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Effects of methanolic macroalgae extracts from *Caulerpa sertularioides* and *Ulva lactuca* on *Litopenaeus vannamei* survival in the presence of *Vibrio* bacteria

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ABSTRACT

Macroalgae are potentially excellent sources of highly bioactive secondary metabolites that are useful for the development of new functional ingredients. This study was conducted to determine whether methanolic extracts from *Caulerpa sertularioides* and *Ulva lactuca* macroalgae might be possible alternatives for the prevention of shrimp vibriosis, which is caused by *Vibrio parahaemolyticus* and *Vibrio alginolyticus*. Macroalgae extracts prepared with methanol as the solvent were evaluated for antibacterial activity with the microplate method. The extracts' effects on the mortality of juvenile *Litopenaeus vannamei* were evaluated at doses of 150 and 300 mg L^{-1}. Two independent assays for *V. parahaemolyticus* and *V. alginolyticus* were performed. The methanolic extract of *C. sertularioides* exhibited activity against *V. parahaemolyticus* and *V. alginolyticus*, and it had minimal inhibitory concentrations of <1000 and < 1500 μg mL^{-1}, respectively. *L. vannamei* mortality in the presence of both The methanolic extract of *C. sertularioides* exhibited activity against *V. parahaemolyticus* and *V. alginolyticus*, and it had minimal inhibitory concentrations of <1000 and <1500 μg mL^{-1}, respectively. and *V. alginolyticus* bacteria significantly decreased after treatment with 300 mg L^{-1} *C. sertularioides* methanolic extract.

1. Introduction

Aquaculture is one of the fastest growing animal food production sectors. In 2012, global aquaculture production consisted of freshwater species (62.9%) and marine species (37%). *Litopenaeus vannamei* shrimp are the most highly produced crustacean worldwide [1]. However, high shrimp mortality has occurred because of viral and bacterial diseases such as white spot syndrome virus (WSSV) and *Vibrio* bacteria [2].

In recent years, marine resources have attracted attention as part of the search for bioactive compounds for the development of new medicines and healthy foods [3–5]. Bioactive compounds are compounds that have a beneficial effect on living organisms, beyond their caloric contribution [6]. More than 18,000 structurally unique bioactive metabolites have been isolated from plants and marine animals. Some of these compounds have been identified on the basis of their antibacterial, anti-inflammatory and anti-carcinogenic properties [7].

Macroalgae are potentially excellent sources of highly bioactive secondary metabolites that may be useful in the development of new functional ingredients. Macroalgae, a large and diverse group of simple, typically autotrophic organisms ranging from unicellular to multicellular forms, can be classified into three primary groups – red algae, brown algae and green algae – on the basis of their pigmentation [8]. The marine algae include green algae (Chlorophyta), which include the *Caulerpa* and *Ulva* genera. These algae are a rich source of important bioactive compounds, such as sterols, hormones, vitamins, and the structural components of biomembranes [9].

Several earlier workers have used different solvent to extract bioactive compounds from seaweeds. Methanol has been found to be the best solvent for majority of the algae in other studies where various solvents are used [10,11].

The use of macroalgae in aquaculture has increased over the past decade because of their ability to remove nutrients from

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monoculture wastewater ponds and to generate biomass that facilitates multitrophic integration [12,13].

In particular, macroalgae are a very important resource with high commercial value as food, forage, soil conditioners and pharmaceutical resources [14,15], as well as nutritional supplements for improving animal health [16–18].

The aim of this study was to evaluate the effects of two methanolic macroalgae extracts on *L. vannamei* survival in the presence of *Vibrio* bacteria.

2. Materials and methods

2.1. Macroalgae meals

Macroalgae (*Caulerpa sertularioides* and *Ulva lactuca*) were collected from the north-west coast of Mexico, in Agiabampo Bay, Sonora (26°22′31″ North and 109°13′37″ West). The samples were dried in a convection oven (SHELL LAB CE3F, Cornelius, Oregon, USA) at 45 °C for 24 h, milled in a pulverizer PULVEX 200, sifted through a 250 μm mesh sieve, and stored at 4 °C until use.

2.2. Methanol extracts from macroalgae

Methanol extracts were obtained from 200 g of dried macroalgae meal in 2 L of methanol at 99.8%. The extractions were performed at room temperature and stirred 3 times daily for 7 days, after which the methanol was evaporated in a vacuum (rotary evaporator Yamato RE301, Santa Clara, CA, USA) at 60 °C until a dry residue was obtained [19]. The extracts were stored at −20 °C.

2.3. Antibacterial activity of C. sertularioides and U. lactuca against Vibrio parahaemolyticus and Vibrio alginolyticus

The *V. alginolyticus* strain was obtained from the DICTUS laboratory in Hermosillo, Sonora, Mexico, and *V. parahaemolyticus* was obtained from the CIBNOR microbial ecology laboratory in La Paz, Baja California Sur, Mexico. These bacteria were reactivated via incubation for a period of 18–24 h at 37 °C in trypticase soy broth.

The antibacterial activities of *C. sertularioides* and *U. lactuca* methanol extracts were determined at the following concentrations for each of the extracts: 2500, 2000, 1500 and 1000 μg mL^{-1}. An inhibition assay was performed in microplates in triplicate. All the extracts at the indicated concentrations were added to the 96-well microplates and were tested against 1×10^6 CFU mL^{-1} bacterial inoculum; gentamicin (50 μg mL^{-1}) was used as a control. Readings were taken at 620 nm in a Biotek microplate reader at 0, 6, 12, 24 and 48 h after the microplates were inoculated with bacteria.

2.4. Effects of methanol algae extracts on L. vannamei survival in the presence of V. parahaemolyticus and V. alginolyticus

Two independent assays were used to determine the effects of *C. sertularioides* and *U. lactuca* extracts on *L. vannamei* survival in the presence of *V. parahaemolyticus* and *V. alginolyticus*. An 18-tank culture system was used with 10 *L. vannamei* juveniles (0.75 ± 0.01 g) treated with one of two extract concentrations (300 and 150 mg L^{-1}), a positive control (extract without bacteria) or a negative control (only bacteria). The juveniles were immersed in 10 L aquariums for 3 h with constant aeration. After 3 h of exposure, the *L. vannamei* juveniles were infected via injection of 1×10^6 CFU g^{-1} *V. alginoyticus* or *V. parahaemolyticus* into the ventral sinus of the cephalothorax. Shrimp mortality was recorded at 6, 12, 24, 72 and 120 h post-infection. A measurement of the immune response of each organism that survived after 120 h was performed.

2.5. Total haemocyte count

Before the haemolymph extraction, a syringe was loaded with 200 μL of a precooled (4 °C) anticoagulant solution (SIC-EDTA, Na_2) (450 mM NaCl, 10 mM KCl, 10 mM HEPES, and 10 mM EDTA, Na_2 at pH 7.3) [20]. Fifty microlitres of the haemolymph anticoagulant mixture was diluted in 150 μL of formaldehyde (6%), and 15 μL was dispensed onto a haemocytometer (Neubauer) to determine the total haemocyte count (THC) under a compound microscope.

2.6. Antioxidant activity

Frozen shrimp muscle was thawed and dissected, and a 100 mg fragment of each tissue was then homogenized with a pestle in 1.5 mL microcentrifuge tubes containing 1 mL of phosphate buffer (50 mM, pH 7.0). The homogenate was centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatant was removed and stored at −50 °C.

Catalase activity was measured by following the kinetics of hydrogen peroxide reduction at 240 nm, with an extinction coefficient of 0.04 mm cm^{-1} [21], and the kinetics was determined by measuring the absorbance at 240 nm and calculating the specific activity (units per milligram of soluble protein).

Superoxide dismutase activity was determined in accordance with the method described by Beauchamp and Fridovich [22], by using nitro blue tetrazolium (NBT) in the presence of riboflavin. The absorbance values were input into an in-house-generated computer programme to calculate the specific activity (units per milligram of soluble protein).

2.7. Statistical analysis

A one-way ANOVA was applied to determine significant differences among treatments. Tukey's multiple range test was used to identify differences among the means. All statistical analyses were performed at the 0.05 significance level in STATISTICA™ 7.0 (StatSoft, Inc., Tulsa, OK, USA).

3. Results and discussion

3.1. Inhibition assay: antibacterial activity of C. sertularioides and U. lactuca against V. parahaemolyticus and V. alginolyticus

The *C. sertularioides* methanolic extracts significantly inhibited the growth of *V. parahaemolyticus* (MIC_{50} < 1000 μg mL^{-1} and MIC_{90} < 1500 μg mL^{-1}) and *V. alginolyticus* (MIC_{50} < 1500 μg mL^{-1} and MIC_{90} > 1500 μg mL^{-1}) in a concentration-dependent manner ($P < 0.05$; Fig. 1). *V. parahaemolyticus* was most susceptible to the *C. sertularioides* extract (Table 1). The antibiotic gentamicin (GM) was used as a positive control, and it completely inhibited bacterial growth in all the bioassays.

Although *C. sertularioides* and *U. lactuca* have not been used for *Vibrio* growth control, Kanjana et al. [3] have treated *Vibrio harveyi* with *Gracilaria fisheri* methanol extract and have obtained an MIC_{50} of 100 μg mL^{-1}, which is significantly lower than the MIC obtained in this study.

Pretto et al. [23] have evaluated *Calophyllum brasiliense* extracts and have found that MIC values of less than 100 μg mL^{-1} exhibit good antimicrobial activity, whereas values of 100–500 μg mL^{-1} have moderate activity. Although 500 to 1000 μg mL^{-1} was considered to be weak activity, more than 1000 μg mL^{-1} was considered to indicate an absence of antimicrobial activity. However, these activity ranges occurred for only Gram-positive bacteria.

Two Gram-negative bacteria were used in this study, and, in