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Evaluating The Prophylactic and Therapeutic Potential of *Ceratophyllum Demersum* Ethanol Extract Against *Aeromonas Sobria* Infection in *Botia Rostrata* (Günther, 1868): A Bioassay-Guided Fractionation Approach

Mainak Mukherjee[1,2], Abhishek Choudhury[1], Suman Bhusan Chakraborty[1,*]

[1]Department of Zoology, University of Calcutta, Kolkata, West Bengal, India.
[2]Department of Zoology, Fakir Chand College, Diamond Harbour, West Bengal, India.

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Corresponding Author
E-mail: sumanbc76@gmail.com

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Abstract

Present study aims to point out the antibacterial phytocomponents in *Ceratophyllum demersum* against *Aeromonas sobria* infection in *Botia rostrata*. In disc diffusion assay, *C. demersum* ethanol extract (CE) showed the highest antibacterial activity (17.66±0.33 mm). In subsequent *in vivo* study, healthy, juvenile *B. rostrata* males (Weight: 2.7±0.5 g; Length: 4.9±0.8 cm) were fed basal and CE-fortified (0.4 g/kg feed) diets for 30 days either before being intraperitoneally injected with *A. sobria* (96h LD_{50} single dose: $1*10^{7.261}$cfu/ml; 25 µl) and basal diet feeding for 7 days (prophylactic) or after bacterial inoculation and basal diet feeding for 7 days (therapeutic). Fish fed CE fortified-diet showed significantly (P<0.05) lower mortality, serum cortisol, cytokines, gill and liver reactive oxygen species (ROS) levels, while significantly (P<0.05) higher innate immune parameters than the basal diet-fed fish. CE seemed equally effective as a prophylactic agent to stimulate innate immunity and provide resistance against *A. sobria*, and a therapeutic agent to reduce *A. sobria* induced physiological alterations. Chromatography and GC-MS analysis disseminated the presence of 1-tetradecene, 1-heptacosanol, ethane 1,1 diethoxy and cyclotrisiloxane hexamethyl in CE, which were described to manifest antibacterial, antioxidative and immunostimulatory potential.

Introduction

Botia rostrata (Gunther, 1868), a hill stream loach, is found mainly in the shallow streams of North East Indian states and contributes to a major share of ornamental fish in the world market. However, bacterial infection may cause the gradual decline of its natural populations (Hossen et al., 2014). Besides, infection with ubiquitous bacteria from aquatic habitat and during intensive culture is common in ornamental fish (Cardoso et al., 2019). *Aeromonas sobria,* a motile aeromonad was earlier reported to infest shallow water of ponds and rivers worldwide. The presence of *A. sobria* has even been reported in *Labeo rohita* and *Hypophthalmichthys molitrix* from aquaculture ponds at Poonch District of Jammu and Kashmir, India (Dar et al., 2016). Pathogenicity of *A. sobria* infection in *B. rostrata* has also been evaluated recently (Mukherjee and Chakraborty, 2024).

The innate parameters are the spearhead of fish immune defense acting as a crucial factor in disease resistance (Xia et al., 2017). Synthetic and natural immunostimulants can augment innate immunity in fish (Kumar et al., 2013). But, large-scale application of such synthetic immunostimulants may have negative effects on the environment as well as human health (Midthun et al., 2021). To overcome this difficulty, plant extracts were targeted as a sustaining alternative to augment innate immunity and also to mitigate bacterial sepsis in fish (Chakraborty & Hancz, 2011).

Ceratophyllum demersum is a cosmopolitan, perennial, obligatory aquatic plant. The submerged macrophyte is easily available and has long been used as aquarium commodity in ornamental fish markets. Previous studies have reported extracts of this plant to show antioxidant and antimicrobial properties and treat different ailments in humans (Emsen & Dogan, 2018). The antimicrobial activity of *C. demersum* has been evaluated *in vitro* against some fish pathogens from the cold waters of Kashmir (Lone et al., 2023). However, as per our knowledge, no studies have yet evaluated the prophylactic and therapeutic potential of the plant against *A. sobria* infection in fish.

Bioactive constituents and their interactions determine the antimicrobial potential of any plant extract (Vaou et al., 2022). Major therapeutic agents may be isolated by a separation technique based on their bioactivity and physicochemical properties (Mukherjee et al., 2022). Therefore, the principal antimicrobial components responsible for the antimicrobial properties of *C. demersum* must be identified using chromatographic techniques.

Considering above all aspects, present study aimed to determine the immunostimulatory and antimicrobial efficacy of *C. demersum* against *A. sobria* infection in *B. rostrata*. Moreover, efforts were made to point out the important antimicrobial phytoconstituents in the plant. It is the first study to identify the bioactive molecules responsible for the antimicrobial property of *C. demersum* and its application in ornamental fish culture.

Material and Methods

Procurement of Bacterial Strain

The bacterial strain *A. sobria*, MTCC 3613, was obtained from MTCC, IMTECH, Chandigarh, India. The lyophilized strain was revived in tryptic soy broth by 24 h incubation at 30°C in a shaking incubator. The rehydrated culture was subsequently streaked on agar plates (nutrient agar + tryptic soy broth medium) and incubated for 24 h at 30°C. This streak plate method was repeated each time to get single bacterial colonies.

Collection and Preparation of Plant Extract

C. demersum was obtained from water bodies of Diamond Harbour (22°11'30" North, 88°11'5'' East), West Bengal, India. Air dried plants were then powdered with a grinder and percolated separately in ethanol, methanol and hexane (sample to solvent ratio 1:2, w/v) for 2 days. Respective extracts were filtered (Whatman grade 42) and the filtrates were evaporated using a rotary vacuum evaporator (Roteva - Equitron Medica Private Limited, India). Each dried extracts dissolved in DMSO (50 mg/ml) were kept in air-tight amber glass bottles and stored at -20°C.

Determination of Antibacterial Efficacy of the Plant Extracts in Vitro

Overnight grown *A. sobria* culture was spread on a sterile petri plate containing nutrient agar medium using a sterile spreader to create a lawn at a final inoculum of 2 x 10^9 cfu/ml. Three 6 mm diameter wells were made in four plates with the help of a sterile cork borer. Three plates were placed separately with ethanol, methanol, and hexane extracts (50 µl) of the plant using sterile pipettes, while the fourth plate (control) was placed with DMSO. Each petri dish was then incubated for 24 hrs at 30°C. Zone of inhibition around the wells were measured in the nearest millimeter. The experimental result was determined by the average of the 3 wells in each plate.

Fish Collection and Acclimatization

Healthy, juvenile *B. rostrata* males were collected from the fish breeding facility at Rajiv Gandhi University, Arunachal Pradesh. Fish were transported to Kolkata in oxygen packed polythene bags and acclimatized for 1 month in aerated cistern (1000 lit). Water in the cistern was maintained at 24.0±0.5°C, pH 7.0-7.5, and 6.5-7.0 mg/l dissolved oxygen. During acclimatization fish were fed *ad libitum* commercially available fish feed (Tetra bits complete, Germany; crude proteins 47.5%) twice every day at 10 am and 6 pm.

Preparation of Plant Extract-Fortified Diets

C. demersum ethanol extract dissolved in DMSO was mixed at desired concentrations with the commercial feed to prepare the plant extract-fortified diet. Control diet was made by adding only DMSO. The pellets were first pulverized, the plant extract was thoroughly and uniformly mixed with the feed, then made wet with deionized water, pelleted again with a pelletizer (diameter 2 mm), and baked at room temperature.

Acute Toxicity Studies

At the onset, an acute oral toxicity test was performed according to OECD 203 guidelines. Juvenile fish (Weight: 2.7±0.5 g; Length: 4.9±0.8 cm) from the acclimated stock were fed *C. demersum* ethanol extract-fortified feed at different concentrations (0, 0.125, 0.25, 0.5, 1, 2, 4 g/kg of feed) in three replicates per concentration (n= 10 fish per replicate) for 4 days. Fish were hand-fed each day at the rate of 2% of fish body weight. Since no mortality was recorded during the four days of experiment, 1/10th of the highest safer dose for the plant extract was selected for further experiments (Karale et al. 2013).

Experimental Design

Next, 375 juvenile, male *B. rostrata* (Weight: 2.7±0.5 g; Length: 4.9±0.8 cm) from the acclimated stock were randomly divided into five treatment groups with 3 replicates per group (n= 25 fish per replicate): i) G1- fed diet without any plant extract for 37 days (negative control), ii) G2- fed diet without any plant extract for 30 days followed by intraperitoneal (i.p.) injection with a single dose of *A. sobria* suspension ($1*10^{7.261}$ cfu/ml; working volume: 25 µl) and again fed basal diet without any plant extract for 7 days (positive control for prophylactic treatment), iii) G3- fed diet fortified with *C. demersum* ethanol extract (0.4 g/kg feed) for 30 days followed by *A. sobria* i.p. injection and fed basal diet for 7 days without any plant extract (prophylactic treatment with plant extract) iv) G4- i.p. injection with *A. sobria* and fed basal diet without any plant extract for 37 days (positive control for therapeutic treatment) v) G5- i.p. injection with *A. sobria* and fed basal diet without any plant extract for 7 days followed by feeding diet fortified with *C. demersum* ethanol extract (0.4 g/kg feed) for 30 days (therapeutic treatment with plant extract). The dose of bacterial inoculation was 96h LD_{50} of *A. sobria* in *B. rostrata* as determined in our earlier study (Mukherjee and Chakraborty, 2024).

The test aquarium of (75 x 30 x 30 cm) size was employed for each experimental set up. Excess diet and detritus were removed daily, and temperature at 24.0-25.0°C, pH at 7.0-7.5, and 6.5-7.0 mg/l dissolved oxygen was maintained throughout the experiment. During the experiment, fish were fed at 2% body weight per day and starved 24 h before final sampling. After experimental tenure, surviving fish were anaesthetized with phenoxy-ethanol (1:20,000, v/v) and blood was collected via a nonlethal caudal puncture. Blood from 4 fish per replicate was pooled in a heparinized tube, while that from another 4 in a non-heparinized tube. Then the fish was dissected to collect liver and gill tissue, and tissue from 4 fish per replicate was pooled and stored at -20^{0}C for biochemical analysis. Collectively, three pools of heparinized and non-heparinized blood, gill and liver tissue samples were obtained from three replicates for each treatment group.

Analysis of Innate Immune Parameters

Heparinized blood samples from each experimental group were processed to analyze different innate immunological parameters. Phagocytotic activity, Sera lysozyme activity and Respiratory burst activity were then measured using standard protocols described earlier (Mukherjee and Chakraborty, 2024).

Analysis of Serum Cortisol Level

Fresh non-heparinized blood samples from each experimental group were centrifuged at 3500g for 6 min at 4°C to collect serum for cortisol analysis using a fish-specific ELISA kit (MyBioSource, San Diego, CA, USA, Catalogue no. MBS9424415) following manufacturer's protocol. The absorbance was measured using a microplate reader (Varioskan LUX Multimode, Thermo Scientific) at 450 nm.

Analysis of Ros Level in Gill and Liver Tissues

ROS level was determined using H_2-DCFDA following the standard protocol described earlier (Mukherjee et al., 2022). ROS detection was performed in FL1 channel (emission filter at 489 nm, BD Accuri C6 flow cytometer, BD Biosciences, USA) and the data were analysed in FlowJo_V10 software.

Analysis of Serum Cytokine Levels

Levels of different cytokines such as TNFα (MBS704369), IFNγ (MBS011958), IL1β (MBS700230), IL2 (MBS2602623), IL6 (MBS015740) and IL10 (MBS282130) in serum from each experimental group were measured using fish-specific ELISA kits following manufacturer's protocol. The absorbance was measured using a microplate reader (Varioskan LUX Multimode, Thermo Scientific) at 450 nm.

Qualitative Estimations of the Plant Extracts

The presence of different phytochemical groups such as tannin, saponin, alkaloid, carbohydrate, glycoside, flavonoid and steroid/terpenoid in *C. demersum* ethanol extract was determined following standard protocols described previously (Mukherjee et al., 2019).

Quantitative Estimations of the Plant Extracts

Total phenolic content in *C. demersum* ethanol extract was determined using the Folin Ciocalteu reagent with gallic acid as standard, while total flavonoid content was determined using colorimetric assay with rutin as standard following methods described earlier (Mukherjee et al., 2019).

Column and Thin Layer Chromatographic Fractionation

C. demersum ethanol extract was first fractioned using column chromatography with Spectrochem SILICAGEL 60–120 mesh. $CHCl_3$:CH_3OH mixture was used with an increasing polarity (100% to 0% $CHCl_3$) as eluting solvent. Fractions were collected in sterile test tubes and plugged in cotton. These fractions were further analyzed in TLC performed on TLC Silicagel 60 F254 pre-coated plates (layer thickness 200 µ; E. Merck, Germany), the mobile phase was C_6H_{14}: CH_3OH (65:35). Then the plates were stained with iodine vapour to determine the R_f from the spots on the plate. The fractions with similar R_f were pooled and evaporated in a vacuum rotary evaporator. Each dried fractions were divided into two parts; one part was kept in air tight amber vial at -20°C for GC-MS analysis and the other part was dissolved in DMSO (50 mg/ml) for testing of antibacterial potential *in vitro* against *A. sobria* following procedures detailed in earlier section.

Gas Chromatography and Mass Spectrometry Analysis

The fractions showing the highest antibacterial potential during *in vitro* study were analyzed using GC-MS QP2020 (Shimadzu, Kyoto, Japan) equipped with HP-5MSI (19091S-433I) column (30 m in length x 0.25 mm inner diameter x 0.25 µm in thickness of film). Pure helium gas was used as the carrier gas at a constant linear velocity of 47.2 cm/s. Total flow, column flow and purge flow were 50 ml/min, 1.69 ml/min and 3 ml/min, respectively. Injector temperature was 300 ◦C, injection volume was 1 µl and injection technique was split less. The interface and ion source temperatures were 300 °C and 200 °C, respectively. The temperature program was the following: 50 °C held for 1 min to 280 °C at 10 °C/min, held for 5 min. Total running time was 40 min. The acquisition was performed in full-scan mode in the mass range of 50–600 m/z, with a scanning rate interval of 0.3 s. The relative quantity of the chemical compounds present in each of the fractions was expressed as percentages based on the peak area produced in the chromatogram. Compounds were identified using GC retention time on column and matching of the spectra with NIST library (NBS75K.L).

Statistical Analysis

All data from quantitative phytochemical tests, *in vitro* minimum inhibition zone measurement, prophylactic and therapeutic feeding experiments were presented as mean ± standard error of the mean (SEM). Normal distribution and equal variance of the data were analysed by Shapiro–Wilk test and Levene's test, respectively. Minimum inhibition zone data and treatment effects on different parameters were analyzed by one-way analysis of variance (ANOVA), followed by a post hoc Tukey test (P-value 0.05). IBM SPSS Statistics Version 20 software was used to analyze data.

Results

Antibacterial Potential of Extracts During in Vitro Study

No zone of inhibition was noticed in the plate treated with DMSO. Ethanol extract of *C. demersum* showed the highest zone of inhibition (17.66±0.33), which was significantly (P<0.05) higher compared to methanol extract (11.66±0.66) and hexane extract (8.66±0.33). Hence, ethanol extract of *C. demersum* was selected for the subsequent *in vivo* experiment.

Mortality and Morbidity of Fish During in Vivo Study

No morbidity and mortality were observed in fish from the treatment group G1. Fish in treatment groups G2 and G4 showed blood clots at the base of the fins and bleeding ulcers at the middle portion of the head. These two groups also showed significantly higher (P<0.05) cumulative mortality (G2: 53.33±0.1%; G4: 46.66±0.22%) compared to other treatment groups. Fish in the prophylactic group (G3) showed no morbidity and the lowest cumulative mortality (30±0.10%) among all the bacteria-exposed groups. On the other hand, fish in the therapeutic group (G5) showed moderate morbidity immediately after bacteria exposure, which healed during the period of feeding with a plant extract-fortified diet. Moreover, G5 showed slightly higher cumulative mortality (33.33±0.11%) compared to G3. Interestingly, mortality in both G3 and G5 was observed only within 7 days of bacteria injection.

Innate Immune Parameters

All innate immune parameters in G2, G3, G4, G5 treatment groups were significantly (P<0.05) lower compared to those in G1. However, all the parameters increased significantly (P<0.05) in fish fed plant extract fortified diet either before (prophylactic, G3) or after (therapeutic, G5) bacterial infection compared to those in bacteria injected fish fed control diet throughout the experiment (G2 and G4). There was no significant variation (P>0.05) in all three innate immunological parameters between treatment groups G2 and G4, and between G3 and G5 (Figure 1).

Cortisol Level

Stress hormone cortisol level was the lowest in fish from control group G1, and it significantly (P<0.05) increased in bacteria infected groups G2 (+104%) and G4 (+103.6%) compared to this control group. A significant decrease (P<0.05) in cortisol level was noticed in prophylactic (G3: -34.7%) and therapeutic (G5: -34.5%) groups compared to G2 and G4, respectively. However, there was no significant difference (P>0.05) in cortisol level between treatment groups G2 and G4, and between G3 and G5 (Figure 2).

Analysis of Ros

ROS level in both gill and liver tissues was significantly (P<0.05) higher in G2 (Gill: +266.5%; Liver: +81.2%) and G4 (Gill: +205.6%; Liver: +58.4%) compared to that in G1. A significant decrease (P<0.05) in ROS level was noticed in prophylactic (G3) and therapeutic (G5) groups compared to G2 (Gill: -40.9%; Liver: -47.8%) and G4 (Gill: -21.3%; Liver: -48.4%), respectively. There was no significant difference (P>0.05) in ROS level only between treatment groups G3 and G5 in liver tissues (Figure 3).

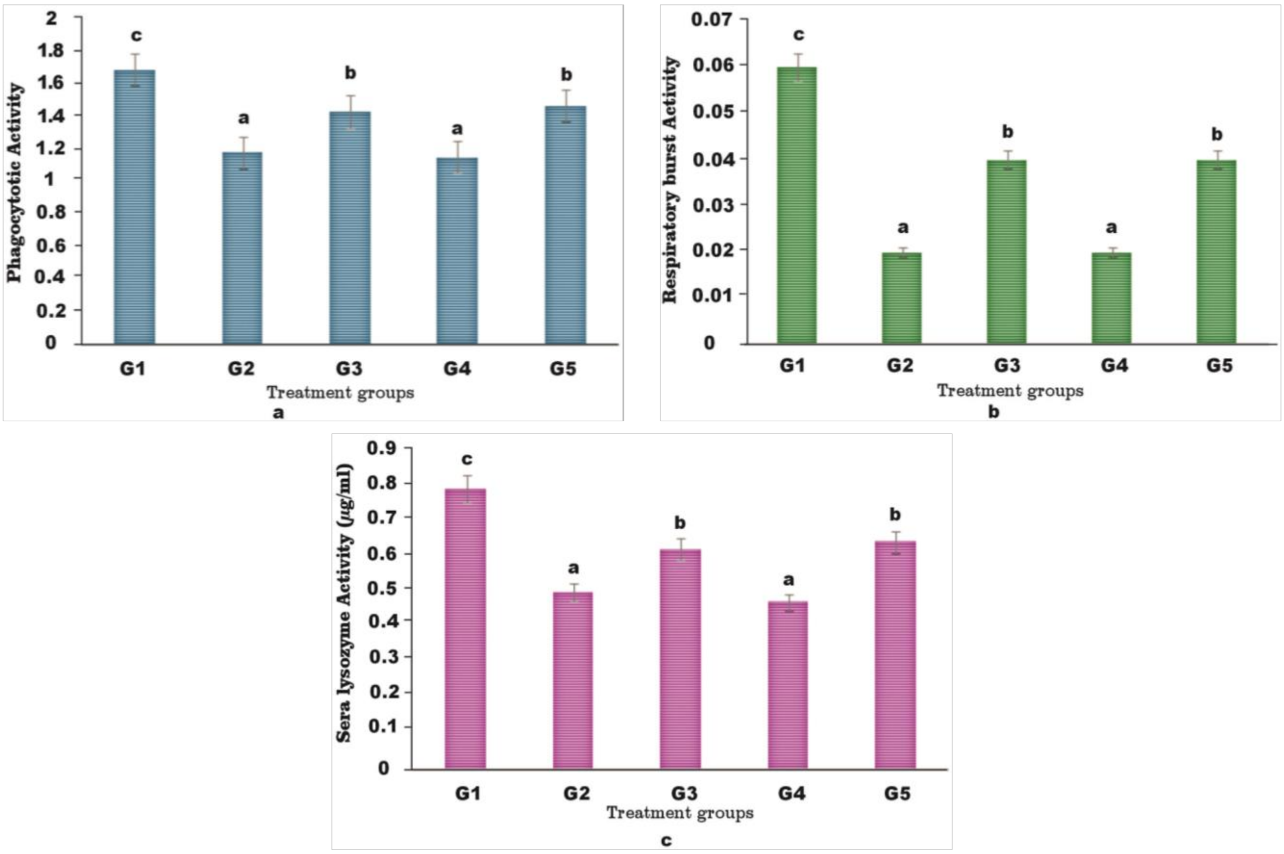

Figure 1. Innate immune parameters (a. phagocytotic activity, b. respiratory burst activity, c. sera lysozyme activity) in different experimental groups during prophylactic and therapeutic modes of treatment with *Ceratophyllum demersum* ethanol extract in *Aeromonas sobria*-infected *Botia rostrata.* Data is represented in form of mean±SEM (n=3). Different superscripts indicate significant difference (P<0.05) in means following post hoc Tukey's test. η^2 = Phagocytotic activity (0.958), Respiratory burst activity (0.987), Sera lysozyme activity (0.957).

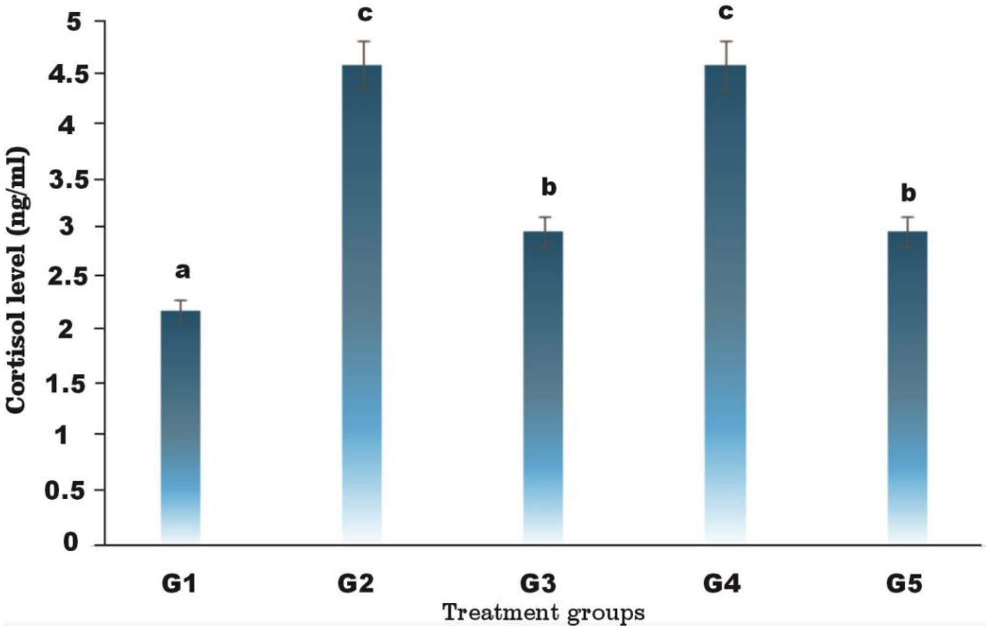

Figure 2. Cortisol level in different experimental groups during prophylactic and therapeutic modes of treatment with *Ceratophyllum demersum* ethanol extract in *Aeromonas sobria*-infected *Botia rostrata.* Data is represented in form of Mean±SEM (n=3). Different superscripts indicate significant difference (P<0.05) in means following post hoc Tukey's test. η^2=cortisol (0.976).

Cytokine Levels

IL1β level significantly (P<0.05) increased in G2 (+42.75%) and G4 (+40.78%) compared to G1. A significant decrease (P<0.05) in IL1β level was noticed in G3 compared to G2 (-27.4%). IL1β level in G5 also decreased, though not significantly (P>0.05) compared to that in G4 (-17.8%). Moreover, there was no significant difference (P>0.05) in IL1β level between G2 and G4, and between G3 and G5. IL2 and IL6 levels in G2 (+55.2% and +34.1%, respectively) and G4 (+49.6% and +37.6%, respectively) were significantly higher (P<0.05) compared to G1. A significant decrease (P<0.05) in IL2 (-23.9%) and IL6 (-14.9%) level was observed in G5 compared to G4. IL2 and IL6 levels in G3 also decreased, though not significantly (P>0.05) compared to that in G2 (-22.2% and -14.5%, respectively). Moreover, there was no significant difference (P>0.05) in IL2 and IL6 levels between G2 and G4, and between G3 and G5. IL10 level significantly (P<0.05) increased in G2 (+44.54%) and G4 (+51.75%) compared to G1. A significant decrease (P<0.05) in IL10 level was observed in G3 and G5 groups compared to G2 (-24.6%) and G4 (-24.8%), respectively. There was no significant difference (P>0.05) in IL10 level between G2 and G4, and between G3 and G5. TNFα level significantly (P<0.05) increased in bacteria infected groups G2 (+49.73%) and G4 (+52.91%) compared to control group G1. TNFα level in prophylactic (G3) and therapeutic (G5) treatment group was lower, though not significantly (P>0.05) compared to bacteria infected control diet fed group G2 (-17%) and G4 (-16.6%), respectively. There was no significant difference (P>0.05) in TNFα level between treatment groups G2 and G4, and between G3 and G5. IFNγ level significantly (P<0.05) increased in bacteria infected groups G2 (+67.34%) and G4 (+68.87%) compared to the control group G1. A significant decrease (P<0.05) in IFNγ level was noticed in prophylactic (G3) and therapeutic (G5) groups compared to G2 (-26.1%) and G4 (-25.1%), respectively. There was no significant difference (P>0.05) in IFNγ level between treatment groups G2 and G4, and between G3 and G5. (Table 1).

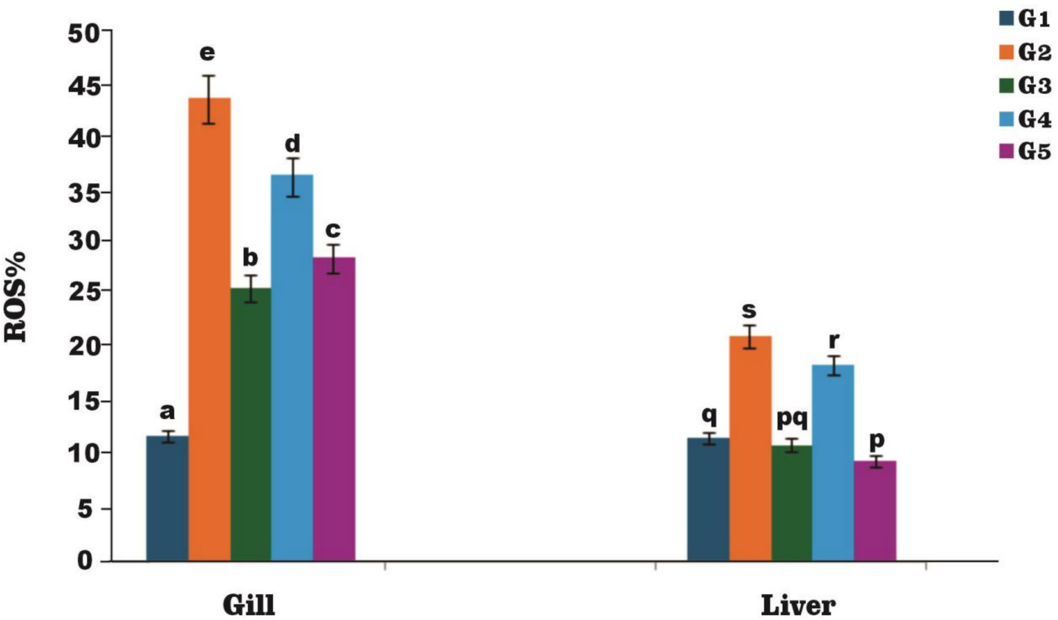

Figure 3. Reactive oxygen species (ROS) percentage in gill and liver tissues in different experimental groups during prophylactic and therapeutic mode of treatment with plant extract in bacteria infected *Botia rostrata.* Data is represented in form of mean±SEM (n=3). Different superscripts indicate significant difference (P<0.05) in means following post hoc Tukey's test. η^2= ROS in gill (0.998), ROS in liver (0.986).

Table 1. Cytokine levels in different experimental groups during prophylactic and therapeutic modes of treatment with *Ceratophyllum demersum* ethanol extract in *Aeromonas sobria*-infected *Botia rostrata.* Data is represented in the form of Mean±SEM (n=3). Different superscripts indicate significant differences (P<0.05) in means following post hoc Tukey's test. η^2=IL1β (0.894), IL2 (0.887), IL6 (0.768), IL10 (0.763), TNFα (0.792), IFNγ (0.763)

GROUP	IL1β (pg/ml)	IL2 (pg/ml)	IL6 (pg/ml)	IL10 (pg/ml)	TNFα (pg/ml)	IFNγ (pg/ml)
G1	101.33±11.56^{a}	178.67±19.91^{a}	99.66±9.26^{a}	104.00±13.61^{a}	126.00±8.71^{a}	130.67±8.97^{a}
G2	144.65±11.21^{b}	277.33±15.87^{b}	133.66±17.29^{b}	150.33±17.29^{b}	188.67±13.28^{b}	218.67±23.13^{b}
G3	105.00±11.54^{a}	215.66±10.27^{ab}	114.33±16.17^{ab}	113.33±31.80^{a}	156.67±12.02^{ab}	161.67±23.51^{a}
G4	142.65±11.21^{b}	267.33±15.88^{b}	137.16±17.29^{b}	157.83±17.30^{b}	192.67±13.28^{b}	220.67±23.13^{b}
G5	117.33±19.24^{ab}	203.33±8.82^{a}	116.66±14.53^{a}	118.66±29.87^{a}	160.67±15.37^{ab}	165.33±20.90^{a}

Qualitative and Quantitative Assay of Plant Extract

Qualitative analysis for phytochemicals in *C. demersum* ethanol extract revealed the presence of saponin, flavonoid, glycoside and alkaloid. Total phenol content in the extract was 1.265±0.161 mg of GAE/g dry weight, while total flavonoid content was 0.80±0.34 mg of RE/g dry weight. A total of 11 fractions were obtained after column chromatography of *C. demersum* ethanol extract. Based on similar TLC R_f values, those 11 fractions were combined to a total of 7 fractions. These 7 fractions were subject to *in vitro* antibacterial assay and minimum inhibition zones were calculated to nearest millimeter. Fraction E (17.66±0.33) and fraction F (16.33±0.66) were found to show the maximum inhibition zone and hence chosen for subsequent GC-MS analysis.

A total of 15 peaks were obtained from fraction E (Figure 4) and total of 12 peaks were obtained from fraction F (Figure 5) during GC-MS analysis. Based on higher peak area %, the major phytocomponents identified from fraction E were 1-tetradecene, 1-hexadecanol, 1-hexacosanol and 1-heptacosanol (Figure 4), and from fraction F were ethane 1,1 diethoxy and hexamethylcyclotrisiloxane (Figure 5).

Discussion

In the present study, a higher incidence of pathological symptoms and mortality was detected in fish fed only the control diet for the entire experiment duration, once challenged with *A. sobria*, consequent to the virulent strain's pathophysiology. Similar to the present observation, the maximum pathogenicity of

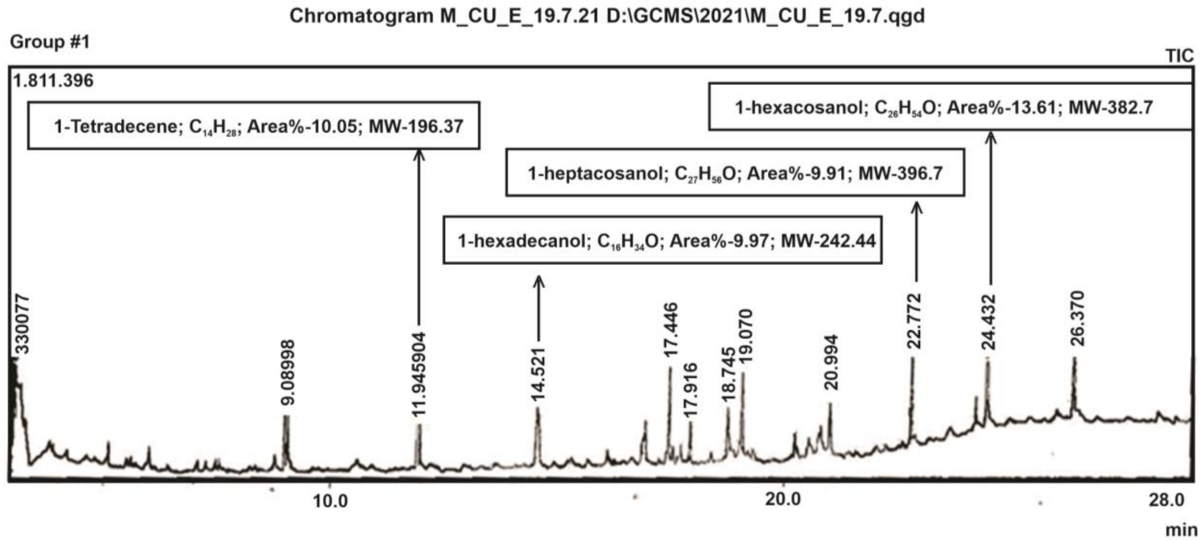

Figure 4. GC-MS Chromatogram of fraction E. 15 peaks were obtained from fraction E Based on higher peak area %, the major phytocomponents identified from fraction E were 1-tetradecene, 1-hexadecanol, 1-hexacosanol and 1-heptacosanol.

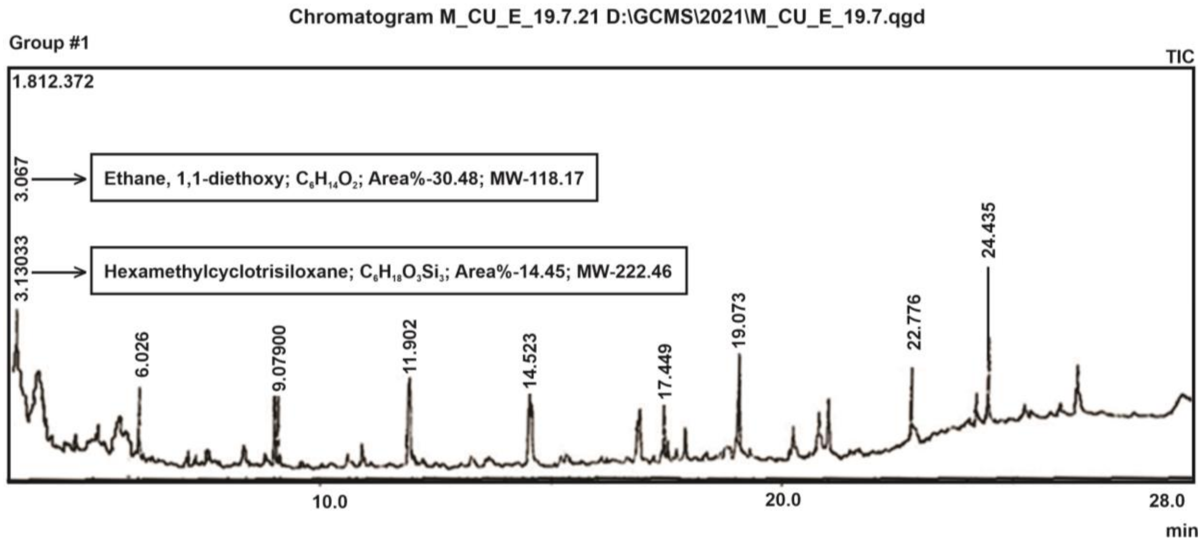

Figure 5. GC-MS Chromatogram of fraction F. 12 peaks were obtained from fraction F. Based on higher peak area %, the major phytocomponents identified from fraction F were ethane 1,1 diethoxy and hexamethylcyclotrisiloxane.

Aeromonas sp. has been spotted during the early days of bacterial exposure in several previous studies (Semwal et al. 2023). *A. sobria*-induced mortality was significantly ($P<0.05$) reduced by dietary administration of the plant extract. This might be due to the antimicrobial effects of the phytochemicals present in the plant extract. Similar decrease in bacteria-induced mortality of different fish species fed diet fortified with various plant extracts have been reported earlier (Semwal et al., 2023). Phenolic compounds were reported to destroy bacterial cell membranes (Chakraborty & Hancz, 2011), which can be correlated to our results as *C. demersum* has been found to be rich in phenolic compounds.

The present results indicated compromised state of innate immunity in fish fed basal diet (groups G2, G4) in face of *A. sobria* challenge. The decrease in phagocytic activity in pathogen-infested groups might be the main cause for the bacteria to create lesions in the fish body. *Aeromonas sp.* can secrete Mn-superoxide dismutase and catalase enzymes. These enzymes guard them from free radicals and reactive oxygen species generated by immune cells, hence inhibiting phagocytic activity (Leclere et al., 2004). During the respiratory burst, O_2 consumption occurs, correlating with cytokine release in fish. Similar to the present context, previous reports have mentioned decreased respiratory burst activity in fish due to *Aeromonas* infection (Mukherjee et al., 2022). A decrease in lysozyme activity in pathogen-exposed groups indicated that suppression of the pathogen by the natural defence mechanisms of the fish might not be possible. Immunostimulants exert their effects by augmenting innate immune parameters and immunomodulatory effects in teleost fish (Chakraborty & Hancz, 2011). Such immunomodulatory effects of the plant extract were also evident in prophylactic (G3) and therapeutic (G5) groups, which showed better innate immune parameters post-bacterial challenge compared to their corresponding control group (G2 and G4, respectively).

The enhanced cortisol concentration indicated a pathogen-induced stress response in fish and might have resulted in reduced innate immunity in G2 and G4.

The role of cortisol in the suppression of immune function may be manifested by the lymphocyte and monocyte-macrophage response observed during an infection episode (Verburg-van Kemenade et al., 2011). Dietary administration of plant extract reduced the stress hormone level in G3 and G5. This observation was similar to an earlier study where dietary administration of β-glucan potentiated the stress response due to transportation and prevented an overshooting of cortisol after subsequent *A. hydrophila* exposure (de Mello et al., 2019). Increased ROS level in gill and liver tissues of G2 and G4 in the present study corroborated the earlier observation that *Aeromonas* infection in fish might increase the intracellular ROS generation and cause oxidative stress (Chen et al., 2020). Phenolic phytoconstituents in *C. demersum* ethanol extract act as potent antioxidants quenching the pathogen-induced free radicals, and thereby reduced ROS levels in fish tissues (Syed et al., 2018).

To scrutinize the innate immune response of fish, pro-inflammatory cytokines can be used as markers. Of late, it has been a subject of acute engrossment in the context of the augmentation of immunity in aquaculture (Sakai et al., 2021). During pathogen recognition, phagocytes release different pro-inflammatory cytokines and interleukins (Sakai et al., 2021). A previous study reported *A. sobria* to produce pro-inflammatory cytokines in mouse macrophages (Zhang et al., 2021). In the present study as well, *A. sobria* exposure caused a massive increase in the levels of all the pro-inflammatory cytokines in *B. rostrata*. The increase of IL-1β level during bacterial infection leads the fish to respond immediately to bacterial disease (Zou and Secombes, 2016; Gallani et al., 2020). On the other hand, IL-10 controls as well as terminates inflammation, acting as pro- as well as anti-inflammatory cytokine. Besides, pro-inflammatory IL-6 is enhanced during bacterial inoculation (Fischer et al., 2007). The increase of IL-2 has also been implicated in the regulation of compromised immunity (Biswas et al., 2013). Er & Dik (2014) confirmed that bacterial inoculation could enhance TNF-α production in organisms, which is in agreement with our study. Both IL-1β and TNF-α induce IFNγ expression, a potential activator of macrophages (Kim and Austin, 2006). The increase in the level of cytokines indicated a prompt attempt by *B. rostrata* to resist pathogenic invasion. On the other hand, plant extracts may control excessive inflammation. In an earlier study with *Oreochromis niloticus*, Neamat-Allah et al. (2021) observed *Aeromonas* infection to increase the production of cytokines, and treatment with white mulberry leaf extract to reduce those enhanced parameters. A similar tendency was observed in our results where dietary administration of plant extract could bring down the increased level of cytokines in bacteria-infected fish during prophylactic and therapeutic modes of treatment. Interestingly, prophylactic application could provide slightly better resilience against *A. sobria* infection compared to the therapeutic mode of application.

The pharmacological effects of different crude plant extracts are often based on synergistic and antagonistic interaction of the phytoconstituents (Phan et al., 2018; Orona-Ortiz et al. 2021). The functional antimicrobial efficacy of *C. demersum* ethanol extract may as well depend upon the combined action of different phytoconstituents present in it.1-Heptacosanol is a long-chain primary fatty alcohol. This compound possesses nematocidal, anticancer, antioxidant, and antimicrobial activities (Everlyne et al., 2015). The antibacterial properties of *C. demersum* ethanol extract may depend on the presence of 1-heptacosanol. Ethane, 1,1-diethoxy and 1 tetradecene demonstrate valuable therapeutic uses encompassing anti-inflammatory and analgesic effects (Al-Wathnani et al., 2012). Cyclotrisiloxane, hexamethyl / metabolite were observed to work against several pathogens except *Shigella dysenteria* (Kingsley and Abraham, 2022). 1-hexadecanol is a long chain primary fatty alcohol, which exhibits inhibitory action against the growth of *Mycoplasma gallisepticum* and *Mycoplasma pneumonia* (Fletcher et al., 1981). 1- hexadecanol was reported to inhibit *Staphylococcus aureus* by Togashi et al., (2007). Hexadecane was identified in seaweeds by Mohy El-Din and Alagawany (2019) exhibiting antibacterial activity. 1-hexadecanol detected in *Pocillopora verrucosa* ethyl acetate crude extract showed antibacterial and antimicrobial activity (Hamed and Hussein, 2020). 1- hexacosanol isolated from leaf extract of *Launaea taraxacifolia* showed inhibitory role against *Staphylococcus aureus* (Tayman et al., 2013). 1-hexacosanol was also isolated from leaf extract of the *Rumex dentatus* and it showed an inhibitory role against *Staphylococcus aureus* (Mohd Rehan et al., 2020).

Conclusion

Dietary fortification with *C. demersum* ethanol extract improved the oxidative and inflammatory status of fish in the face of bacterial infection. Moreover, the plant extract seemed equally effective as a prophylactic as well as a therapeutic agent to stimulate innate immunity and provide resistance against *A. sobria*. Six potential anti-bacterial bioactive components such as 1-tetradecene, 1-heptacosanol, 1-hexacosanol, 1-heptacosanol, ethane 1, 1 diethoxy and hexamethylcyclotrisiloxane are present in *C. demersum* ethanol extract. Large-scale trials are needed to determine the commercial prospect of using the plant extract as an immunostimulating and antimicrobial agent in ornamental fish culture.

Ethical Statement

The investigation was performed according to the guidelines of the Care and Use of Laboratory Animals published by US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was also permitted by Institutional Animal Ethics Committee, University of Calcutta (Registration #885/ac/05/CPCSEA).

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Author Contribution

Mainak Mukherjee: Data curation, Formal Analysis, Investigation, Methodology, Writing- original draft; Abhishek Choudhury: Data Curation, Methodology; Suman Bhusan Chakraborty: Supervision, Resources, Writing- review and editing.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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