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Evaluation of antibacterial activity and immunostimulant of red seaweed *Chondrococcus hornemanni* (Kuetzing, 1847) against marine ornamental fish pathogens

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PEER REVIEW

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Comments
This study is a valuable research works at which authors have demonstrated the antibacterial and immunostimulant properties of the crude and semi-purified fraction of the seaweed C. hornemanni against ornamental fish pathogens. The results are interesting and suggest that the C. hornemanni has better antimicrobial and immunostimulant efficacy against bacterial infection and improve the immune response.
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ABSTRACT

Objective: To evaluate the antibacterial activity and immunostimulant of red seaweed *Chondrococcus hornemanni* (*C. hornemanni*) (Kuetzing, 1847) against marine ornamental fish pathogens.
Methods: In the present study, seaweed extract of *C. hornemanni* showed significant antimicrobial activity against two fish pathogens and the partially purified potential compound was characterized by GC-MS. Purified seaweed extract was injected to the clownfish, *Amphiprion sebae* to study the innate immune response of these fishes.
Results: The extracts was found effective and had more than 80% inhibitory activity against *Aeromonas hydrophila* (20 mm) and *Vibrio parahaemolyticus* (19 mm) at a concentration (25 mg/mL) shown higher antimicrobial activity. The white blood cell count and respirator burst activity was significantly increased in the experimental tanks (E1) and (E2) when compared with control.
Conclusions: Hence, it is concluded that the seaweed extracts of *C. hornemanni* had potential bioactive compounds and act an immunostimulant and improve the immune response to fish.

KEYWORDS
Seaweed, Fish pathogen, Antimicrobial activity, GC-MS analysis, Immunostimulants

1. Introduction

Global imports on marine and fresh water fishes including invertebrates in 2007 have been valued at USD 327 million. The recent information exposed that the trade value of marine origin has increased from USD 9 million in 2003 to USD 29 million in 2007. More than 84 species of marine ornamental fishes which come under the group such as clowns, damsels, cardinals and pseudochromis are being proved to be reared in captivity. In marine ornamental fishes, about 95% are from the wild, whereas 5% are captive-bred[1]. Whereas in the case of fresh water fishes, it is just opposite. It is critical to develop and enforce the effective ornamental fishery management plans and regulations. In captive breeding of ornamental fishes, few constraints have been considered as instrumental which have to be properly

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monitored and overcome. Bacterial disease outbreaks impose significant problems in ornamental fish production.

Seaweeds are primitive non flowering plant without root, stem and leaves. They are one of the commercially important marine renewable resources. Varieties of marine macro-algae are commonly referred to as seaweeds. Macroalgae can be classified as red algae (Rhodophyta), brown algae (Phaeophyta) and green algae (Chlorophyta) depending on their nutrient and chemical composition[2]. Marine algae are known to produce their novel secondary metabolites and also reported that the variety of metabolites present in seaweeds. They serve as an important source of bioactive natural substances and they contain different vitamins, minerals, trace elements, protein, iodine, bromine and bioactive substances. Seaweeds are also serving as excellent sources of food and medicine[3].

Today, there are several reports dealing with the antimicrobial activity of solvent extracts obtained from marine algae. Research and utilization of marine algae in the field of pharmaceuticals have increased in recent years[4,5]. Isolation and identification of antibiotic from marine algae has proceeded rapidly and screening of all classes of marine algae for their potential antibiotic value is considered as novel products from seaweeds[6].

The use of immunostimulants as an alternative to the drugs, chemicals and antibiotics currently being used to control fish diseases is growing, partially because immunostimulants, in contrast to vaccines, enhance the innate (or non-specific) immune response[7,8]. Immunostimulants can be administered by injection, bathing and orally and the latter method is appearing to be the most practicable[9]. The best-known immunostimulants are components of the bacterial cell walls, such as lipopolysaccharides and glucans[10], but the synthetic compounds, polysaccharides, vitamins, animal and plant extracts can also enhance the non-specific immune response of fish[11].

2. Materials and methods

2.1. Sample collection and preparation

Healthy seaweed, *Chondrococcus hornemanni* (*C. hornemanni*) was collected by snorkeling at the Agatti Island waters, Lakshadweep, Southwest Coast of India. Followed, the seaweeds were washed thoroughly with sea water to remove extraneous materials and brought to the laboratory in plastic bags containing sea water to prevent evaporation. Latter, it is shade dried and ground to powder using mortar and pestle. The powdered sample is stored for further study.

2.2. Extraction of active compounds

About 30 g of dried seaweed powder was submerged in 100 mL of methanol and placed at 35 °C in a shaker being operated at 120 r/min for 24 h to allow full extraction of the active compounds. After 24 h, the extract was filtered using Whatman No. 1 filter paper fitted in a suction pump followed by centrifugation at 5000 r/min for 20 min. The supernatant was concentrated under vacuum on a rotary evaporator at low temperature to get crude extracts. These extracts were stored in refrigerator until use.

2.3. Purification of extract by column chromatography

The methanol extracts of the seaweed (1 g) were applied in a silica gel (230-400 mesh) column packed with hexane and eluted with hexane and chloroform 9:1 to 1:9 and 100% chloroform followed by chloroform and methanol 9:1 to 1:9 and 100% methanol.

2.4. Test microorganisms

The bacterial fish pathogens such as *Vibrio parahaemolyticus* (*V. parahaemolyticus*) and *Aeromonas hydrophila* (*A. hydrophila*) were obtained from the marine ornamental fish hatchery, Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences Annamalai University, Parangipettai, Tamil Nadu, India. The bacterial strains were inoculated in nutrient broth and incubated at 30 °C for 24 h and further sub-cultured followed; the pure culture is used for anti-bacterial analysis.

2.5. Agar diffusion method

Antibacterial activity was determined by agar diffusion method. Mueller Hinton Agar plates were prepared and swabbed with 18 h cultured fish pathogen. Making use of template, the well (6 mm) was punctured with the help of gel puncture. Latter, the extracts were loaded on each well. Chloramphenicol was used as a positive control and solvent was used as a negative control. They were incubated at 30 °C for 24 h in incubator. The test was performed in triplicates. The zone of inhibition was measured by antibiotic zone scale.

2.6. Characterization of active compounds by gas chromatograph-mass spectrometer (GC-MS) analysis

Extracted fraction was subjected to GC-MS (Perkin Elmer) analysis. Phyco-constituents were detected using a Perkin Elmer Clarus 500 series GC system and capillary column Elite-5ms (5% Phenyl, 95% dimethylpolysiloxane, Column length: 30 m Column id: 250 μm) was used with helium at a 1 mL/min^{-1} used as a carrier gas. GC oven temperature was programmed from 70 °C at 6 °C to 150 °C (2 min), at 6 °C to 290 °C (5 min). The split ratio was adjusted to 1:20 and injection volume was 2 μL. The injection and detector temperature was 250 °C. The GC-MS electron ionization mode was 70 eV. Mass range was from m/z 45-450 amu.

2.7. Experimental setup

Experimental setup was created and named as control, E1 and E2. Fifteen fishes were stocked in three tanks (duplicates for each tank) 15 nos. per each tank. The E1 and E2 fishes were injected with seaweed extracts.

2.8. Immunostimulant assay of seaweed extract

The innate immune mechanisms in *Amphiprion sebae* (*A. sebae*) [(20±2) g body weight] was tested by following the

method of Harikrishnan *et al*[12]. The fishes were kept in 100 L tanks and named as control, E1 and E2; they were injected LD_{50} concentration of 25 mg/0.1mL. The corresponding control fish received 0.1 mL of distilled water. The experiment was done in separately with replicated tanks.

2.9. White blood cell (WBC) count

Three fishes were randomly collected from each experimental and control tank. The blood sample was collected at a regular interval of 0, 3, 6 and 9 d. About 0.5 mL blood was collected from the caudal vein using 1 mL sterile syringe and preserved to perform WBC count. About 1 in 100 dilution of the blood was made in phosphate saline buffer (PBS, 0.02 mol/L, pH 7.3) and counts were carried out using haemocytometer and expressed as cells/mL.

2.10. Nitroblue tetrazolium activity

Blood sample of 50 μL was placed in a microtitre plate well and then equal amount of 0.2% nitroblue tetrazolium (NBT) solution was added and incubated for 2 h at room temperature. Followed, the NBT added wells were washed with methanol and removed the solvent completely and than 125 μL of 2 mol/L potassium hydroxide and 150 μL of dimethyl sulfoxide were mixed and measured the OD value in spectrophotometer at 620 nm[12].

2.11. Challenging study

To determine the LD_{50} concentration and pathogenicity, the pathogens were cultured in nutrient broth (50% sea water) at 37 °C for 24 h and then the cells were separated by centrifugation at 6000 r/min for 10 min. Supernatant was discarded and pellet containing cells was suspended in phosphate buffer (pH 7.4) and preserved at 4 °C. Bacterial cells in phosphate buffer were serially diluted as 3×10^6 CFU/mL. The experimental fishes were given a single intraperitoneal injection of the bacterial pathogens of 250 μL in PBS/fish and the control was injected PBS solution.

3. Results

3.1. Screening of antibacterial activity

The seaweed extracts showed significant antimicrobial activity against all the bacterial pathogenic bacteria. The 1st fraction of the extracts was found effective and had more than 80% inhibitory activity against *A. hydrophila* (20 mm) and *V. parahaemolyticus* (19 mm) at a concentration (25 mg/mL) shown higher antimicrobial activity with *C. hornemanni* extracts (Table 1). The antibacterial activity was attributed due to the presence of bioactive compounds. Higher inhibitory activity was observed in *V. parahaemolyticus* and *A. hydrophila* during challenging study in the experimental tank E1 and E2.

3.2. Characterization of active compound

Based on the GC-MS result, a total of eight bioactive compounds were identified from the partially purified fractions of *C. hornemanni* (Figure 1). The identification was performed based on the peak areas, molecular weight and molecular formula. The 1,7,7-trimethylbicyclo and tetrachlorotricyclo were recorded at the retention time of 28.25 and 24.53 with 41.88%, and 26.62 of peak value followed isoamyl acetate (11.56%), adamantane (3.58%), butyrophenone (2.51%), germacrene A (1.65%), 9-(methylthio)- (1.33%), cyclopropane (0.97%) at the retention time of 26.28, 19.59, 14.11, 30.42, 24.00 and 16.46 respectively very less amount of compound was found in *C. hornemanni* (Table 2). These 8 compounds were found active molecules responsible for the inhibition of tested bacteria.

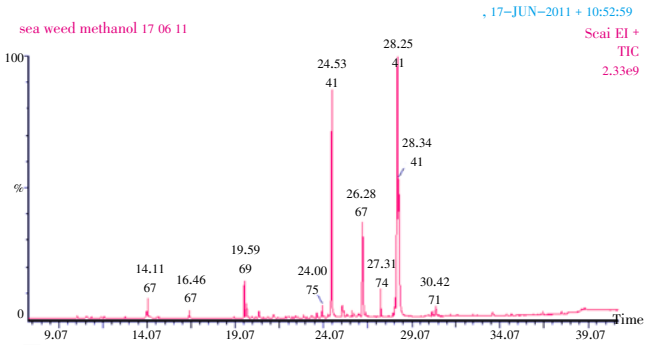


Figure 1. GC-MS chromatogram shows 8 major peaks from partially purified compound.

3.3. Total WBC count

The WBC count was significantly increased in the fishes available in the experimental tanks (E1 and E2) when compared with control. Experimental tank (E1) was observed 4.55×10^5 cell/mL and it was observed in 4.80×10^5 cell/mL in tank E2 . In both the tanks, the WBC count was frequently increased on 3rd day and 6th day and significantly increased on 9th day (Figure 2).

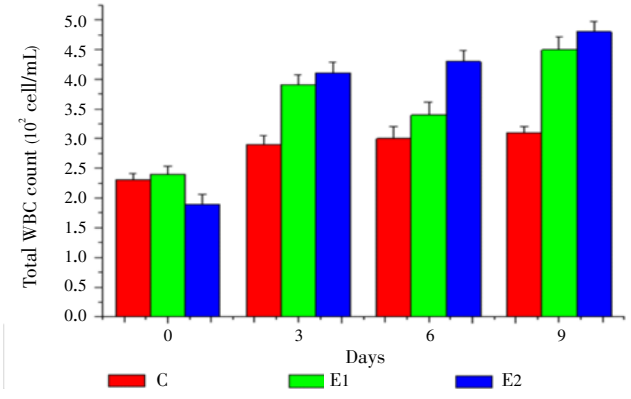


Figure 2. Total WBC count of control and experimental tanks (E1) and (E2).

Table 1
Antibacterial screening of purified fractions against fish pathogens.

Test organism	Zone of inhibition extract (mm)	Positive control (chloramphenicol) (mm)	Negative control (methanol) (mm)
A. hydrophila	20	28	0
V. parahaemolyticus	19	29	0

Table 2
Analysis of active fraction of *C. hornemanni* in GC-MS.

Name of the compound	Molecular formula	Retention time	Molecular weight	Peak area (%)
Butyrophenone	$C_{10}H_{12}O$	14.11	148.00	2.5173
Cyclopropane, 1-chloro-2,2-dimethyl-3-(3,3-dimethyl-1-butynyl)-	$C_{11}H_{17}Cl$	16.46	184.00	0.9787
Adamantane	$C_{10}H_{16}$	19.59	136.23	3.5806
9-(methylthio)-	$C_{16}H_{26}S$	24.00	250.00	1.3350
5,5,10,10Tetrachlorotricyclo[7.1.0.0(4,6)]decane	$C_{10}H_{12}Cl_4$	24.53	272.00	26.6200
Isoamyl acetate,3-methylbut-1-yl ethanoate	$C_7H_{14}O_2$	26.28	130.19	11.5680
1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one	$C_{10}H_{16}O$	28.25	152.23	41.8870
Germacrene A	$C_{15}H_{24}$	30.42	204.00	1.6711

3.4. Respirator burst activity (NBT assay)

Respirator burst activity (NBT assay) was measured for all tanks at 3 d interval. On 6th day, the E1 group of tank showed similar activity as E2 tank and the same tank showed gradual increase of NBT activity. On 9th day, the NBT activity of E2 tank was found more or less similar to E1 tank. The maximum level of activity was showed on 9th day of experiment which is recorded as 0.75 in E2 tank and 0.70 in E1 (Figure 3).

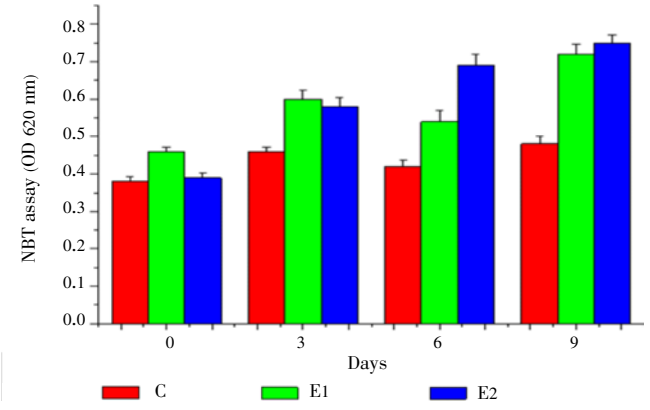
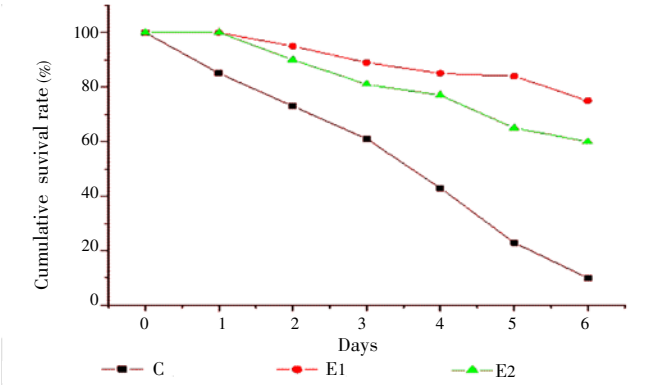


Figure 3. The respirator burst activity (NBT assay) of three groups.

3.5. Cumulative survival rate

After 9th day of experiment, the fishes were challenged with *V. parahaemolyticus* and *A. hydrophila* and the cumulative survival rate was registered (Figure 4). The control tank fishes showed lesions in gill. The cumulative survival rate of the experimental period in the infected tank showed 10%. Cumulative survival of E1 group fishes showed 79% and E2 group fishes showed 70%.



Figuew 4. Cumulative survival rate of challenge with *V. parahaemolyticus* infected.

4. Discussion

Seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities. Lipid-soluble extracts from marine macroalgae have been investigated as a source of substances with antibacterial properties. Moreover, several different organic solvents have been used to screening algae for antibacterial activity[13]. Similar to the results found in the current study, methanol was the best solvent for extracting the bioactive compounds; meanwhile, it gave the highest antimicrobial activity against the selected four fish pathogenic bacteria.

A high antimicrobial activity from seaweed extracts has been reported against Gram negative and Gram positive bacteria[14]. Ely *et al.* have reported the methanolic extract of *Cladophora prolifera*[15], which had moderate bactericidal activity against *Staphylococcus aureus* and *Vibrio cholerea*. Taskin *et al.* have investigated the antibacterial activities of the extracts from six algae species prepared by methanol extracts against six pathogenic bacteria[16]. Umamaheswari *et al.* observed the antibacterial activity of marine macro alga *Chaetomorpha aerea* collected from Vellar estuary and the maximum antibacterial potential was recorded from ethanol extract against *Pseudomonas aeruginosa* and the minimum was noted in methanol extracts against *Micrococcus* sp. and *Salmonella typhii*[17]. In the present investigation, the purified fractions of methanol extracts of *C. hornemanni* have high inhibitory activity against two fish pathogenic bacteria such as *V. parahaemolyticus* and *A. hydrophila*. Bansemir *et al.* have reported the antibacterial activities of the extracts from 26 algal species prepared by dichlorometane, methanol and water extracts against five fish-pathogenic bacteria[18].

Konig and Wright and Bansemir *et al.* studied the characterization and compound identification using GC-MS for the active fraction and they reported that the fatty acids exhibited antimicrobial activity against different species of bacteria and fungus pathogens from the red algae such as *Laurencia obtuse*, *Laurencia rigida* and *Laurencia chondrioides*[19,20]. In the present study, eight major peak were observed in GC-MS analysis like 1,7,7-trimethylbicyclo, tetrachlorotricyclo, isoamyl acetate, adamantane, butyrophenone, germacrene A and 9-(methylthio), cyclopropane.

The major components of the innate immune system (nonspecific) are macrophages, monocytes, granulocytes and humoral elements[21]. Immunostimulants and adjuvants

used as fish vaccines are of interest, as they offer an alternative to drugs, chemicals and antibiotics. Herbal based immunostimulants are capable of enhancing immune responses and reducing losses from viruses, bacteria and parasitic infections in Carp[22]. Immunostimulants can be applied via injection, bathing or oral administration, the latter seems to be the most practicable[12]. In the present study, immunostimulant effect of seaweed extract was performed in *A. sebae* at the dose of 25 mg/0.1 mL through intraperitoneal administration and the immunological parameters were measured at 0, 3rd, 6th and 9th days interval. Harikrishnan *et al.* have reported that the administration of seaweed extract through intraperitoneal injection is enabled the immunostimulant quick effect in fish[23]. Furthermore they reported the oral administration of immunostimulant is slowly absorbed by the fish.

The WBC plays an important role in the immune response of fish, particularly in inflammation[24]. In the present study, the seaweed extracts injected in fish WBC were significantly increased in both E1 and E2 tanks compared to control. Furthermore, the study conducted by Das *et al.* revealed that WBC was increased in Rohu *Labeo rohita* fish fed with *Euglina viridis*[25]. Similarly, the WBC were increased in the Rock bream, *Oplegnathus fasciatus* fed with herbal, probiotics and mixed diets fed groups WBC significantly increased on week 1 and 3 when compared to control[26].

In the present study, highest respiratory burst activity was calculated. However, extract injected fish produced an increase intracellular O_2-production in E1 and E2 tanks (reduction of NBT) after 3rd to 9th days, whereas control was attains its stationary level. Jang *et al.* reported that *in vitro* treatment with glycyrrhizine isolated from *Glycyrrhiza glabra* enhanced the respiratory burst activity of macrophages and the proliferative responses of lymphocytes from rainbow trout[27]. Ethanol herbal extract also increase the intracellular respiratory burst activity of goldfish phagocytic cells on second week. This effect was observed in large yellow croaker and common carp after feeding them with a diet containing a mixture of *Astragalus membranaceus* and *Astragalus sinensis* extracts[28,29].

The result on LD_{50} values for *A. hydrophila* and *V. parahaemolyticus* after 9 d respectively with 250 μL (3×10^6 CFU/mL) of cells for (20±2) g sized fishes. Clown fish *A. sebae* were infected with two predominant bacterial pathogens such as *A. hydrophila* and *V. parahaemolyticus*. Symptoms were observed during infection studies as hemorrhages and congestion of blood circulation, tail rot and pectoral fin hemorrhages and hemorrhages in mouth after 3 d of infection respectively. Similar result was reported after feeding large yellow croaker with glucan and challenge against *Vibrio harveyi*[30]. After challenge with *A. hydrophila* and *V. parahaemolyticus*, there was increased survival in experimental tank and poor survival in control tank. The cumulative survival rate of E1 tank showed 79% and E2 showed 70% and infected control 10% respectively. Similar to the above findings, cumulative survival rate infected fish are usually increased after treatment with various immunostimulants[8,11].

Result of the present study evidenced the admirable immunostimulant activity of active compounds from the seaweed *C. hornemanni*. Further, complete purification and characterization of the active compounds and its field evaluation are needed for this seaweed compounds like 1,7,7-trimethylbicyclo, Tetrachlorotricyclo, Isoamyl acetate, Adamantane, butyrophenone, germacrene A, 9-(methylthio), cyclopropane. These compounds are responsible and premising hopeful for the development of high-quality immunostimulant activity. Potential studies also need to identify the exact compound from the mixture of bioactive compounds for the management of bacterial diseases in aquaculture.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

The occurrence of bacterial infection in marine ornamental fish is becoming a serious problem worldwide. The use of immunostimulants as an alternative to the antibiotics is of great concern to enhance the innate immune response.

Research frontiers

The drug from sea is an emerging field of research. The authors have successfully carried out the antibacterial and immunostimulant activity of the seaweed extract and analysed the same by GC-MS.

Related reports

There are several reports reported the antimicrobial activity of solvent extract of marine algae [4,5,6] and the immunistimulant activity of herbal extract was determined in the fishes [7,8,9]. But, the immunostimulant activity of the seaweed C. hornemanni in ornamental fish has not determined yet.

Innovations and breakthroughs

Result of the present study evidenced the admirable immunostimulant activity of active compounds from the seaweed C. hornemanni. Further, The compounds responsible for the immunostimulant activity have also identified.

Applications

It is beneficial to know the potential application of W. ugandensis in medicine. The results of the present study suggest the use of the plant as an alternative for the

treatment of candidiasis.

Peer review

This study is a valuable research works at which authors have demonstrated the antibacterial and immunostimulant properties of the crude and semi-purified fraction of the seaweed C. hornemanni against ornamental fish pathogens. The results are interesting and suggest that the C. hornemanni has better antimicrobial and immunostimulant efficacy against bacterial infection and improve the immune response.

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