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Water hyacinth (*Eichhornia crassipes*) leaves enhances disease resistance in *Channa punctata* from *Vibrio harveyi* infection

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Abstract

Background: *Channa punctata*, Indian spotted snakehead, has a great economic value in south and south-east Asia being an important protein source for humans. Fish cultures are affected due to various bacterial and viral infections. *Vibrio harveyi* is a fish pathogenic bacteria which causes several outbreaks throughout the world and leads to huge mortalities. In this study, leaves of *Eichhornia crassipes* (water hyacinth) were used to investigate its immunostimulatory potential in *Channa punctata*.

Results: The immunostimulatory effects of water hyacinth leaves were studied in fish fed with 2.5% and 5% supplementary feed (experimental groups) in comparison to normal feed (control groups). Gas chromatography mass spectrometry (GC-MS) analysis of *E. crassipes* methanol extract showed presence of various components which have immunostimulatory, antioxidant, antibacterial, and anti-inflammatory activities. The antibacterial activity, antioxidant potential, and presence of phenol and flavonoids in methanol and ethanol extracts supported its use in fish feed. The healthy acclimatized fish were challenged with *V. harveyi* weekly. Liver function tests, alkaline phosphatase levels, and immunoglobulin content in the experimental groups were improved with respect to those in the positive control group. The spleen and head kidney were obtained at the final day of experiment, and macrophages were isolated; higher percentage of phagocytosis and phagocytic index indicated enhanced cell-mediated immune response in fish due to supplemented feed.

Conclusion: Plant-infused feed with leaves of *E. crassipes* can be recommended as a regular feed supplement to enhance fish immunity and disease resistance against the *V. harveyi* infection.

Keywords: *Eichhornia crassipes*, Antibacterial, Antioxidant, Immunostimulant, GC-MS, *Channa punctata* fish

Background

Aquaculture of fin fish, crustaceans, and mollusks is one of the fastest growing areas, where different kinds of marine and freshwater fish have been cultured, resulting in increase in worldwide production of cultured fish every year (Tidwell & Allan, 2001). Fish in water are continuously exposed to ubiquitous

population of viruses, bacteria, and protozoans, among which few are potentially infectious to fish as well as to humans.

Since ancient time, medicinal system with rich herbal traditions has been used by Chinese, Egyptian, Greek, Indian, Mesopotamian, Roman, and Tibetan populations (Hoareau & DaSilva, 1992; Meena, Bansal, & Kumar, 2009). Today around 80% of the world's population uses traditional medicines as their primary health care service. More than 50,000 of plant species are believed to be used for medicinal purposes worldwide (Gewali, 2008). Presence of various components including secondary metabolites like alkaloids,

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flavonoids, tannins, terpenes, and polyphenols have protective pharmacological targets for the prevention against Alzheimer's disease, cancer, inflammatory pains, malaria, and microbial infections.

Water hyacinth (*Eichhornia crassipes*) is useful in bioenergy production (Bergier, Salis, Miranda, Ortega, & Luengo, 2012; Huang, 2015) and waste water treatment (Huang, 2015; Todd & Josephson, 1996). In addition, it is used as vegetables in many countries due to its high carotene content and the presence of various secondary metabolites like terpenoids and alkaloids (Lalitha, Sripathi, & Jayanthi, 2012). It also has the potential to absorb various harmful water pollutants like lead, mercury, and other carcinogenic chemicals. Their concentrations could be 10,000 times more than the surrounding water in *E. crassipes*.

It is a very fast-growing plant, which covers the pond very rapidly and affects the ecology of the pond. A study suggested that ponds with 10–15% *E. crassipes* covering had a decreased number of phytoplanktons (due to nutritional competition) and zooplanktons (McVea & Boyd, 1975) and hence a much lower fish production due to disturbed pond ecology.

Pathogens can enter the fish body through various routes and cause infection. Infection depends directly on the virulence strength and quantity of potential pathogen, host animal health, and environment. The most common concern of a fish culturist is the rapid multiplication of pathogens in host and water bodies which is responsible for fast transfer of infection to other individuals, resulting in uncontrollable mortalities in hatcheries, culturing farms, and natural habitats. This not only results in huge economic loss but also makes fish unfit for human consumption.

Vibrio harveyi is a gram-negative bacteria mainly found in marine environment, but few studies also reveal their presence in freshwater, which causes vacuities, skin ulcer, gastro-enteritis, eye lesions and luminous vibriosis, loss of limb functions, and appendage degradation in many fish including teleost's, mollusks, and crustaceans. *V. harveyi* has been responsible for various disease outbreaks throughout the world and cause economic losses to fish farmers as well as aqua-industries (Chatterjee & Haldar, 2012; Zhou et al., 2012).

Antibiotics for fish have been developed which are chemotherapeutic agents with fast rate of action (Rodgers & Furones, 2009). Besides the prevention of animal diseases, this may lead to emergence of antibiotic resistance in bacteria, accumulation of drugs in animals, and low nutritional value which deteriorate food quality and cause consumer refusal (Cabello, 2006; Samanidou & Evaggelopoulos, 2007).

The present study has been focused to determine the biotic potential and immunomodulatory role of *E. crassipes*, methanolic extract against *Vibrio harveyi* in *Channa punctata* fish fed fishmeal supplemented with 2.5% and 5% plant powder.

Methods

Preparation of plant extracts

Healthy leaves from the *E. crassipes* were collected from their natural habitats and washed thoroughly in running tap water. The leaves were shade dried at room temperature, ground to fine powder, and sieved. Five grams of fine powder was soaked separately in ethanol and methanol. The solutions were stirred overnight on a magnetic stirrer at room temperature. The slurry obtained was then filtered through a Whatman filter paper; filtrates were dried using a rotary evaporator and used for determination of various biological activities and gas chromatography mass spectrometry (GC-MS)/MS analysis.

Determination of antibacterial activity

Antibacterial activity of ethanol and methanol extracts was determined by disc diffusion assay (DDA) against *Vibrio harveyi*. Sterilized LB agar plates (1.5%) were prepared with 2% NaCl, and bacteria were seeded with a standard inoculum of 1×10^8 cells. Sterile circular paper discs (thickness 2 mm; diameter 6 mm) were placed on agar plate and were impregnated with 100 μ l plant extract prepared at six different concentrations (200, 100, 50, 25, 12.5, and 6.25 μ g per disc) in 0.2% dimethyl sulphoxide (DMSO). Ampicillin and ciprofloxacin were used as positive control while DMSO (0.2%) was used as a negative control.

Antioxidant assays

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

Antioxidant property of *E. crassipes* leaf extract was determined using the Brand-Williams, Cuvelier, and Berset (1995) method modified by Miliauskas, Venskutonis, and Van Beek (2004). Ten microliters of freshly prepared methanol sample (0.5 mg/ml) was added to 300 μ l of DPPH solution (6×10^{-5} M in methanol) in a 96-well microtiter plate. Mixture was incubated at 37 °C for 20 min, and absorbance was taken at 515 nm. For control, 10 μ l of methanol was added to 300 μ l DPPH solution. Free radical scavenging property of plant extract was calculated as percentage inhibition using the standard formula $[(A_B - A_S)/A_B] \times 100$, where A_B is the absorbance of blank and A_S is the absorbance of sample (Sarikurcu, Ozer, Cakir, Eskici, & Mete, 2013). The assay was performed similarly for the ethanol extract, with ethanol as the carrier blank control. Serial double dilution of

ascorbic acid was used as a positive standard (20 to 0.078 mg/ml). Samples were run in quadruplicates in this assay.

FRAP (ferric-reducing ability of plasma) assay

10 μ l of respective plant extract solution (0.5 mg/ml) was mixed with 30 μ l of distilled water and 300 μ l of freshly prepared FRAP solution (containing 10 parts of 300 mM acetate buffer (pH 3.6), 1 part of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and 1 part of 20 mM ferric chloride). Samples were incubated at 37 °C for 30 min. A standard curve was prepared by serial double dilution of ferrous sulphate (20.0 to 0.009 mg/ml) as substrate. Absorbance was recorded at 593 nm. For negative control, acetate buffer was used instead of a sample.

Estimation of total flavonoids

Flavonoid content of methanol and ethanol extracts of *E. crassipes* were estimated using the Dowd method as modified by Arvouet-Grand, Vennat, Pourrat, and Legret (1994). One milliliter of 2% aluminum tri-chloride solution (prepared in methanol) was mixed with 1 ml of plant extract. After 10 min of incubation at RT, absorbance was recorded at 415 nm. For control (carrier blanks), 1 ml of sample was replaced by methanol/ethanol solution (Sarikurkcu et al., 2013). Concentrations of flavonoids in extracts were calculated using serial double dilution of quercetin as standard (8.33–0.032 mg/ml).

Determination of total phenolic content (TPC)

Phenolic content in methanol and ethanol extracts of *E. crassipes* leaf were determined according to Djerdane et al. (2006). Thirty microliters of extracts (2 mg/ml) was dissolved in 135 μ l distilled water and 30 μ l Folin-Ciocalteu's Phenol reagent (2x). Ninety microliters μ l of 2% sodium carbonate solution was added after 3 min, and the mixture was incubated for 2 h in the dark at 25 °C with intermittent shaking. Absorbance was recorded at 760 nm, and a standard curve was obtained using serial double dilutions of Gallic acid (20–0.5 μ g/ml) (Sarikurkcu et al., 2013).

Gas chromatography mass spectrophotometry (GC-MS) analysis

Dried methanol extract of *E. crassipes* was dissolved in HPLC grade methanol (1 mg/ml). Samples were filtered through a 0.22- μ m syringe filter, and GC-MS/MS analysis was performed at AIRF-JNU, New Delhi, India. One microliter of sample was loaded by an automatic programmed syringe injector in the GC-MS instrument.

GC-MS data was then interpreted using NIST/NIH/EPA Mass spectral Database with NIST05 (National Institute of Standards and Technology) MS program v.2.0d and WILEY08 libraries. Unknown components were also identified with the help of spectrum in NIST and Wiley libraries according to their retention time. The names, chemical formulas, molecular mass, and structure of identified compounds were determined. Chemical and biological activities of identified compounds were found using the online search portal of Dr. Duke's Phytochemical and Ethnobotanical Databases, NCBI-Pubchem, ChemSpider from Royal Society of Chemistry, and various literatures.

Preparation of fish feeds

Control fish feed was prepared using fish meal, wheat flour, cod liver oil, vitamins, and mineral premixes mixed at definite proportion (Verma, Rani, Sehgal, & Prakash, 2012). For experimental feed, dried powder of *E. crassipes* leaf was added to control feed substituting wheat flour at 2.5% and 5% proportions. Feed was prepared manually in the form of fine pellet, air dried and stored in an air tight box to prevent bacterial /fungal growth.

Fish maintenance and experimental setup

C. punctata weighing approximately 150–155 g were obtained from the local market New Delhi, India. Fish were placed in 150-L glass tanks, fed on control artificial feed, and acclimatized to laboratory conditions: temperature 25 \pm 1 °C and alternate light and dark intervals of 12 h each. Fish were fed daily in the morning and evening with 3% of their body weight. Water of the aquaria was replaced daily with fresh dechlorinated water maintained at laboratory condition (25 \pm 1 °C). Healthy acclimatized fish were selected for the study on the basis of their skin luster and absence of body lesions. Fish were divided into four groups: group A (negative control; un-injected group fed on supplemented feed), group B (positive control; group injected with *V. harveyi* and fed on non-supplemented feed), and groups C and D (groups injected with *V. harveyi* and fed on experimental feed formulated with 2.5% and 5% supplemented feeds containing *E. crassipes* leaf powder, respectively). Fish were fed on their respective feed 15 days prior to the first challenge. Each group was further subdivided into 4 subgroups (1, 2, 3 and 4). Prior to challenge with bacteria, blood was collected from each subgroup of fish to obtain non-immunized serum after anesthesia to fish using 2-phenoxyethanol at 1 ml/L concentration. After blood collection, the fish were immediately transferred to 2 successive baths of dechlorinated water to make them

Table 1 Antibacterial activity of ethanol and methanol extracts of *E. crassipes* against *V. harveyi*

| | Concentration | Methanol | Ethanol |
|-----------------------------|---------------|----------------------------|----------------------------|
| <i>Eichhornia crassipes</i> | 20 mg/ml | 1.23 ± 0.02 ^{c,n} | 0.83 ± 0.02 ^{b,m} |
| | 10 mg/ml | 0.88 ± 0.02 ^{b,n} | 0.70 ± 0.03 ^{b,m} |
| | 5 mg/ml | 0.73 ± 0.02 ^{a,n} | 0.63 ± 0.01 ^{a,m} |
| Ampicillin | 10 mg/ml | 0.60 ± 0.00 | 0.60 ± 0.00 |
| Ciprofloxacin | 10 mg/ml | 2.17 ± 0.04 | 2.13 ± 0.03 |
| DMSO | -- | 0.60 ± 0.00 | 0.60 ± 0.00 |

Values are represented in mean ± SEM. Values with different superscripts are significantly different. Values with superscripts "a" to "c" represent differences at various concentrations of the extract, while "m" and "n" show difference in activity of ethanol and methanol extracts at respective concentrations

immediate sensitive. The fish were injected with *V. harveyi* (1×10^7 cells) intraperitoneally at day 0. In successive weeks (days 7, 14, and 21), the blood was collected from one subgroup in each group, and the remaining fish were injected with bacteria. For blood collection, fish were temporary anesthetized with 2-phenoxyethanol (1 ml/L), and the blood was collected from the caudal artery using syringes fitted with 22-gauge needle and euthanized through decapitation. After that the fish were decapitated immediately, and organs like the spleen and kidney were excised and kept at -20°C until use. The blood was allowed to clot at RT for 2 h and then kept overnight at 4°C . Serum was separated by centrifugation at 3000g for 10 min and stored at -20°C until use. The dead animals were submitted to the dead animals management authority for autoclaving and proper disposal as per CPCSEA guidelines.

Serum biochemical analysis

Isolated serum from each fish groups collected at different time intervals was used for determination of AST, ALT, and ALP using respective diagnostic kits in 96-well plates following the kit's manufacturer's instructions discussed by Verma et al. (2012). Total protein in the serum samples was also measured

using Lowry's method (Lowry, Rosebrough, Farr, & Randall, 1951).

Phagocytic assay

Phagocytes were isolated (Verma et al., 2012; Verma, Rani, Sehgal, & Prakash, 2015) from the anterior kidney and spleen on day 21 and diluted to density at 5×10^4 cells/ml. Two hundred microliters of isolated cell suspension was evenly spread over pre-washed glass slides and incubated for 1 h at 25°C in 5% CO_2 . Three hundred microliters of heat-killed *Saccharomyces cerevisiae* cells (3 mg/ml) was spread over adhered phagocytes after washing with PBS (pH 7.4). Slides were incubated for 1 h at 25°C in 5% CO_2 . Slides were then washed with PBS (to remove unbound cells), fixed in methanol, and stained with Giemsa stain. One hundred cells from each slide were counted to determine phagocytosis. Percentage phagocytosis and phagocytic index were calculated (Verma et al., 2012).

Estimation of immunoglobulin levels using sandwich ELISA

Serum samples from all groups were diluted (1:3) in 50 mM carbonate buffer (pH 9.6) and added to individual wells of immunoassay plates (Greiner Bio-one, UK) and incubated for 12–14 h at 4°C . Unbound serum proteins were removed by 3 successive washings with wash buffer (PBS having 0.05% Tween 20) at RT. The unbound sites were blocked by adding 300 μl of 1% casein (prepared in PBS) and incubated at 25°C for 2 h. The wells were washed thrice with wash buffer; 300 μl of sonicated *V. harveyi* solution (1×10^5 cells/ml in PBS) was added to each well of the plate and incubated for 2 h at RT. Wells were again washed with wash buffer. One hundred microliters of rat anti-*V. harveyi* Ig (1:1000 diluted in PBS-T; raised in Wistar rats) was added to the wells and incubated at 25°C for 2 h. After 3 washings with wash buffer, HRP-conjugated secondary antibody (rabbit anti-rat IgG; 1:1000 diluted in PBS-T) was added and incubated at 25°C for the next 90 min. Unbound antibodies were washed, and 125 μl substrate solution (0.05% o-

Table 2 Antioxidant potential and total flavonoids in methanol and ethanol extracts of *E. crassipes* leaf powder through DPPH and FRAP assays

| | DPPH assay (% scavenging activity) | FRAP assay (mM Fe^{2+} /mg extract) | Total flavanoids (μg QEs/mg extract) |
|------------------|------------------------------------|--|--|
| Methanol extract | 48.15 ± 1.02 ^b | 231.89 ± 2.95 ^b | 30.46 ± 1.71 ^a |
| Ethanol extract | 30.89 ± 1.21 ^a | 192.24 ± 0.83 ^a | 36.54 ± 0.76 ^b |
| Ascorbic acid | 92.66 ± 1.02 | 1428.28 ± 146.20 | -- |
| Gallic acid | 85.80 ± 3.76 | 2907.48 ± 299.71 | -- |

Values are represented as mean ± SEM. Antioxidant potential of methanol and ethanol extracts were compared by calculating *p* values using *t* test, and *p* values less than 0.05 were considered significant. Dissimilar superscripts "a" and "b" in each assay represent that they are statistically different. Ascorbic acid and gallic acids were used as positive controls for DPPH and FRAP assays, respectively

Table 3 Total phenols in methanol and ethanol extracts of *E. crassipes*. Amounts are represented in equivalence to other standard antioxidants containing phenolic group (except ascorbic acid)

| μg equivalent/mg extract | Methanol extract | Ethanol extract |
|-------------------------------------|-------------------------------|-------------------------------|
| Quercetin | 18.20 \pm 1.22 ^a | 26.37 \pm 1.01 ^b |
| Gallic acid | 16.75 \pm 1.08 ^a | 24.06 \pm 0.90 ^b |
| BHT | 60.12 \pm 1.25 ^a | 78.82 \pm 2.53 ^b |
| Ascorbic acid | 49.13 \pm 1.06 ^a | 54.65 \pm 1.52 ^b |

Values are represented as mean \pm SEM. *p* values less than 0.05 are considered statistically significant. Values having different superscripts "a" and "b" in the same row are statistically different

phenylene-diamine-dihydrochloride in 0.1 M citrate buffer, pH 5.0 containing 0.09% H₂O₂) was added to each well. After 15 min, 35 μl of 1 M oxalic acid was added to stop reaction and absorbance was measured at 495 nm using ELISA plate reader (ECIL, India).

Statistical analysis

Experimental data was pooled, and statistical analysis was performed using ANOVA followed by Newman-Keuls' multiple range test. The values were represented as mean \pm standard error, and values with *p* value 0.05 or less were considered statistically significant.

Results

Antibacterial activity of methanol and ethanol extracts from leaves of *E. crassipes* examined by disc diffusion

assay showed inhibition on the growth of *V. harveyi* at different concentrations (20, 10, and 5 mg/ml). Ciprofloxacin used as positive control showed maximum inhibition, *V. harveyi* showed resistance against ampicillin, and no inhibition was observed in negative control (Table 1).

The antioxidant property of methanol and ethanol extracts, identified by percentage scavenging activity through DPPH assay and ferric-reducing ability, showed appreciable results in reference to standards. Methanol extract from the leaves of *E. crassipes* showed higher DPPH and FRAP assay values which suggest substantial antioxidant potential (Table 2).

Presence of total flavonoids and total phenols were detected in both the extracts. On estimation ethanol extract showed higher amount of total flavonoids (present in microgram of quercetin equivalents per milligram of extract; Table 2). Similarly, total phenol in ethanol extract of *E. crassipes* showed higher values in comparison to methanol extract (Table 3).

Gas chromatography mass spectrometric analysis (GC-MS) showed presence of various components in the methanol extract of leaves from *E. crassipes* (Fig. 1). Palmitic acid (24.18%), 9-hexadecenal (10.29%), neophytadiene (8.42%), 3-undecanone (7.36%), stearic acid (6.35%), vitamin E (5.85%), stearic acid methyl ester (5.33%), and stigmasterol (5.25%) were the major compound constituents comprising more than 73% of the total compounds. Various studies have shown that these compounds exhibit antibacterial, antioxidant, anti-inflammatory, and immunostimulating properties (Table 4).

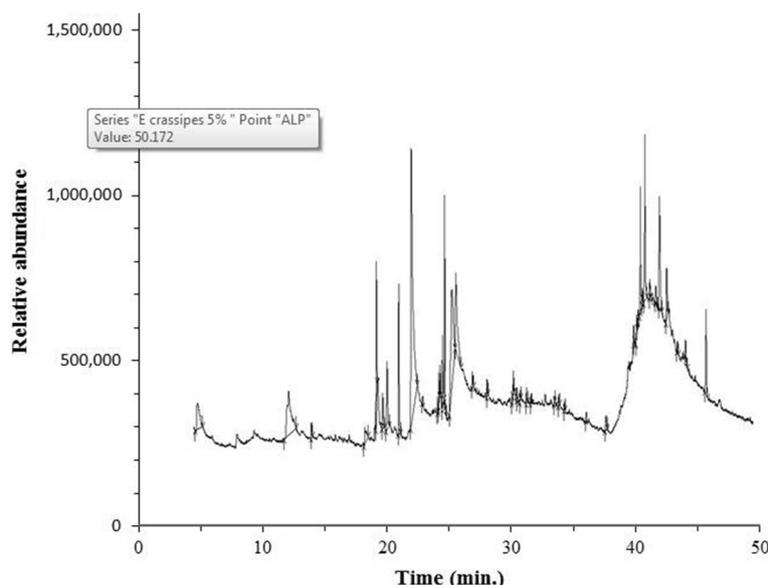


Fig. 1 GC chromatogram of methanol extract of *E. crassipes* (leaf)

Table 4 Represents the compounds identified by gas chromatography mass spectrophotometry analysis of methanol extract of *E. crassipes* (leaf)

| Name | Retention time | Area% | Chemical formula |
|-----------------------------------|----------------|-------|--|
| 4-Methyl-m-dioxane-(amine) | 4.626 | 3.04 | C ₅ H ₁₀ O ₂ |
| 3-Undecanone | 12.013 | 7.36 | C ₁₁ H ₂₂ O |
| 1-Hexadecene | 13.868 | 0.42 | C ₁₆ H ₃₂ |
| 1-Nonadecene | 18.179 | 1.04 | C ₁₉ H ₃₈ |
| Neophytadiene | 19.104 | 8.42 | C ₂₀ H ₃₈ |
| Methyl palmitate | 20.919 | 4.24 | C ₁₇ H ₃₄ O ₂ |
| Palmitic acid | 21.921 | 24.18 | C ₁₆ H ₃₂ O ₂ |
| Myristic acid, methyl ester | 22.822 | 0.15 | C ₁₅ H ₃₀ O ₂ |
| Linoleic acid, methyl ester | 24.055 | 0.35 | C ₁₉ H ₃₄ O ₂ |
| Oleic acid, methyl ester | 24.156 | 0.83 | C ₁₉ H ₃₆ O ₂ |
| Phytol | 24.447 | 2.56 | C ₂₀ H ₄₀ O |
| Stearic acid, methyl ester | 24.617 | 5.33 | C ₁₉ H ₃₈ O ₂ |
| 9-Hexadecenal | 25.202 | 10.29 | C ₁₆ H ₃₀ O |
| Stearic acid | 25.528 | 6.35 | C ₁₈ H ₃₆ O ₂ |
| Palmitic acid (β)-monoglyceride | 26.868 | 0.72 | C ₁₉ H ₃₈ O ₄ |
| Methyl palmoxirate | 30.092 | 0.07 | C ₁₈ H ₃₄ O ₃ |
| Alpha.-monostearin | 30.182 | 0.89 | C ₂₁ H ₄₂ O ₄ |
| Cyclocarbosilane | 30.461 | 0.4 | C ₃ H ₁₂ Si ₃ |
| Eicosanoic acid, methyl ester | 31.244 | 0.42 | C ₂₁ H ₄₂ O ₂ |
| Di-n-octyl phthalate | 31.606 | 0.25 | C ₂₄ H ₃₈ O ₄ |
| Squalene | 36.052 | 0.22 | C ₃₀ H ₅₀ |
| 3-Bromocholest-5-ene | 37.666 | 0.57 | C ₂₇ H ₄₅ Br |
| Stigmast-5-en-3-ol, oleate | 40.439 | 2.99 | C ₄₇ H ₈₂ O ₂ |
| 22,23-Dibromostigmasterol acetate | 40.612 | 0.42 | C ₃₁ H ₅₀ Br ₂ O ₂ |
| Vitamin E | 40.798 | 5.85 | C ₂₉ H ₅₀ O ₂ |
| Epicedrol | 41.196 | 0.5 | C ₁₅ H ₂₆ O |
| Stigmasterol | 41.978 | 5.25 | C ₂₉ H ₄₈ O |
| Stigmast-5-en-3-ol | 42.561 | 2.94 | C ₂₉ H ₅₀ O |
| 4,22-Stigmastadiene-3-one | 43.427 | 0.36 | C ₂₉ H ₄₆ O |
| Cholest-4-en-3-one | 44.095 | 0.58 | C ₂₇ H ₄₄ O |
| 3,5-Di-tert-butylphenol | 45.758 | 2.98 | C ₁₄ H ₂₂ O |

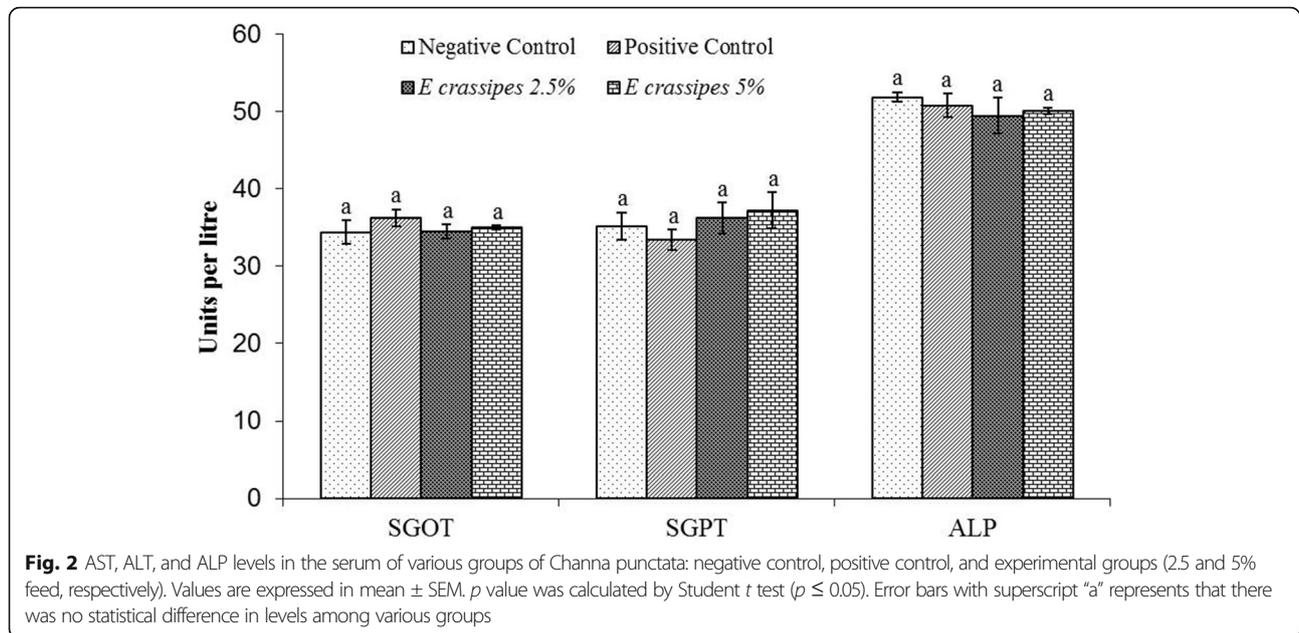
Non-significant changes were observed in AST, ALT, and ALP levels in serum among all groups of fish, after 15 days of initial feeding with respective feeds (Fig. 2).

Phagocytic activity as shown in Fig. 3 was calculated in macrophages isolated from the spleen and head kidney from all groups on the 21st day of the experiment. Significant changes were observed in the positive control as well as in the experimental groups (3 and 4), when challenged with *V. harveyi* in comparison to the negative control group. However, highest levels of percentage phagocytosis and phagocytic index was observed in the experimental groups fed on supplemented feed containing 5% of *E. crassipes* leaf powder. The phagocytic index

of spleen macrophages showed insignificant difference within the experimental groups (3 and 4) but showed significant difference with control groups (1 and 2).

The total serum protein showed increased levels in the positive control group (group 2) and experimental groups (groups 3 and 4) in comparison to the negative control group (group 1) at days 7, 14, and 21. The highest levels of total serum protein was observed in the experimental group fed on 5% supplemented feed at all times during the study (Fig. 4).

Figure 5 depicts the immunoglobulin levels in the serum of all groups at different time intervals. No time-dependent changes were observed in the negative



control group. However, significant differences were observed in the immunoglobulin levels in the positive control as well as in the experimental groups (groups 3 and 4). The highest levels of immunoglobulins were observed in group 4 fed on 5% *E. crassipes* supplemented feed.

Discussion

Synthetic vaccines and antibodies are being used for the prevention of disease in the aquaculture sector. The use of vaccines and antibodies are laborious, expensive, and specific for particular host and pathogen and may lead to compromised immune system of fish. The use of antibiotics in aquaculture has also increased but this may also lead to the emergence of antibiotic resistance against bacteria (Mishra, Oviedo-Orta, Prachi, Rappuoli, & Bagnoli, 2012) and could yield fish with lower nutritional value, less biomass, and bio-accumulation of drugs or harmful chemicals (Mishra et al., 2012). This causes huge economic losses due to consumer refusal and hence less commercial value. This has urged aquaculturists and sensitized them toward the health of aquatic organisms. There is a realization to discover some more potential, herbal immunostimulants to minimize the stress due to various pathogens without any harmful effects.

Various studies have reported extracts from plants to exhibit antibacterial activity against various gram-positive and gram-negative bacteria (Bartfay, Bartfay, & Johnson, 2012; Marasini et al., 2015; Mariri & Safi, 2014). Many of these extracts have equivalent or better antibacterial activity than standard antibiotics. Fish pathogenic bacteria like *V. harveyi* may causes severe

damage in the liver, the kidney, and appendage degradation, suppressing the immune system in fish (Hashem & El-barbary, 2013). *V. harveyi* showed ampicillin resistance; however, *E. crassipes* extract showed a zone of inhibition at different concentrations. Shanab, Shalaby, Lightfoot, and El-Shemy (2010) showed that methanol extract was more effective in comparison to ethanol extract and also reported the antimicrobial (antibacterial and anti-fungal) activity of crude extract of *E. crassipes* against various other bacteria.

Stress-related diseases in organisms are known to cause damage to different organs and may ultimately lead to death. Stress causes the release of oxygen free radicals. In order to minimize this, there is a need of antioxidants which help reduce free radicals. DPPH and FRAP assays showed the presence of antioxidant compounds. Methanol extract showed higher value in comparison to ethanol extract in both assays (Table 3).

The amount of total flavonoids and phenol present in the extracts were also evaluated in the reference with other phenolic compounds or phenol-derived compounds. Tables 3 and 4 suggest that there were high amounts of flavonoids and phenolic compounds in the ethanol extract. Literature review suggests that phenols, flavonoids, tannin, terpenes, and other important compounds are present as secondary metabolites in plants (Thomas & Krishnakumari, 2015; Toure, Bouatia, Idrissi, & Draoui, 2015). These compounds inhibit reactive oxygen species and free radicals, prevent mutations, and have antibacterial, anti-viral, antioxidant, anti-inflammatory, and anti-cancer as well as cardio-protective activities.

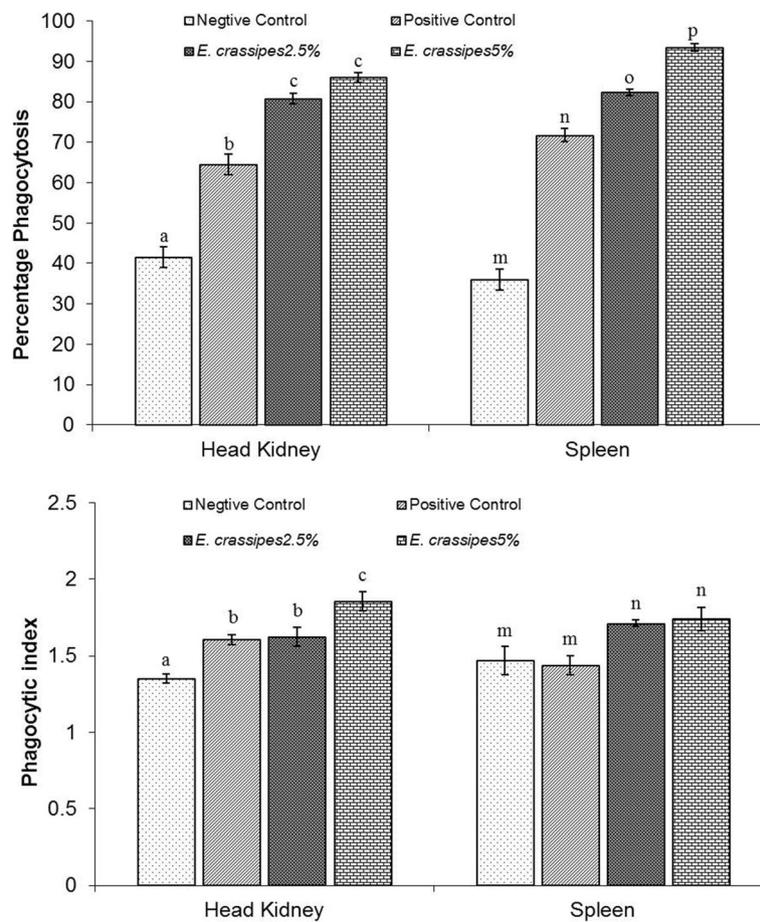


Fig. 3 Phagocytic activity of macrophages isolated from the spleen and head kidney of *Channa punctata*: **a** percentage phagocytosis and **b** phagocytic index. Values are expressed in mean \pm SEM. *p* values are calculated using one-way ANOVA followed by Student Newman-Keuls' multiple range test. *p* values \leq 0.05 considered as significantly different. Error bars with dissimilar superscripts differ statistically

E. crassipes on GC-MS analysis showed the presence of many secondary metabolites which have capabilities like antibacterial and anti-viral properties, including protection against many diseases: anti-tumor, anti-cancer, neuroprotective, allergenic, oxidative stress inhibitor, NO-inhibitor, and cardio-protective (Hassan et al., 2011; Somasagara et al., 2012; Valenzuela, Imarai, Torres, & Modak, 2013).

Early investigations revealed the presence of harmful metabolites and chemicals in plants that cause adverse effects on the health of the organism (Ardo et al., 2008; Lavecchia, Rea, Antonacci, & Giardi, 2013). Enzyme-based liver function test (ALT and AST) and alkaline phosphatase levels were checked to identify if there were any detrimental effects of *E. crassipes* in *Channa punctata*. The tests revealed that there were no significant changes in the test samples of the fish fed on supplementary feed (Fig. 2).

In the present study, macrophages isolated from the spleen and head kidney showed significant increase in

phagocytosis (percentage phagocytosis and phagocytic index) in the experimental groups when compared with the control groups. This suggested enhanced cell-mediated immune response in fish (El-Boshy et al., 2014; Sirimanapong et al., 2015; Verma, Rani, Sehgal, & Prakash, 2013).

Antibodies form the major component of the humoral immune system, and they have been well documented to play an adaptive role in neutralizing and destroying the invading pathogens in all class of organisms, including fish. Increase in the titer of these specific antibodies helps in neutralization and speedy removal of antigen introduced in the host body. The fish challenged with *V. harveyi* showed enhanced antibody titer in a time-dependent manner. Higher levels of antibodies observed in the experimental groups showed an immunomodulatory activity of plant when introduced in fish feed (Verma et al., 2015).

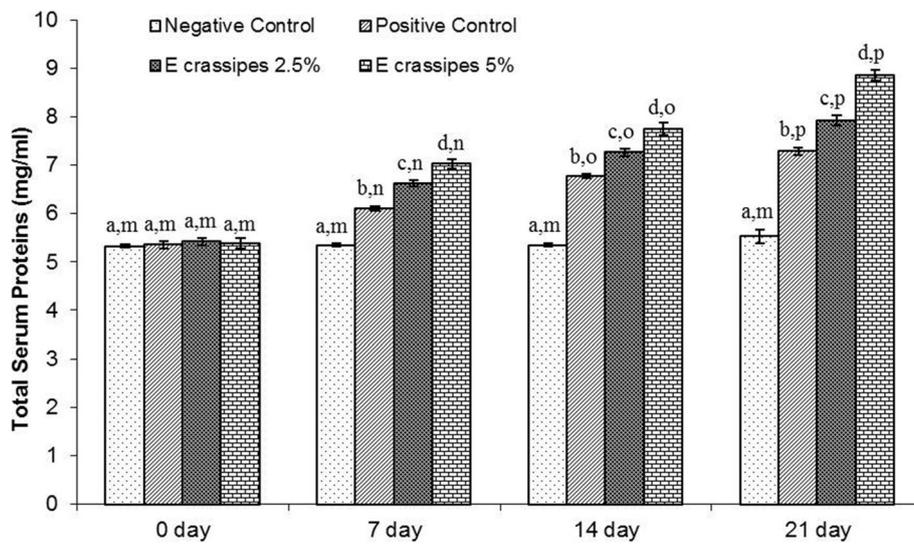


Fig. 4 Total serum proteins in serum samples of various groups of *C. punctata* on days 0, 7, 14, and 21. Values are expressed in mean \pm SEM. *p* values were calculated using one-way ANOVA followed by Student Newman-Keuls' multiple range test, and *p* values \leq 0.05 are statistically different. Error bars with dissimilar superscripts differ significantly. Values compared within four groups, specific time point is represented by superscripts "a" to "d," and within groups at various time points is represented by superscripts "m" to "p"

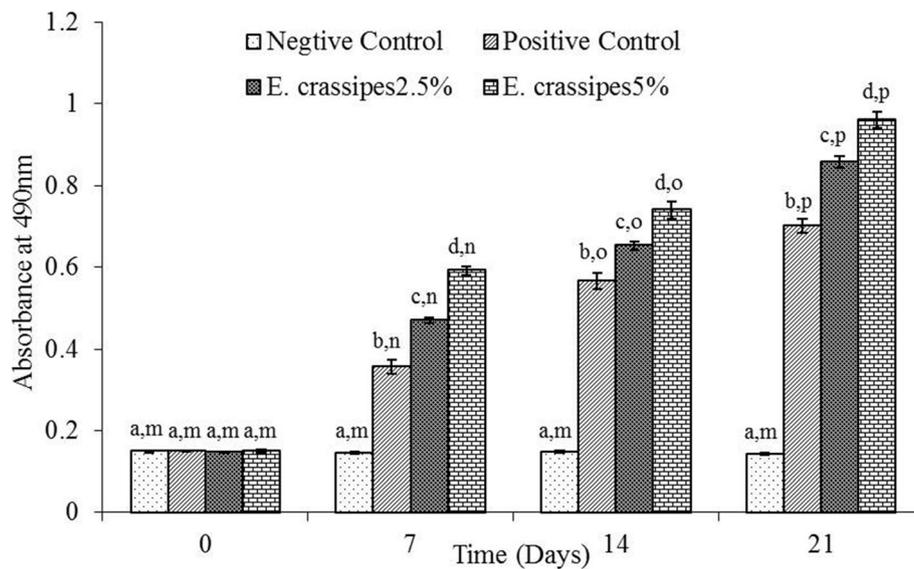


Fig. 5 Immunoglobulin levels in the serum of *C. punctata* at various time intervals (days 0, 7, 14, and 21). Values are expressed in mean \pm SEM. *p* values were calculated using one-way ANOVA followed by Student Newman-Keuls' multiple range test, and *p* values \leq 0.05 are statistically different. Error bars with dissimilar superscripts differ significantly. Values compared within four groups, specific time point is represented by superscripts "a" to "d," and within groups at various time points is represented by superscripts "m" to "p"

Conclusion

E. crassipes is a fast-growing weed plant which is easily available and did not have any detrimental effect on fish when supplemented in its feed. It also stimulated both humoral and cell-mediated immune response in *Channa punctata*. Therefore, we recommend field trials of feed supplemented with *E. crassipes* so that it can be recommended for development of disease resistance in *C. punctata* against *V. harveyi*.

Abbreviations

E. crassipes: *Eichhornia crassipes*; *C. punctata*: *Channa punctata*; *V. harveyi*: *Vibrio harveyi*; GC-MS: Gas chromatography mass spectrometry; DDA: Disc diffusion assay; DMSO: Dimethyl sulphoxide; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; FRAP: Ferric-reducing ability of plasma; TPTZ: 2,4,6-Triphenyl-5-triazine; PBS: Phosphate buffer saline

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Authors' contributions

VKV, OP, and SRK designed the experimental work and performed experimentation, KVR wrote the manuscript and NS supervised the whole experiment. All authors discussed the result and read and approved the final manuscript.

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Availability of data and materials

The raw files for GC-MS and other parameters can be available by the corresponding author on request.

Ethics approval and consent to participate

The experimentation on *Channa punctata* fish was approved by the Animal Ethics Committee, University of Delhi. Investigations were performed following INSA, New Delhi Guidelines.

Consent for publication

Not required.

Competing interests

The authors declare that they have no competing interest.

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