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# Effect of Herbal Antibacterial Extracts on the Gut Floral Changes in Indian White Shrimp *Fenneropenaeus indicus*

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## Abstract

*Antibacterial methanolic extracts of Murraya koeniji, Psoralea corylifolia and Quercus infectoria were screened against the bacterial pathogens such as Pseudomonas aeruginosa, Staphylococcus aureus and Vibrio harveyi isolated from infected shrimp. Minimum Inhibitory Concentration (MIC) and Minimal Bacterial Concentration (MBC) were carried out against V. harveyi with three extracts and three different diets were prepared by supplementing equal proportion of the active fractions, namely Control (0), HD1 (500), HD2 (1000 mg/Kg). Diets were individually fed to the Fenneropenaeus indicus adults for 30 days. The survival, growth and bacterial resistance differed significantly (P<0.05) from the control group to experimental groups. Gut bacterial flora was analyzed qualitatively, quantitatively before and after feeding the herbal diets. The bacterial isolates of shrimp gut predominantly consisted of Aeromonas Sp; Photobacterium Sp; Proteus Sp; E.coli ; Salmonella Sp; Vibrio Sp and Yersinia Sp. After herbal diet treatment, gut floral changes were observed due to the antibacterial effects. The active principles of the herbal extracts suppressed the bacterial gut flora such as Salmonella sp., Proteus sp., Yersinia sp. and Aeromonas sp., etc.*

**Keywords:** Herbal extracts, antibacterial, *Fenneropenaeus indicus*, Gut flora

## Introduction

Bacterial diseases are the major problem affecting shrimp hatcheries and mass mortalities in shrimp hatchery are associated with luminous bacterial disease [1]. Vibriosis is one of the major diseases in shrimp aquaculture, [2] causing devastating mortality in the farm as well as in hatchery stage. *Vibrio harveyi* and *V. campbelli* have spread disease in shrimp larva [3] *V. penaeicida* and *V. parahaemolyticus* are affected in shrimp juveniles and adults [4].

Intestinal bacteria, such as *Aeromonas* and *Vibrio* sp often cause opportunistic pathogen [5]. Gut micro flora plays an important role in the digestive process, and disease susceptibility of marine feeders [6].

Oxley et al. [9] reported that the wild and cultured prawns (eg. *Fenneropenaeus merguiensis* (De Man, 1888)) harbor a diverse bacterial flora, which includes the dominant genera like *Aeromonas*, *Plesiomonas*, *Photobacterium, Pseudoalteromonas*, *Pseudomonas* and *Vibrio*. The similarity existing in the intestine bacterial flora of cultured prawns suggests the host specificity of intestinal microbial colonization. An understanding of the host intestinal bacterial floral interactions is of much significance for the development of a healthy cultivation environment and also to optimize the potential species growth.

Antibiotics are administered to farmed shrimp primarily to prevent the bacterial diseases [10]. Though antibiotics and several other chemicals give positive effects, they cannot be recommended due to their residual, expensive, resistance strain development in bacteria and other side effects. Recently, studies on antibiotic resistance were noticed in shrimp aquaculture. Baticados et al. [11] reported that luminous strains of *Vibrio harveyi* and *V. splendidus* isolated from shrimp larvae are resistant to erythromycin, kanamycin, pencicillin G, and streptomycin.

Plants are the storehouses and sources of safer and cheaper chemicals. Herbal preparations are prescribed to cure various diseases, as they contain active principles. The herbal preparations are known to have an important role in disease control due to their antioxidant and antimicrobiological activities [12]. The natural plant products have been used to promote the various activities like antistress, promoting growth, 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. [13, 14]. The present study focuses on screening and characterization of the selected Indian herbal extracts having antibacterial activity against the shrimp gut associated pathogens, and to study the different combinations of active extracts to enhance the immune system in the shrimp aquaculture system.

## Material and Method

**Antibacterial herbal extracts and Screening:** An antibacterial herb such as *Murraya koenigii, Psoralea corylifolia* and *Quercus infectoria* were extracted (15) using organic percolation extraction by methanol at 48 hrs. The filtered and condensed extracts were screened against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Vibrio harveyi* isolated from infected shrimps by agar disc diffusion method [16].

**MIC and MBC:** The Minimum inhibitory concentration (MIC) was determined as the lowest concentration, and the highest dilution, which completely inhibited the growth of bacteria. To determine the minimum bactericidal concentration (MBC), the two lowest concentrations which inhibited bacterial growth were plated out on a nutrient agar and incubated at 37 $^0$C for 24 hrs. The MIC and MBC were carried out against *V. harveyi* only.

**Gut Floral Studies of *F. indicus:*** Wild *F. indicus* weighing 10.0 ± 1 g were collected from the shrimp farm, Manakudy, Tamilnadu, India and brought to the laboratory, acclimatized to laboratory condition and stocked in a FRP tank (5000 l capacity). For assessing gut flora, gut was removed aseptically, weighed and washed with 85 % sterile saline and homogenized. The homogenate was diluted in sterile seawater and plated in nutrient agar and incubated at 37$^0$ C. The colonies were counted quantitatively and the single colonies were picked up and then streaked into slants. The isolated colonies were identified by morphological, physiological and biochemical confirmations (17) as well as based on the characteristics described in Bergey's Manual of Systematic Bacteriology (18).

**Herbal antibacterial diet preparation*:*** Antibacterial diets were prepared according to the method described by M. Boonyaratpalin, 1993 [19]. The basal diet contained 45.1% protein; 7.2% lipid; 14.6% ash; 7.1% moisture and 3% fibres. Equal proportions of the methanolic extracts of three herbals were mixed with the basal ingredients at the concentration of 500 mg (HD 1) and 1000 mg/kg (HD 2) diets. The diet contained 45.1% protein, 7.2% lipids, 14.6% ash, 7.1% moisture, and 3% fiber. Ingredients were mixed thoroughly with sufficient water, cold extruded, cut into pellets, air dried, and stored at room temperature.

**Experimental Set-up and feeding:** Healthy uniform sized *F. indicus* adults weighing, 7.5 ± 0.5 g were stocked in triplicate to fiber glass tanks (500 l capacity) of two experimental groups (HBD1 and HBD2) and a control group with continuous flow-through water and constant aeration system. The water quality parameters such as temperature (27 ± 1.0 $^0$C), salinity (28 ± 1.5 ‰), and pH (8.2±0.1) were maintained every day. Survival also monitored throughout the experimental period. Shrimp were fed 10% of their body weight three times a day. Uneaten food and waste materials were removed daily before feeding.

**Bacterial Challenge and cumulative mortality:** At every ten days interval (days 10, 20 and 30), 10 shrimps were bath challenged with virulent *V. harveyi* at the more than $10^7$ Cfu/ ml in the 100 l glass aquaria. The survival and pathological signs were observed at 5 days of post challenge.

**Survival and growth parameters:** Survival was monitored every day after feeding. Average daily weight gain was calculated by deducting the initial weight from the final weight. The specific growth rate (SGR) was calculated by using the formula:

$$SGR\ (\%) = \frac{(\text{Ln } W_2 - \text{Ln } W_1)}{(t_2 - t_1)} \times 100$$

Where, Ln =Natural logt, $W_2$ = Final weight at time $t_2$ , $W_1$ = Initial weight at time $t_1$ .

**Influence of antibacterial extracts on gut micro floral changes:** After feeding, experimental and control diets to *F. indicus* adults, five random samples were taken and the gut bacterial flora were analysed by quantitatively and qualitatively by standard protocol.

**Data analysis:** Data were statistically analyzed by one way ANOVA. F values were observed, at $P < 0.05$ levels following the statistical analysis described by [20].

## Result and Discussion

The three methanolic antibacterial extracts, *P. corylifolia, M. koenigii* and *Q. infectoria* effectively suppressed the shrimp bacterial pathogens isolated from the infected *F. indicus* gut. The averaged zone of inhibition was observed ranging between 9 to 14 mm against the selected bacterial pathogens. *Q. infectoria* and *P. corylifolia* effectively controlled the pathogens and had the zone of inhibition of 12 to 14 mm (Table1).

**Table 1.** Antibacterial screening of the methanolic herbal extracts against shrimp pathogens.

| **Pathogens** | **Zone of inhibition (mm)** | | |
|---|---|---|---|
| | ***P. corylifoila*** | ***M. koeniji*** | ***Q. infectoria*** |
| *P. aeruginosa* | 12. 30 ± 0.94 | 9.30 ± 1.04 | 13.30 ± 1.94 |
| *S. aureus* | 12.0 ± 0.55 | 10.33 ± 1.63 | 12.00 ± 2.05 |
| *V.harveyi* | 14.0 ± 0.95 | 12.45 ± 0.63 | 14.00 ± 1.50 |

The MIC and MBC of the three extracts against the pathogens were given in the Table 2. The herbal extracts, *P. corylifoila* and *Q. infectoria* had the MIC of 40 µg against the *V. harveyi* whereas in *M. koeniji* only 60 µg were observed.

**Table 2.** Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the methanolic extract against *V. harveyi.*

| Con (µg) | Methanolic herbal extracts | | | | | |
|---|---|---|---|---|---|---|
| | *P. corylifoila* | | *M.koenigi* | | *Q. infectoria* | |
| | MIC | MBC | MIC | MBC | MIC | MBC |
| 10 | | ++++ | | ++++ | | +++ |
| 20 | | +++ | | ++++ | | +++ |
| 30 | | ++ | | ++++ | | ++ |
| 40 | 40 | + | | +++ | 40 | + |
| 50 | | - | | ++ | | - |
| 60 | | - | 60 | + | | - |
| 70 | | - | | - | | - |

- : No Growth; +: Minimum Growth; ++: Intermediate Growth; >+: Maximum Growth

Similar work done by [21] sixteen kinds of Thai traditional herb; *Ocimum sanctum*, *Cassia alata, Tinospora cordifolia, Eclipta alba, T. crispa, Psidium guajava, Clinacathus nutans, Andrographis paniculata, Momordica charantia, Phyllanthus reticulatus, P. pulcher, P. acidus* were tested for their antibacterial efficacy against 10 strains of *Vibrio* sp. isolated from diseased shrimp. Methanolic extracts of the three ayurvedic antimicrobial herbs (*S. trilobatum, A. panniculata* and *P. corylifolia*) suppress bacterial activity at the rate of 80µg $disc^{-1}$ against the pathogens *P. aeruginosa*, *S. typhi*, *S. aureus* and *Vibrio* spp. (22). Anita (23) studied the antibacterial activity of herbal plant extracts *Acalypha indica, Adhatoda vasica, Anacardium occidentale, Azadirachta indica, Lawsonia inermis, Psidium guajava, Rosa damascena* and *Tridax procumbans* against the fish pathogens of *Vibrio anguillarum, V. alginolyticus* and *V. harveyi.*

The percentage of qualitative bacterial gut flora isolated from *F. indicus* adult before antibacterial herbal treatment and its phenotypic confirmation were given in the Fig1 and Table 3 respectively.

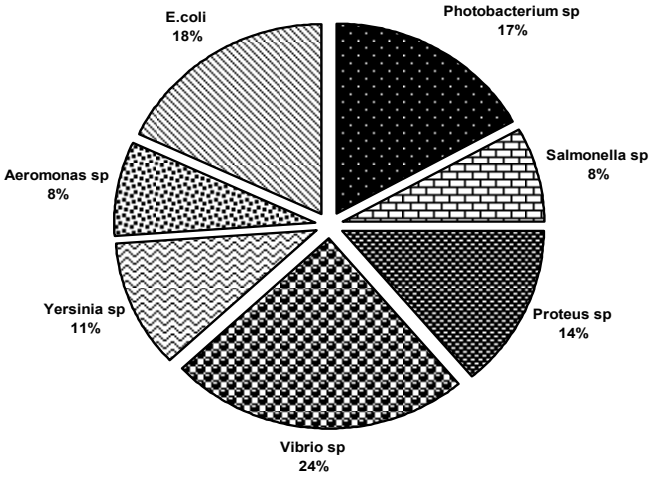


**Figure 1.** Qualitative bacterial gut flora (%) of *F. indicus* adult before antibacterial herbal treatment

The gut floral data revealed that, *Vibrio* sp (24%) dominated and followed by *E. coli* (18%), *Photobacterium* sp (17%), *Proteus* sp (14%) and *Yersinia* sp (11%). The lowest gut flora is 8% in the *Salmonella* sp as well as the *Aeromonas* sp respectively. Several bacteriological surveys were performed in different shrimp with stages sp by (24) using

molecular identification and found out that *Vibrio* sp is the dominant one and followed by *photobacterium* sp. A bacterial flora study was carried out in *Macrobrachium rosenbergii* gut in India and 367 randomly selected isolates were characterized and identified. Gram-positive bacteria such as *Micrococcus*, *Bacillus*, Coryneforms, *Arthrobacter*, faecal coliforms and *Enterococci* were found (25).

**Table 3.** Morphological and biochemical characteristics of the Major bacterial genera isolated from the gut of the wild Indian white shrimp *F. indicus*

| Sl. No | Morphological / Biochemical Tests | | Gut floral isolates | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 |
| 1. | Gram staining | | -ive | -ive | -ive | -ive | -ive | -ive |
| 2. | Simple Staining | | Rod | Rod | Rod | Rod | Rod | Rod |
| 3. | Negative Staining | | Rod | Rod | Rod | Rod | Rod | Rod |
| 4. | Acid Fast staining | | -ive | -ive | -ive | -ive | -ive | -ive |
| 5. | Capsule Staining | | -ive | -ive | -ive | -ive | -ive | -ive |
| 6. | Motility | | M | NM | M | NM | M | NM |
| 7. | Oxidase | | + ive | + ive | + ive | + ive | + ive | + ive |
| 8. | Catalase | | -ive | -ive | -ive | -ive | -ive | -ive |
| 9. | Indole | | +ive | -ive | -ive | -ive | -ive | -ive |
| 10. | Methyl- Red | | +ive | +ive | -ive | -ive | -ive | +ive |
| 11. | Voges-Proskaeur | | + ive | + ive | + ive | + ive | - ive | + ive |
| 12. | Simmons | | + ive | - ive | + ive | + ive | - ive | - ive |
| 14. | Starch | | + ive | + ive | - ive | - ive | - ive | + ive |
| 15 | Triple Sugar Iron | Slant | Alkaline | Alkaline | Alkaline | Alkaline | N | Alkaline |
| | | Bud | Acid | Acid | Acid | Acid | N | Acid |
| | | Gas | N | N | N | N | N | N |
| | | Slant | N | N | P | P | P | N |
| 16 | Urease | | + ive | + ive | + ive | + ive | + ive | + ive |

1. Photobacterium sp; 2.Salmonella sp; 3. Proteus sp; 4. Photobacterium sp; 5. Vibrio sp; 6. Yersinia sp; 7. Aeromonas sp

Figure 2 and Table 4 revealed that, the gut flora was changed qualitatively and quantitatively and it might be due to the action of antibacterial active principles. The methanolic active extracts inhibited the growth of specific bacterial gut sp. Mostly the bacterial pathogens were inhibited by the active extracts. The extracts may inhibit the transcription and leads to arrest the protein synthesis of the bacteria. For the total bacterial count, before herbal treatment the load was $5.8 \times 10^5$ Cfu/g, but the load decreased to $4.7 \times 10^4$ and $2.8 \times 10^3$ Cfu/g due to the herbal action. The same manner was reflected in the Vibrio count also. The control group the *Vibrio* count was $1.75X\ 10^5$ and it decreased to the minimum of $1.35\ X\ 10^2$ Cfu/g. The methanolic extracts effectively controlled the pathogens such as *Salmonella*, *Vibrio* sp, *Yersinia* and *Aeromonas* sp in the *F. indicus in vivo*. Herbal diets prepared from five different herbs such as *Adathoda vasika*, *Murraya koenigii, Ocimum basilicum, Psoralea corylifolia* and *Qurcus infectoria* were effectively suppressed the pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus, Aeromonas hydrophila, Vibrio harveyi*, and *V. parahaemolyticus* in the *P. monodon* immune system (26).

**Table 4.** Total bacterial count and Total Vibrio count (Cfu /g) in *F. indicus* gut at different intervals after antibacterial herbal treatment

| Sampling intervals (d) | Bacterial Count (Cfu/g) | | | | | |
|---|---|---|---|---|---|---|
| | Total bacterial count | | | Vibrio count | | |
| | HD-0 | HD-1 | HD-2 | HD-0 | HD-1 | HD-2 |
| 10 | $6.5 \times 10^6 \pm 1.5 \times 10^2$ | $4.2 \times 10^5 \pm 1.0 \times 10^2$ | $2.7 \times 10^4 \pm 0.5 \times 10^2$ | $2.3 \times 10^5 \pm 1.0 \times 10^1$ | $1.3 \times 10^3 \pm 1.0 \times 10^1$ | $1.0 \times 10^3 \pm 0.3 \times 10^1$ |
| 20 | $7.0 \text{ X } 10^6 \pm 1.0 \times 10^2$ | $6.5 \times 10^4 \pm 1.6 \times 10^2$ | $5.3 \times 10^3 \pm 1.0 \times 10^2$ | $1.8 \times 10^5 \pm 0.30 \times 10^2$ | $1.0 \times 10^3 \pm 0.5 \times 10^2$ | $0.5 \times 10^3 \pm 0.30 \times 10^2$ |
| 30 | $5.8 \times 10^5 \pm 0.7 \times 10^2$ | $4.7 \times 10^4 \pm 0.5 \times 10^2$ | $2.8 \times 10^3 \pm 0.5 \times 10^2$ | $1.75 \times 10^5 \pm 0.5 \times 10^1$ | $1.75 \times 10^2 \pm 1.1 \times 10^1$ | $1.35 \times 10^2 \pm 0.5 \times 10^1$ |

In fig. 2 can be noticed that the load was very much decreased and some of the bacteria such as S*almonella* and *Yersinia* sp. were totally absent due to the antimicrobial herbal action. Sivaram et al. (14) successfully controlled *Vibrio* and improved the immunocompetence of grouper *E. tauvina* larviculture using herbal methonolic extracts. Methanolic extracts of three ayurvedic antimicrobial herbs (*S. trilobatum, A. panniculata* and *P. corylifolia*) suppressed bacterial activity and had the MIC of 80µg $disc^{-1}$ against the pathogens *P. aeruginosa*, *S. typhi*, *S. aureus* and *Vibrio* spp. (22). Rao and Parekh (27) reported that certain seaweed extract have great antimicrobial activity against gram positive and gram negative bacteria. The results of Immanuel et al. (28) on bacterial load in muscle and hepatopancreas tissues of shrimp *P.indicus* juveniles showed that it was very high in control (without treatment) group. Chinese medicinal herbs such as *Astragalus membranaceus* and *Lonicera japonica* have effectively improved the immune system and controlled the *Aeromons hydrophila* in *Oreochromis niloticus* (29). Rutin, a plant active compound has improved the biochemical, immunological and haematological parameters in *Litopenaeus vannamei* during the stress conditions by *Vibrio alginolyticus* (30).

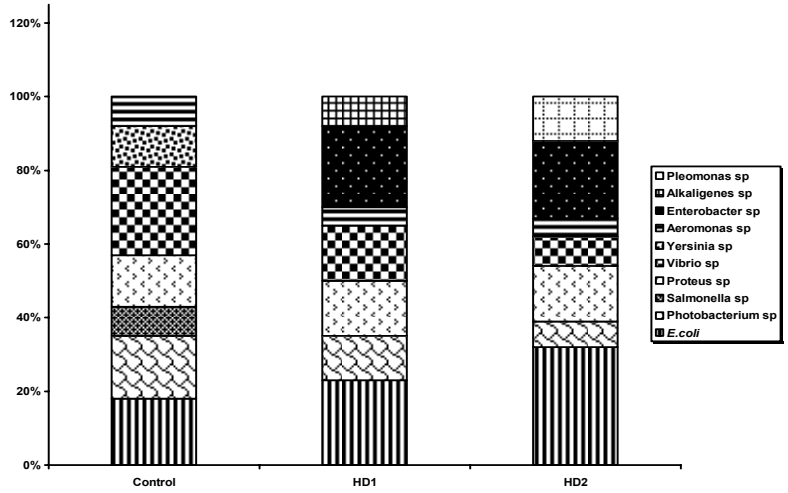


**Figure 2.** Gut floral changes observed in *F. indicus* before and after antimicrobial herbal treatment

The cumulative mortality and growth parameters of herbal antibacterial diets fed *F. indicus* were given in the Fig 3 and Table 5 respectively.

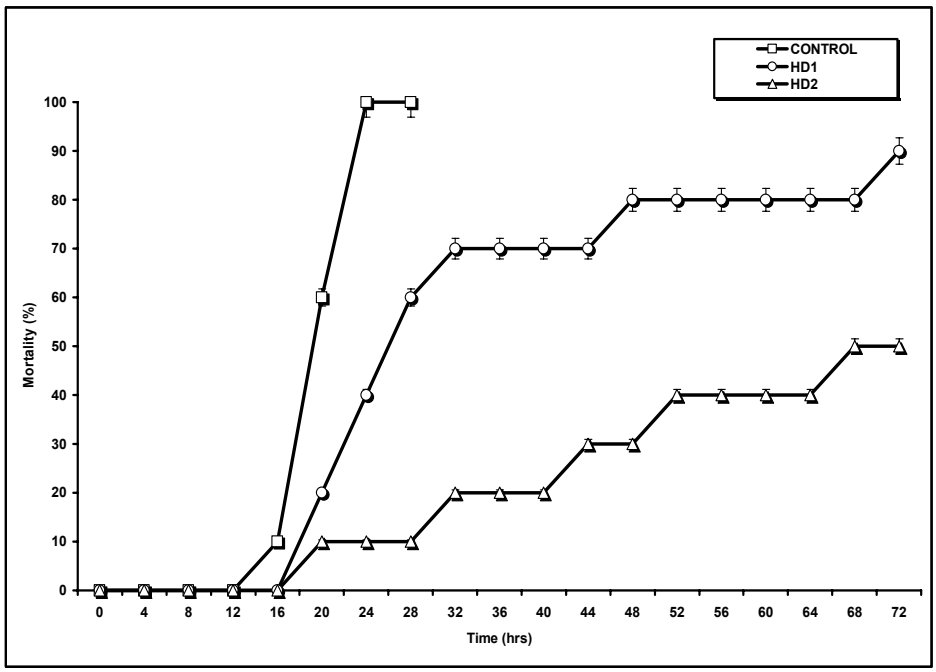


**Figure 3.** Cumulative mortality (%) of *F.indicus* fed with antibacterial herbal diets and challenged with *V. harveyi* after 30 days/

**Table 5.** Growth characteristics of the of *F. indicus* fed with the antibacterial herbal after 30 days

| Treatment | Length (cm) | | Wet weight (g) | | Weight gain (g) | Specific growth rate (%) | Survival (%) |
|---|---|---|---|---|---|---|---|
| | Initial | Final | Initial | Final | | | |
| Control | 7.5 ± 0.12 | 8.5 ± 0.216 | 7.5 ± 0.16 | 8.1 ± 0.12 | $0.60^{a}$ ± 0.8 | $0.11^{a}$ ± 0.08 | 70 |
| HBD1 | 7.1 ± 0.16 | 8.1 ± 0.26 | 8.5 ± 0.20 | 9.3 ± 0.16 | $0.80^{b}$ ± 0.47 | $0.13^{b}$ ± 0.04 | 80 |
| HBD2 | 6.9 ± 0.24 | 7.6 ± 0.16 | 8.5 ± 0.286 | 9.4 ± 0.32 | $0.90^{c}$ ± 0.91 | $0.14^{c}$ ± 0.09 | 85 |

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

The *F. indicus* succumbed to death cent percent with in 28 hrs when no antibacterial active principles incorporated in the diets against bacterial challenge. Surprisingly the herbal antibacterial diets help to increase the survival significantly ($P<0.05$) and of 90 % at 72 hrs in HD1 and 50 % in HD 2 diets respectively. The herbal diet HD1 helps to reduce 20 % of the mortality and the HD2 helps to reduce mortality of maximum 40 %. The growth parameters such as weight gain and specific growth are also significantly ($P < 0.01$) increased from the control to experimental groups. Citarasu [31] found the *P. monodon* post larvae (PL 1 – 25) reared in bacteria inoculated water showed the increased bacterial load in the intestine and tissue parts. At the same time, the methanolic herbal extracts helped to reduce the bacterial load and he found *P. corylifolia* enriched *Artemia* fed group *P. monodon* post larvae got better results of decreased bacterial load. Edahiro et al. (32) reported that yellowtail treated orally with glycyrrhizin showed increased protection against *E. seriola* infection. *Emblica officinalis, Cyanodon dactylon, Adathoda vasica*, the herbal immunostimulants improved the

immune system and reduced the microbial infection in the gold fish *Carassius auratus* (33) and similar work was carried out by Magdelin (34) on the ornamental fish *Poecilia sphenops* using herbal immunostimunants. Methanolic extracts of *Ocimum sanctum, Withania somnifera* and *Myristica fragrans* herbs significantly improved the immune parameters such as phagocytic activity, serum bactericidal activity, Albumin–globulin (A/G) ratio and leukocrit against *Vibrio harveyi*, grouper juvenile, *Epinephelus tauvina* larviculture. Sivaram et al. (14). Also the methanolic extracts of five different herbal medicinal plants like *Cyanodon dactylon, Aegle marmelos, Tinospora cordifolia, Picrorhiza kurooa* and *Eclipta alba* effectively controlled WSSV infection in the shrimp *P. monodon* (35). The antibacterial active principles of the herbals may lyse the cell wall, block the protein synthesis and DNA synthesis, inhibit the enzyme secretions and interfere with the signalling mechanism of quorum sensing pathway (36)

In the present work, the antibacterial active fractions incorporated diets very effectively suppressed the bacterial growth in the gut and reduced the pathogenesis. Due to the immense characteristics such as antimicrobial and immunostimulant capabilities of the herb, they are highly promising to the aquaculture industry. The new treatment system will be eco-friendly and effective in controlling the microbial diseases and the non-resistant strain development in bacteria.

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