

Immunostimulating influence of herbal biomedicines on nonspecific immunity in Grouper *Epinephelus tauvina* juvenile against *Vibrio harveyi* infection

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Received: 5 July 2007 / Accepted: 28 December 2007 / Published online: 19 January 2008
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Abstract Herbals such as *Cynodon dactylon*, *Piper longum*, *Phyllanthus niruri*, *Tridax procumbens*, and *Zingiber officinalis* were extracted with acetone, benzene, butanol, and petroleum ether and screened against the pathogen *Vibrio harveyi* isolated from the infected Grouper *Epinephelus tauvina*. Among the different solvent extractions screening to *V. harveyi*, petroleum ether extracts were suppressed significantly ($P < 0.05$). Equal proportions of the all-plant extracts were mixed with the artificial feeds at concentrations of 100, 200, 400, and 800 mg kg⁻¹ of diet and fed to grouper juveniles of 20 ± 2 g average weight for a period of 60 days. Every 20 days, fish juveniles were challenged with *V. harveyi* and the immune response was studied. The herbal diets significantly ($P < 0.05$) increased the survival, growth, and immune responses compared to the control group. The herbal diets were significantly improved ($P < 0.01$) in immune parameters such as phagocytic activity and albumin–globulin (A–G) ratio. Among the different concentrations of the herbals in the diet, the 400 mg kg⁻¹ diet was the most effective in the experiment.

Keywords Antibacterial · Herbals · *Epinephelus tauvina* · Immunostimulants · *Vibrio harveyi*

Introduction

Groupers are commercially important marine food fish in several countries, especially in south-east and middle-east Asia. Since market demand for groupers is larger than supply from captures and fisheries and considering the fast-growing economic importance of

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grouper culture in recent years, many countries are nowadays concentrating on developing commercial grouper farming technology (Al-Thobaity and James 1996). Many commercial grouper fish farms depend mainly on juveniles collected from the wild due to hatchery constraints and poor larval survival, which limits commercial expansion of grouper farming in south-east Asian countries (Chen 1979; Tseng 1983).

In the early 1990s grouper production in Hong Kong was about 3,000 t/year. In the last few years, production has dropped to 1,000 t/year due to environmental problems such as poor water conditions leading to stresses and disease outbreaks (Sadovy 2000). A number of diseases for groupers have been reported and the main causative agents are the typical group of parasitic organisms, bacteria, and viruses. Pathogenic bacteria cause severe losses in hatchery and grow-out culture systems which are usually related to poor management and water quality. Fish are easily stressed by improper handling and can become infected with *Flexibacteria*, leading to skin damage and tail rot. Gram-negative bacteria, especially *Vibrio parahaemolyticus* and *V. alginolyticus*, cause haemorrhagic septicaemia in grouper in nursery and grow-out cage systems. The Gram-positive bacteria *Streptococcus* sp. also causes systemic infection in grouper. Affected fish exhibit signs of weakness, abnormal swimming, and occasionally corneal hemorrhage (Danayadol et al. 1995). A range of bacterial diseases has also been reported, including *Vibrio* spp., *Pseudomonas* sp., *Pasteurella piscicida* and *Flexibacter* sp. Bacteriosis caused by *Pseudomonas* sp. among *E. tauvina* cultured in Malaysia was first reported by Nash et al. (1987), where all age groups were affected during an outbreak in November/December to February/March in 1982–1986. Mortalities ranged from 20% to 60%. Affected fish showed extensive haemorrhagic erosions and ulcerations of the skin, fins, and tail. Also *V. harveyi* (CMB57) and other microbiota such as *Streptococcus*, *Pseudomonas*, *Corynebacterium*, *Staphylococcus*, and *Proteus* spp. were isolated from the body fluid and blood of the infected Grouper *E. tauvina*. Among the isolated microbiota from the body fluid and blood, *V. harveyi* dominated the other bacteria found in the body fluid (Sheeja et al. 2003; Sivaram et al. 2004).

Chemotherapy is widely used to control infectious parasitic, bacterial, and fungal diseases. It has been used for the control of parasitic and bacterial diseases of grouper. However, experience demonstrates that there are problems in chemical treatment of fish diseases; in some cases the treatments can be harmful because of associated stress. In Singapore, Chong and Chao (1986) reported some cases of drug overdose leading to fish death and other detrimental side-effects. Formalin overdose results in severe gill damage. Ulcerative dermatitis develops with repeated treatment with nitrofurazone, leading to fish death. Application of chemotherapeutants has created problems with toxicity, resistance, residues, and possibly some public health and environmental consequences. Their efficacy under certain aquatic conditions (open-water systems) remains questionable, and they can be costly. The use of chemicals in treating health problems has also been complicated by the misleading advice provided to the farmers by feed and chemical companies regarding the use of antibiotics and other therapeutic drugs.

Some chemotherapeutic agents of plant origin that have been isolated promise to deal with drug-resistant bacteria (Richard and Anthony 1983). Compounds such as volatile oils, tannins, phenolic compounds, saponins, alkaloids polysaccharides, and polypeptides were shown to be effective alternatives to antibiotics. The screening of plant extracts and natural products for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents (Press 1996) as well as serve in drug discovery from natural products for primary lead compounds (Lawrence 1999). Traditional antibacterial therapy is going through a crisis due to the rapidly increasing development of resistance to existing agents. Such resistance has an impact on all areas of chemotherapy. Plant-derived

phytomedicines have great promise in the treatment of infectious diseases. Plant-based antimicrobials represent a vast untapped source for medicines and have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side-effects that are often associated with synthetic antimicrobials. Herbal products promise a cheaper source for therapeutics, greater accuracy than chemotherapeutic agents, and a viable solution for all problems which grouper culture faces today.

Several antimicrobial, antistress, immunostimulant, growth-promoting plant products significantly influenced the fish/shrimp larviculture (Citarasu et al. 1998, 2002, 2003a, 2003b). Sivaram et al. (2004) successfully controlled the *Vibrio* pathogen, and improved the immune system of grouper larviculture using herbal methanolic extracts. These findings suggest that phytochemicals could be an alternative to the chemotherapeutic molecules and safe to use in aquaculture. The present study focus on screening of selected antibacterial/immunostimulant herbals against the *V. harveyi* bacterial pathogen isolated from infected grouper and apply the products to grouper larviculture.

Materials and methods

Herbals

Five herbs with antimicrobial/immunostimulant characteristics were selected following Nadkarni (1995). The detailed descriptions are given in the Table 1.

Source of the bacterial culture

V. harveyi was isolated from the ulceration (wounds) of the infected Grouper, *E. tauvina*. Triplicate samples were aseptically blended with alkaline peptone water (Hi media) with 3% NaCl and incubated at 37°C overnight for enrichment. The culture was plated on thiosulfate citrate bile salt sucrose (TCBS) agar (HIMEDIA, India). Further it was identified by morphological and biochemical conformations (Farmer and Hickman-Brenner 1992) as well as based on the characteristics described in Bergey's Manual of systematic bacteriology (Holt et al. 1994). The isolates were oxidase positive, sensitive to vibriostatic agents, grown in nutrient broth (HIMEDIA, India) with 3% and 6% NaCl and TCBS agar. Serological tests were also done with standard anti-*V. harveyi* sera for species confirmation (Table 2). The pure cultures were stored in TCBS slants at 4°C for further experiments.

Extraction and screening of the herbals against *V. harveyi* using different solvents

Dried herbal powders were soaked in various solvents such as acetone, benzene, butanol, and petroleum ether individually for 48 h (Eloff 1998) in equal ratio (1:1). The slurry was then filtered, washed to remove nonsoluble fractions, and the filtrate was centrifuged (20,000 *g* for 30 min) for clarification. The clarified extracts were condensed at 35°C until the solvent residue had evaporated. Each 500 µg of condensate was impregnated in 5-mm-diameter sterile paper discs (Himedia, India) and screened against *V. harveyi* through replicates of the disc diffusion test as described by Bauer et al. (1966). Dried antimicrobial discs with impregnated herbal extracts were carefully dispensed with uniform distances

Table 1 Detailed descriptions of the selected medicinal herbs

| Botanical name | Common name | Family | Useful parts | Active principles | Biological effect |
|-----------------------------|--|---------------|--------------|---|---|
| <i>Cynodon dactylon</i> | Bermuda grass | Poaceae | Whole plant | Cynodin, hydrocyanic acid, and triticin | Antimicrobial, antidiabetic, immunostimulant |
| <i>Piper longum</i> | Indian long pepper | Piperaceae | Dried seeds | Piperine, piperlonguminine, sylvatine, guineensine, piperlongumine, and filifline | Antibacterial and appetizer |
| <i>Phyllanthus niruri</i> | Stone-breaker | Euphorbiaceae | Whole plant | Phyllanthin, phylochrysin, phyltetralin, and quercitrin | Antimicrobial liver corrective |
| <i>Tridax procumbens</i> | Coat buttons, tridax daisy, wild daisy | Asteraceae | Whole plant | <i>b</i> -Sitosterol | Antibacterial and wound healing |
| <i>Zingiber officinalis</i> | Ginger | Zingiberaceae | Rhizome | Zingiberene, gingerol, and camphene | Antimicrobial, appetizer, and immunostimulant |

Table 2 Morphological and biochemical characteristics of the pathogen *V. harveyi* isolated from the Grouper *E. tauvina*

| Characters | Reactions |
|--|-------------|
| Morphology | Curved rods |
| Gram's stain | – |
| Motility | + |
| Swarming | – |
| Swarming on TSA (+2% NaCl) | – |
| Growth in 6% NaCl | + |
| Growth in 12 % NaCl | – |
| Oxidase (Kovacs) | + |
| Indole production | + |
| Voges-Proskauer test | – |
| D-glucose acid | + |
| Arginine dihydrolase (2nd and 7th day) | – |
| Adonitol fermentation | – |
| D-Xylose fermentation | – |
| Erythritol fermentation | – |
| Luminescence | Variable |
| Cytochrome oxidase | + |
| Nitrate reduction | + |
| O/129 sensitivity | + |
| Thornley's arginine dihydrolase | – |
| Lysine decarboxylase | + |
| Ornithine decarboxylase | + |
| Voges-Proskauer reaction | – |
| L-Arabinose ^a | – |
| M-Inositol ^a | – |
| D-Arabinose ^a | + |
| Sucrose ^a | Variable |

^a Used as sole carbon source
TSA Tryptic soy Agar

over Muller Hinton agar surface and correct implantation was assured by applying gentle pressure over the disc. Control tests were carried out using sterile discs without impregnation of the herbal extracts. All plates were kept at 35°C for 24 h incubation. After the incubation time, plates were studied for inhibitory zone formation of antimicrobial extracts on microbial lawns in agar surface.

Experimental culture

Healthy Grouper *E. tauvina* juveniles weighing approximately 20 ± 2 g were caught from the Gulf of Mannar, India. They were acclimatized and kept in quarantine tanks for the period of 15 days to assess their disease-free health status. During this time, they were fed commercial feeds. After acclimatizing, five triplicated ($5 \times 3 = 15$) experimental groups were stocked, having an average body weight of 22 ± 1 g in 500 l capacity, flow-through aquaria with a water flow rate of 1 l/min. The water quality parameters were maintained constantly (salinity 30 ± 0.5 ppt, temperature $28 \pm 1.5^\circ\text{C}$, DO >6.0 mg/l, ammonia-nitrogen <0.22 mg/l).

Feed preparation and feeding

An equal proportion of all five petroleum ether herbal extracts was incorporated into five experimental diets, containing 0 (control), 100, 200, 400, or 800 mg/kg of the extract mixture. Diets were prepared following the basal ratio of Boonyaratpalin (1993). The basal diet contained 45.1% protein, 7.2% lipid, 14.6% ash, 7.1% moisture, and 3% fibre. To prepare the feeds, ingredients were mixed thoroughly and 4% gelatin solution containing the active principles at the appropriate concentration was added along with the oil ingredients. Sufficient water was added and the pH was adjusted to 7 ± 0.1 . The mixture was then cold extruded, cut into pellets, air dried, and stored at room temperature.

Fish were fed with 5% of their body weight, every day until the end of experiment (60 days). The standard growth rate (SGR) was calculated by using the formula:

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

where, \ln is the logarithm function, W_2 is the final weight at time t_2 , and W_1 is the initial weight at time t_1 .

Challenge experiment

On the 20th, 40th and 60th day of feeding experiment, five fishes were challenged with a lethal dose of *V. harveyi* (1×10^8) (isolated from the infected *E. tauvina*, cultured and maintained using Tryptic soy broth and agar with 2% NaCl W/V) intramuscularly, transferred to a 250 l aquaria, and observed for 5 days for pathological signs.

Immunological parameters

Every 20 days, 24 h after final feeding, five randomly chosen fish from each experiment and control group, were taken and anaesthetized with 50 mg MS-222/dm³ of water, and used for blood collection. Blood was collected from caudal vein, with 1 ml plastic syringe rinsed with anticoagulant, and a part of the blood was transferred immediately, added to an equal volume of 10% tri sodium citrate, and stored at 4°C. The remaining blood was kept at room temperature for 1 h without anticoagulant to collect the serum and stored at –40°C.

For phagocytosis assay, 10^7 cells of formalin-killed *V. harveyi* were added to 0.1 ml of pooled blood samples from two fishes in a sterile microplate and incubated for 30 min at 25°C after thorough mixing in the well. After incubation, the blood-bacteria suspension was mixed gently and 50 μ l of this suspension was smeared on three glass slides. After air drying, the smears were fixed in 95% ethanol, redried, and stained with May–Grunwald's Giemsa. The phagocytic cells and phagocytosed bacteria were counted (Park and Jeong 1996).

For the study of serum bactericidal activity, two fishes from each groups were injected with 0.1 ml/kg body weight with live *V. harveyi* suspension (10^5 cells/ml) and blood samples were collected 10 and 90 min after injection. One hundred microliters of blood was serially diluted and plated in the TCBS medium (Hi media, India). The albumin–globulin ratio (A–G) of the sera was calculated following the method of Sahoo et al. (1999). The serum samples were analyzed for total protein following the dye-binding

method of Bradford (1976), using bovine serum albumin as standard, for albumin by the bromocresol green method, and for globulin by subtracting the albumin value from the total protein value. Finally, the albumin–globulin ratio (A–G) was calculated.

Statistics

Data were analyzed using one-way analysis of variance (ANOVA) following Zar (1974), to discover the significant difference at the 5% ($P < 0.05$) level.

Results

Screening of herbal against the dominant pathogen *V. harveyi*

Among the different solvent extracts, those of benzene and petroleum ether (Fig. 1) effectively controlled the pathogen *V. harveyi* isolated from the infected grouper. The zones of inhibitions for benzene were 12.83 and 14.16 mm of *C. dactylon* and *P. longum*, respectively. Also the petroleum ether extracts exhibited 13, 12, and 14.5 mm inhibition zones for *C. dactylon*, *P. niruri*, and *T. procumbens*, respectively.

Bacterial challenge

In all challenges of grouper juveniles (20th, 40th, and 60th day) by *V. harveyi*, the control group had the highest and fastest mortality. Survival was significantly increased ($P < 0.05$) for the groups fed diets incorporating the herbal immunostimulant. Only a slight variation in survival was observed after the 20th day challenge among the different concentrations of

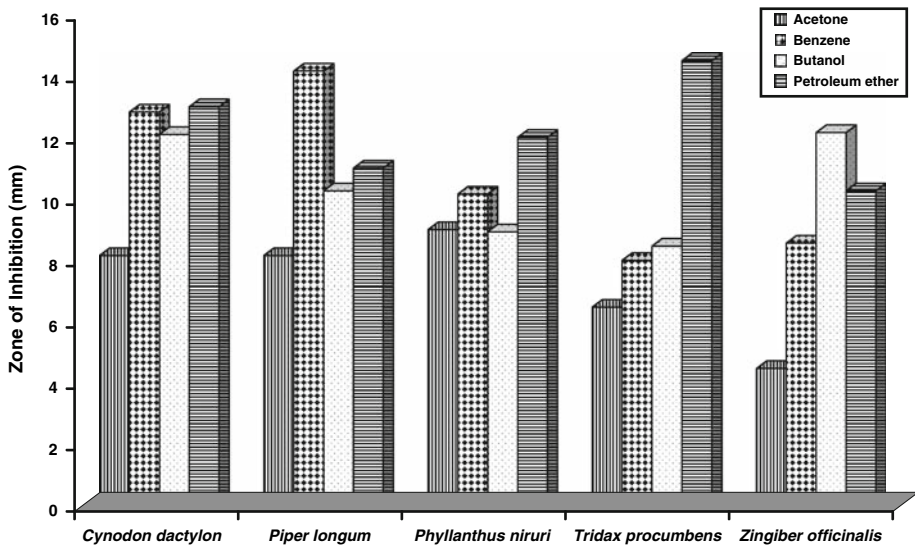


Fig. 1 Screening the antibacterial activity of different organic solvent extracts of the selected herbs on the dominant pathogen *V. harveyi* isolated from infected Grouper *E. tauvina*

herbal diets. The maximum survival of 32.66% was observed for the 400 mg/kg diet. Surprisingly, on the 60th day after challenge, the herbal diets yielded very good results and attained survival rates of 78.33%, 84.66%, 92.0%, and 93.33% for the 100, 200, 400, and 800 mg/kg diets, respectively (Fig. 2).

Growth performance

Percentage standard weight gain (SGR) was calculated from the difference between the final and initial weight of the each fish group (Table 3). The SGR of the control group was 0.70%. This was significantly increased ($P < 0.05$) to 1.22, 1.37, 1.47, and 1.47% in the 100, 200, 400, and 800 mg/kg diets, respectively. The gain was increased a fold from the control group to the 400 and 800 mg/kg diets, respectively.

Immunological parameters

The percentage phagocytic activity showed a significant variance ($P < 0.01$) between the control group and the groups fed the herbal diets in different days of challenging. Only

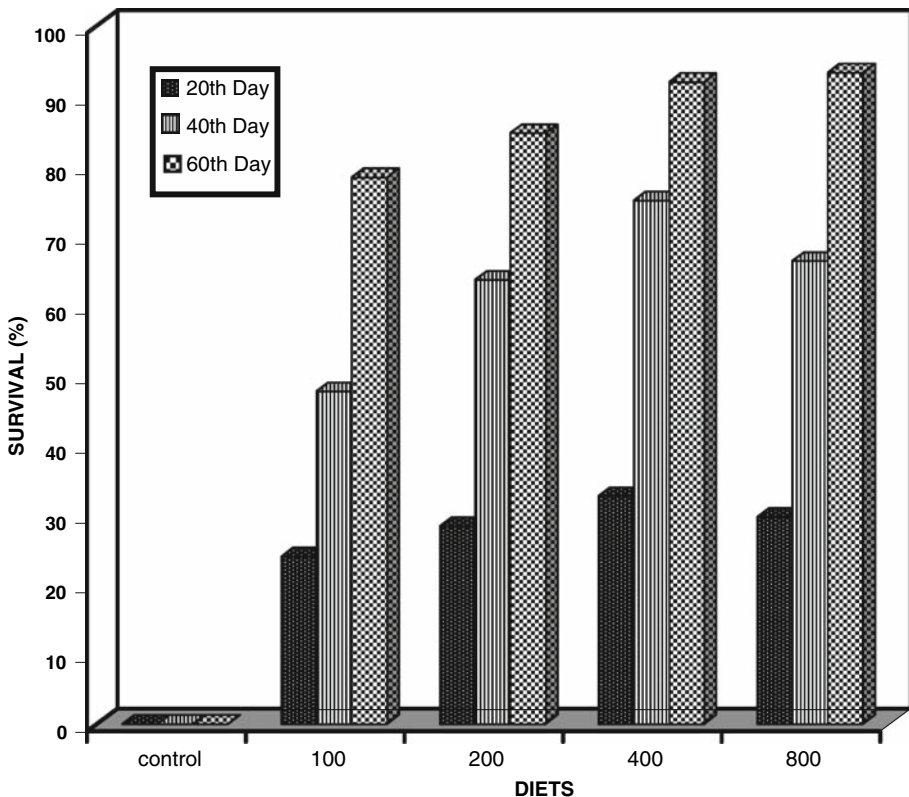


Fig. 2 Percentage survival after challenge with *V. harveyi* for the Grouper *E. tauvina* juveniles fed diets incorporating the herbal immunostimulant

Table 3 Growth characteristics of Grouper *E. tauvina* juveniles fed diets containing different concentrations of the herbal immunostimulant for the period of 60 days

| Diet (mg/kg) | Initial wt. (g) | Final wt. (g) | SGR (%) |
|--------------|-----------------|---------------|---------------------------|
| Control | 21.9 ± 0.36 | 33.46 ± 0.61 | 0.70 ^a ± 0.00 |
| 100 | 21.96 ± 0.50 | 45.76 ± 0.28 | 1.22 ^b ± 0.071 |
| 200 | 21.76 ± 0.25 | 49.8 ± 49.8 | 1.37 ^c ± 0.078 |
| 400 | 22.22 ± 0.25 | 53.83 ± 1.83 | 1.47 ^d ± 0.05 |
| 800 | 21.66 ± 0.28 | 52.43 ± 0.76 | 1.47 ^d ± 0.001 |

Values with the same lower-case superscript do not differ significantly from each other ($P < 0.05$) based on one-way ANOVA

around 30% of the *V. harveyi* is phagocytosed in the control group. The herbals extracts increased the ingestion of the bacteria significantly ($P < 0.05$). The highest value of 77% was observed for the 400 mg/kg diet on the 60th day after challenge (Table 4).

The serum bactericidal activity and albumin–globulin (A–G) ratio are tabulated in Table 5. Compared to the control group, a significant ($P < 0.01$) increase in the serum bactericidal activity was observed for the diets incorporating herbal extract. For the 400 mg/kg diet, the increase was nearly 70%. Also, significantly ($P < 0.01$) increased A–G ratios of 4.13, 3.52, and 3.36 were observed in the groups fed 400, 800, and 200 mg/kg herbal diets, respectively. The control group attained a value of only 1.65.

Discussion

In aquaculture, chemotherapeutic agents such as commercial antibiotics and disinfectants are commonly employed for disease management, although this is not advisable due to cost effectiveness, environmental hazards, and the antibiotic resistance developed by many pathogens (Kruse and Soram 1994). To overcome this present situation, herbal medicine can be used as an effective antimicrobial drug. These are the ancient Indian systems of health care and longevity (Citarasu et al. 2002), but are still rejected by the scientific community due to a lack of standardization (Ponni 2002). In the present study, four different extracts of the five herbs effectively controlled the pathogen *Vibrio harveyi*. This was supported by Jinish (2002), who extracted the plants *Terminalia bellerica*, *Ocimum sanctum*, *Daemia extensa*, *Andrographis paniculata*, *Solanum suratense*,

Table 4 Phagocytic activity (%) of Grouper *E. tauvina* juveniles fed diets containing the herbal immunostimulant for the period of 60 days

| Diet (mg/kg) | Phagocytic activity (%) | | |
|--------------|---------------------------|---------------------------|---------------------------|
| | 20th Day | 40th Day | 60th Day |
| Control | 26.16 ^a ± 1.04 | 30.50 ^a ± 1.80 | 30.33 ^a ± 1.52 |
| 100 | 43.66 ^b ± 1.52 | 48.50 ^b ± 2.08 | 51.00 ^b ± 2.00 |
| 200 | 56.33 ^c ± 1.52 | 62.16 ^c ± 1.04 | 65.66 ^c ± 3.05 |
| 400 | 68.99 ^d ± 2.01 | 73.5 ^d ± 1.5 | 77.0 ^d ± 1.0 |
| 800 | 65.0 ^d ± 1.0 | 71.33 ^d ± 2.08 | 71.66 ^d ± 3.05 |

Values with the same lower-case superscript do not differ significantly from each other ($P < 0.05$) based on one-way ANOVA

Table 5 Albumin–globulin ratio (A–G) and the serum bactericidal activity of Grouper *E. tauvina* juveniles fed diets incorporating herbal immunostimulant after the end of the experiment

| Diet (mg/kg) | Albumin–globulin ratio (A–G) | Serum bactericidal activity |
|--------------|------------------------------|-----------------------------|
| Control | 1.64 ^a ± 0.02 | 4.83 ^a ± 0.20 |
| 100 | 3.10 ^b ± 0.10 | 8.53 ^b ± 0.13 |
| 200 | 3.35 ^b ± 0.04 | 12.33 ^c ± 0.05 |
| 400 | 4.15 ^c ± 0.03 | 15.44 ^d ± 0.14 |
| 800 | 3.54 ^b ± 0.04 | 12.62 ^c ± 2.08 |

Values with the same lower-case superscript do not differ significantly from each other ($P < 0.05$) based on one-way ANOVA

Tinospora cardifolia, *Withania somnifera*, and *Myristica fragrans* and tested the extracts against the gut microbiota isolates from the Grouper *E. tauvina*, and found them to have strong antimicrobial activity. Navarro et al. (1996) showed that methanolic plant extracts from *Eucalyptus globules*, *Punica granatum*, *Artemisia mozicana*, and *Bovvonia arborea* possess strong in vitro antibacterial activity against the *Staphylococcus aureus*, *E. coli*, *Pseudomonas* sp., and *Candida* sp. In the present study, *C. dactylon* and *P. niruri* extracted by petroleum ether exhibited good antibacterial activity. Also Jinish (2002) reported independently that the plant extracts have antimicrobial activity against fish pathogenic bacteria.

Interestingly, the fish juveniles fed the herbal immunostimulant diets had above 90% survival after the 60th day challenge and 41% higher weight than the fish fed the control diet. Itami et al. (1998) examined the potency of oral administration of peptidoglycan derived from *Bifidobacterium thermophilum* to kuruma shrimps through an artificial pellet diet. After 95 days, the shrimps were challenged by *Vibrio penaeicidae*. The survival and other immune parameters were significantly improved compared to the control group. Citarasu et al. (2002) developed an *Artemia*-enriched herbal diet for *Penaeus monodon* with the combination of five herbs, which significantly increased growth and survival during stress conditions. Also, feeding with *W. somnifera*, *Ferula asafoetida*, and *Mucuna pruriens* remarkably accelerated the spawning rate, fecundity, and larval quality in hatcheries of *P. monodon* (Babu and Marian 2001). Several herbal principles have been tested for their growth-promoting activity in aquatic animals (Citarasu et al. 2002; Jaya-prakas and Eupharsia 1996).

Ecoactiva, a glucon-based immunostimulant preparation, was effective in Snapper *Pagrus auratus*; especially significant was the enhancement of macrophage superoxide anion production. In the present study, in the grouper juveniles fed the 400 and 800 mg/g diets, the haemocytes engulfed the pathogens (phagocytic activity) more than twice as much as in the control group. Also the herbal diets (200, 400, and 800 mg/g) significantly influenced the albumin–globulin ratio, and tripled the serum bactericidal activity. The presence of antimicrobials and immunomodulatory principles in our experimental herbals (Nadkarni 1995) stimulate the immune system. Sivaram et al. (2004) screened the methanolic extracts of ten antibacterial herbs against *V. harveyi* isolated from infected cultured greasy Grouper (*E. tauvina*) and mixed selected herbal extracts in the artificial diets and fed the juvenile groupers. Among the ten plants, *Ocimum sanctum*, *Withania somnifera*, and *Myristica fragrans* showed significant improvements in immune parameters such as phagocytic activity, serum bactericidal activity, albumin–globulin (A–G) ratio, and leucocrit value.

Immunostimulant effects of the dietary intake of various medicinal plant extracts such as *Viscum album*, *Urtica dioica*, and *Zingiber officinalis* on rainbow trout (*Oncorhynchus mykiss*) were investigated by Dügenci et al. (2003). In this experiment, fish fed diets containing herbals improved the nonspecific defense mechanisms, including extracellular and intracellular respiratory burst activities, phagocytosis in blood leukocytes, total plasma protein level, specific growth rate (SGR), and condition factor (CF). It is well known that fish treated with immunostimulants show increased phagocytosis as well as respiratory burst activity (Sakai 1999; Blazer et al. 1989). Siwicki et al. (1990) reported that oral administration of levamisole increased the phagocytic index of phagocytic cells. Additionally, it was reported that feeding yeast products *Candida utilis* and *Saccharomyces cerevisiae* gave rise to enhanced phagocytic activity of blood leukocytes in rainbow trout. Albumin–globulin ratio is a measurable humoral component of the nonspecific defences. The reduction of A–G in aflatoxin-treated glucan-fed fish might be due to increased total serum globulin level, with significance for the protective mechanisms of fish. Also the immunostimulants β -1,3 glucan and aflatoxin B1 (AFB1) improved the specific immunity against *Aeromonas hydrophila* challenge in *Labeo rohita* fish (Sahoo and Mukherjee 2001).

The different parameters of the present work revealed that these herbal immunostimulants are very effective against the *V. harveyi* infection in the juvenile grouper and further work should be done on the isolation and characterization of the active compounds from these herbals.

Acknowledgements One of the authors, Dr. T. Citarasu, gratefully acknowledges the Department of Science and Technology (DST), Ministry of Science and Technology, Govt. of India, for its financial support, in the form of a research grant (Young Scientist Fellowship—SR/FT/L-27/2005).

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