International Journal of Aquatic Biology (2015) 3(3): 155-160
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.NPAJournals.com

Original Article

Antibacterial activities of bioactive compounds extracted from Marine algae *Gracilaria salicornia* against *Aeromonas hydrophila*

Somayeh Rasooli[1], Masoud Sattari*[1], Zohreh Ramezanpour[2], Javid Imanpour Namin[1]

[1]Department of Fisheries, Faculty of Natural Resources, University of Guilan, Sowmeh Sara, Iran.
[2]Department of Ecology, International Sturgeon Research Institute, Rasht, Iran.

Abstract: Herbal medicinal products have attracted significant research interest in recent years. Considering the efficiency of algae products in controlling pathogenic bacteria and also easy access to large resources of algae, this study was conducted to evaluate the effects of methanolic, chloroformic and aqueous extracts of *Gracilaria salicornia* against *Aeromonas hydrophila*, a heterotrophic, Gram-negative, rod-shaped bacterium found mainly in warm climate. Algae samples were collected from Qeshm Island coastlines and transferred to the laboratory. Standard methods were used to obtain the algae extract. Antibacterial activities of various extracts were tested against the bacterium using well diffusion assay method. Significant differences were observed in antibacterial activities of different extracts ($P<0.05$). The diameter of zone of growth inhibition varied in correlation with concentration of the extracts (50, 100, 200 and 300 mg.ml^{-1}). The best inhibition zone was observed at 100, 200 and 300 mg.ml^{-1} methanolic and 300 mg.ml^{-1} aqueous extracts.

Article history:
Received 13 February 2015
Accepted 22 April 2015
Available online 25 June 2015

Keywords:
Red algae
Extract
Fish pathogen
Antibacterial
Gracilaria salicornia

Introduction

Application of herbal medicinal products with minimal environmental impacts, without causing resistance in bacteria and easy availability have attracted considerable research interests (Aoki, 1992; Marino and Spiewak, 1999; Maunchid et al., 2005; Perrucci, 1994). In addition, high costs of using antibiotics in aquaculture, environmental concerns and difficulties in prescribing antibiotics have resulted in herbal medicinal products to be considered as a replacement for commercial antibiotics (Raa, 1996; Iwama, 1996).

Algae are low calorie food sources rich in vitamin, minerals, protein, steroids, polysaccharides and fiber (Ito and Hori, 1989; Lahaye, 1993) and used in traditional medicine since 3000 BC (Smit, 2004). They also contain substances for developing new antibiotics (Faulkner, 2002; Newman et al., 2003). Although, the effects of algae extracts on Gram positive and negative bacteria are influenced largely by species, organs used for extraction, target microorganisms, season and growth condition (Paniagua et al., 2009; La Barre et al., 2010; Kellmann et al., 2010; Guven et al., 2010).

The genus *Gracilaria* is a member of red algae with a life cycle in three phases inhabiting tropical and sub-tropical regions (Guiry, 2011; Skriptsova et al., 2001). Some biological functions have been reported for bioactive compounds extracted from this genus of algae, including anti-oxidative, anti-inflammatory, anti-fungal, anti-viral, anti-spasm and cytotoxic activities. These bioactive compounds may be used to treat disorders in nervous system, cardio vascular and blood circulatory system. They may also be used as anti-hyperglycemic and contraceptive agents (De Almeida et al., 2011). These algae have also been subject of few studies in Iran (Gharanjik et al., 2000; Saeidnia et al., 2009;

* Corresponding author: Masoud Sattari
E-mail address: msattari@guilan.ac.ir

Nasir et al., 2011; Plubrukarn eet al., 2012; Zandi et al., 2007; Jassbi et al., 2013; Saeidnia et al., 2011). *Aeromonas hydrophila* is a Gram negative, motile, facultative aerobic and anaerobic bacteria found in water bodies and also in digestive systems of healthy fishes. Under stressful conditions, these bacteria are caused mortality in freshwater fishes through dermal and visceral hemorrhagic septicemia and enteritis (Tavakoli and Akhlagi, 2009). Biological and antibacterial activities of bioactive compounds obtained from marine macro-algae have been subjects of several studies worldwide (Sastry and Rao, 1994) including studies against *A. hydrophila* (Rebecca et al., 2012; Al-Laham and Al-Fadel, 2014; Aruna et al., 2010; Vijayabaskar and Shiyamala, 2011). In addition, antibacterial activities of some vascular plants against *A. hydrophila* have been studied in Iran (Ali Shahi et al., 2009; Ali Shahi, 2010). Considering the efficiency of bioactive compounds obtained from algae to control pathogenic bacteria and availability of a large amount of algae in northern and southern parts of Iran, the present study was conducted to evaluate the effect of methanolic, chloroformic, aqueous extracts of *G. salicornia* against *A. hydrophila*.

Materials and methods

Samplings were carried out during high tide at Kaveh Harbour in the Qeshm Island (south Iran at 55'57ºE and 26'56ºN) from late October 2013 till December 2014. The algae samples were washed in sea water and cleaned of epiphytic algae, sand, debris and transported to the laboratory in the ice box.

Methanolic extract: Ten grams of the algae powder (powdered by liquid nitrogen) were covered in filter paper and placed in soxhlet apparatus containing 100 ml methanol (80%) in a mantle at 65°C (boiling point of the solvent). The extractions were repeated four successive times (approx. 4 hrs). The obtained extract was placed in 50 ml falcon tubes and kept at 4°C for further experiment (Souza, 2011).

Chloroformic extract: To obtain chloroformic extract, 100 ml of raw methanolic extract and 100 ml chloroform were mixed in a container. Within few minutes, two separate phases were formed. The chloroform phase was removed after 3 days and stored at 4°C for further tests. The obtained extracts were centrifuged, passed through Whatman filter paper and freeze dried. The used solvent for extraction was evaporated and the remaining part was kept at -17°C for antibacterial tests.

Aqueous extract (Polysaccharide): Algae powder with proportion of 1:10 was boiled in distilled water (100°C) under reflux for 2 hrs. The hot extract was passed through filters with 24 micrometer mesh size. The filtered aliquot was condensed using a rotary evaporation condenser to the lowest volume, called row polysaccharide extract.

Concentrations of methanolic and chloroformic extracts: Methanolic extracts of 50, 100, 200 and 300 $mg.L^{-1}$ were prepared by dissolving algae samples in 0.5 ml dimethyl sulfoxide (DMSO) solvent. The desired concentrations of the extracts were stored at -17˚C for further tests.

Concentrations of polysaccharide extract: Polysaccharide extracts of 50, 100, 200 and 300 $mg.L^{-1}$ were prepared. Algae aqueous extract were dissolved in 0.5 ml distilled water and the desired concentrations were kept at -17˚C in labeled sterile micro tubes for further tests.

Antibacterial tests: *Aeromonas hydrophila* isolates was obtained from fish health laboratory of International Sturgeon Research Institute. Antibacterial activities of algae extracts were assayed using well diffusion assay method. Bacterial cultures (nutrient agar and nutrient broth) were incubated at 37°C for 24 hrs. Then, 30 µL of bacterial strain cultured in nutrient broth was uniformly added on nutrient agar followed by punching 3 holes (4-mm in diameter) aseptically in the medium using the tip of pipette. These holes were loaded with different concentrations of the extracts and incubated at 37˚C for 24 hrs. The zones of inhibition were measured after 24 hrs using a digital caliper (Indu, 2006; Sharma and Sharma, 2011). The whole process was carried out aseptically. Commercial Tetracycline and DMSO were used as positive and

Table 1. Antibacterial activities of *G. salicornia* extract against *A. hydrophila*, triply positive (+++), doubly positive (++), positive (+) and negative (-).

Concentration mg.ml^{-1}	Methanolic extract	Chloroformic extract	Aqueous extract
50	+++	++	++
100	+++	++	++
200	+++	++	++
300	+++	++	+++
Positive control	+++	+++	+++
Negative control	-	-	-

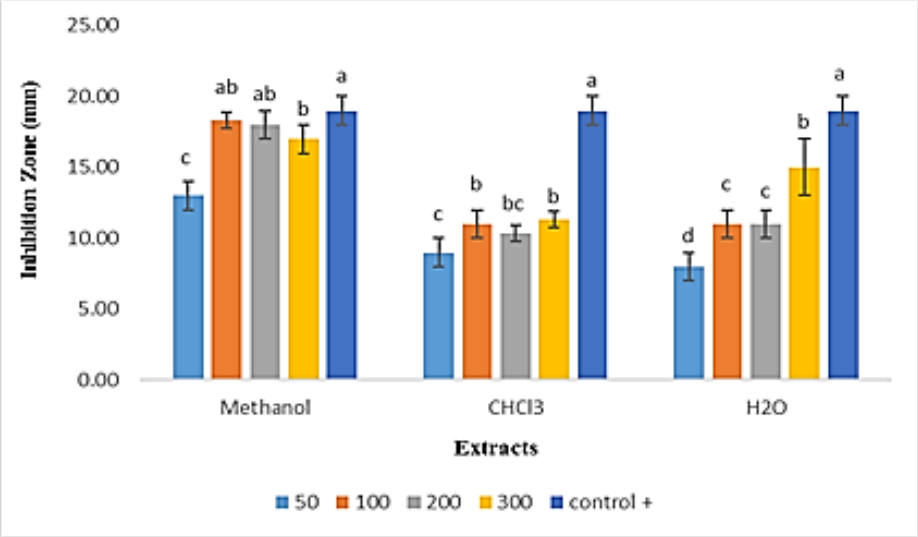

Figure 1. Comparison of antibacterial activities of algae extract with various concentrations and commercial antibiotic against *A. hydrophila*.

negative controls, respectively.

Data analyses: Data are presented as mean ± SD of triplicate samples. One-Way ANOVA was used to test the significance of differences between four algae extract concentrations and also between algae extracts and commercial antibiotics. Duncan post hoc test was used to assess differences among sample means. Statistical package of SPSS Ver. 16 was used for data analysis.

Diameter of the inhibition zone was measured according to Thomson et al. (1985). Zones of inhibition around the wells were measured and scored as follow: triply positive (+++) if a zone of inhibition was greater than 7 mm, doubly positive (++) if greater than 2 mm, positive (+) if less than 2 mm, and negative (-) if no inhibition of bacterial growth was occurred (Thompson et al., 1985).

Results

The results of antibacterial tests of the algae extracts against *A. hydrophila* are presented in Table 1. Different responses were observed and the bacterium was sensitive to commercial antibiotic (positive control). Negative control (DSMO) did not have any effect on the bacterium.

The results revealed significant differences among activities of various concentrations of the methanolic, chloroformic and aqueous extracts (P<0.05). A significance differences between antibacterial activities of algal extracts and the positive control were also found except 100 and 200 mg.ml^{-1} methanolic (Fig. 1). Antibacterial effect of all studied concentrations of the methanolic extracts as well as 300 mg.ml^{-1} aqueous extract were significantly higher than those of other extracts (Fig. 1).

Discussion

In the present study, all concentrations of the methanolic extracts and 300 mg.ml^{-1} aqueous extract

showed considerable antibacterial activities. Other concentrations of aqueous and chloroformic extracts showed poor antibacterial activities compared to the methanolic extracts. Antibacterial activities of 100 and 200 $mg.ml^{-1}$ methanolic and aqueous extracts did not have significant differences with commercial antibiotics, hence they can be categorized as similar as Tetracycline i.e. commercial antibiotics.

Hornsey and Hide (1974) studied antibacterial activities of 151 species of marine algae and reported no such activity for *Gracilaria* (Hornsey and Hide, 1974). In another work, polar and non-polar extracts of *Gracilaria* species exhibited some effects on certain pathogenic bacteria (De Almeida et al., 2011). Adaikalaraj et al. (2012) studied the potentials of antibacterial activities in red algae Gracilariacaea and reported higher activity for methanolic extracts (Adaikalaraj et al., 2012). Rebecca et al. (2012) also reported antibacterial activities of methanolic extract of *Centroceiod* sp. against *A. hydrophila* (Rebecca et al., 2012). The findings of this study confirm, the results of those above mentioned works regarding antibacterial activities of *Gracilaria* extract.

The present study is the first report on antibacterial activities of bioactive compounds extracted from *G. salicornia* against *A. hydrophila*. According to the results, *G. salicornia* extracts can be used to control pathogenic microorganisms in aquaculture. From ecological point of view some metabolites produced by marine plants could be useful in growth and resistance of fish against certain diseases. Marine algae uphold an advantage to develop antibacterial agents for application in aquaculture. From economic viewpoint, it is possible to establish standard methods for extracting various bioactive compounds from algae with higher antibacterial activities.

Acknowledgments

Authors extend their sincerest gratitude to Mr. Rasa a senior lecturer at the Department of Biology and Mrs. Fatemeh Ghanbari, PhD students of Organic Chemistry of the Department of Chemistry at University of Guilan for their supports.

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