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Antimicrobial activity of seaweeds extracts against pathogenic bacteria in aquaculture

Atividade antimicrobiana de extratos de algas frente a bactérias patogênicas na aquicultura

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RESUMO
O presente trabalho teve como objetivo investigar a atividade antimicrobiana dos extratos metanólicos das algas *Haematococcus pluvialis*, *Kappaphycus alvarezii*, *Sargassum filipendula* e *Undaria pinnatifida* em cepas padrão e em bactérias patogênicas de organismos aquáticos. O método de microdiluição em caldo foi utilizado para determinação da concentração inibitória mínima (CIM) dos extratos. Além disso, foi determinado o perfil de suscetibilidade das bactérias frente a 12 antimicrobianos pelo método de disco-difusão em ágar. Os testes de atividade antimicrobiana mostraram que apenas os extratos de *S. filipendula* e *U. pinnatifida* inibiram todas as bactérias testadas, sendo *U. pinnatifida* o extrato com maior eficiência contra os patógenos. Também os testes mostraram predileção da atividade antimicrobiana da microalga de água doce *H. pluvialis* por micro-organismos de ambientes marinhos, enquanto as algas marinhas, *K. alvarezii*, *S. filipendula* e *U. pinnatifida*, mostraram-se mais eficazes na inibição do crescimento de cepas patogênicas de água doce. A multirresistência foi verificada em todas as cepas patogênicas isoladas testadas (*Pseudomonas sp.*, *S. agalactiae*, *V. alginolyticus* and *V. anguillarum*). Os resultados sugerem que os extratos das algas exerceram atividade antimicrobiana frente as cepas de bactérias patogênicas da aquicultura, sendo o extrato da *U. pinnatifida* o que inibiu o crescimento dos micro-organismos com as menores concentrações.

Palavras-chave: *H. pluvialis*, *K. alvarezii*, *S. filipendula*, *U. pinnatifida*, resistência múltipla.

ABSTRACT
The present study aimed to investigate the antimicrobial activity of *Haematococcus pluvialis*, *Kappaphycus alvarezii*, *Sargassum filipendula* and *Undaria pinnatifida* methanolic extracts in pathogenic bacteria from aquatic organisms and standard strains. Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the extracts. In addition, susceptibility profile of two isolated bacteria to 12 antimicrobials was determined by agar diffusion method. The antimicrobial activity tests showed that only the extracts of *S. filipendula* and *U. pinnatifida* inhibited all the bacteria tested being *U. pinnatifida* the extract with more efficiency against the pathogens. Also the tests showed predilection of the antimicrobial activity of freshwater microalgae *H. pluvialis* by microorganisms from marine environments, while the marine waters seaweeds, *K. alvarezii*, *S. filipendula* and *U. pinnatifida*, have been shown to be more effective in inhibiting the growth of freshwater strains. Multiresistance was verified in all isolated pathogenic strains tested (*Pseudomonas* sp., *S. agalactiae*, *V. alginolyticus* and *V. anguillarum*). These results suggest that seaweed methanolic extracts exert antimicrobial activity even in multiresistant strains of pathogenic aquaculture bacteria, being *U. pinnatifida* extract the only that inhibited bacteria growth with the lowest concentrations.

Keywords: *H. pluvialis*, *K. alvarezii*, multiple resistance, *S. filipendula*, *U. pinnatifida*.

1 INTRODUCTION

Aquaculture is considered an important industry, reaching a total annual production higher than 80 million tons (FAO, 2019). In 2011, in Brazil, the activity increased 31.1% compared to the previous year, totaling 628 thousand tons of fish (FAO, 2019). Despite its growth, aquaculture industry faces difficulties due to the presence of infectious diseases, which cause harm to the producers and can make the activity less profitable (TAVECHIO; GUIDELLI; PORTZ, 2009). Therefore, the administration of antimicrobials has been used to eliminate or inhibit the growth of pathogenic microorganisms and, consequently, to reduce mortality of aquatic organisms (WATTS et al., 2017).

However, indiscriminate and erroneous use of antimicrobials, such as exposure to antibiotics at sub inhibitory concentrations, can lead to the emergence of resistance, both in human intestinal bacteria and bacteria of aquatic organisms, enabling the dissemination of resistance genes in several bacterial populations (CABELLO et al., 2016; LIU; STEELE; MENG, 2017). In Brazil, the Ministry of Agriculture, Livestock and Supply (2013) has already banned the use of some antibiotics such as: chloramphenicol and nitrofurans (IN nº 09, 06/27/2003), quinolones and sulfonamides (IN nº 26, 9/07/2009), spiramycin and erythromycin (IN No. 14, 05/17/2012) as feed additive in animal production. This fact shows the constant need for researches and the development of new biologically active substances as alternative to chemotherapeutics that act inhibiting pathogens, preventing diseases, as well as growth promoters and without side effects (DAWOOD; KOSHIO; ESTEBAN, 2018; PÉREZ-SÁNCHEZ; MORA-SÁNCHEZ; BALCÁZAR, 2018).

Seaweeds are characterized as an important source of bioactive compounds due to its ability to produce secondary metabolites with a broad spectrum of biological activities. Compounds with antimicrobial, antifungal, vermifuge, antimicrobial and anti-oxidant activity were detected in green, red or brown seaweed tissues (PAL; KAMTHANIA; KUMAR, 2014; MICHALAK; CHOJNACKA, 2015; PÉREZ; FALQUÉ; DOMÍNGUEZ, 2016). With increasing resistance of pathogens to antibiotics, the use of seaweeds extracts could be an effective natural alternative with less side effects and toxicity than antibiotics.

The aim of the present study was to evaluate *in vitro* the antimicrobial activity of methanolic extracts of *H. pluvialis*, *K. alvarezii*, *S. filipendula* and *U. pinnatifida* against aquaculture pathogenic bacteria.

2 MATERIAL AND METHODS

The experiments were carried out at the Marine Shrimp Laboratory (Laboratório de Camarões Marinhos - LCM) and at the Morphogenesis and Plant Biochemistry Laboratory, both belonging to the Federal University of Santa Catarina (Universidade Federal de Santa Catarina - UFSC), in southern Brazil.

Biological material

Sargassum filipendula samples were collected in Praia do Sambaqui (27º29'22.1''S 48º32'17.1''W), Florianópolis, Santa Catarina State, Brazil, in May 2015. *Kappaphycus alvarezii* and *Haematococcus pluvialis* biomass were collected from the seaweed and microalgae sections of the LCM; while samples of the exotic seaweed *Undaria pinnatifida* were provided by the Argentine company Soriano S/A.

Methanolic extracts preparation

With the exception of the microalgae *H. pluvialis*, seaweeds went through a cleaning process to be frozen at -20ºC and then lyophilized. The process is based on washing with distilled water three times, 1 minute bath in 0.5 mol L^{-1} ammonium formate solution and rinsing with distilled water also three times. To obtain methanolic extract, the biomass of lyophilized seaweed was ground in liquid nitrogen in a pistil mortar (Annex 3). Aliquots of 10 g of biomass ground of each seaweed were homogenized in Becker with 100 mL of 80% methanol and extracted for 1 h. The extracts obtained were centrifuged (4000 rpm, 10, room temperature) and the supernatant was carefully collected. The solvent was evaporated in a rotary evaporator under vacuum at a temperature of 55°C. Aqueous residue was filtered on Whaltman filter paper and stored in amber glass vessel at -20°C. For negative control, it was used methanol 80%, evaporated in a rotary evaporator under vacuum at a temperature of 55°C. Being the aqueous residue (625 mg mL^{-1}concentration) used in *in vitro* tests.

UV-visible scanning spectrophotometry

Seaweed extract samples UV-visible spectral profile was determined from an exploratory scan. To obtain optical absorption measurements of bioactive compounds, present in the methanolic extracts, a UV-Vis Spectrophotometer (Gold Spectrum lab-53 UV-Vis spectrophotometer, BEL photonics, Brazil) was used, operating in between 200 ηm to 700 ηm region.

Microorganism's strains

Bacterial strains used for in vitro microbiological tests were *Vibrio harveyi* (ATCC 14126), *Vibrio parahaemolyticus* (ATCC 17802), *Vibrio vulnificus* (LAM 64), *Vibrio alginolyticus* (BCCM 2068) and *Vibrio anguillarum* (ATCC 19264) as bacterial strains pathogenic for marine shrimp; and *Pseudomonas aeruginosa* (ATCC 0053), *Pseudomonas* sp. (GU113077) and *Streptococcus agalactiae* (CP018623) as pathogenic strains for freshwater fish. *Pseudomonas* sp. and *S. agalactiae* were isolated from and outbreak disease in fish and *V. alginolyticus* and *V. anguillarum* were isolated from marine shrimp and cod respectively. .*Escherichia coli* ATCC 25102, *Staphylococcus aureus* ATCC 25923 and Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300 were tested as standard strains.

For strains activation, aliquot of 100 µL of cryopreserved isolates at -20°C in Brain Heart Infusion (BHI) and glycerol solution was inoculated into 10 mL tube containing BHI broth for strains of fresh water and BHI supplemented with 3% NaCl for *Vibrio* strains and incubated at 30ºC for 24h.

Microbiological tests

Minimum inhibitory concentration (MIC) was achieved through the broth microdilution method in 96-well microplates (Annex 4). In the tests with the seaweed extracts against microorganisms *E. coli* ATCC 25102, *P. aeruginosa* ATCC 0053, *Pseudomonas sp.* GU113077, *S. aureus* ATCC 25923, *S. aureus* methicillin-resistant (MRSA) ATCC 43300 and *S. agalactiae* 100 µL of PB medium (Poor Broth 1% peptone, 0.5% NaCl, pH 7.4) were added each well of the flat bottom 96-well microplate and 100 µL of each methanolic extract in the first well. Subsequently, a serial two-factor dilution was performed up to the 12th well. At last, 20µL of pathogenic microorganisms, mentioned above, was added to each well at an adjusted concentration of $1x10^3$ $CFU.mL^{-1}$ according to McFarland nephelometric scale. The microplates were incubated at 30 °C for 24 h. Tests with *Vibrio* strains were performed with the same procedure, replacing PB culture medium with PWS (Peptone Water Saline 1% peptone, 3% NaCl, pH 7.0). CIM assays were performed in triplicate.

For comparison purposes the degree of susceptibility of the isolated of *Pseudomonas sp., S. agalactiae, V. alginolyticus* and *V. anguillarum* it was performed antimicrobial susceptibility testing (TSA) through the disk diffusion method in Mueller Hinton agar (Himedia Laboratories®) following the modified Kirby-Bauer method (BAUER et al., 1966). The following antibiotics were used (LaborClin®) belonging to the classes: (1) beta-lactams -

penicillin (10 IU), ampicillin (10 mg) associated with tazobactam piperacillin (100/10mg) and amoxicillin with clavulanic acid (20/10 mg) (2) aminoglycosides - gentamicin (10mg), amikacin (30 μg) and tobramycin (10 mg), (3) fluoroquinolones - enrofloxacin (5 μg), ciprofloxacin (5 μg), norfloxacin (10 mg), levofloxacin (5 μg) and nalidixic acid (30 μg), (4) phenicols - chloramphenicol (30 μg), (5) macrolide - erythromycin (15 μg) and azithromycin (15 μg), (6) sulfa drugs - sulfamethoxazole plus trimethoprim (1.25/23,75 μg) and (7) tetracyclines - tetracycline (30 μg), (8) cephalosporins - ceftazidime (30 μg), cefoxitin (30 μg), cefepime (30 μg) and cephalexin (30 μg), (9) lincosamides - clindamycin (2 mg), (10) carbapenems - meropenem (10 mg) (11) glycopeptide - vancomycin (30 μg) and (12) ansamycins - rifampicin (5 μg). The reading of the disc diffusion method was performed by measuring the inhibitory halos of each disk and comparing the values presented in an appropriate table according to the manufacturer of the disc (LaborClin®), thereby determining the sensitivity or resistance of the bacterium antimicrobials tested. The multiresistance observed was evaluated in accordance with Youn et al. (2011), which defined the multidrug resistance to antibiotics as the resistance to more than three different classes of antimicrobials. Reference sample of *S. aureus* ATCC 25923 were used as quality control of the discs.

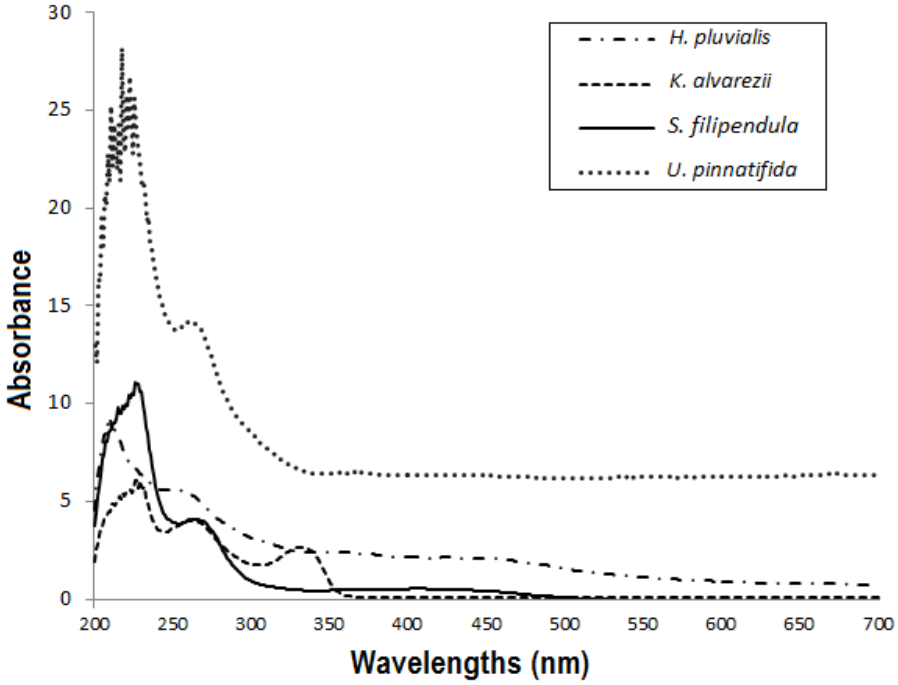
3 RESULTS

UV-visible scanning spectrophotometry

It is possible to observe that the spectral profile of the extracts of *H. pluvialis*, *K. alvarezii* and *U. pinnatifida* presented groups of chromophores that absorbed light between 200 ηm and 700 ηm, while the *S. filipendula* extract had absorption bands between 200 ηm and 520 ηm (Figure 1).

From the two spectral profiles of UV-visible scanning of the samples under study, one can infer a significant presence of phenolic compounds, considering the observed values of maximum absorbance typical of this class of metabolites.

Figure 1: Profile spectrophotometric scanning (λ = 200 ηm-700 ηm) of methanolic extracts of *H. pluvialis, K. alvarezii, S. filipendula* e *U. pinnatifida*.



Microbiological tests

The results obtained in the *in vitro* antimicrobial activity tests showed that the extracts of *S. filipendula* and *U. pinnatifida* inhibited all the bacteria tested (Table 1.). Nevertheless, with *S. filipendula* it was necessary to use higher concentrations to obtain an inhibitory effect against most strains. In contrast, the extract of *U. pinnatifida* was the most efficient since a lower amount of this extract was necessary to obtain an inhibitory effect; however, the extract did not show higher efficiency against *V. harveyi* or *V. parahaemolyticus* comparing with the other seaweed extracts. Additionally, *H. pluvialis* extract was more efficient by inhibiting *V. harveyi* and *V. anguillarum*, but had no inhibitory effect against the two *Staphylococcus* strains. It was observed that *K. alvarezii* extract needed larger amounts of extract to obtain inhibition against bacteria, being *V. alginolyticus* the only resistant to the inhibitory effect of this seaweed. Negative control did not inhibit bacterial growth.

Table 1: Profile of antimicrobial resistance tested and inhibition behavior against extracts from 12 strains of microorganisms.

Strains	*H. pluvialis* MIC (mg/mL)	*K. alvarezii* MIC (mg/mL)	*S. filipendua* MIC (mg/mL)	*U. pinnatifia* MIC (mg/mL)	Antibiotic resistance profile
E. coli ATCC 25102	78,125	156,25	39,062	19,531	NT
P. aeruginosa ATCC 0053	78,125	78,125	156,25	39,062	NT
S. aureus ATCC 25923	NI	312,5	156,25	19,531	NT
S. aureus MRSA ATCC 43300	NI	312,5	156,25	19,531	NT
V. harveyi ATCC 14126	19,531	312,5	78,125	78,125	NT
V. parahaemolyticus ATCC 17802	78,125	156,25	156,25	156,25	NT
V. vulnificus LAM 64	78,125	156,25	156,25	39,062	NT
Pseudomonas sp. GU113077	39,062	39,062	78,125	2,442	Amp/Cfx/ Cfo/Cpm/ Nal/Sut/Tob
S. agalactiae CP018623	39,062	312,5	78,125	19,531	Cip/Cpm/ Gen/Sut
V. alginolyticus BCCM 2068	39,062	NI	78,125	19,531	Amc/Ami/ Amp/Cfx/ Cfo/Enro/ Gen/Tob
V. anguillarum ATCC 19264	19,531	312,5	156,25	19,531	Amc/Ami/ Amp/Cip/ Cfx/Cfo/ Enro/Gen/ Ppt/Tob

NI = did not inhibit the growth of the strain tested. NT = no antimicrobial susceptibility test (TSA) was performed because it was a standard strain. Amp = ampicillin, Amc = amoxicillin with clavulanic acid, Ami= amikacin, Cfx = cephalexin, Cfo = cefoxitin, Cpm = cefepime, Cip = ciprofloxacin, Nal = nalidixic acid, Sut = sulfazotrim, Gen = gentamicin, Ppt= piperacillin with tazobactam, Tob = tobramycin.

The antimicrobial susceptibility profile of four isolates of aquatic organisms using the disc-diffusion method, showed that *V. anguillarum* was resistant against 40% of the tested antibiotics followed by *V. alginolyticus* and *Pseudomonas* sp. with 32% and 28% of resistance respectively. The strain of *S. agalactiae* was the most sensitive showing resistance only to four antibiotics (18%). All isolated bacteria presented resistance to at least one antibiotic from the groups quinolones, aminoglycosides and mainly from the group of beta-lactams in the case of Gram-negative strains (Table 1).

4 DISCUSSION

The bioactive compounds present in the different types of algae are responsible for conferring the biological activity of these organisms of such great metabolic diversity (PAL;

KAMTHANIA; KUMAR, 2014). In the present study, we can observe through UV-visible spectrophotometry the presence of the class of phenolic compounds in the methanolic extracts of the four species of algae tested. According to Adam et al. (1998) the presence of such compounds justifies the antimicrobial activity of the extracts. In the spectral region of 290 to 380 ηm the absorption of phenolic compounds occurs, but also the absorption of proteins and nucleic acids occurs (BACHEREAU; MARIGO; ASTA, 1998). However, due to the use of 80% methanol solution to effect extraction, it is possible that the proteins are precipitated or degraded. Thus, we suggest the presence of the class of phenolic compounds and we can consider that these molecules are responsible for conferring the seaweeds extracts their antimicrobial potential in vitro against the pathogenic bacteria for aquaculture.

Regarding the microbiological tests, extract of the microalgae *H. pluvialis* obtained MIC results of less than 78,125 $mg.mL^{-1}$ against Gram negative. In addition, it demonstrated efficiency in both freshwater and seawater bacteria, emphasizing that growth against the *V. anguillarum* marine water strain was inhibited at the concentration of 19,531 $mg.mL^{-1}$, the lowest concentration observed in relation to the others extracts. The antimicrobial activity observed probably occurs due to short chain fatty acids, such as butanoic acid, methyl lactate and simple phenols. These are present in the astaxanthin, carotenoid found in high levels in *H. pluvialis*, responsible for the inhibition of bacteria and fungi, besides possessing antioxidant activity (WANG; WILLÉN; WADSTROM, 2000; SANTOYO et al., 2009). Rao et al. (2010) found a MIC from 400 ppm of *H. pluvialis* extract against Gram-positive and Gram-negative bacterial strains.

Among the extracts of algae tested, the highest antimicrobial potential, due to the ability to inhibit the strains with the lowest concentrations in the broth microdilution method, was the extract of *U. Pinnatifida*, evidencing its possible applicability in freshwater and marine environments, and also in Gram-positive and Gram-negative strains. In addition, the freshwater and marine bacteria in which the extract showed efficiency in inhibition were considered multiresistant according to Youn et al. (2011), therefore, have greater difficulty of inhibition. According to Silva et al. (2013), marine seaweeds demonstrated antibacterial activity against virulent and antibiotic resistant Vibrio species, a finding that corroborates the results obtained in this study. Cabral (2012) evaluated ethanolic extracts (60%, 80% and 100%) of *U. pinnatifida*, 100% of which showed inhibitory activity against *Klebsiella pneumoniae* and *Listeria monocytogenes*, with MIC between 19.53 and 39.06 $mg.mL^{-1}$, values close to those observed in MIC over Gram-negative bacteria tested in the present study. However, the solvents used are different, and 80% methanol is the most suitable solution for extraction of phenolic

compounds, which are indicated as one of several bioactive compounds responsible for inhibition of bacterial growth (VIEIRA et al., 2010).

The antimicrobial activity of the extract of *S. filipendula* observed in this study occurred from the concentration of 156.25 mg mL^{-1}, obtaining antimicrobial activity against 100% of the strains tested being more efficient against the *E. coli* strain (39,062 mg mL^{-1}). Sastry and Rao (1995) point to the bioactive molecule dioctyl phthalate (PDO) as responsible for the antimicrobial activity of *Sargassum wightii* extract against *S. aureus*, *Proteus vulgaris*, *E. coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Shigella sonnie*, *V. cholerae* and *P. aeruginosa*. The *Sargassum dentifolium* extract with the solvent dichloromethane, tested in vitro by Shanab (2007), by the disc-diffusion method, presented 12 mm halos for *Bacillus subtilis* and *Streptococcus fuecalis*, and 11 mm for *E. coli* and *Staphylococcus albus*. In addition to the inhibitory action under bacterial growth reported in the literature, alginic acid, a polysaccharide present in the cell wall of brown seaweed, isolated from *S. wightii*, demonstrated potent anti-inflammatory and antioxidant action (PARSAEIMEHR; CHEN, 2013).

The seaweed *K. alvarezii* has in its biochemical composition the presence of carbohydrates, proteins, lipids, fatty acids, amino acids, sterols and phenols (RAJASULOCHANA; DHAMOTHARAN; KRISHNAMOORTHY; 2010). However, terpenoid, florotanin and phenol secondary metabolites are compounds that are responsible for the antimicrobial activity of marine seaweeds in the presence of pathogenic microorganisms (PRABHA; PRAKASH; SUDHA, 2013). As an example, the results observed in the present study, where the extract of *K. alvarezii* was able to inhibit the bacterial growth of 11 bacteria evaluated from 312.5 $mg.mL^{-1}$. Sivakumar et al. (2014), although they used the disk diffusion assay, which was considered to be less accurate (NASCIMENTO et al., 2007), indicate an inhibition halo of 8.6 mm in the concentration of 300 µg of crude extract of *K. alvarezii* compared to *V. harveyi* isolates from *Penaeus monodon* larvae. This result corroborates with those observed in the present study, where the extract of *K. alvarezii* was able to inhibit the growth of 80% (4/5) of the tested Vibrio strains, including *V. harveyi* species. The only strain tested that *K. alvarezii* extract did not inhibit was *V. alginolyticus*. Such bacteria are reported in the literature as one of the pathogens responsible for causing in *K. alvarezii* to ice-ice disease. Thus, we suggest that the observed inactivity of the *K. alvarezii* extract against the *V. alginolyticus* strain is associated with ice-ice disease.

In this study, we observed the predilection of the antimicrobial activity of freshwater microalgae *H. pluvialis* by microorganisms from marine environments, while the marine waters seaweeds, *K. alvarezii*, *S. filipendula* and *U. pinnatifida*, have been shown to be more effective

in inhibiting the growth of freshwater strains. Freshwater microorganisms did not present defense mechanisms to the active biomolecules present in marine seaweeds, as well as the saltwater bacteria exposed to the extract of the freshwater microalgae *H. pluvialis*, since these compounds do not belong to their habitat, thereby, inhibition was observed. In this way, it is suggested that this is the reason for such a predilection. Manivannan et al. (2011) observed a similar characteristic in his study, where marine seaweeds *Turbinaria conoides*, *Padina gymnospora* and *Sargassum tenerrimum* demonstrated selective antimicrobial activity by natural freshwater microorganisms.

The MIC of the seaweeds extracts for the 11 species of bacteria tested ranged from 2,442 to 312,5 $mg.mL^{-1}$, that is, a higher concentration of extracts was required to exert antimicrobial activity when compared to the concentrations of similar studies performed. However, authors such as Hood et al. (2013) support the impossibility of a direct comparison between antimicrobial activity investigations of extracts and essential oils due to the lack of standardization of the methods.

About the susceptibility profile of the four isolates observed in the disc-diffusion method, the results obtained are similar to those described by Gastalho et al. (2014) which indicate a more common resistance of Gram-negative isolates of aquatic organisms to the beta-lactam antibiotics due to a higher beta-lactamase activity. All isolated bacteria were resistant to at least one antibiotics from the group of quinolones, aminoglycosides and beta-lactams. Youn et al., (2011) describe that resistance to three or more antimicrobials from different classes indicates a multidrug resistance. According to Guardabassi et al. (2010), this result is an alert for the emergence of multiresistant strains in aquaculture. These findings justify the importance of implementing measures to control the use of synthetic antimicrobials in aquaculture. It should be noted that among the isolated strains only *V. alginolyticus* showed resistance to the extract of *K. avarezii*. The other isolated strains were sensitivity to the rest of the tested extracts. However, there was no relationship between antimicrobial multiresistance and resistance to seaweeds extracts tested by us.

In conclusion, the results of this study demonstrated that the methanolic extracts of *H. pluvialis*, *K. alvarezii*, *S. filipendula* and *U. pinnatifida* exert antimicrobial activity *in vitro* against Gram-positive and Gram-negative bacteria pathogenic to aquatic organisms. Among the evaluated extracts, the extract of *U. pinnatifida* had the highest antimicrobial potential against the bacterial strains evaluated. Although the isolated strains presented multiresistance to antibiotics, they were sensitivity to the extracts indicating that they could be an alternative to the control of bacterial diseases in aquaculture.

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