Chemical diversity and antifouling activity of geniculate calcareous algae (Corallinales, Rhodophyta) from Brazil

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Marine biofouling is a natural process by which many organisms colonize and grow in submerged structures causing serious economic consequences for the maritime industry. Geniculate calcareous algae (GCA; Corallinales, Rhodophyta) produce bioactive secondary metabolites and are a promise for new antifouling compounds. Here, we investigated the antifouling activity of four GCA species (*Amphiroa beauvoisii*, *Amphiroa flabellata*, *Cheilosporum sagittatum* and *Jania crassa*) from Brazilian litoral against macro and microorganisms. Simultaneosly, we have developed metabolomic approach to study seaweeds chemical profiles using gas chromatography coupled to mass spectrometry (GC-MS) and data analysis by Principal Component Analysis and the molecular networking analysis through the global natural products social molecular networking platform (GNPS). Our results revelead that all extracts were active against marine bacteria, with *C. sagittatum* (CsSI) extract being the most active. For the mussel *Perna perna*, the extract of *C. sagittatum* (CsSI) was the most active, with a 100% inhibition. In terms of toxicity, only the extract of *J. crassa* (JcP) showed a 20% mortality rate. Chemical profiles of GCA extracts were qualitatively and quantitatively different, with the steroid (3β) cholest-5-en-3-ol the as the major compounds identified in all extracts, except in *C. sagittatum* extract (CsSI). In addition, intra-interspecific chemical variabilities were observed among GCA extracts of different populations, which could explain the variability in antifouling activity. The present study contributed with new information about the chemical substances produced by this group of seaweeds and showed its antifouling potential. These GCA species may be the subject of future studies to obtain new bioactive substances with potential biotechnological in the maritime area.

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Abstract

Marine biofouling is a natural process by which many organisms colonize and grow in submerged structures causing serious economic consequences for the maritime industry. Geniculate calcareous algae (GCA; Corallinales, Rhodophyta) produce bioactive secondary metabolites and are a promise for new antifouling compounds. Here, we investigated the antifouling activity of four GCA species (*Amphiroa beauvoisii*, *Amphiroa flabellata*, *Cheilosporum sagittatum* and *Jania crassa*) from Brazilian litoral against macro and microorganisms. Simultaneosly, we have developed metabolomic approach to study seaweeds chemical profiles using gas chromatography coupled to mass spectrometry (GC-MS) and data analysis by Principal Component Analysis and the molecular networking analysis through the global natural products social molecular networking platform (GNPS). Our results revelead that all extracts were active against marine bacteria, with *C. sagittatum* (CsSI) extract being the most active. For the mussel *Perna perna*, the extract of *C. sagittatum* (CsSI) was the most active, with a 100% inhibition. In terms of toxicity, only the extract of *J. crassa* (JcP) showed a 20% mortality rate. Chemical profiles of GCA extracts were qualitatively and quantitatively different, with the steroid (3β) cholest-5-en-3-ol the as the major compounds identified in all extracts, except in *C. sagittatum* extract (CsSI). In addition, intra-interspecific chemical variabilities were observed among GCA extracts of different populations, which could explain the variability in antifouling activity. The present study contributed with new information about the chemical substances produced by this group of seaweeds and showed its antifouling potential. These GCA species may be the subject of future studies to obtain new bioactive substances with potential biotechnological in the maritime area.

Subjects: Biodiversity, Biotechnology, Plant Science, Aquatic and Marine Chemistry, Biological Oceanography

Keywords: Geniculate calcareous algae, Crude extract, Chemical composition, Metabolomics, Multivariate analysis, Biofouling

Introduction

Marine biofouling is the process of colonization and growth of sessile organisms on submerged surfaces such as ship hulls, platforms, pipes and buoys (*Maréchal & Hellio, 2009*).

After the adsorption of organic particles on these surfaces, the formation of the bacterial biofilm occurs, which in turn facilitates the proliferation of other microorganisms (*Wahl, 1989*; *Dang et al., 2007*; *Martín-Rodríguez et al., 2015*). Subsequently, this biofilm may also facilitate the colonization and growth of macro-organisms such as mussels, seaweeds, barnacles and bryozoans occurs (*Whal, 1989*; *Martín-Rodríguez et al., 2015*). The biofouling of these micro and macrofoulings causes serious impacts on the marine industry worldwide, as it affects the efficiency of maritime transport due to increased roughness and corrosion of vessels (*Ali et al., 2020*; *Ferreira et al., 2020*), generates an increase in maintenance costs (*Cao et al., 2011*) and fuel consumption (*Ali et al., 2020*). In addition to being one of the main vector of introduction of exotic/invasive species (*Davidson et al., 2016*; *Vimala, 2016*; *Ali et al., 2020*).

As the main strategy adopted to minimize the impacts of biofouling in the shipbuilding industry, biocides containing arsenic, mercury, lead and tributyltin (TBT) were used to kill or inhibit the colonization of fouling organisms (*Ali et al., 2020*). However, these biocides showed a high level of environmental contamination and risks to marine organisms (*Silva et al., 2018*; *Ali et al., 2020*; *Han et al., 2021*). Therefore, after proving the high toxicity of TBT to target and non-target marine species, its application in the coating of ships for use as a biocide was banned in 2008 (*Batista-Andrade et al., 2018*). Other biocides with less toxic formulations were used to control biofouling, such as Diuron, Irgarol 1051and Sea-Nine 211, for example. However, studies with these substances alone or mixed still report their negative effects on several marine organisms (*Wang et al., 2011*; *Batista-Andrade et al., 2018*).

Seaweeds are a rich source of bioactive substances and target of biotechnological studies. In the natural environment, they produce a variety of chemical substances, known as secondary metabolites or natural products, capable of preventing the growth of epibiont organisms (*Da Gama et al., 2008; 2014*; *Othmani et al., 2015*; *Qian et al., 2015*; *Carvalho et al., 2016*; *Sánchez-Lozano et al., 2019*). In this context, can be an efficient alternative with antifouling potential. The production of these metabolites might change according to the influence of several factors, such as temperature (*Sudatti et al., 2011)*, location (*Plouguerné et al., 2010*; *Stengel, Connan & Popper, 2011*), season (*Stengel, Connan & Popper, 2011*; *Mansur, 2020*), and exposure to ecological interactions (*Stengel, Connan & Popper, 2011*).

The geniculate calcareous algae (GCA; Corallinales, Rhodophyta) are included in this group of seaweeds, whose thalli are composed by alternating calcified and uncalcified segments,

in contrast with non-geniculate calcareous algae (NGCA) that have an entirely calcified thallus (*Johansen, 1981*). Both calcareous algae are worldwide distributed (*Foster, 2001*; *Harvey et al., 2005*; *Riosmena-Rodríguez, Nelson & Aguirre, 2017*) and are distinguished from the other red algae by the presence of calcium carbonate ($CaCO_3$) in the cellular walls in the form of calcite (*Nash & Adey, 2017*).

The GCA are producers of a variety of chemical substances, such as fatty acids (*Cikos et al., 2021*), sterols (*Caf et al., 2019*) and hydrocarbons (*Ahmed et al., 2011*). Tannins, flavonoids, alkaloids and carotenoids (*Akbary, Adeshina & Jahanbakhshic, 2020*; *Cikos et al., 2021*) have also been found in the chemical profile in these seaweeds. These substances are responsible for different biological activities (*Raj et al., 2019*; *Cikoš et al., 2021*; *Mofeed et al., 2022*; *Righini et al., 2021*), including antifouling activity (*Medeiros, Da Gama & Gallerani, 2007*; *Kantida et al., 2012*; *Deepa, Srikumar & Padmakumar, 2014*).

Although this group is numerous and widely distributed, there have been few studies focused on understanding the chemical composition and biotechnological applications of GCA. Considering the importance of the bioactive substances produced by this group and the scarcity of studies carried out on the Brazilian coast. The present study aimed to analyze the intra/interspecific chemical profile and the antifouling potential of the crude extracts of four species of GCA (*Amphiroa beauvoisii* J.V.Lamouroux, *Amphiroa flabellata* Harvey, *Cheilosporum sagittatum* (J.V.Lamouroux) Areschoug and *Jania crassa* J.V.Lamouroux) collected in Arraial do Cabo, Brazil.

Materials & Methods

Study area and sampling sites

Arraial do Cabo is located on the coast of Rio de Janeiro State, on the Southeastern Brazil (*Fig. 1*). The region is influenced by localized summer and spring upwelling events associated with the local wind regime and bathymetry (*Castelao, 2012; Belem, Castelao & Albuquerque, 2013*). Despite these upwelling events, which bring cold nutrient-rich subsurface waters, the average sea surface temperatures inside the Arraial do Cabo Bay are still predominantly >20°C (*Guimaraens & Coutinho, 1996*; *Candella, 2009*). Thus, while the surrounding rocky shores are characterized largely by tropical reef communities, the Arraial do Cabo region represents a

unique site with the co-occurrence of both tropical and subtropical marine species (*Laborel, 1970*; *Lanari & Coutinho, 2014*).

The seaweeds samples were collected manually, in the intertidal and infralittoral regions by SCUBA diving in different sites of Arraial do Cabo (*Fig. 1*) in the summer of 2018. The study sites included: Fenda de Nossa Senhora, Prainha, Praia do Forno, Saco do Cherne (rocky shore and articuliths beds, *Tâmega et al., 2017*; *2021*), Praia dos Anjos, Saco dos ingleses and Ponta da Cabeça. A total of nine seaweeds samples were collected, five of the species *A. beauvoisii*, two of the species *J. crassa*, one of the species *C. sagittatum* and one of the species *A. flabellata*. Subsequently, they were washed in seawater to remove sand and associated organisms, frozen and lyophilized. Seaweeds samples are housed in the scientific collection in the Instituto de Estudos do Mar Almirante Paulo Moreira (IEAPM, *Table 1*).

Figure 1

Table 1

Preparation of seaweeds extracts

The freeze-dry seaweeds were extracted in a mixture of ethyl acetate and methanol (EtOAc: MeOH 1:1 v/v) in a proportion of 3.5 mL of solution for 1g of dry weight of the sample. The extraction of each seaweed was performed three times, in which each interval had 2h/ 16h (overnight)/ 2h maceration period, respectively. Prior to each standing time, the material sonicated for 30 minutest. Subsequently, the extracts were filtered by gravity and concentrated under reduced pressure.

Chemical profiles of extracts

The extracts obtained were analyzed by gas chromatography coupled to mass spectrometry (GC-MS) to assess the chemical diversity of GCA. Before analysis, the extracts were diluted in dichloromethane (HPLC, Tedia) and filtered through 0.45 μm PTFE filters (Millipore, EUA) to remove any insoluble constituents. Subsequently, the solvent was evaporated, the samples were lyophilized and the remaining material was resuspended in ethyl acetate (HPLC grade, Tedia) at a concentration of 1mg/mL. GC-MS analysis was carried out

using Shimadzu CG-2010 equipment coupled to the QP-2010 ultra-mass spectrometer, comprising an AOC-20i auto-injector and a 30 m x Φint 0.25 mm Rtx-1MS column.

The column flow rate was 1.20 mL/min in split mode, with a ratio of 1/5 and helium as carrier gas. The injector temperature was 280 °C and the column was heated to 150 °C for 3 minutes, followed by a temperature ramp to 300 °C (rate of 6 °C/min) and at the end remaining at 300 °C for 5 minutes, totaling 33 minutes of analysis. The mass detector was used in electron impact mode (70 eV), with interface temperature at 300 °C and ion source at 200 °C. Chemical profiles were analyzed based on their mass spectra and retention time. Chemical substances were identified by comparing their mass spectra with those available in the NIST 11 library, taking into account similarity ≥ 85%.

The chromatograms obtained through GC-MS were converted into computable document format (CDF) and inserted into the global natural products social molecular networking (GNPS) platform, creating a molecular network (*Wang et al., 2016*). In addition, a metadata table was created to help identify the substances present in each crude extract and compare them. The results were processed in the Cytoscape program, allowing to identify similarities of chemical classes between the studied GCA substances. The cosine value ≥ 0.7 was taken into account in the identification of substances. The data obtained on this platform were compared to the NIST 11 library.

Antibacterial activity

For antibacterial bioassays we modified the methodology described by *Devi et al. (2011)* The antibacterial activity of all extracts at the natural concentration was tested against strains associated marine fouling: four strains of Gram-negative marine bacteria (*Polaribacter irgensii*, *Pseudoalteromonas elyakovii*, *Pseudomonas fluorescens* and *Vibrio aestuarianus*) and one strain of Gram-positive marine bacteria (*Shewanella putrefaciens*). The antibacterial tests were performed by disc diffusion assay (n=5), at an optical density (O.D) of 1.5–1.8 at 630 nm.The extracts were solubilized in ethyl acetate and methanol (1:1 v/v) and applied to sterile discs (5 mm in diameter) made of filter paper (Whatman nº. 1) (*Table 2*). Disks with the antibiotic streptomycin (Sigma-Aldrich) were used as a positive control (n=5) at a concentration of 10 mg/g. Posteriorly to a period of 24 h incubation at 30 °C, the diameter (mm) of the inhibition halo around the disks was measured using the ImageJ program (version 1.52a).

Table 2

Antifouling activity against the mussel *Perna perna*

The assay of antifouling activity against the mussel *Perna perna* was modified from the method described by *Da Gama et al. (2003)*. Specimens were collected in the coastal area of Ponta da Cabeça (Praia Grande), Arraial do Cabo, Brazil. Specimens were carefully separated and cleaned. The specimens were selected when fulfilling three criteria: shell length between 1.6–2.0 cm, in addition to active exposure of the feet and capability to crawling.

The extracts were solubilized in ethyl acetate and methanol (1:1 v/v) at the natural concentration (*Table 2*) and incorporated into filter paper discs (5 cm). After drying the filters, they were placed at the bottom of glass petri dishes (60x15 mm). Discs soaked only in seawater were used as null control. Each plate was filled with 12 ml of seawater and three specimens.

A total of 10 replicates were used for each treatment and control. After 24 hours of experiment, the number of byssus fixed by the mussels in each experimental condition was evaluated. At the end of the tests, the mussels were placed in containers with filtered seawater, at a temperature of 22 ºC, salinity of 35 and constant aeration for 24 hours. After this period, the response of the individuals to touch, tissue loss and open valve were followed to measure the toxic effect of the extracts.

Data analysis

Multivariate analyse was performed to investigate the possible chemical profile variability in extracts of GCA. The COWtool software (Correlation Warping Algorithm) was used to perform the baseline correction of each chromatogram and to correct the peak retention time (*Nielsen et al., 1998*). The matrix with all chromatograms aligned was constructed through Principal Component Analysis (PCA) using the Rstudio language and environment (http://www.R-project.org) with the “ChemometricsWithR” package installed (*Wehrens, 2011*). The activity against marine bacteria was expressed in millimeters (mm), while for the mussel *P. perna*, the values of byssus fixed on the plates were expressed in percentage.

For the experiment with *P. perna*, the values of byssus fixed on the plates were converted into percentages for further analysis. The assumptions of normality and homogeneity required

for ANOVA were verified using the Shapiro-Wilk and Cochran C tests, respectively. The one-way ANOVA was used in the antibacterial experiment to compare the values of inhibition halo between control (positive) and treatments (extracts) for the same bacteria; and in the with *P. perna* experiment to compare the percentage of byssus fixed between control (negative) and treatments (extracts). Significant differences ($p < 0.05$) were post-hoc calculated by the Tukey's test. These analyses were performed using the program Statistica 8.

Results

Chemical profile of extracts

The chromatograms obtained through GC-MS analysis showed intra/interspecific chemical variability, both qualitatively and quantitatively of the analyzed extracts (*Figs. 2–3*). More complex profiles were observed in the extracts of *J. crassa* (JcP) and *A. flabellata* (AfPG), while the least complex were in the extracts *A. beauvoisii* (AbSCB) and *C. sagittatum* (CsSI), with respect to the amount of compounds. The molecular network created on the GNPS platform also evidenced this variability grouping of similar classes, such as sterols (1), fatty acid esters (2), fatty alcohols (3), hydrocarbons (4) and fatty acids (5) (*Fig. 4*). A total of 17 substances were identified through the mass spectra of the GNPS platform and NIST 11 in the extracts of GCA (*Table 3*).

Substances with relative area ≥ 2% (of each extract) were pointed out in their respective chromatograms. The steroid (3β) cholest-5-en-3-ol (peak 12) was the most abundant substance identified in all samples of *A. beauvoisii* (AbFNS–21.70%), (AbP–37.11%), (AbPF–39.07%), (AbSCB–35.84%) and (AbSCC–28.20%); in the both samples of *J. crassa* (JcP–16.62%) and (JcPA–12.63%) and *A. flabellata* (AfPC–19.49%). In the extract of *C. sagittatum* (CsSI), palmitic acid (peak 6) was the most abundant substance, with 31.35% area.

Figure 2

Figure 3

Figure 4

Table 3

Principal component analysis (PCA)

The chemical profiles of the nine GCA extracts were obtained by GC-MS and compared using principal component analysis (PCA). In this study, the two principal components explained 78.9% of the total chromatographic variation (PC1=65.0% and PC2=13.9%) (*Fig. 5A*). The negative axis of PC1 grouped extracts from *A. beauvoisii* (AbFNS, AbP and AbSCC), *J. crassa* (JcP and JcPA), *A. flabellata* (AfPC) and *C. sagittatu*m (CsSI). On the other hand, the positive axis grouped the extracts of *A. beauvoisii* (AbPF and AbSCB), showing the intraspecific chemical variability in relation to the extracts of *A. beauvoisii.* The compounds responsible for this distribution were mainly palmitic acid (6) and the sterol (3β) cholest-5-en-3-ol (12) (*Fig. 5B*). Meanwhile, the negative axis of PC2 grouped extracts from *A. beauvoisii* (AbPF), *J. crassa* (JcPA) and *C. sagittatu*m (CsSI). On the contrary, the positive axis grouped extracts from *A. beauvoisii* (AbFNS, AbP, AbSCC and AbSCB), *J. crassa* (JcP) and *A. flabellata* (AfPC). In addition to the two compounds already mentioned for PC1, the sterol (3β, 5α)-ergosta-7-en-3-ol (14) (*Fig. 5C*) was also important in the distribution of samples in this component, showing intra/interspecific chemical variability.

Figure 5ABC

Antibacterial activity

All crude extracts showed antibacterial activity against bacterial strains tested. The extracts, when compared to each other, showed significantly different performance in inhibiting all bacterial strains*,* except against the bacteria *V. aestuarianus* (*Table 4*). In general, there were no significant differences in the performance of the extracts when compared to the same GCA species, except for extracts from *A. beauvoisii* seaweed collected in Saco do Cherne (AbSCB and AbSCC). When comparing these extracts, the AbSCC, collected in the coastal habitat, showed a significantly greater inhibition halo for the bacterial strains *P. fluorescens*, *P. irgensii* and *S. putrefaciens*, than the AbSCB, collected in the articuliths beds. For the other two bacteria tested, *P. elyakovii* and *V. aestuarianus*, the extracts did not show significant differences in inhibition.

The *C. sagittatum* extract (CsSI) stood out from the others, presenting a significantly larger inhibition halo than all other extracts when tested against the bacteria *P. elyakovii*.

Table 4

Antifouling activity against the mussel *Perna perna*

All extracts significantly inhibited the byssus fixation from the mussel *P. perna* compared to the seawater control (ANOVA, $F = 50.40$, $p < 0.001$) (*Fig. 6A*). When comparing the extracts, it was also possible to verify significant differences in their antifouling activities against mussels. In plates with *C. sagittatum* extract (CsSI) there was no byssus fixation, and its antifouling activity was significantly higher than for all other extracts. On the other hand, the AbSCC extract was significantly less active against the target organism than all other extracts, showing a byssus inhibition of 57.18%. Inhibition of fixed byssus for all other extracts did not differ significantly between them.

Regarding the toxicity test, only the JcP extract showed a toxic effect against *P. perna*, with 20% mortality for the mussel, a value significantly higher than the other extracts that did not show mortality (ANOVA, $F = 13.50$, $p < 0.001$) (*Fig.6B*).

Figure 6AB

Discussion

In the present study, the chromatographic and spectroscopy analyses of the GCA species collected in Arraial do Cabo, showed intra and interspecific variability in the extracts' composition, even being collected in closely located areas and with similar oceanographic conditions. For instance, some metabolites were identified in all extracts (e.g. heptadecane, (3β)-cholest-5-en-3-ol, palmitic acid), with variations in peak area and relative abundance (implying a quantitative variation). Whilst, compounds such as (Z)-7-hexadecenal were only detected in *J. crassa* (JcPA e JcP) and *A. flabellata* (AfPC) species, (qualitative variation). On the other hand, pentadecanoic acid was absent in *A. beauvoisii* extracts from Prainha (AbP) and Saco do Cherne (AbSCC and AbSCB), but present in *A. beauvoisii* from Fenda de Nossa Senhora (AbFNS) and Praia do Forno (AbPF), which revealed an intraspecific qualitative variability.

Different chemical classes were found in the extracts of GCA through analysis by GC-MS and identification by the used databases (NIST and GNPS). Fatty acids, hydrocarbons, sterols and fatty alcohols were the most recurrent classes of metabolites observed in the study. The analysis by molecular networking performed on the GNPS platform was an important metabolomics tool, allowing the observation of the grouping of families of substances within the same chemical class and contributing to the identification of substances along with the NIST library.

Marine macroalgae are capable of synthesizing an extensive amount of compounds with a wide range of structural and functional diversity (*Biris-Dorhoi et al., 2020*). Several environmental factors such as temperature (*Sudatti et al., 2011*), nutrient availability (*Stengel, Connan & Popper, 2011*) and different ecological interactions (*Stengel, Connan & Popper, 2011*) in the environment are known to influence the production of these compounds. In the maritime area of Arraial do Cabo there are two distinct morphological features, in which the inner portion, the Bay of Arraial do Cabo, is characterized as a sheltered zone with warmer waters, while the outer part is strongly influenced by wave action and upwelling seasonal. In the context, decreases the water temperature (< 18 °C) and increases the availability of nutrients in this external part (*Guimaraens & Coutinho 1996*; *Candella, 2009*; *Batista et al*., *2020*). Although the GCA collections were carried out both in the internal (sheltered) and external (exposed) parts of Arraial do Cabo and during the summer period, when the influence of upwelling is more marked in the region, quantitative and qualitative variability was observed in the chemical profile of the extracts collected for areas with similar environmental conditions.

The sterol (3β)-cholest-5-en-3-ol was the most abundant compound identified in the databases (GNPS and NIST), present in almost in all extracts and ranging from 12.63% to 39.07%, with exception of *C. sagittatum* (CsSI). The production of this compound was also reported as the most abundant in the methanolic extract of *J. rubens* (Linnaeus) J.V.Lamouroux (24.25%) collected from Egypt (*Ahmed et al., 2011*). On the other hand, in the extract (methanol and hexane, 1:1 v/v) from *Amphiroa anceps* (Lamarck) Decaisne, not as one of the major substances, showing only 2% of area (*Mofeed et al., 2022*). Our study is the first to present the production of sterol (3β)-cholest-5-en-3-ol for the genus *Arthrocardia* and *Cheilosporum*, contributing with more information about the chemical composition of these GCA genus. This class of metabolite is essential to the cellular structure of several organisms, and it is also

associated with different biological activities, such as antioxidant, antiviral and antitumor activity, for example. (*Alassali et al., 2016*, *Thirumurugan et al., 2018*; *Fagundes & Wagner, 2021*).

Palmitic acid, also found in all extracts, being more abundant in the *C. sagittatum* extract (CsSI) compared to the others, showed 31.35% of area. *Venkatesalu et al. (2012)* analyzed the composition of fatty acids in different species of marine macroalgae, including geniculate calcareous algae. The results presented greater abundance of palmitic acid in the species *Cheilosporum spectabile* Harvey ex Grunow (11.72%), *Amphiroa foliacea* J.V.Lamouroux (91.56%) and *Amphiroa* sp. (92.92%) collected in spring, during monsoon and post-monsoon, respectively. Palmitic acid was also identified as the most abundant substance in the species *A. anceps* - 57.57% (*Jayasreesn et al., 2013*) and *J. rubens* - 34.22% (*Caf et al., 2019*). Studies with fatty acids showing potential biological activity has become, every time, more attractive. Antibacterial (*Desbois & Smith, 2009*; *Casillas-Vargas et al., 2021*), antioxidant (*Henry, 2002*), antifungal (*Guimarães & Venancio, 2022*) and antifouling activity (*Goto et al., 1992*; *Gao et al., 2014*) are some of biotechnological activities reported to this class of metabolite.

The variability in the activity against five bacterial strains was also observed among GCA extracts. Marine bacteria are often used in assays to assess antibiofilm activity, as the inhibition of specific species can directly affect the colonization of fouling organisms (*Dobretsov et al., 2009*; *Da Gama, 2014*). In the test performed against bacterial strains was possible to verify the significant differences in the extracts of *A. beauvoisii* in which the sample collected on the rocky shore of Saco do Cherne (AbSCC) achieved better results, which can be explained by the chemical composition this extract. Meanwhile, the material sampled at the same location, but from a different habitat (articuliths beds of Saco do Cherne, AbSCB) presented a weaker response. The differences in the performance of the results between the chemical extracts of the *A. beauvoisii* samples may be related to the habitat in which the samples occur. The intertidal rocky shore habitat is susceptible to daily stress with variations in irradiance, temperature, desiccation and water movement (*Fields et al., 1993; Helmuth, 1999; 2002; Massa et al., 2009*). On the other hand, articuliths beds of Saco do Cherne (15–18m depths) it is a more stable habitat with lower environmental variations (*Tâmega et al., 2021*).

The inhibitory activity of the ethanolic extract *A. anceps* against marine bacteria of the genus *Vibrio* was also presented by *Deepa, Srikumar & Padmakumar, 2014*. Studies against

pathogenic microorganisms were also found for the genus *Amphiroa*. *Vlachos et al. (1997)* presented results of the activity of ethanolic extract of *Amphiroa ephedraea* (Lamarck) Decaisne against four species of fungi and eleven species of bacteria, being more active in the inhibition of *Bacillus subtilis* EL39 (20–25mm). On the other hand, inactivity or low inhibition of extracts of *A. beauvoisii, Amphiroa cryptarthrodia* Zanardini and *Amphiroa rigida* J.V.Lamouroux against different biological models (bacteria, viruses and fungi) was observed in the study developed by *Ballesteros, Martín & Uriz et al. (1992).* Extract of *A. anceps* (MeOH/Hex 1:1), containing 1,2-benzenedicarboxylic acid, diisooctyl ester (30.4%) and pentadecanoic acid, 14-methyl-, methyl ester (29.5%) as the most abundant substances, showed antibacterial activity against *Salmonella typhimurium, Staphylococcus aureus* and *Escherichia coli*, and antiviral activity against rotavirus and coxsackievirus B3 (*Mofeed et al., 2022*).

Considering the extracts of *J. crassa* it was also possible to verify the inhibitory activity against strains of marine bacteria. However, the results did not show significant statistical differences between them (JcP and JcPA), except against the bacteria *S. putrefaciens*, in which the sample collected in Prainha (JcP) was more active in compared to that collected at Praia dos Anjos (JcPA). This observation, according the results of the chemical profile, showed an intraspecific variation in the chemical composition of these samples. The literature also reports the biological potential of the genus *Jania* making use of other models of pathogenic microorganisms. Ethanolic extract of *Jania adhaerens* J.V.Lamouroux showed a mild activity against four strains of marine bacteria, including the genus *Pseudomonas* (also evaluated in this work) with an inhibition zone of 0.5mm (*Kantida et al. 2012*). In the study performed by *Sasikala & Geetha (2017)*, the methanolic extract of the species *J. rubens* was one of the most active against different bacterial strains, mainly against *Enterococcus faecalis* and *Streptococcus pyogenes*. For the species *J. crassa*, in specific, only the study developed by *Soares et al. (2012)* reports inhibitory action of its extract (dichloromethane/methanol 1:1) against two types of Herpes viruses.

The extract of C*heilosporum sagittatum* (CsSI) presented one of the most satisfactory inhibitory responses against four species of marine bacteria (*P. elyakovii, P. fluorescens, P. irgensii* and *S. putrefaciens*). Its chemical composition, presenting palmitic acid as the most abundant compound, may be associated with this activity. Methanolic extract of *Cheilosporum spectabile* also showed activity against two species of diatoms (*Navicula subinflata* and

Nitzschia palea) (*Deepa, Srikumar & Padmakumar, 2014*). Studies against pathogenic microorganisms were also found for the genus *Cheilosporum*. *Vlachos et al. (1997)* also observed that the extract of *C. sagittatum* inhibited the growth of four species of fungi and twelve species of bacteria, having the most remarkable responses against *Bacillus subtilis* EL39, *Micrococccus* sp. and *Staphylococcus aureus.* In the study made by *Stirk et al. (2003),* it was also possible to verify the activity of the ethanolic extract of the genus *Cheilosporum* against strains of Gram positive and negative bacteria. Studies on the biotechnological potential of this genus are scarcer. In this sense, the current study presents another promising alternative for the use of natural products from macroalgae with antibacterial potential.

In addition to, the antifouling experiments using the mussel *P. perna* have shown this mollusc as an excellent model organism for fouling studies, as it demonstrates a fast and clear response to bioactive substances (*Da Gama et al., 2003*; *2008; Barbosa et al., 2007*; *Plouguerné et al., 2010*). Marine fouling organisms use the byssal threads to firmly attach themselves to various submerged structures, such as ship hulls, causing serious economic problems for the shipbuilding industry (*Wang et al., 2017*). In the present study, crude extracts obtained from the different calcareous algae, inhibited the byssus production, in which the extract of *C. sagittatum* (CsSI) presented the highest levels of biological activity. *J. crassa*, collected in Prainha (JcP), was the only extract that showed considerable toxic effects over the target organism with 20% mortality. In a similar experiment, *Medeiros et al. (2007)* reported antifouling potential against the mussel *P. perna* from the crude extract of four species of macroalgae, including one species of *Jania* genus. *J. rubens* was seen as one of the most active species against the mollusc but, in contrast, mortality was not observed.

Conclusions

The results obtained for the extracts of the species *A. beauvoisii*, *A. flabellata*, *C. sagittatum* and *J. crassa* collected in Arraial do Cabo are unprecedented in terms of chemical composition, intra/interspecific chemical variability and activity against the tested models. In addition, the present study contributed with new information about the chemical substances produced by this group of seaweeds and showed its antifouling potential. These GCA species may be the subject of future studies to obtain new bioactive substances with potential application in the maritime area.

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Competing interests

The authors declare there are no competing interests.

Author contributions

* Ellen A. de S. Oliveira conceived and designed the experiments, performed the experiments, analyzed the data, prepared the figures and/or tables and drafted the work or revised it critically for important content.
* Juliana A. de S. Oliveira performed the experiments, analyzed the data, prepared the figures and/or tables and drafted the work or revised it critically for important content.
* Priscila R. de Araújo conceived and designed the experiments, performed the experiments, analyzed the data and drafted the work or revised it critically for important content.
* Frederico T. de S. Tâmega analyzed the data and drafted the work or revised it critically for important content.

* Ricardo Coutinho analyzed the data and drafted the work or revised it critically for important content.

* Angélica R. Soares conceived and designed the experiments, analyzed the data and drafted the work or revised it critically for important content.

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Figure 1

Geographic location of collection sites for geniculate calcareous algae in different areas (in blue) of Arraial do Cabo/RJ.

Figure 1 : Geographic location of collection sites for geniculate calcareous algae in different areas (in blue) of Arraial do Cabo/RJ: Fenda de Nossa Senhora (FNS), Prainha (P), Praia do Forno (PF), Saco do Cherne - rocky shore (SCC), Saco do Cherne - articuliths bed (SCB) Praia dos Anjos, Saco dos ingleses e Ponta da Cabeça.

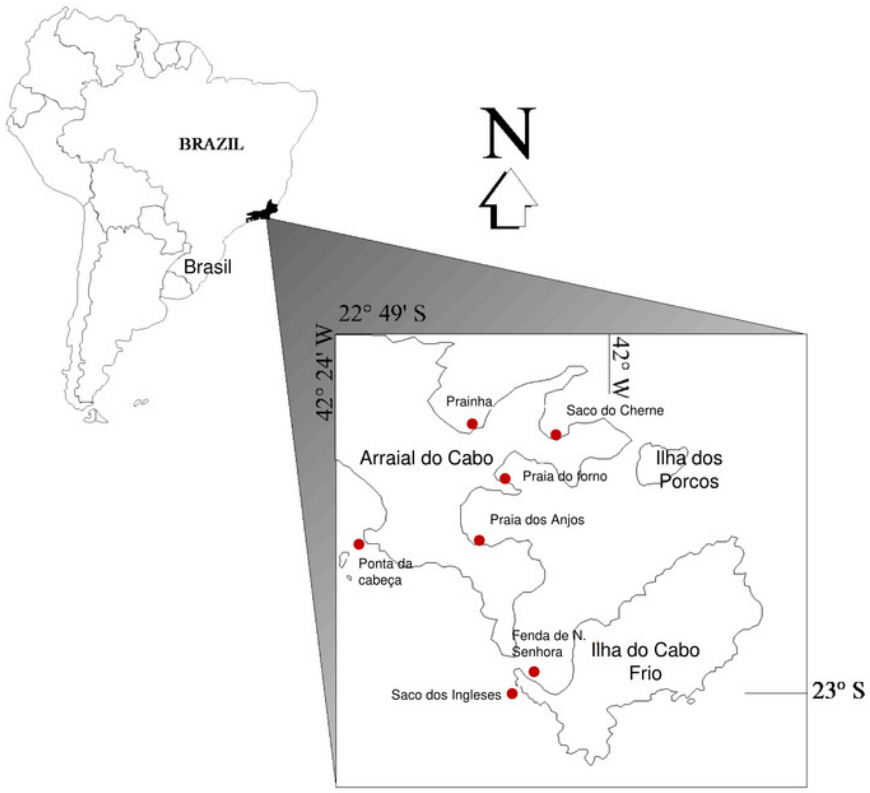

Figure 2

Chromatograms of GCA extracts obtained by GC-MS.

Figure 2: Chromatograms of GCA extracts obtained by GC-MS. AbFNS (*A. beauvoisii* - Fenda de Nossa Senhora); AbP (*A. beauvoisii* - Prainha); AbPF (*A. beauvoisii* - Praia do Forno); AbSCB (*A. beauvoisii* - Saco do Cherne, articuliths bed); AbSCC (*A. beauvoisii* - Saco do Cherne rocky shore). The numbers indicate the most abundant substances (relative area ≥ 2%).

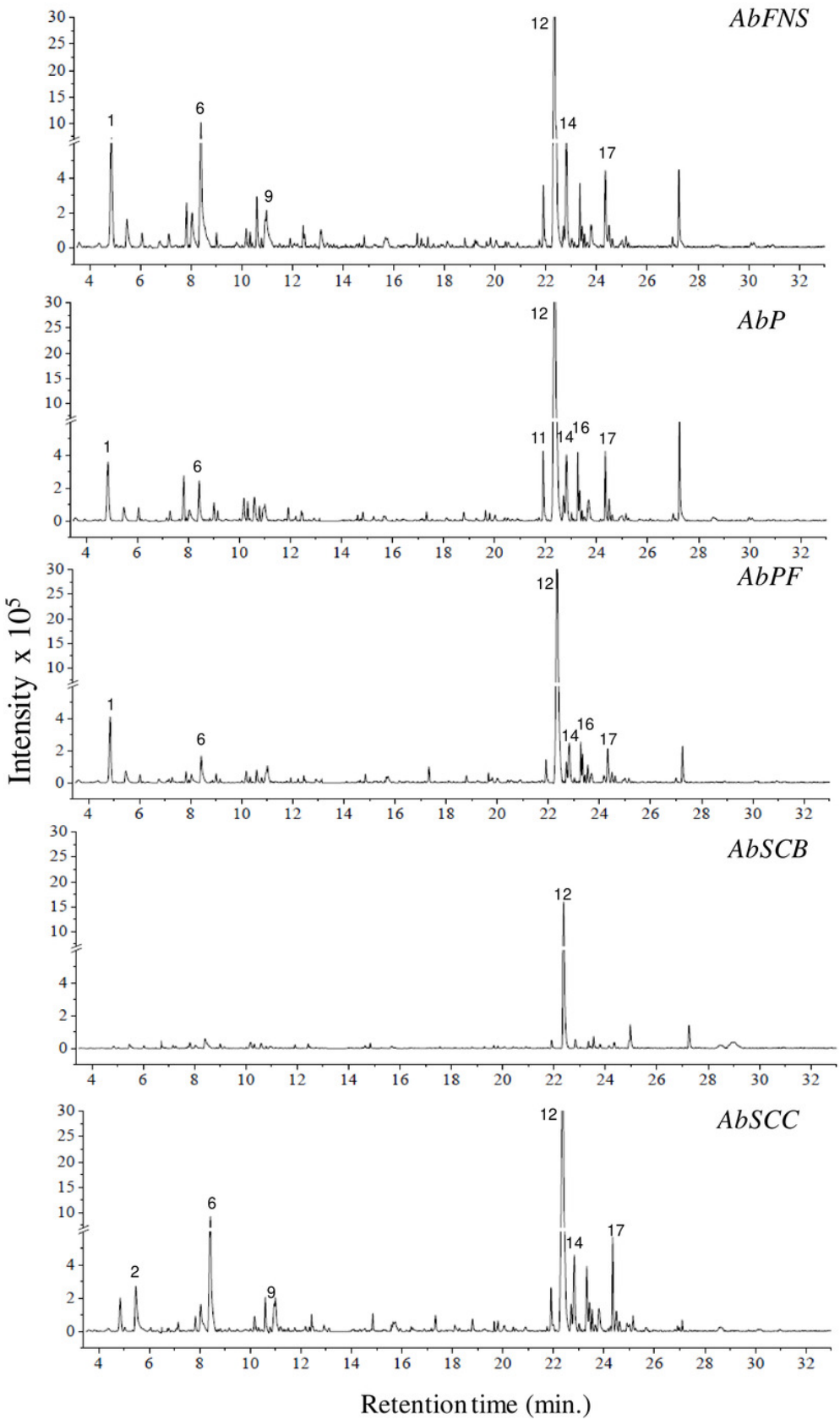
AbFNS
AbP
AbPF
AbSCB
AbSCC
Intensity x 10^5
Retention time (min.)

Figure 3

Chromatograms of GCA extracts obtained by GC-MS.

Figure 3: Chromatograms of GCA extracts obtained by GC-MS. JcPA (*J. crassa* - Praia dos Anjos); JcP (*J. crassa* - Prainha); CsSI (*C. sagittatum* - Saco dos Ingleses) e AfPC (*A. flabellata* - Ponta da Cabeça). The numbers indicate the most abundant substances (relative area ≥ 2%).

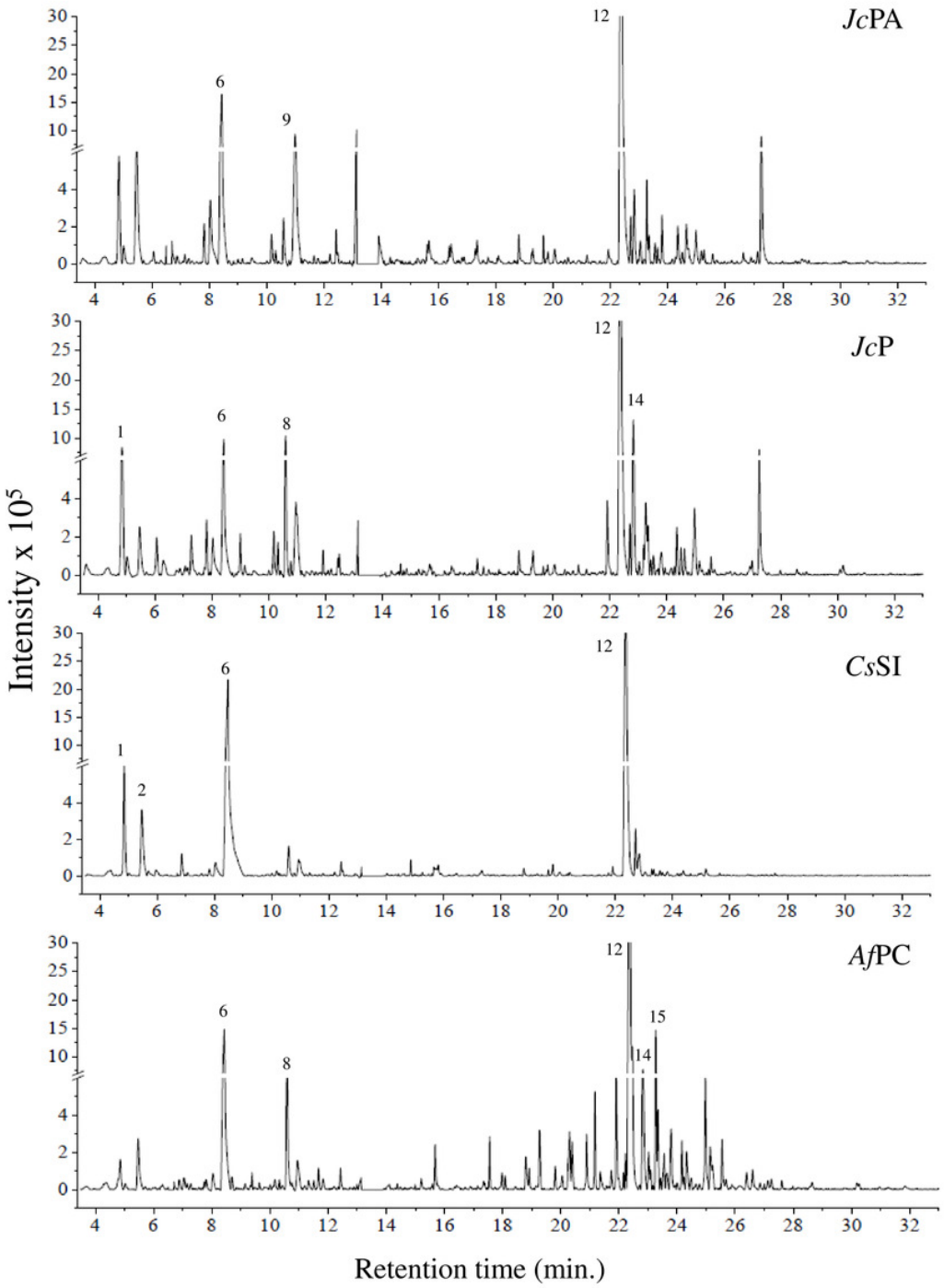
JcPA
JcP
CsSI
AfPC
Intensity x 10^5
Retention time (min.)

Figure 4

Molecular network obtained on the GNPS platform from extracts of GCA.

Figure 4: Molecular network obtained on the GNPS platform from extracts of GCA. Each number represents a different molecular family: Sterols (1), fatty acid esters (2), fatty alcohols (3), hydrocarbons (4) and fatty acids (5). AbFNS (*A. beauvoisii* - Fenda de Nossa Senhora); AbP (*A. beauvoisii* - Prainha); AbPF (*A. beauvoisii* - Praia do Forno); AbSCB (*A. beauvoisii* - Saco do Cherne, articuliths bed); AbSCC (*A. beauvoisii* - Saco do Cherne costão); JcPA (*J. crassa* - Praia dos Anjos); JcP (*J. crassa* - Prainha); CsSI (*C. sagittatum* - Saco dos Ingleses) and AfPC (*A. flabellata* - Ponta da Cabeça).

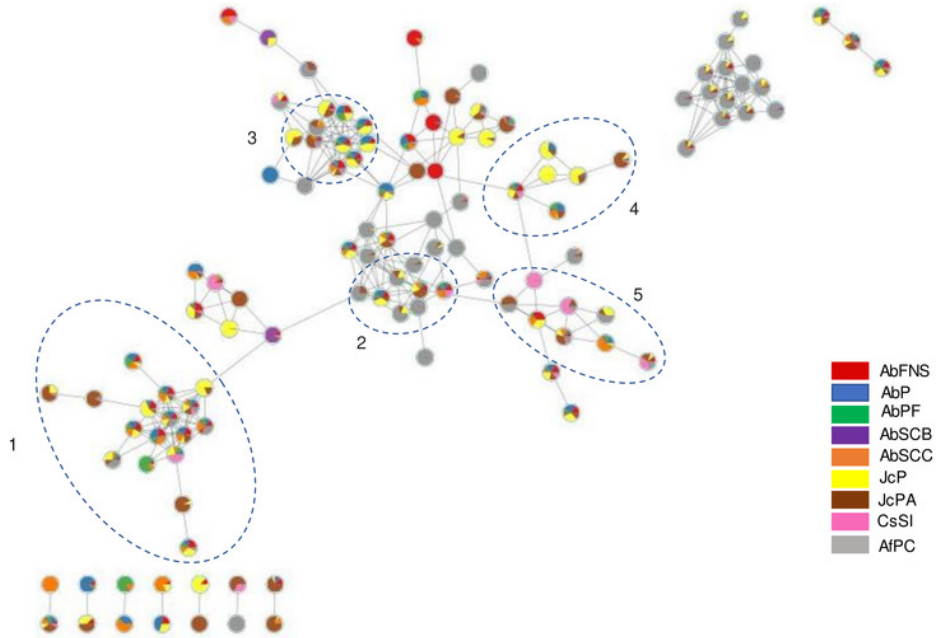

Figure 5

Interrelation of the chemical profiles of different GCA species obtained by GC-MS through PCA analysis.

Figure 5: Interrelation of the chemical profiles of different GCA species obtained by GC-MS through PCA analysis (PC1 = 65% and PC2 = 13.9%). Different symbols represent each species evaluated in this study. AbFNS (*A. beauvoisii* - Fenda de Nossa Senhora); AbP (*A. beauvoisii* - Prainha); AbPF (*A. beauvoisii* - Praia do Forno); AbSCB (*A. beauvoisii* - Saco do Cherne banco); AbSCC (*A. beauvoisii* - Saco do Cherne rocky shore); JcPA (*J. crassa* - Praia dos Anjos); JcP (*J. crassa* - Prainha); CsSI (*C. sagittatum* - Saco dos Ingleses) and AfPC (*A. flabellata* - Ponta da Cabeça).

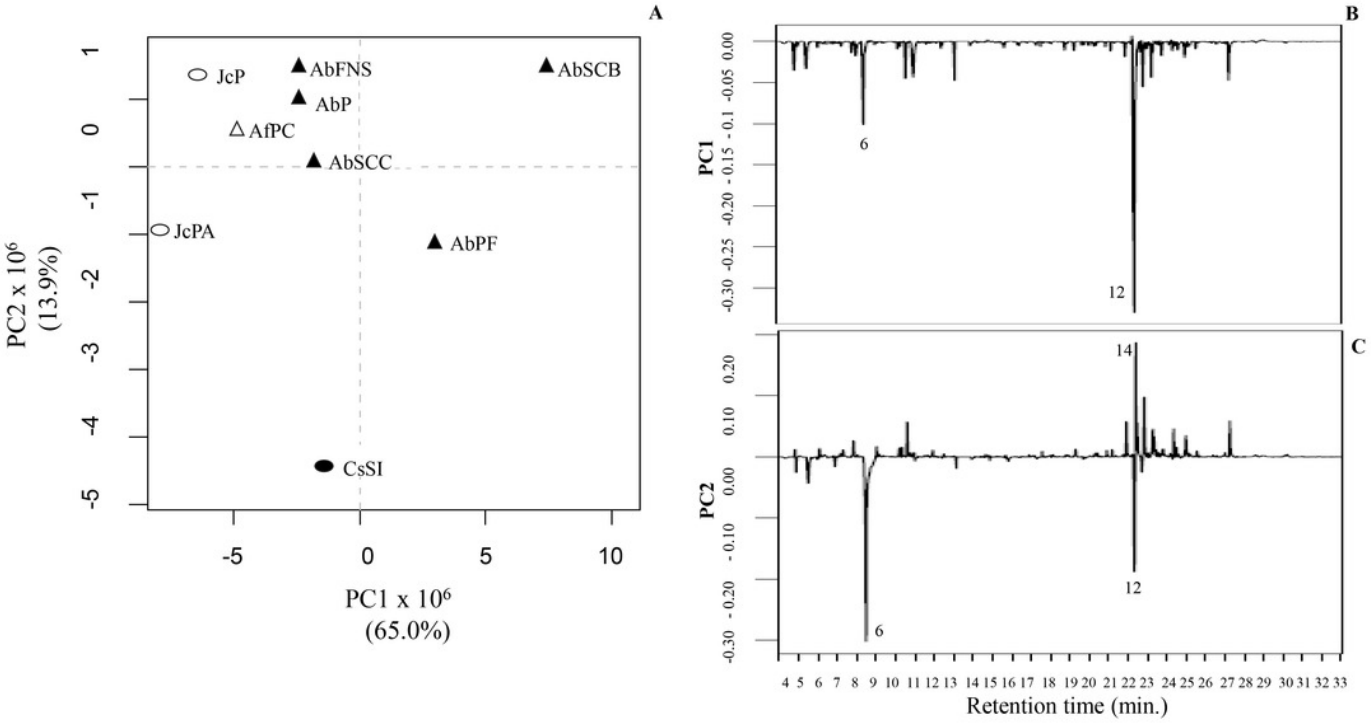

Figure 6

Effect of GCA extracts on byssus production (A) and mortality (B) by the mussel *P. perna*.

Figure 6: Effect of GCA extracts on byssus production (A) and mortality (B) by the mussel *P. perna*. Mean and standard deviation in percentage of byssus production after 24h. B: Mean and standard deviation in percentage of individual mortality after 24h. ANOVA followed by Tukey's test. Letters indicate significant differences between treatments. AbFNS (*A. beauvoisii* - Fenda de Nossa Senhora); AbP (*A. beauvoisii* - Prainha); AbPF (*A. beauvoisii* - Praia do Forno); AbSCB (*A. beauvoisii* - Saco do Cherne banco); AbSCC (*A. beauvoisii* - Saco do Cherne rocky shore); JcPA (*J. crassa* - Praia dos Anjos); JcP (*J. crassa* - Prainha); CsSI (*C. sagittatum* - Saco dos Ingleses) and AfPC (*A. flabellata* - Ponta da Cabeça), and C (control - seawater).

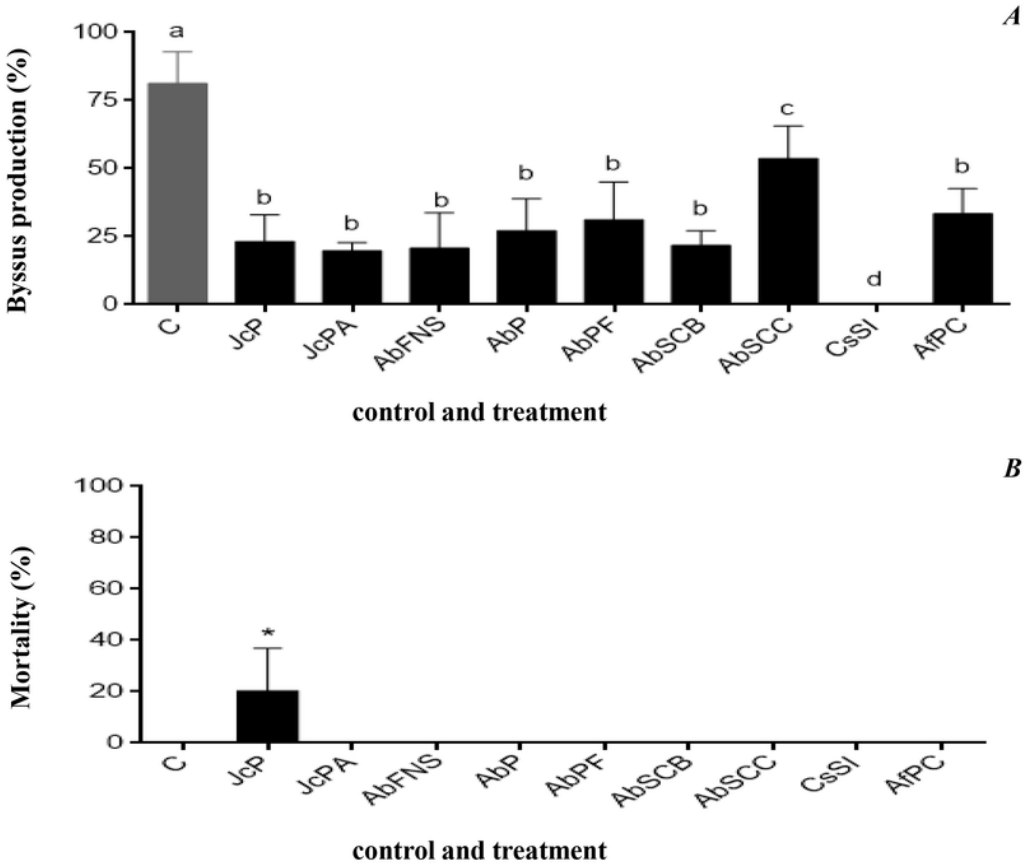
A
Byssus production (%)
100
75
50
25
0
a
b
b
b
b
b
b
c
d
b
C
JcP
JcPA
AbFNS
AbP
AbPF
AbSCB
AbSCC
CsSI
AfPC
control and treatment
B
Mortality (%)
100
80
60
40
20
0
*
C
JcP
JcPA
AbFNS
AbP
AbPF
AbSCB
AbSCC
CsSI
AfPC
control and treatment

Table 1(on next page)

Species of GCA sampled and their collection sites.

Species	Code	Collection site	Wet weight (g)	Coordinates	Voucher number
Amphiroa beauvoisii	AbFNS	Fenda de Nossa Senhora	400,00	22°57'39.4"S, 42°01'12.0"W	3372
Amphiroa beauvoisii	AbP	Prainha	474,40	22°58'06.1"S, 42°00'54.2"W	3373
Amphiroa beauvoisii	AbPF	Praia do Forno	184,95	22°59'58.9"S, 42°00'40.2"W	3374
Amphiroa beauvoisii	AbSCB	Saco do Cherne (banco)	192,24	22°57'37.1"S, 42°00'25.5"W	3375
Amphiroa beauvoisii	AbSCC	Saco do Cherne (costão)	265,53	22°57'37.1"S, 42°00'25.5"W	3376
Cheilosporum sagittatum	CsSI	Saco dos Ingleses	207,48	23°00'01.1"S, 42°00'46.8"W	3377
Jania crassa	JcPA	Praia dos Anjos	213,37	22°58'43.9"S, 42°01'06.5"W	3378
Jania crassa	JcP	Prainha	309,11	22°57'39.4"S, 42°01'12.0"W	3379
Arthrocardia flabellata	AfPC	Ponta da cabeça	117,45	22°58'36.1"S, 42°02'06.9"W	3380

Table 2(on next page)

Natural concentration of GCA extracts applied in the antifouling activity assay with marine bacteria.

1

Samples	Natural concentration (mg/g)
AbFNS	38
AbP	31
AbPF	39
AbSCB	21
AbSCC	28
CsSI	26
JcPA	24
JcP	37
AfPC	21

Table 3(on next page)

Substances from GCA extracts annotated by GC-MS and identified by NIST library (≥ 85%) and//or GNPS platform (cosine ≥ 0.7). X: substances identified by both database (NIST and GNPS); Y: substances identified only in the NIST database.

Peak	*tR (min)*	Phytochemical components	Molecular formula	NIST	GNPS	Relative area (%)								
						JcP	JcPA	AbFNS	AbP	AbPF	ASCB	ASCC	CsSl	AfPC
1	4.86	Heptadecane	$C_{17}H_{36}$	X	X	2.36	1.05	4.45	3.58	5.38	0.3	1.41	4.61	0.76
2	5.47	Tetradecanoic acid	$C_{14}H_{28}O_2$	X	X	0.79	1.6	1.13	0.82	0.99	0.55	2.17	3.21	1.41
3	6.91	Pentadecanoic acid	$C_{15}H_{30}O_2$	Y		0.33	0.07	0.6	-	0.43	-	-	1.2	0.39
4	7.82	Hexadecanoic acid methyl ester	$C_{17}H_{34}O_2$	X	X	0.58	0.23	0.9	1.5	0.52	0.69	0.39	0.17	0.15
5	8.04	Palmitoleic acid	$C_{16}H_{30}O_2$	X	X	0.87	1.09	1.84	0.47	0.48	0.43	1.87	0.89	0.63
6	8.43	Palmitic acid	$C_{16}H_{32}O_2$	X	X	3.25	4.36	7.93	2.2	2.13	1.9	8.05	31.35	9.03
7	10.20	(Z.Z)-9.12-Octadecadien-1-ol	$C_{18}H_{34}O_2$	X	X	0.5	0.21	0.44	0.94	0.77	1.08	0.52	0.13	0.33
8	10.59	Phytol	$C_{20}H_{40}O$	Y		2	0.53	1.22	1.02	0.8	0.69	1.02	0.91	2
9	11.00	Oleic acid	$C_{18}H_{34}O_2$	X	X	1.59	5.14	2.28	1.78	1.94	-	2.54	1.02	1.41
10	21.18	(Z)-7-Hexadecenal	$C_{16}H_{30}O$	X	X	0.04	0.04	-	-	-	-	-	-	1.33
11	21.91	((3β)cholesta-5.22-dien-3-ol	$C_{27}H_{44}O$	X	X	0.69	0.09	1.26	2.22	1.24	0.88	1.13	0.23	1.66
12	22.36	(3β) cholest-5-en-3-ol	$C_{27}H_{46}O$	X	X	16.62	12.63	21.7	37.11	39.07	35.84	28.2	27.53	19. 49
13	22.70	Desmosterol	$C_{27}H_{44}O$	X	X	0.37	0.41	0.81	0.75	1	-	0.51	1.56	0.05
14	22.82	(3β. 5α)-ergosta-7-en-3-ol	$C_{27}H_{46}O$	X	X	3.15	0.52	4.24	3.08	2.97	1.76	3.51	1.22	3.08
15	23.27	Oleic anhydride	$C_{36}H_{66}O_3$	X	X	0.92	0.45	0.16	0.03	0.13	-	0.12	0.32	3.86
16	23.34	Stigmasterol	$C_{29}H_{48}O$	X	X	0.56	0.18	1.57	2.25	2.13	0.83	1.78	0.26	1.37
17	24.35	Sitosterol	$C_{29}H_{50}O$	X	X	0.59	0.28	2.04	2.53	2.03	0.99	3.14	0.06	1.06

1

Table 4(on next page)

Effect of GCA extracts on bacterial growth after 24h.

ANOVA followed by Tukey's test. Letters indicate significant differences between treatments. AbFNS (*A. beauvoisii* - Fenda de Nossa Senhora); AbP (*A. beauvoisii* - Prainha); AbPF (*A. beauvoisii* - Praia do Forno); AbSCB (*A. beauvoisii* - Saco do Cherne articuliths bed); AbSCC (*A. beauvoisii* - Saco do Cherne rocky shore); JcPA (*J. crassa* - Praia dos Anjos); JcP (*J. crassa* - Prainha); CsSI (*C. sagittatum* - Saco dos Ingleses) and AfPC (*A. flabellata* - Ponta da Cabeça), and C^+ (positive control – streptomycin).

1

	Macroalgae extracts and control										
Bacteria species	AbFNS	AbP	AbPF	AbSCB	AbSCC	CsSI	JcP	JcPA	AfPC	**C+**	*F*
Pseudoalteromonas elyakovii	3.20 ± 0.85 [c]	3.57 ± 0.59 [c]	3.25 ± 0.62 [c]	3.67 ± 0.52 [c]	3.25 ± 0.62 [c]	4.61 ± 0.46 [b]	3.14 ± 0.67 [c]	3.50 ± 0.84 [c]	2.94 ± 0.55 [c]	7.04 ± 0.72[a]	68.32
Pseudomonas fluorescens	4.16 ± 0.63 [b]	3.31 ± 0.36 [c]	3.49 ± 0.47 [c]	2.90 ± 0.66 [c]	4.36 ± 0.45 [b]	3.57 ± 0.74 [b]	3.16 ± 0.78 [c]	2.82 ± 0.55 [c]	2.82 ± 0.67 [c]	9.46 ± 0.84[a]	150.9
Polaribacter irgensii	2.58 ± 0.89 [c]	3.00 ± 0.45 [c]	2.77 ± 0.42 [c]	2.87 ± 0.55 [c]	4.41 ± 0.53 [b]	4.18 ± 0.38 [b]	3.04 ± 0.39[c]	3.16 ± 0.74 [c]	4.32 ± 0.63 [b]	6.05 ± 0.48[a]	77.52
Shewanella putrefaciens	4.24 ± 0.43 [b]	4.20 ± 0.57 [b]	2.80 ± 0.87 [b]	2.99 ± 0.99 [c]	3.93 ± 0.44 [b]	4.26 ± 0.40 [b]	4.12 ± 0.62 [b]	3.02 ± 0.92[b]	3.85 ± 0.66 [b]	9.80 ± 0.48[a]	128.6
Vibrio aestuarianus	3.09 ± 0.41 [b]	2.78 ± 0.49 [b]	2.54 ± 0.48 [b]	2.46 ± 0.52 [b]	2.93 ± 0.80 [b]	3.15 ± 0.53 [b]	2.69 ± 0.53 [b]	2.42 ± 0.42 [b]	2.43 ± 0.86 [b]	6.51 ± 0.52[a]	73.74