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Antimicrobial Activities of Marine Macroalgal Lipidic Extracts Against Fish Pathogenic *Vibrio* Species of Kakinada Coastal Region

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ABSTRACT

Antibacterial seaweeds against pathogenic *Sibrio* species were the focus of this study, which aimed to find an alternative to the routinely used antibiotics in aquatic farming by identifying antibacterial seaweeds (Red algae and Green algae) from Kakinada marine coast. Tests of the antibacterial activity of chloroform/ methanol lipidic extracts of six seaweed species against pathogenic *Vibrio* species were performed using the disc diffusion method. Lipidic algal extracts from marine algae were shown to have a variety of sensitivity profiles. *Vibrio* species infections can be prevented and treated with antibacterial compounds found in seaweeds, either as an adjunct or as a complete alternative to conventional antibiotics. While the chloroform and methanol extracts of *G. longissima* and *C. linum* are the most promising, they were also effective against *V. alginolyticus, V. vulnificus* and *V. ordalii*, respectively. It is hypothesized that some of the lipids found in the seaweeds studied could be used as an enrichment or creative feed for fish in aquaculture facilities and this remains an open possibility for all of the species under study. Research findings from this study also support possible use of seaweed extract, as an antibacterial compound and aquaculture feed.

Keywords Antibacterial compounds, Kakinada marine coast, Seaweeds, *Vibrio* spp.

INTRODUCTION

Diseases originating from microorganisms in the mariculture industry can result in large financial losses around the world and the emergence of antibiotic-resistant microorganisms has led to an increased demand for new antibacterial compounds with minimal side effects. Seaweeds have recently received a lot of attention as a potential source of antibacterial metabolites. Aquaculture farming faces excessive mortality and blemishes on fish skin caused by illnesses of microbial origin due to intensive farming (Sanches-Fernande *et al.* 2022). *Vibriosis* is a frequent disease in aquaculture caused by bacteria belonging to the *Vibrio* genus due to various factors, particularly the source of fish, environmental factors (including water quality and farm management) and the virulence factors of *Vibrio*. The most prevalent disease outbreaks in seafood and shellfish, known as *Vibriosis* are caused by bacteria, most of the genus *Vibrio*, such as *V. harveyi*, *Vibrio anguillarum*, *V. ordalii, V. vulnificus, V. parahaemolyticus, V. alginolyticus* and

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V. salmonicida (Cavallo and Stabili 2002, Cavallo *et al.* 2013). These bacteria can also accumulate in the gastrointestinal tracts of the cultured fishes, posing a major health risk to humans. As an instance, one of the most potential food borne pathogens, *V. vulnificus,* is responsible for the majority of cases of gastroenteritis. *Mytilusgallo provincialis* from Indian aquaculture farms is frequently infected with *V. parahaemolyticus* (Normanno *et al.* 2006). The use of antibiotics a therapy purpose has unwanted side effects such as toxicity to reared organisms and discharge of chemical traces into our environment. These antibiotic remnants may constitute a potential risk to the health of both animals and humans (Cabello 2006). Several frequently used antibiotics are ineffective against various dangerous strains of *Vibrio*, notably *V. harveyi*, *V. parahaemolyticus* and *V. splendidus* (Kumar *et al.* 2021). As a consequence of this, there is an increasing market demand for novel antibacterial agents that are safe for use in aquaculture (Thanigaivel *et al.* 2016).

Metabolites with unique structural features and biological effects can be found in abundance in marine species (Ghallab *et al.* 2022, Arrieche *et al.* 2022). These researchers have identified and evaluated a wide variety of marine chemicals, some of which may be useful as novel medications (Mohamed *et al.* 2021). Despite its medicinal potential for treating a wide range of ailments, seaweeds have long been employed in traditional folk medicine. Strikingly, several research studies have established that primary and secondary metabolites found in the green, brown and red algae may indeed be useful bioactive compounds for the pharmaceutical industry because of their *in vitro* ability to neutralize bacteria and many other epibionts (e.g., helminthic activity, cytostatic and antiviral, antibiotic and antibacterial activity) (Pratiwy and Arifah 2021). For instance, some which include macrolides, cyclic peptides and proteins, polyketides, sesquiterpenes, terpenes and fatty acids, which have been demonstrated to exhibit antibacterial action both against Gram-negative bacteria and Gram-positive (Kolanjinathan *et al.* 2009). In order to perform these activities, there are various variables, including the type of seaweed, the thallus area, microbial abundance and seasonality are being taken in to consideration (Thanigaivel *et al.* 2016). However, marine algal extracts have been shown to inhibit human infections more frequently than fish pathogens (Monteiro *et al.* 2021, Zammuto *et al.* 2022, Jusidin *et al.* 2022).

In this study the lipidic extracts of five marine macroalgae are investigated (e.g., *Cheatomorpha, Gracilaria gracilis, Cladophora rupestris*, *Gracilariopsis longissima, Ulva prolifera*), which were significantly tested against several well-known fish pathogenic *Vibrio* species (e.g., *V. paraheamolyticus, V.harveyi, V. vulnificus, V. alginolyticus, V. splendidus*, *V. ordalii* and *V. salmonicida*). Many commercially significant both warm and cold – water marine fish species are infected by these diseases, which have a devastating financial impact on fish farming operations in aquaculture in Kakinada marine coast in Andhra Pradesh.

MATERIALS AND METHODS

Investigated algal species

In the present study,seaweeds collected from the sites of Kakinada marine coast by random catchment and cleaned thoroughly with seawater and then brought them to the laboratory. Three Rhodophyta and three Chlorophyta species were investigated. In the unattached seaweed population, at a depth of around 5 meters, found *Gracilariadura* (Gracilariaceae). Both unattached *Gracilaria gracilis* and *Gracilariopsis longissima* dominating thalli were found at a depth of around 60 cm. Unattached seaweed communities in the Kakinada are dominated year-round by *Chaetomorphalinum* (Cladophoraceae), a Cladophorales-family member that forms “mattresses” up to 55 cm thick, densely populated by a diverse range of invertebrates. At a depth of roughly 55 centimeters, it was retrieved we have determined the algal species from Kakinada marine coast. The attached form of *Ulva prolifera* (Ulvaceae) were obtained in the second Inlet at a depth of 40 centimeters, in an area where a small river provides freshwater input. According to (Cavallo *et al.* 2013), the species was classified based on its morphological characteristics as followed by the standard set of keys for biochemical identification of environmental *Vibrio* species (Alsina and Blanch 1994).

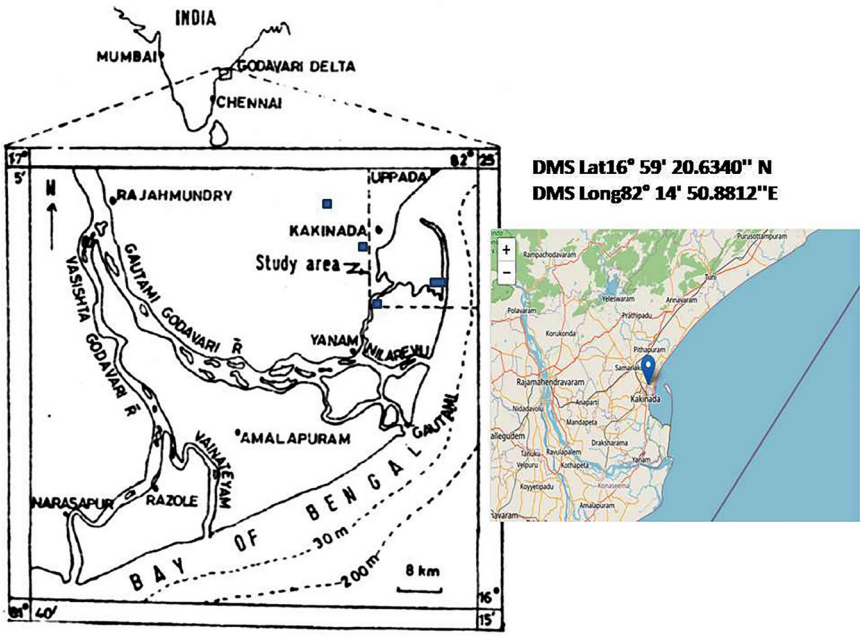

Fig. 1. Map of the Kakinada marine coast, Andhra Pradesh. ( ) indicates the sampling stations of seaweed and pathogenic *Vibrio* species.

Isolation of test organisms from marine fishes

The strains of *Vibrio* species identified in the study are as follows *Vibrio ordalii, Vibrio salmonicida, Vibrio alginolyticus, Vibrio splendidus, Vibrio harveyi* and *Vibrio vulnificus* were isolated from seawater samples of the Kakinada marine coast of Andhra Pradesh (Fig. 1). Identification of *Vibrio* species were done by colonial appearance and biochemical tests.

Preparation of lipidic extracts from marine macroalgae

In order to eliminate necrotic sections from algae samples, a mixture of ethanol and sodium hypochlorite was used to remove epiphytes and other marine creatures. To remove any remaining material, the samples were thoroughly washed in sterile milli-Q water. To extract the chloroform/methanol (2:1 fractions at 50-55°C for 24 hrs) from the freshly cleaned algal material, the soxhlet equipment was used to extract 3 g of each sample from the selected macroalgal species. At a predetermined temperature 55°C and 0.27 kP pressure extraction solvents were evaporated under a vacuum. The antibacterial activity of algal extracts was next tested using the paper disc diffusion method (Bauer 1966), which involved dissolving 5 mg of extracts in 1 mL of ethanol.

Antimicrobial activity

The Kirby Bauer disk diffusion technique was used to measure antimicrobial activity (Bauer 1966). Each algal extract was impregnated with 10, 20, 30, 40 and 60 µL of sterile paper discs, 7 mm in diameter (Whatman International Ltd) and left to air-dry at room temperature for 5 h. The discs were then tested for the presence of bacterial spores. Discs impregnated only with 60 µL of solvent were used as a negative control; the positive control was displayed by the *Vibriostatic* agent O/129 at a concentration of 10 g to verify that residues of the solvents used for extraction could affect antibiotic activity ($MeOH/CHCl_3$, chloroform anhydrous >99% with ethanol as a stabilizing agent, methanol >99.8%t ; (Sigma–Aldrich) Assays were carried out on autoclaved marine agar plates seeded with 100 microliters of the test bacterium suspension (about 10^8 CFU per milli liter), For 24 hrs at 30°C, Mueller Hinton No 2 agar plates were incubated with

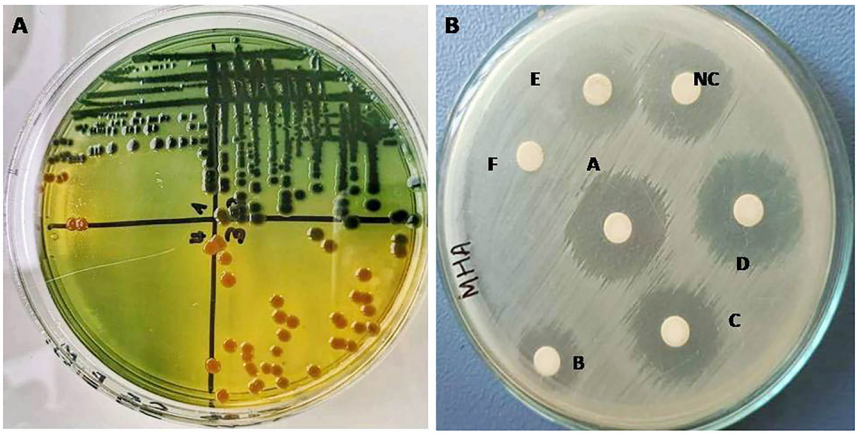

Fig. 2. 2A. *Vibrio* colonies in TCBS agar. 2B. Kirby Bauer's disc diffusion assay by lipidic extracts of seaweeds. *Gracilariopsis longissimi* lipidic extracts against selected pathogenic marine bacteria. (A) *Vibrio parahaemolyticus*, (B) *V. alginolyticus* (C) *V. ordalii,* (D) *V. salmoicida*, (E) *V. harveyi,* (F) *V. ordalii* (NC= Negative control), = Disc impregnated with marine algal extract.

impregnated discs and control discs on top of each other. Antibacterial action was evident in the clear zone around the discs. The diameter of the clear zone was used as the diameter of the microbial growth inhibition (measured in millimeters). Each bacterial strain was tested in three separate batches. Statistical analysis used in this study is standard deviation.

RESULTS AND DISCUSSION

Antibacterial activity was found in all six algae species (3 Rhodophyta and 3 Chlorophyta species) tested in this study (Figs 2–3). Only 10 µL of each algal extract (50 µg of dry extract) was needed to establish this inhibitory action against *V. paraheamolyticus, V. ordalii, V. salmonicida, V. alginolyticus, V. splendidus, V. harveyi* and *V. vulnificus*. Using 30 µL of each algal extract, the data shown in Fig. 3 represents the findings. According to the data in this Figure, all the algal extracts tested inhibited *Vibrio splendidus* or *V. harveyi* growth at low level when compared to other *Vibrio* species. *Gracilariopsis longissima* demonstrated considerably stronger ($P<0.01$) inhibitory activity against *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* with inhibition diameters of 14, 13 and 12 mm respectively, according to statistical analysis (standard deviation with <4%). Strikingly *V. salmonicida* and *V. ordalii* were also inhibited by this *G. longissima* species, but the inhibition zones were significantly effective for *V. paraheamolyticus*, *V. alginolyticus* (14 and 12 mm respectively). The inhibition zones of *Chaetomorphalinum* against *V. vulnificus* were similar to that of *G. longissima*. Similarly, *V. ordalii* exhibited the same diameter of inhibition, and it was comparable with *V. vulnificus*. Compared to *G. longissima, C. linum* and *Cladophoraru pestris* demonstrated a significantly reduced bioactivity ($P<0.01$) against *V. alginolyticus, V. splendidus* and *V. harveyi*. Comparable diameters of inhibition of growth was also seen when *Gracilaria dura* extract was tested against *V. vulnificus* and *V. dura.* Finally, the lipidic extracts of *Gracilaria longissima* were more active against *V. salmonicida*, *V. paraheamolyticus*, *V. alginolyticus* and *V. ordalii* whereas *Ulva prolifera* extracts were effective against *V. paraheamolyticus* and *V. ordalii*, respectively. Not matching with figure according to statistical analysis, bacterial inhibition diameters were all the same except for those produced by *G. longissima* and *C. linum*, which were significantly higher ($P< 0.05$) compared to other algal extracts.

Seaweed species collected from the Kakinada marine fish production tanks were tested to determine whether they may inhibit the growth of some *Vibrio* species to see if they could be used as an alternative to commonly used antibiotics in aquaculture. The chloroform/methanol extracts from the six seaweeds examined in this study indicated varying levels of bioactivity as well as varying sensitivities of the

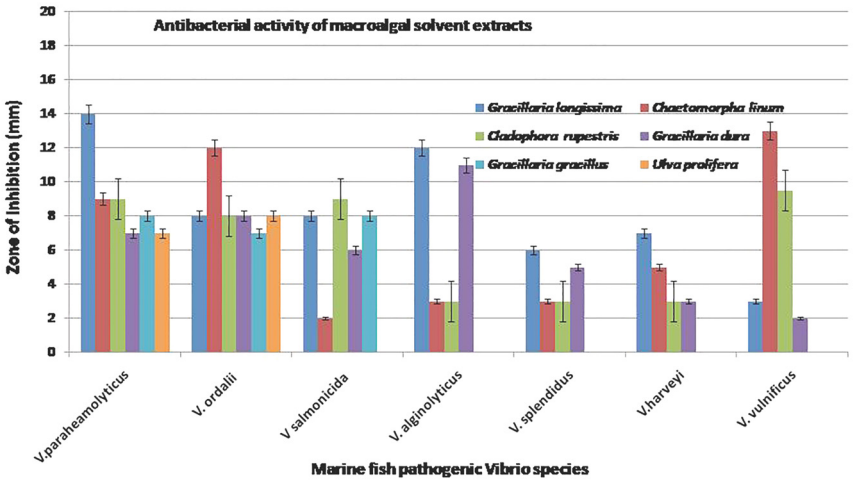

Fig. 3. Antibacterial activity of chloroform/methanol extracts (30 µL) of seaweeds collected in the Kakinada, Andhra Pradesh and its zone of inhibition in mm.

Vibrio strains under investigation. Among all the antibacterial extracts tested, *Gracilariopsis longissima* demonstrated the largest spectrum of efficacy, showing activity against four different *Vibrio* species, albeit at varying levels of inhibition. Bacteriostatic activity against *V. alginolyticus* and *V. vulnificus* was previously proven by earlier work (Stabili *et al.* 2012, 2019) using a *G. longissima* chloroform/methanol lipidic extracts from Kakinada marine macroalgal species. *G. longissima* had the most efficacy, however, *Chaetomorpha linum's* extract was only effective against two strains of *vibrio,* namely *V. vulnificus* and *V. ordalii.* Several Gram-negative and Gram-positive bacteria were inhibited by extracts of *C. linum* from an Indian lake, but *V. cholerae* remained unaffected (Patra *et al.* 2009). There don't appear to be any other papers in the literature discussing the possibility of using this species as a source for biologically active compounds. Because *C. linum* is often found in lagoon unattached communities throughout the world (Plus *et al.* 2005, Stabili *et al.* 2012), the present study's good findings may provide an excellent foundation for future research on the species.

It was found to have observed significant bioactivity against three of the examined *Vibrio* species (*V. ordalii*, *V. vulnificus*) (Fig. 3) although, with less of an inhibitory capacity than the preceding macroalgal species, *Cladophora rupestris*. The dichloromethane extract of Atlantic French thalli proved potent against Gram-negative bacteria, the solid extracted from macroalgae *C. rupestris* collected in Spanish waters was inactive against Gram-positive bacteria (Salvador *et al.* 2007). Both studies did not include *Vibrio* species. It was found that *Cladophoraglomerata* from India has significant action against numerous *Vibrio* species (i.e., *V. vulnificus, V. anguillarum* and *V. parahaemolyticus*) against fish pathogenic *V. fischeri* (Yuvaraj *et al.* 2011). There was also mild to medium action against *V. alginolyticus, V. vulnificus* and *V. parahaemolyticus* in the methanol extract of Indian *C. albida* (Manilal *et al.* 2015).

However, for these new aquaculture medicines uses, indicates that the chloroform/methanol extracts of *Cladophora rupestris* from Kakinada's marine coast exhibited antibacterial activity against three of the six *Vibrio* species examined. In order to test the antibacterial activity of species in this genus against *Vibrio* spp. with various extraction methods, these results provide a strong impetus for further research. *Gracilaria dura* extracts were shown to be effective in this investigation against two different *Vibrio* species. Finally, extracts of *Ulva prolifera* and *Gracilaria gracilis* which were solely efficient against one *Vibrio* species, were the least effective. Different species of the same genus have different antibacterial activity

levels, which has been widely documented (Francavilla *et al.* 2013, Capillo *et al*. 2018).

Many studies have been done on the *Gracilaria* genus's bioactive content (De Almeida *et al.* 2011) with references cited therein. However, the toxicity level varies depending mostly on seaweed species, microorganism species and extraction methods employed. A recent study found that the ethanolic extracts of the Indian *Gracilaria corticate* plant were extremely effective against *V. parahaemolyticus* and *V. cholerae*, but less effective against *S. flexneri* and *P. aeruginosa.* In a study on the antibacterial activity of *Gracilaria fisheri*, prevented the *Vibrio* harveyi infections in the black tiger shrimp *Penaeus monodon* (Kanjana *et al.* 2011). We also found that the ethanol extract had the strongest antibiotic activity against *V. harveyi* of the four tested solvents, but the hexane, chloroform and methanol extracts had the weakest antimicrobial activity. In Iberian thalli of *G. dura,* however no *Vibrio* species were inhibited by the solid extracts prepared by pounding the fresh material without any solvent and showed low medium activity against Gram-negative and Gram-positive bacteria and yeasts, (Salvador *et al*. 2007). The extracts obtained with diethyl ether evidenced the best results against yeasts, Gram-negative and Gram-positive bacteria, among some of the extracts of fresh *G. gracilis* from the Turkish coast generated using different solvents (TÜney *et al.* 2006).

By showing bioactivity against *Vibrio* species in their thalli, both *G. gracilis* and *G. dura* from Kakinada now have a wider range of antibacterial activity than initially assumed. It was shown that methanol and acetone extracts of *Ulva prolifera* from Alexandria harbour in Egypt were effective against many Gram-negative and Gram-positive bacteria, however, no *Vibrio* species were tested. Extraction with 95% ethanol in Korea of the same species yielded an excellent antioxidant activity, making it suitable for the production of therapeutic goods (Cho *et al.* 2011, Moreira *et al.* 2022). The Kakinada marine intensive aquaculture tanks could lead to a new study into various uses for this species.

Vibrio ordalii was demonstrated to be the most sensitive to the extracts of each of the marine algae studied, except for *G. gracilis*, when it came to *Vibrio* species. However, only a few of the dozens of seaweed extracts examined reduced the growth of *V. alginolyticus, V. vulnificus* and *V. salmonicida*. *V. splendidus* and *V. harveyi*, on the other hand, were unaffected by any Kakinada seaweed extract. To find seaweed extracts that are effective against fish pathogenic *Vibrio* spp., we reviewed the literature on this topic. In order to uncover new bioactive compounds against fish and crustacean infections in aquaculture plants, several *Vibrio* fish infections were tested internationally using seaweed extracts. Nonetheless due to the paucity of knowledge about the seaweed bioactivities against the *Vibrio* species were studied in this work.

The methanolic extract of the red algae *Gracilaria corticata* in India and *Ceramium rubrum* in Germany were found to inhibit *V. alginolyticus* (Dubber and Harder 2008). Methanol may be an appropriate extraction technique for a certain class of compounds found in this division, if that is indeed the case. We hypothesize that different lipophilic substances may have antibacterial properties because the solvents utilized in our study were able to extract lipids (Stengel *et al.* 2011). Ethanol extracts of *Asparagopsis taxiformis* from the Sicily channel marginally inhibited *V. harveyi* and *V. vulnificus* (Genovese *et al.* 2012). The extract of the Indian thalli of *Gracilaria fisheri*, which demonstrated a strong inhibition against *V. harveyi* when dissolved in ethanol, was also employed (Kanjana *et al.* 2011).

CONCLUSION

Vibrio species infections can be prevented and treated with antibacterial compounds found in seaweeds from the Kakinada, either as an adjunct or as a complete alternative to conventional antibiotics. While the chloroform and methanol extracts of *G. longissima* and *C. linum* are the most promising, they were also effective against *V. alginolyticus, V. vulnificus* and *V. ordalii*, respectively, when used in combination. It's possible that some of the lipids found in the seaweeds studied could be used as an enrichment or creative feed for fish in aquaculture facilities, and this remains an open possibility for all of the species under study.

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