.

3. Results

3.1. Antioxidant activity

The antioxidant activities of all the herbal extracts are given in Table 2. The total phenolics, FRAP and DPPH assays for antioxidant activity of different herbals extracts differs significantly ($P < 0.05$). The maximum total phenolic, FRAP and DPPH activity was noticed in *Syzygium cumini* extract followed by *Cassia auriculata* extract and the minimum activity of all the three assays were observed in *Acalypha indica* extract.

3.2. Antibacterial activity

The MIC and MBC of different herbal extracts are given in Table 3. The herbal extracts showed better MBC and MIC against the gram

Table 3
MBC (μg mL^{-1}) and MIC (μg mL^{-1}) of different herbal extracts against gram positive and negative bacteria.

Bacteria	*Vibrio parahaemolyticus*		*Staphylococcus aureus*		*Aeromonas hydrophila*	
Herb spp.	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})	MIC (μg mL^{-1})	MBC (μg mL^{-1})
Phyllanthus acidus	25.00	50.00	12.50	25.00	12.50	25.00
Tephrosia purpurea	12.50	50.00	6.25	25.00	25.00	50.00
Murraya koenigii	6.25	25.00	3.12	6.25	6.25	12.50
Moringa oleifera	25.00	50.00	6.25	25.00	12.50	50.00
Cassia auriculata	3.12	6.25	1.56	3.12	3.12	6.25
Sesbania grandiflora	50.00	100.00	12.50	50.00	50.00	100.00
Syzygium cumini	3.12	6.25	0.78	1.56	1.56	6.25
Ficus religiosa	25.00	100.00	6.25	12.50	25.00	50.00
Solanum nigrum	50.00	100.00	12.50	50.00	25.00	50.00
Cassia angustifolia	50.00	100.00	25.00	50.00	50.00	100.00
Acalypha indica	50.00	100.00	12.50	50.00	25.00	50.00

MIC - minimum inhibitory concentration.
MBC - minimum bacterial concentration.
Concentration of herbal extract is 50 μg mL^{-1}.

positive bacteria, *Staphylococcus aureus* than the gram negative bacterial strains, *Vibrio parahaemolyticus and Aeromonas hydrophila*. Over all, *Syzygium cumini* and *Cassia auriculata* showed better MBC and MIC than any other extract, whereas *Acalypha indica* exhibited poor MBC and MIC among the all extracts.

3.3. Water quality parameters

The physico-chemical parameters of water were within the optimum range during the entire period of culture. The range of all the physico-chemical parameters were as follows: dissolved oxygen 6.0–6.3 mg L^{-1}, temperature 25.9–27.7 °C, pH 8.1–8.3, ammonia nitrogen 0.05–0.15 mg L^{-1}, nitrate nitrogen 0.81–1.29 mg L^{-1}, nitrite nitrogen 0.074–0.083 mg L^{-1} and salinity 30–32‰ was present throughout the experimental period.

3.4. Growth parameters

The growth related parameters such as weight gain percentage, SGR, FCR, PER and FER are given in Table 4. The weight gain percentage was found to be significantly different ($P < 0.05$) among the various treatment groups but no significance exists between ID and CD/ID groups. Highest weight gain percentage was recorded in ID group, followed by CD/ID group. Though the SGR of the different treatments differ significantly ($P < 0.05$), there was no significant difference present between ID and CD/ID groups. The lowest SGR value was found in CD group and the maximum SGR was observed in ID group. The FCR of different treatments varied significantly ($P < 0.05$). The lowest and best FCR was recorded in ID group and the highest and poor FCR was found in CD group which was significantly different from other group. The mean PER value was significantly different ($P < 0.05$) among the different treatment groups. The highest PER value was recorded in ID group and the least PER value was found in CD. The FER value of different treatment groups varied significantly ($P < 0.05$) with the highest in the ID which was not significantly different from CD/ID group and the lowest value was noticed in CD group.

3.5. Non-enzymatic antioxidants

The vitamin C and E level in the hepatopancreas and muscle tissues of *L. vannamei* before and after challenge with *V. parahaemolyticus* is given Fig. 1. The vitamin C and E levels in both tissues differ significantly ($P < 0.05$) in pre-challenge as well as post-challenge. The level of vitamin C and E was higher in hepatopancreas. The vitamin C and E level was getting decreased after challenge with *V. parahaemolyticus*. The level of vitamin C and E was found highest in ID group and lowest in CD group both in before and after artificial infection. There was a significant difference ($P < 0.05$) between pre- and post-challenge vitamin C and E levels in all treatments.

Table 4
The growth performance of *L. vannamei* fed with different experimental diets.

Treatment	Weight gain (%)	SGR (%)	FCR	PER	FER	Survival (%)
CD	$54.12^{a} \pm 1.90$	$0.96^{a} \pm 0.04$	$2.60^{c} \pm 0.15$	$1.09^{a} \pm 0.07$	$0.39^{a} \pm 0.02$	78.33 ± 1.90
ID	$78.17^{c} \pm 2.98$	$1.28^{c} \pm 0.05$	$2.14^{a} \pm 0.14$	$1.33^{c} \pm 0.06$	$0.46^{c} \pm 0.01$	88.33 ± 2.98
CD/ID	$74.49^{c} \pm 3.73$	$1.23^{c} \pm 0.05$	$2.23^{ab} \pm 0.11$	$1.27^{bc} \pm 0.05$	$0.45^{bc} \pm 0.01$	90.00 ± 3.73
2CD/ID	$61.85^{b} \pm 1.74$	$1.06^{b} \pm 0.03$	$2.38^{b} \pm 0.13$	$1.20^{b} \pm 0.08$	$0.42^{b} \pm 0.02$	85.00 ± 1.74
P value	0.001	0.001	0.001	0.001	0.02	0.293

Mean values in the same column with different superscript (a, b, c) differ significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.

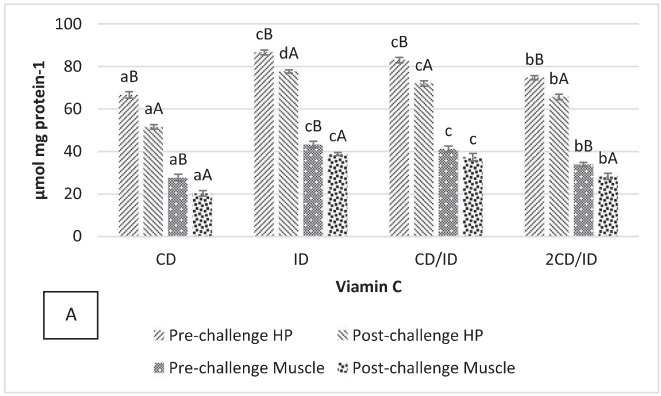
$$\text{Weight gain (\%)} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100.$$

$$\text{Specific Growth Rate (SGR)\%} = \frac{\text{Log}_e\text{final weight} - \text{Log}_e\text{initial weight}}{\text{Number of days}} \times 100.$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Feed consumption (dry weight)}}{\text{Body weight gain (wet weight)}}.$$

$$\text{Protein Efficiency Ratio (PER)} = \frac{\text{Net weight gain (wet weight)}}{\text{Protein fed (dry weight)}}.$$

$$\text{Survival (\%)} = \frac{\text{Total number of fish harvested}}{\text{Total number of fish stocked}} \times 100.$$



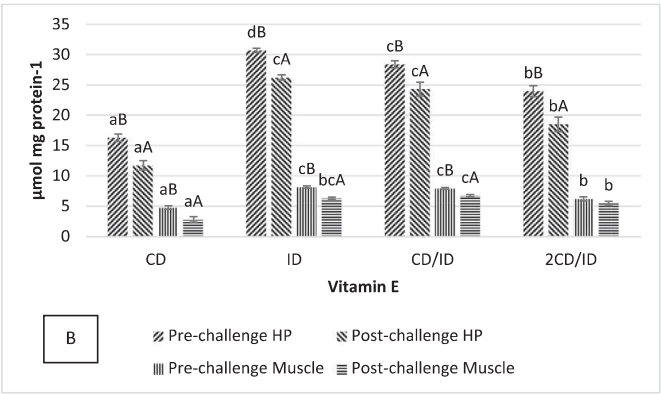


Fig. 1. Vitamin C (1A) and E (1B) level in hepatopancreas and muscle tissues of *L. vannamei* before and after artificial infection.
Mean values of similar pattern bar with different superscript (a, b) differ significantly ($P < 0.05$).
Mean values in the experimental groups with different superscript (A, B) between pre- and post-challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3; HP-Hepatopancreas.

3.6. Enzymatic antioxidants

Among the different treatment groups, the activity of SOD enzyme in hepatopancreas, gill and haemolymph were significantly ($P < 0.05$) different in pre- and post-challenge condition (Table 5). Highest SOD activity was observed in hepatopancreas followed by gill and serum. In hepatopancreas and gill samples, the highest SOD activity was found in CD/ID group and lowest activity was recorded in control (CD) group in both pre-and post-challenged status. During post-challenge in all the treatments with respective of hepatopancreas, gill and haemolymph, the activity of SOD enzyme notably increased. There was a significant difference ($P < 0.05$) between pre- and post-challenge serum SOD enzyme activity in all treatments. During pre- and post-challenge the activity of hepatopancreas, gill and haemolymph CAT activity varied significantly ($P < 0.05$) among the different treatments (Table 5). CAT also followed the same trend as of SOD in hepatopancreas, gill and haemolymph in both pre- and post-challenge condition. There was no significant difference in the activity of both enzymes in all the three samples in ID and CD/ID treatments during pre- and post-challenge condition.

3.7. Haemolymph respiratory burst, prophenol oxidase, lysozyme and phagocytic activity

A significant difference was observed in the haemolymph respiratory burst, prophenol oxidase, lysozyme (Fig. 2) and phagocytic activity (Fig. 3A) of shrimps in different treatments both in pre- and post-challenge groups. Highest prophenol oxidase and respiratory burst activity was observed in alternate day feeding of control and immunostimulant incorporated diet (CD/ID) and the lowest value was recorded in control group (CD). The value of prophenol oxidase and respiratory burst was significantly different between pre- and post-challenged groups of all the treatment groups ($P < 0.05$). The maximum and minimum value of haemolymph lysozyme and phagocytic activity were observed in ID and CD groups respectively, in both pre- and post-challenge status. While comparing the pre-challenged with post-challenge group, the values of haemolymph prophenol oxidase, respiratory burst, lysozyme activity and phagocytic activity were in increasing trend in post-challenge condition.

3.8. AST and ALT

The activities of AST and ALT enzymes in hepatopancreas and muscle showed significant ($P < 0.05$) difference among the treatment group and are given in the Table 6. Hepatopancreas tissue showed better activity of AST and ALT than muscle tissue. In both tissues, the highest activity of AST and ALT enzyme was observed in CD group and the lowest activity was noticed in ID groups. In both tissues AST was found higher than ALT enzyme. While compare with pre-challenge condition, the post-challenge activity of AST and ALT were higher in

Table 5
The level of enzymatic antioxidants in different treatments before and after artificial infection.

Enzyme	Condition	Tissue	CD	ID	CD/ID	2CD/ID	P value
SOD	Pre-challenge	HP	$64.93^{a} \pm 1.69$	$87.38^{c} \pm 2.89$	$90.13^{c} \pm 2.61$	$73.04^{b} \pm 1.86$	0.001
	Post-challenge	HP	$59.82^{a} \pm 1.14$	$93.51^{c} \pm 2.10$	$94.34^{a} \pm 2.14$	$73.29^{b} \pm 1.01$	0.001
	Pre-challenge	Gill	$22.35^{aA} \pm 1.46$	$33.37^{bc} \pm 1.96$	$33.93^{c} \pm 2.49$	$27.25^{abA} \pm 1.40$	0.007
	Post-challenge	Gill	$28.67^{aB} \pm 0.41$	$39.08^{d} \pm 0.86$	$35.95^{c} \pm 1.07$	$32.10^{bB} \pm 0.66$	0.001
	Pre-challenge	HL	$4.63^{a} \pm 0.39$	$12.18^{b} \pm 0.73$	$11.60^{bA} \pm 0.85$	$6.98^{aA} \pm 0.15$	0.001
	Post-challenge	HL	$4.38^{a} \pm 0.21$	$14.19^{c} \pm 0.70$	$16.88^{cB} \pm 0.55$	$10.36^{bB} \pm 0.59$	0.004
Catalase	Pre-challenge	HP	$4.64^{a} \pm 0.22$	$12.42^{b} \pm 0.70$	$12.87^{b} \pm 0.57$	$8.13^{aA} \pm 0.33$	0.001
	Post-challenge	HP	$4.30^{a} \pm 0.34$	$15.31^{c} \pm 1.21$	$16.96^{c} \pm 0.90$	$10.36^{bB} \pm 0.30$	0.001
	Pre-challenge	Gill	$2.40^{a} \pm 0.25$	$4.84^{cA} \pm 0.20$	$4.64^{cA} \pm 0.32$	$3.15^{bA} \pm 0.09$	0.001
	Post-challenge	Gill	$2.23^{a} \pm 0.11$	$6.06^{cB} \pm 0.26$	$5.89^{cB} \pm 0.28$	$3.97^{bB} \pm 0.10$	0.001
	Pre-challenge	HL	$1.43^{a} \pm 0.09$	$3.08^{bA} \pm 0.10$	$3.41^{b} \pm 0.21$	$1.89^{a} \pm 0.11$	0.004
	Post-challenge	HL	$1.97^{a} \pm 0.06$	$4.11^{bB} \pm 0.17$	$4.62^{b} \pm 0.41$	$2.19^{a} \pm 0.14$	0.001

SOD specific activity is expressed in 50% inhibition of epinephrine auto oxidation mg^{-1} protein min^{-1}.
Catalase specific activity is expressed in nmol H_2O_2 decomposed min^{-1} mg^{-1} of protein.
Mean values in the same row with different superscript (a, b, c) differ significantly ($P < 0.05$).
Mean values in the experimental groups with different superscript (A, B) between pre- and post-challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.
HP-Hepatopancreas; HL-Haemolymph.

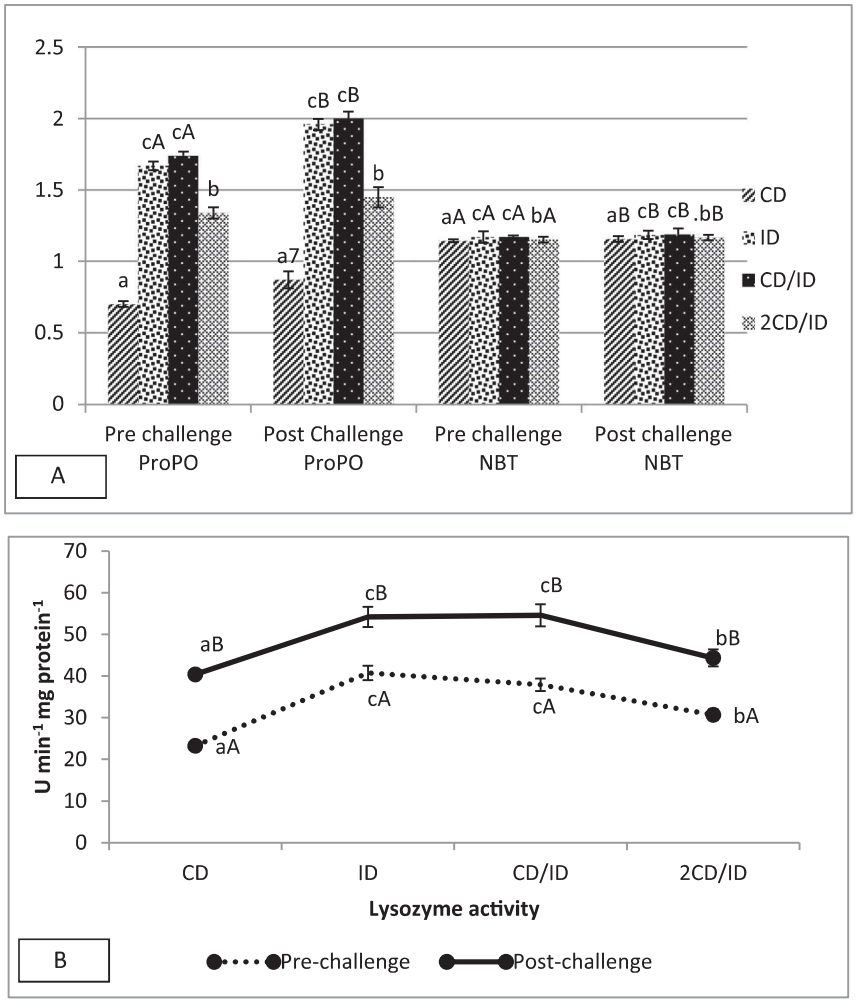


Fig. 2. A: Prophenol oxidase and respiratory burst activities of *L. vannamei* before and after artificial infection.
Mean values in the bars of different parameters with different superscript (a, b) differ significantly ($P < 0.05$).
Mean values in the experimental groups with different superscript (A,B) between pre- and post-challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.
B: Haemolymph lysozyme activity of *L. vannamei* fed with SCLP incorporated diet, before and after artificial infection.
Mean values of line with different superscript (a, b) differ significantly ($P < 0.05$).
Mean values in the experimental groups with different superscript (A, B) between pre- and post-challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.

both tissues.

3.9. Haemolymph biochemical profile

The pre- and post-challenge haemolymph protein, albumin, globulin content and A:G ratio of different treatments were significantly different ($P < 0.05$) among themselves except pre-challenge albumin (Table 7). The highest and lowest protein and globulin levels were found in ID and CD treatments respectively, in both pre- and post-challenge condition. The A:G ratio was significantly different ($P < 0.05$) in both conditions and best A:G ratio was noticed in ID group and the least one in control group both in before and after challenge with *V. parahaemolyticus*. While comparing the pre-challenge group with the post-challenge group, the values of total protein, globulin and A:G ratio were in decreasing trend, whereas the albumin exhibited opposite trend in post-challenge condition but no trend in pre-challenge condition.

3.10. Haematological parameters

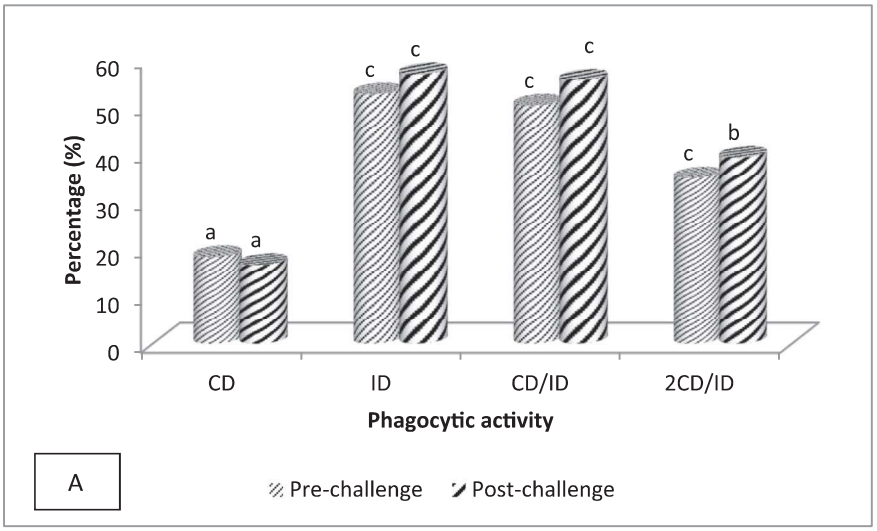
Dietary SCLP had significant ($P < 0.05$) effect on total haemocyte count and granular haemocyte count both in pre- and post-challenge condition in *L. vannamei* (Fig. 3B). The highest and lowest THC and GHC were observed in ID group and control group (CD) both in pre- and post-challenge condition. While comparing the values of pre-challenge THC and GHC with post-challenge condition, the values were in decreasing trend. There was significant difference ($P < 0.05$) found among the various treatment groups in both pre- and post-challenge condition for THC and GHC.

3.11. Disease resistance

The mortality was observed up to 5 days from post-challenge. Percentage of survival (%) of *L. vannamei* after challenging with virulent strain of *V. parahaemolyticus* in different experimental groups was represented in Fig. 4. There was significant difference between the survival rate of control (CD) and SCLP incorporated diet fed groups ($P < 0.05$). But there was no significant statistical difference observed among the ID and CD/ID groups. The least survival was recorded in control (CD) group whereas highest survival was noticed in ID group.

4. Discussion

Based on the in vitro examination of antioxidant and antimicrobial properties, *Syzygium cumini* leaf powder was selected as an immunostimulant to incorporate in the diet of *L. vannamei*. Since early 2000, in most of the Asian countries culture of *L. vannamei* has been taken up from the monotonous culture of black tiger prawn. *L. vannamei* is preferred over *P. monodon* mainly because of its tolerance to wide range of salinity, temperature, omnivorous feeding habits, less protein requirement, availability of SPF/SPR seeds and high stocking density. *L. vannamei* is being cultured in ultra-high densities under intensive management, which might lead them to highly susceptible to several bacterial diseases particularly vibriosis and early mortality syndrome which is caused by the virulent strains of *Vibrio parahaemolyticus*. Averting the bacterial infectious diseases can be possibly achieved by



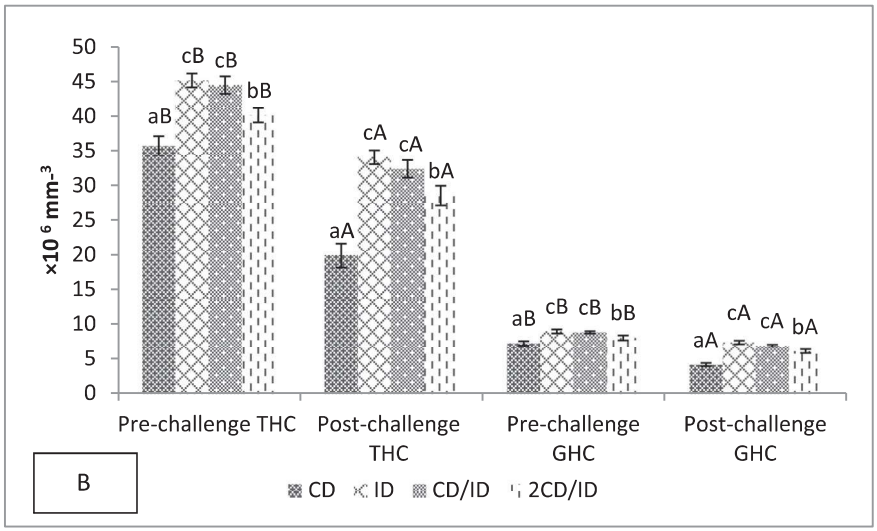


Fig. 3. A: Haemolymph phagocytic activity of *L. vannamei* fed with SCLP incorporated diet, before and after artificial infection. Mean values of the similar pattern bars with different superscript (a, b) differ significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.
B: The total haemocyte count (THC) and granular haemocyte count (GHC) of *L. vannamei* before and after artificial infection. Mean values in the bars of same parameter with different superscript (a, b) differ significantly ($P < 0.05$).
Mean values in the experimental groups with different superscript (A, B) between pre- and post-challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.

immunostimulant feeding regimes in the shrimp culture. In this context the potent plant based natural immunostimulant, *Syzygium cumini* leaf powder was selected to prevent *V. parahaemolyticus* infection. Further, to optimize the duration of immunostimulant feeding, different feeding frequencies were trialed to determine its required frequencies of immunostimulatory diet feeding for better performance.

In the present study, *L. vannamei* fed with dietary immunostimulant showed improved growth performance (weight gain percentage (%), specific growth rate (%) and protein efficiency ratio) in all treatment groups compared to control group. This is in agreement with the results of Yogeeswaran et al. (2012) in *P. monodon* fed with *Cynodon dactylon* as immunostimulant. Similarly, Mona et al. (2015) found better growth rate and specific growth in *Procambarus clarkii* fed with *Cynodon dactylon*. The higher growth may be because of improved digestion,

Table 6
The activity of tissue level AST and ALT enzymes of *L. vannamei* in different treatments before and after artificial infection.

Treatment	AST				ALT			
	Pre-challenge		Post-challenge		Pre-challenge		Post-challenge	
	Muscle	HP	Muscle	HP	Muscle	HP	Muscle	HP
CD	11.80^{cA} ± 0.83	25.14^{cA} ± 1.01	15.42^{cB} ± 0.62	32.78^{cB} ± 0.84	8.43^{cA} ± 0.60	22.24^{bA} ± 1.20	11.43^{cB} ± 0.64	30.50^{cB} ± 0.61
ID	7.54^{a} ± 0.47	17.17^{a} ± 0.95	8.05^{a} ± 0.51	20.48^{a} ± 0.78	5.25^{aA} ± 0.13	14.55^{a} ± 0.67	7.07^{abB} ± 0.42	18.00^{a} ± 1.12
CD/ID	9.55^{b} ± 0.46	18.52^{aA} ± 0.48	10.42^{b} ± 0.48	22.48^{aB} ± 0.45	5.79^{ab} ± 0.18	16.55^{aA} ± 0.41	6.48^{a} ± 0.26	19.49^{aB} ± 0.59
2CD/ID	9.79^{b} ± 0.30	21.32^{bA} ± 0.53	11.22^{b} ± 0.55	26.70^{bB} ± 0.59	6.74^{b} ± 0.29	19.94^{bA} ± 0.43	8.43^{b} ± 0.75	24.96^{bB} ± 1.06
P value	0.004	0.001	0.001	0.001	0.002	0.001	0.001	0.002

HP-Hepatopancreas.
AST is expressed as U min^{-1} mg $protein^{-1}$ and ALT is expressed as U min^{-1} mg $protein^{-1}$.
Mean values in the same column with different superscript (a, b) differ significantly ($P < 0.05$).
Mean values in the experimental groups with different superscript (A, B) between pre- and post-challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.

Table 7
The biochemical composition of haemolymph of *L. vannamei* before and after challenge with *V. parahaemolyticus*.

Treat	Total protein (mg mL^{-1})		Albumin (mg mL^{-1})		Globulin (mg mL^{-1})		A/G ratio	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
CD	$61.79^{aB} \pm 2.43$	$42.63^{aA} \pm 1.14$	$22.38^{B} \pm 1.09$	$12.04^{aA} \pm 0.70$	$39.41^{aB} \pm 2.14$	$30.59^{aA} \pm 1.67$	$0.57^{cB} \pm 0.03$	$0.40^{bA} \pm 0.02$
ID	$80.01^{cB} \pm 1.93$	$65.43^{cA} \pm 2.19$	$20.81^{B} \pm 0.96$	$14.55^{bA} \pm 0.74$	$59.19^{dB} \pm 3.24$	$50.88^{dA} \pm 2.09$	$0.35^{aB} \pm 0.01$	$0.29^{aA} \pm 0.01$
CD/ID	$75.56^{cB} \pm 2.36$	$62.68^{cA} \pm 1.99$	$21.32^{B} \pm 1.15$	$15.65^{bA} \pm 0.52$	$54.24^{cB} \pm 2.21$	$47.03^{cA} \pm 1.61$	$0.39^{aB} \pm 0.02$	$0.33^{aA} \pm 0.01$
2CD/ID	$71.34^{bB} \pm 3.29$	$57.85^{bA} \pm 1.77$	$22.42^{B} \pm 0.87$	$16.57^{bA} \pm 0.48$	$48.92^{bB} \pm 1.89$	$41.28^{bA} \pm 1.44$	$0.46^{b} \pm 0.02$	$0.38^{b} \pm 0.02$
P Value	0.001	0.001	0.147	0.010	0.001	0.003	0.001	0.003

Mean values in the same column with different superscript (a, b) differ significantly ($P < 0.05$).
Mean values in the experimental groups with different superscript (A, B) between pre- and post-challenge group under each treatment vary significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3; A/G ratio - Albumin:Globulin ratio.

$$\text{Protein (mg mL}^{-1}) = \frac{\text{Absorbance of Test (T)}}{\text{Absorbance of Standard (S)}} \times 5.$$

$$\text{Albumin (mg mL}^{-1}) = \frac{\text{Absorbance of Test (T)}}{\text{Absorbance of Standard (S)}} \times 5.$$

$$\text{Globulin (mg mL}^{-1}) = \text{Total protein (mg mL}^{-1}) - \text{Albumin (mg mL}^{-1}).$$

$$\text{A/G ratio} = \frac{\text{Albumin (mg mL}^{-1})}{\text{Globulin (mg mL}^{-1})}.$$

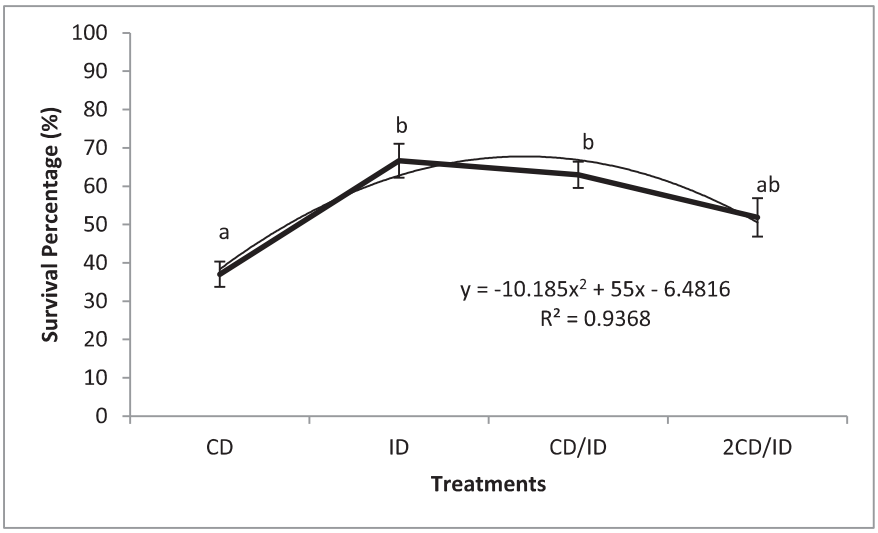


Fig. 4. Percentage of survival of *L. vannamei* after challenge with *V. parahaemolyticus*
The line diagram with different superscript (a, b) differ significantly ($P < 0.05$).
Data expressed as Mean ± SE n = 3.

$$\text{Survival \%} = \frac{\text{Number of shrimps survived after challenge}}{\text{Number of shrimps challenged}} \times 100.$$

pathogen resistance and nutrient absorption which have been translated into higher weight gain in the experimental animals. The SCLP incorporated diet showed improved feed intake and feed conversion efficiency due to better feed attraction and palatability in experimental shrimps because of the presence of essential oils. Correspondingly, Ching (2008) reported significantly higher growth, feed utilization and survival of shrimps fed with diets dietary oregano oil compared to control diet fed groups.

Vitamin E and C are the most important non-enzymatic antioxidants present in the biological system (Jha et al., 1995). Vitamin C being a reducing and antioxidant agent, involves in several biochemical reactions mainly reversible oxidation and reduction within the cells, and directly reacts with O_2^- and OH^- and various lipid hydroperoxides (Yu, 1994). Vitamin C plays an important role in the metabolism of α-tocopherol by reduction of α-tocopheroxyl radicals and regenerates them to α-tocopherol (Packer et al., 1979). In the present study, the immunostimulant fed groups showed better level of vitamin C in tissues of both pre- and post-challenge than the control group (CD). This result strongly indicates that supplementation of SCLP provides better stress resistance in the experimental animals and prevents its excessive utilization particularly in immunostimulant fed groups and hence, the availability of the tissue level vitamin C in immunostimulant fed groups was more and thus provides enhanced antioxidant status. Similarly, Radhakrishnan et al. (2014) revealed that post-larvae of *Macrobrachium rosenbergii* fed with diet containing *Spirulina platensis*, *Chlorella vulgaris* and *Azolla pinnata* showed significantly enhanced levels of vitamin C concentration in the tissues than the control group.

Vitamin E is an important antioxidant present in cell membranes and protects the membranes of cells and organelles from oxidation by reacting with free radicals (Sies and Murphy, 1991). Vitamin E can enhance specific and cell-mediated immunity against infection and macrophage phagocytosis in fishes (Wise et al., 1993). The present result showed higher level of vitamin E concentration in tissues of *L. vannamei* which indicates the protective role of vitamin E against peroxidation. Our findings are in line with Radhakrishnan et al. (2014) who have observed that post-larvae of giant freshwater prawn, *M. rosenbergii* fed with diet containing *S. platensis*, *C. vulgaris* and *A. pinnata* showed significantly increased levels of vitamin E concentration in the hepatopancreas and muscle tissue when compared with the control group.

Superoxide dismutase (SOD) is an enzymatic antioxidant, which specifically scavenge superoxide radicals and hence involves in the protective mechanisms of tissue through oxidative process and phagocytosis. Considerable raise in the superoxide anion activity is considered to be favorable with respect to increased immunity in the host but too much hike may be detrimental (Cheng and Wang, 2001). In the present study, increased level of SOD in the SCLP fed experiment groups than the control group indicates that SCLP incorporation improves the

free radical scavenging activity of the host through adequate synthesis of SOD enzyme. Several research findings support the present result. Chen et al. (2014) revealed that dietary *Petalonia binghamiae* extract in *L. vannamei* enhances the SOD activity and protect the host against *Vibrio alginolyticus*. Mona et al. (2015) studied the protective effect of *Cynodon dactylon* in *Procambarus clarkii* and found that at 3% level it improves the SOD level after 6 weeks of administration and progress the immunity of the host.

Catalase is another antioxidant enzyme, which reduces H_2O_2 to water and oxygen and thus protects the cells from oxidative damage caused by reactive oxygen species (ROS) (Atli et al., 2006). In the present study, catalase activity was higher in SCLP fed treatment groups than the control group in pre- as well as post-challenge. This is in agreement with the results of Pacheco et al. (2011) who studied the elevation of catalase activity in brown shrimp, *Farfantepenaeus californiensis* fed with various immunostimulants and challenged with White Spot Syndrome Virus (WSSV).

The haemolymph transaminases mainly aspartate aminotransferase and alanine aminotransferase levels were found to vary based on the health status, environmental condition, stress, pollution and disease infection (Mohankumar and Ramasamy, 2006). Generally, stress condition and disease will elevate the AST and ALT levels. In the present study, the AST and ALT activity in the SCLP fed experimental groups showed lower level than the control group during pre- and post-challenge. Similarly, the result of Fouzi et al. (2012) supports the present results during post-challenge where the AST and ALT levels were elevated than the pre-injected condition in *P. monodon* injected with WSSV.

Haemolymph protein is linked with a potent innate immune response and is vital component for sustaining the function of healthy innate immune system of crustaceans (Vazquez et al., 2009). In the current experiment, the shrimps fed with SCLP showed better total protein, albumin and globulin composition than the control group and during post-challenge all the groups were represented with lower level of total protein than their pre-challenge counter parts. The present result is in line with Mathew et al. (2009) who found similar effect in *P. monodon* artificially infected with WSSV. Comparable to this result, Fouzi et al. (2012) noted lower level of haemolymph total protein content in *P. monodon* after challenge with WSSV. The increased level of globulin in the present study indicated that SCLP fed shrimps are having improved immune protection than the control groups which was also supported by augmented level of phagocytic activity and percentage of survival.

Prophenol oxidase (ProPO) is a modified form of complementary system and major innate immune system exists in invertebrates to provide defense against microbial pathogens through encapsulation, melanization and function as a non-self recognition system (Beck and Habicht, 1996). According to Yeh et al. (2009) proPO was found to be involved in acute phase immune defense against *V. alginolyticus* in *L. vannamei*. In the present study, proPO activity in SCLP fed experimental groups showed higher activity than the control group in both pre-challenge and post-challenge. This is in line with the results of Yogeeswaran et al. (2012) who found that *P. monodon* fed with herbal immunostimulants showed higher proPO activity than the control groups in different period of study. Correspondingly, in *Fenneropenaeus chinensis*, the expression of proPO activity got increased after challenge with *V. anguillarum* (Gao et al., 2009). According to Fagutao et al. (2009), gene silencing of proPO in kuruma shrimp, *Marsupenaeus japonicus* showed increased bacterial counts in the haemolymph and increased mortality even without bacterial challenge. They have also found that proPO-depleted shrimp confirm lower haemocyte counts than control shrimps and along with notably down-regulated expressions of antimicrobial peptides including lysozyme.

Lysozyme being a cationic enzyme acts on β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in peptidoglycans of bacterial cell walls that enables lysozyme to lyse the invading bacterial pathogens (Alexander and Ingram, 1992). Lysozyme activity is revealed as a first line innate immune system in crustaceans, which results in disease prevention against the incursion of detrimental bacterial pathogens. The present result is in accord with the findings of Chandran et al. (2016) who reported that poly herbal formulations (contains extracts of *Ocimum sanctum*, *Withania somnifera*, *Tinospora cordifolia* and *Emblica officinalis*) enhances the lymphocyte lysozyme activity and in turn improves the immunity of *P. monodon*.

Phagocytosis is the most vital mechanism of crustacean defense system and phagocytic haemocytes are able to demolish invading particles and micro-organisms directly (Sritunyalucksana and Soderhall, 2000). Harikrishnan et al. (2011b) have reported that dietary administration of *Solanum nigrum* extract improves the non-specific immune response particularly phagocytic activity and disease resistance in *P. monodon* against *V. harveyi*. Correspondingly, in the present study phagocytic activity was higher in SCLP fed at different frequencies than the control group in pre- and post-challenge condition.

Respiratory burst is a process of intense oxygen consumption during phagocytosis in which leukocytes increase their oxygen consumption through NADPH oxidase and generate various ROS such as hydrogen peroxide (H_2O_2), superoxide anion radical (O_2^-), singlet oxygen ($^1O_2$) and the hydroxyl radical (OH^-). These ROS are highly toxic and thus develop the source of potent antibacterial system in the host (Klebanoff, 1999). These superoxide anions are the first product released during respiratory burst and hence, measurement of O_2^- released during this process is an accurate method for quantifying the intensity of a respiratory burst (Song and Hsieh, 1994). The present result is comparable with the results of Harikrishnan et al. (2011b) who have registered improved respiratory burst activity and resistance against *V. harveyi* in *P. monodon* fed with dietary *S. nigrum* extract than the control group.

In crustaceans, hardening of exoskeleton, clotting and purging of foreign bodies are taken care by haemocytes (Song and Hsieh, 1994). According to Cheng and Chen (2001) haemocyte abundance depends upon physiological conditions such as the process of ecdysis, reproduction and health as well as nutritional status of the shrimp. Endured stress and disease can reduce haemocyte count significantly. According to Mathew et al. (2009), apoptosis of haemocytes and its accumulation at the site of infection for phagocytosis of foreign materials and pathogens may be a strong reason for decline in THC level. In the present study, the THC count is higher in SCLP fed experimental shrimps than the control group and during post-challenge the THC got reduced than pre-challenge. This result is in agreement with Citarasu et al. (2006) who have reported that challenging the shrimp *P. monodon* with WSSV reduces the THC and immunostimulant fed shrimps showed higher THC than the control group. Rajasekar et al. (2011) found that *Cardiospermum halicacubum* extract at 1% level showed higher THC and protect the host from *V. parahaemolyticus*.

The experimental shrimps challenged with virulent strain of *V. parahaemolyticus* showed mortality in all the treatments and control group had more mortality than the SCLP fed experimental groups at different frequencies. Maximum survivorship was noticed in SCLP daily fed group (ID) which may be possibly due to better level of proPO, phagocytic, lysozyme, respiratory burst, SOD, catalase activity and tissue level non-enzymatic antioxidants. The present result is in line with the results of Harikrishnan et al. (2011b) who found that *P. monodon* fed with dietary *S. nigrum* showed more resistance against *V. harveyi* and significantly better survivorship than the control group.

5. Conclusion

The presence of active antibacterial compounds such as alkaloids, glycosides and polyphenolic compounds (gallic acid and ellagic acid) in the experimental feed containing 1% *Syzygium cumini* leaf powder enhanced the production of proPO, improved the function of macrophages, cytokine synthesis, increased respiratory burst activity, lysozyme activity and phagocytic activity in *L. vannamei*. It increases the

response of enzymatic and non-enzymatic antioxidants in the shrimp and protects them against the stressful condition and thus the host stays healthy. Therefore, it averts the infections caused by opportunistic pathogens especially effective against virulent strains of *V. parahaemolyticus* in Pacific white shrimp. Alternate day feeding of SCLP incorporated feed was proved to be as effective as daily feeding of SCLP incorporated diet. Hence, alternate day feeding of SCLP diet could be preferred over continuous feeding of SCLP diet in view of its cost effectiveness. Therefore, *Syzygium cumini* leaf powder can be used as a promising alternative for antibiotics due to its antibacterial, immune-modulating and growth promoting potency, economic viability and abundancy in nature. Further, in future it is necessary to determine the exact quantity of active principles of *Syzygium cumini* leaf powder in order to account for natural changes in its composition during different seasons and hence serve as useful inputs in effective application of aquafeed formulation.

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