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Phytochemical study and bioactivity of *Gracilaria cornea* extracts: antimicrobial, chromatographic and kinetic tests

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Bioactivity and Chemical Profile of *Gracilaria Cornea* Extracts: antimicrobial, chromatographic and kinetic tests

Dissertation presented to the Postgraduate Program in Modeling and Simulation of Biosystems at the State University of Bahia, as a partial requirement for obtaining the degree of Master of Science, M.Sc.

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CO-ORDINATION: Prof. Dr. Alexandre do Nascimento Silva

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"PHYTOCHEMICAL STUDY AND BIOACTIVITY OF GRACILARIA CORNEA EXTRACTS: ANTIMICROBIAL, CHROMATOGRAPHIC AND KINETIC TESTS"

JESSICA FREITAS SOUZA

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SUMMARY

The motivation for this study comes from the need to search for new antimicrobial agents due to the emergence of resistant microorganisms and fatal opportunistic infections. The aim of this study was to investigate the antimicrobial activity and chemical profile of the extracts of two species of Gracilarias collected on Vilas do Atlântico beach in Lauro de Freitas-Ba. Algae represent one of the most diverse and numerous organisms in the oceans. Its representatives are of great environmental importance and have also been highlighted for their ability to metabolize chemical compounds of great interest to society. From the genus Gracilaria, one of the most representative, two species were chosen that occur most frequently: *Gracilaria domingensis and Gracilaria cornea*. No reports on the phytochemical aspects and biological activity of the extracts of these species were found for the state of Bahia. Therefore, in this study, the algae were evaluated for their antimicrobial activity and phytochemical aspects. The extracts were obtained in hexane, ethyl acetate and ethanol by cold maceration. The microdilution method was used to determine the minimum inhibitory concentration (MIC) against the bacteria. The extracts (Hexane, Ethyl Acetate and Ethanol) of both algae (*G. domingensis and G. cornea*) did not alter the cell viability of Escherichia coli bacterial strains. However, incubation with the ethyl acetate extract of the *G. cornea algae* showed a drop in cell viability in proportion to the increase in concentration, indicating the bioactivity of this extract. The secondary metabolites present in the extracts were determined by chromatographic analysis in the presence of chemical developers. The active fractions or isolated components were analyzed using Nuclear Magnetic Resonance (NMR) experiments. The result of the chromatogram of the compounds showed mentionable scores of 20% area, identifying the component, n-Hexadecanoic acid, with a 99% degree of confidence compared to the mass spectral reference library. The NMR spectra of the fractions show a typical set of H signals for fatty components.

Keywords: Gracilaria, Antimicrobial, Biosystems, Phytochemical aspects, Metabolites.

ABSTRACT

The motivations for this study stem from the need to search for new antimicrobial agents due to the emergence of resistant microorganisms and fatal opportunistic infections. And the objective is to determine the antimicrobial activity and the chemical profile of the extracts of two species of Gracilarias collected at Praia de Vilas do Atlântico in Lauro de Freitas-Ba. Algae represent one of the most diverse and numerous organisms in the oceans. Its representatives have great environmental importance and have also been standing out for their ability to metabolize chemical compounds of great interest to society. From the genus Gracilaria, one of the most representative, two species with the highest occurrence were chosen: *Gracilaria domingensis* and *Gracilaria Cornea*. For the state of Bahia, no reports were found on phytochemical aspects and biological activity of extracts from these species. Therefore, in this work, algae were evaluated for their antimicrobial efficiency and their phytochemical aspects. The extracts were obtained in Hexane, Ethyl Acetate and Ethanol by cold maceration. The microdilution method was used to determine the minimum inhibitory concentration (MIC) against the bacteria. The extracts (Hexane, Ethyl Acetate and Ethanol) from both algae (*G. domingensis* and *G. cornea*) did not alter the cell viability of the Escherichia coli bacterial strains. However, incubation with the ethyl acetate extract of the algae *G. Cornea* showed a drop in cell viability proportionally to the increase in concentration, indicating the bioactivity of this extract. The secondary metabolites present in the extracts were determined through chromatographic analyzes in the presence of chemical developers. The active fractions or isolated components were analyzed based on Nuclear Magnetic Resonance (NMR) experiments. The result of the chromatogram of the compounds, presented mentionable scores of 20% of area, identifying the component, n-Hexadecanoic acid, with a confidence level of 99% compared to the mass spectral reference library. In NMR, the spectra of the fractions consist of a set of signals typical of a fatty component.

Keywords: Gracilaria, Antimicrobial, Biosystems, Phytochemical Aspects, Secondary Metabolites.

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LIST OF ABBREVIATIONS AND ACRONYMS

°C: Degrees Celsius
µL: Microliter
Δ: Delta
ΔC:Chemical shift in ppm in the 1H NMR spectrum
AcEOt:Ethyl acetate
ATCC: American Type Culture Collection.
CC:Column Chromatography
CCD: Thin layer chromatography
CDCl3: Deuterated chloroform
CH2Cl2: Dichloromethane **CCD:**Thin Layer Chromatography **CIM:**Minimum Inhibitory Concentration
CN: Negative control.
CP: Positive control. **CLSI:**ClinicalandLaboratory Standards Institute **DMSO:**Dimethyl Sulfoxide
d: Dupleto
dd: double duplet
1H NMR: Hydrogen nuclear magnetic resonance
Hex: Hexane
Hz: Hertz
95%CI: 95% Confidence Interval **IMI**: International Mycological Institute **L:** Liter
MC: Culture medium **MeOH**: Methanol
MHz: Megahertz
mL: Milliliters.
M:Multipleto

Mg:Milligrams

NCCLS: National Committee for Clinical Laboratory Standards.

WHO: World Health Organization.

Ppm: Parts per million

PN:Natural products

A: Resistant

S: Susceptible.

SE: Stock solution.

UFBA: Federal University of Bahia. **CFU**:
Colony Forming Units. **UNEB**: Bahia State
University. **UV**: Ultraviolet.

µg: Micrograms.

SUMMARY

1. INTRODUCTION

The motivation for this study comes from the need to search for new antimicrobial agents due to the increase in the number of resistant microorganisms and fatal opportunistic infections. According to Alves (2014), algae are organisms capable of providing bioactive compounds with the potential to become new chemical agents that can contribute to the production of new drugs, pharmaceutical products and biologically functional ingredients.

With more than 70% of the Earth's surface, the oceans represent the largest habitat on Earth and a prolific resource of organisms with high biological and chemical diversity (LINDEQUIST, 2016). They are potentially one of the main sources for research into new chemical compounds. This could represent new possibilities for combating different morbidities that affect human health. As well as providing nutraceuticals for the food industry and elements for the cosmetics and pharmaceutical industries.

According to Lidequist (2016), although most drugs are still derived from terrestrial plants, a considerable number of secondary metabolites belong to marine organisms. However, studies on the evaluation of their biological potential and information on their secondary metabolites have large gaps. Considering that many biotic and abiotic factors can interfere with the amount and type of metabolite produced by algae, the chemical profile can present different aspects for the same species collected in different habitats, so even algae that have already been evaluated should be carefully observed when collected in different regions (SOBOYA, 2016).

Although there have been advances in science and accessibility to new bioactive substances, many diseases still cause concern and take the lives of humans and animals. Cancer, the increase in the number of bacterial strains that are resistant to the antibiotics in use, even the latest ones

generation, the degenerative diseases that affect modern society, as well as certain diseases neglected by society, such as leishmaniasis, are examples of these.

The immense coastline that covers the Brazilian coast, especially that of the state of Bahia, presents a diversity of habitats, with unique ecological niches and a wide distribution of different species of algae NUNES (2005 and 2008). Seaweed is known for producing a wide variety of bioactive secondary metabolites, such as terpenes and aromatic compounds. Many of which have been reported to have Anticoagulant, Anti-inflammatory, Antibacterial, Antifungal, Antitumor, Antigenotoxic, Antimalarial, Antioxidant, Trypanocidal and Leishmanicidal activity (ALIANÇA, 2012). Thus, algae are producers of some of the main bioactive compounds with high biological potential, they represent one of the last frontiers for the discovery of new chemical compounds AL-SAIF et al, 2014).

Studies such as those by NUNES (2005 and 2008); SANTOS, (2016) and ALVES *et al* (2010) show that academic research on algae is growing in Brazil, but especially in Bahia, most of it is related to ecological, taxonomic and antioxidant aspects. It is therefore of the utmost importance to know the biological potential and identify the active components present in macroalgae extracts.

Algae are divided into three large groups according to the presence of predominant pigments: brown algae (phaeophytes) have a greater amount of the pigment phycoxanthin, red algae (rhodophytes) produce a large amount of the pigment phycoerythrin and green algae (chorophytes) have chlorophyll as their main pigment.

Among these phyla, rich in bioactives, Rhodophyta or red algae are abundant and diverse in the Brazilian aquatic ecosystem. Floristic studies on benthic marine rhodophytes began in the 1950s, and it is worth noting that the state of Bahia has the largest number of red algae in Brazil.

of benthic marine rhodophyte taxa in the Brazilian Northeast and the third for Brazil (NUNES, 2005).

The genus Gracilaria, the subject of this project, is the most representative in terms of the number of taxa. In addition to taxonomic studies, Gracilarias have been highlighted for the biological activities presented by polysaccharides (carrageenans and agarans) extracted from these algae. They have antibacterial and antifungal action RIOS et al. (2009). However, reports on the activity of secondary metabolites for algae of this genus are more restricted.

Therefore, considering the potential of macroalgae and the scarcity of studies of this nature for the genus Gracilaria, this study evaluated the biological activity of the extracts of two species of Gracilaria: *Gracilaria domingensis and Gracilaria cornea, as* well as determining their phytochemical aspects.

So our problem was, if algae have active agents against microorganisms, then is there any antimicrobial activity in the extract of *Gracilaria cornea* from Villas do Atlântico beach?

1.1 General Objective

The aim of this study was to determine the antimicrobial activity and chemical profile of the extracts of two species of Gracilarias collected on the coast of Bahia.

1.2 Specific objectives

The specific objectives of the work are:

- Determine the concentration of the extract that inhibits bacterial activity
- Fractionating the active extract and selecting and testing fractions
- Trace the kinetics of the antimicrobial activity of the active fraction
- Chemical characterization of the active fraction

2. THEORETICAL FRAMEWORK

2.1 NATURAL MARINE PRODUCTS

Considering the global pharmaceutical market, natural products are and are likely to remain the most important source of new bioactive substances (Felicio *et al*., 2012*). In their study,* Newman and Cragg (2019) show that natural products and/or natural product structures played a highly significant role in the discovery of 1,881 approved agents in the development process between 1981 and 2019. These data further motivate the research of the present work in the search for new metabolites of marine natural products.

Nowadays, it is increasingly known and exposed that marine organisms have significant biochemical potential, they have a rich biodiversity that is partially exploited, so there is a great chance that these organisms will make new contributions to biotechnology through the discovery of new chemical compounds that can be used to create new pharmaceutical products.

Studies on marine natural products have become attractive due to the presence of secondary metabolites that are structurally very different from those found in terrestrial plants, with new carbon skeletons and unusual combinations of functional groups (Silva, P.M., 2009; Simões *et al.,* 2004). Marine macroalgae have stood out and contributed to the discovery of new marine natural products. Compounds isolated from seaweed have demonstrated various biological activities, such as antibacterial activity, antioxidant potential, anti-inflammatory properties, anticoagulant activity, antifungal, anti-obesity, anticancer and antiviral action against HIV, among others (Viana, L.D., 2021; Barbosa *et al.* 2014; Seca & Pinto, 2018). However, there is a need for further studies on the biological potential of the algae under study and information on its secondary metabolites. In addition to the rich biodiversity of marine algae, there are also variations in environmental conditions such as: high light intensity, UV rays, temperature extremes, ionizing radiation, pollutants and pathogens, and the adaptation mechanisms used by them that induce the

different responses, including the production of metabolites with great structural diversity (Silva, P.M., 2009). Macroalgae can be used to find new sources of pharmaceuticals, cosmetics and biofuels, as well as substances with interesting applications in the chemical field and in multiple sectors of the food industry (Felicio *et al.,* 2012*).* Viana (2009), mentions that the most exploited compounds in algae are fatty acids and sterols, carotenoids, phycocolloids, lectins, mycosporine-type amino acids (MAA), halogenated compounds and polyketides. These compounds may also be feasible to explore in the algae extract under study.

2.2 BACTERIAL RESISTANCE

According to the World Health Organization (WHO), bacterial resistance to antimicrobials occurs when bacteria become less susceptible to the effects of drugs that would normally be effective against them, so treatments become unable to control or end infections, making them chronic and fatal. This is due to the excessive or inappropriate use of antimicrobials, which allows bacteria to adapt and develop resistance mechanisms. This is a serious problem, as it can compromise the treatment of infections and increase morbidity and mortality.

Antimicrobial resistance (AMR) represents a growing threat to public health worldwide and requires action from all sectors of government and society. AMR is expected to cause 10 million deaths a year by 2050 (PAHO/WHO, 2021). This is one of the greatest public health challenges, with a significant impact on human and animal health (ANVISA, 2017).

According to PAHO (2021):

> Since 2010 there has been a strong commitment from FAO, WOAH and PAHO to combat AMR, working together to mitigate the risks in the interconnection between human health, animal health and the environment. In this context, the organizations have joined forces to implement the 'Working Together to Combat Antimicrobial Resistance' project to ensure a coherent "One Health" approach recognizing the multidimensionality and the need for an intersectoral response that is required to tackle the problem of AMR. The overall strategic objective of the project supported and funded by the European Union (EU) is to contribute to combating AMR through the implementation of National AMR Action Plans, working with seven Latin American partner countries: Argentina, Brazil, Chile, Colombia, Paraguay, Peru and Uruguay.

Recently, Vale (2021) described that being a natural process (Figure 1), bacterial resistance to antimicrobials occurs over time from the interaction of these microorganisms with the environment in which they live. They become resistant and can survive in living and non-living beings. Such as: humans, animals, food, vegetables, water, soil and air. These bacteria develop various mechanisms of resistance to antimicrobials, including: altering the site at which the antimicrobial acts, managing to change the permeability of the selective membrane, pumping the antimicrobial out of them, as well as producing enzymes that destroy the antimicrobials. These are the main mechanisms used by resistant bacteria to survive

and multiply and can pass on their resistance characteristics to other bacteria.

However, some human actions contribute to the cycle of bacterial resistance happening more quickly, such as: diseases that are treated incorrectly with antimicrobials, patients who don't follow the correct dosage and timing of treatment, the release of resistant bacteria from toilets through basic sanitation into the environment, poor access to drinking water, the inappropriate use of antimicrobials in farming, the disposal of drug waste, leading to water contamination, and the lack of infection control in environments such as hospitals and even farms.

Antimicrobial agents are essential for preserving human health, animal health and plant protection. This phenomenon, called antimicrobial resistance, jeopardizes the effectiveness of treatment for a growing number of infections caused by viruses, bacteria, fungi and parasites (PAHO, 2021).

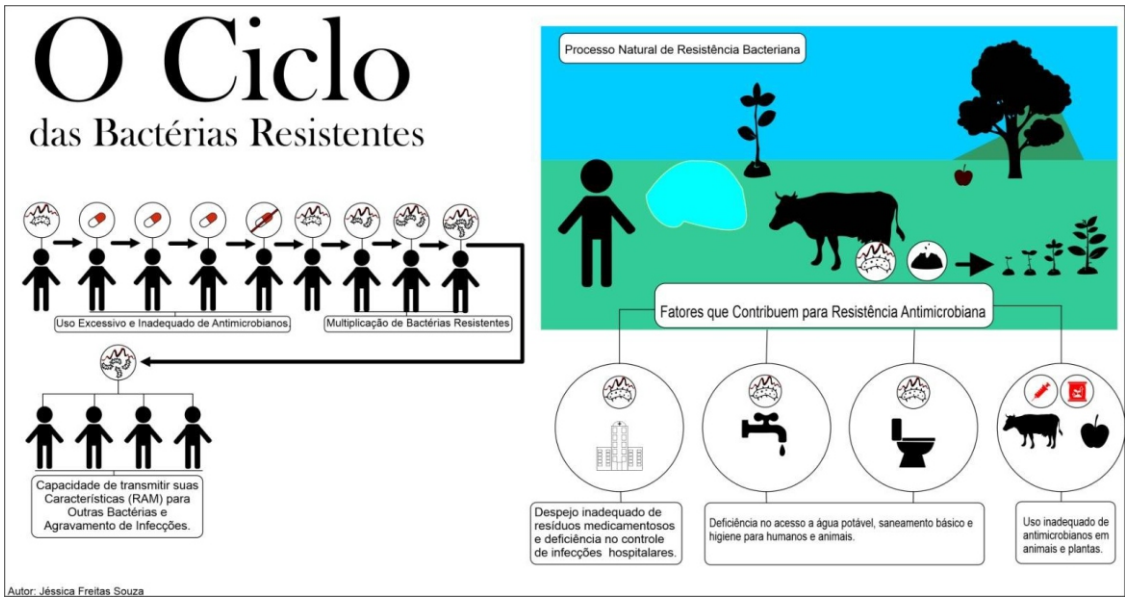


Figure 01. Cycle of resistant bacteria. Source: Author

According to the World Health Organization - WHO (2020), antimicrobial resistance is considered by the WHO to be one of the top ten global public health threats facing humanity. It affects human, animal, plant and environmental health, putting food security, international trade and economic development at risk. Resistance

antimicrobials also lead to increased health costs, hospital admissions, treatment failures, serious illness and death.

One of the mechanisms used by bacteria is the inactivation of the drug caused by the production of enzymes that degrade or inactivate the antibiotic, which is called the enzymatic mechanism of resistance (COSTA and JUNIOR, 2017). According to Guimarães and Pupo (2010), researchers have focused on new approaches aimed at improving the processes of discovering the mechanisms of action of candidates for resistance to antimicrobial drugs. This also includes new techniques and improvements for isolating secondary compounds with bioactivity against resistant microorganisms, and they emphasize that the search must be active and continuous.

Cordeiro and Brito (2012) point out that antimicrobials are an indispensable class of drugs; without them there would be losses in the life expectancy that we have achieved over the decades, but the current ones are under threat due to bacterial resistance, which has led to an increase in the need for new drugs and new classes of antibiotics, both for hospital-acquired and community-acquired infections.

In this way, marine organisms could represent a possible source of new chemical compounds. Clardy and Walsh (2004) state that the exploration of the marine environment has had a significant impact on the chemistry of natural products against resistant bacteria, especially the isolation of secondary metabolites from red algae. The author considers this biological diversity to be intriguing structurally and biologically. They also emphasize that this biological diversity, combined with modern analytical techniques and synthetic organics, could be promising, thus contributing to the fight against AMR.

This growing interest in using natural marine products such as those extracted from algae may increase the availability of molecules that can make a positive contribution to combating microorganisms that are highly resistant to existing antimicrobials.

2.3 GRACILARIAS: Gracilaria cornea and Gracilaria domingensis

The species *Gracilaria Cornea* (J.Agardh) and *Gracilaria domingensis* (Sonderex Kützing) (figure 02) belong to the phylum Rhodophyta, division Rhodophyceae, class Florideophyceae order Gracilariales and family Gracilariaceae. They represent an important source of metabolites characterized by a variety of compounds with rare chemical structures (SILVA 2009).

Among the algae that produce agar-agar, *Gracilaria cornea*, a species with a cylindrical thallus (LYRA *et al* 2011), stands out as a promising source of agar-type galactans (COURA *et al* 2015). Research such as that by Coura *et al* 2012 into *Gracilaria cornea* reveals significant biological activities, the study finding that its total sulfated polysaccharide (Gc-TSP) has antinociceptive and anti-inflammatory effects in vivo without toxicological significance. This is of great value to science.

The alga *Gracilaria domingensis* is also used as fresh food in the human diet, being collected and exported to the Japanese food market. Currently, the main economic activity related to these two algae is the exploitation of agar, a phycocolloid of high economic value (MIRANDA 2009 *apud* MACCHIAVELLO,1999).

In addition to producing agar, the genus Gracilaria has attracted the interest of researchers due to the biological potential that has been observed for secondary metabolites present in extracts of species of this genus. Studies such as those by Aliança (2012), Rios *et al*. (2009) and Newman and Cragg (2019) show that these metabolites have anti-coagulant, anti-inflammatory, antibacterial, antifungal, antitumor, anticancer, antigenotoxic, antimalarial, antioxidant, trypanocidal and leishmanicidal effects. However, more studies on the activity of secondary metabolites for algae of this genus are still scarce compared to terrestrial plants (LINDEQUIST, 2016).

Gracilaria córnea *Gracilaria domingensis*

Figure 02. Source: Marques and Souza 2022. Exsiccates of *Gracilaria cornea* (left) and *Gracilaria domingensis* (right).

Due to the increase in resistant infections, the limitation of the drug of choice and the lack of specific antibacterials against various pathogens have driven the urgent need to search for new antibacterial compounds KASANAHA *ET AL* (2018). Another study by the same author with *Gracilaria edulis*, revealed the biological potential of the extract and pointed out through GC-MS that fatty acids and sterol were the compounds responsible for antibacterial activity against *Vibrio sp*, which infect humans and animals, including fish. SHEKHAR (2012) also pointed out in his research that the ethanol extract of the red algae *Gracilaria edulis* had antibacterial activity against *Escherichia coli, Enterobacter aerogenes, Pseudomonas aeruginosa, Staphylococcus aureus and Streptococcus faecalis.* Studies such as those by DAYUTI (2022) show that the activity of antibacterial compounds extracted from *Gracilaria verrucosa* had a positive effect on inhibiting pathogenic bacteria such as *E. coli* and *S. typhimurium*.

The constant demand for new antibacterial bioactive compounds, justifying this need, the research of ASHWAG (2014) with the species *Gracilaria changii*, found high potential inhibition of the growth of *S. aureus (STR 5) and S. aureus (N8)* after treatment of patients in Saudi Arabia and Malaysia. Likewise, the studies by SREENIVASAN (2010) with this same alga (*Gracilaria changii*), using the methanolic extract, showed good growth inhibition.

antimicrobial activity, resulting in the complete inhibition of bacterial cells against *P. aeruginosa. Further* reinforcing the need for more research to discover new bioactive antibacterial compounds, GOVINDASAMY *et al* (2012) isolated and characterized new compounds from *G.corticata*, which showed a broad spectrum of antibacterial activity and their extract inhibited all the bacteria tested (*Streptococcus pneumonia, Staphylococcus aureus, Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans and Candida sp*) in different activity ranges. The author Arulkumara (2017) in his study with the methanolic extracts of the algae *Gracilaria corticata* and *Gracilaria edulis,* revealed the presence of numerous bioactive metabolites (sulphurous acid, 2-ethylhexyl isohexyl ester, eugenol benzene and phthalic acid), and these showed activity against *B. subtilis*.

On the other hand, research on *Gracilaria cornea* and *Gracilaria domingensis* as promising biological activity with antibacterial substances and specially isolated bioactive compounds is still poorly documented. Therefore, in this study we focused on evaluating the antimicrobial potential, the phytochemical aspects of the extracts of *Gracilaria cornea* and *Gracilaria domingensis* (against the microorganisms *Escherichia coli, Micrococcus luteus and Bacillus subtilis*) and identifying their bioactive compounds.

3. MATERIALS AND METHODS

To define the experimental project and carry out the laboratory tests, a process flow was established, as shown in the conceptual map in Figure 3.

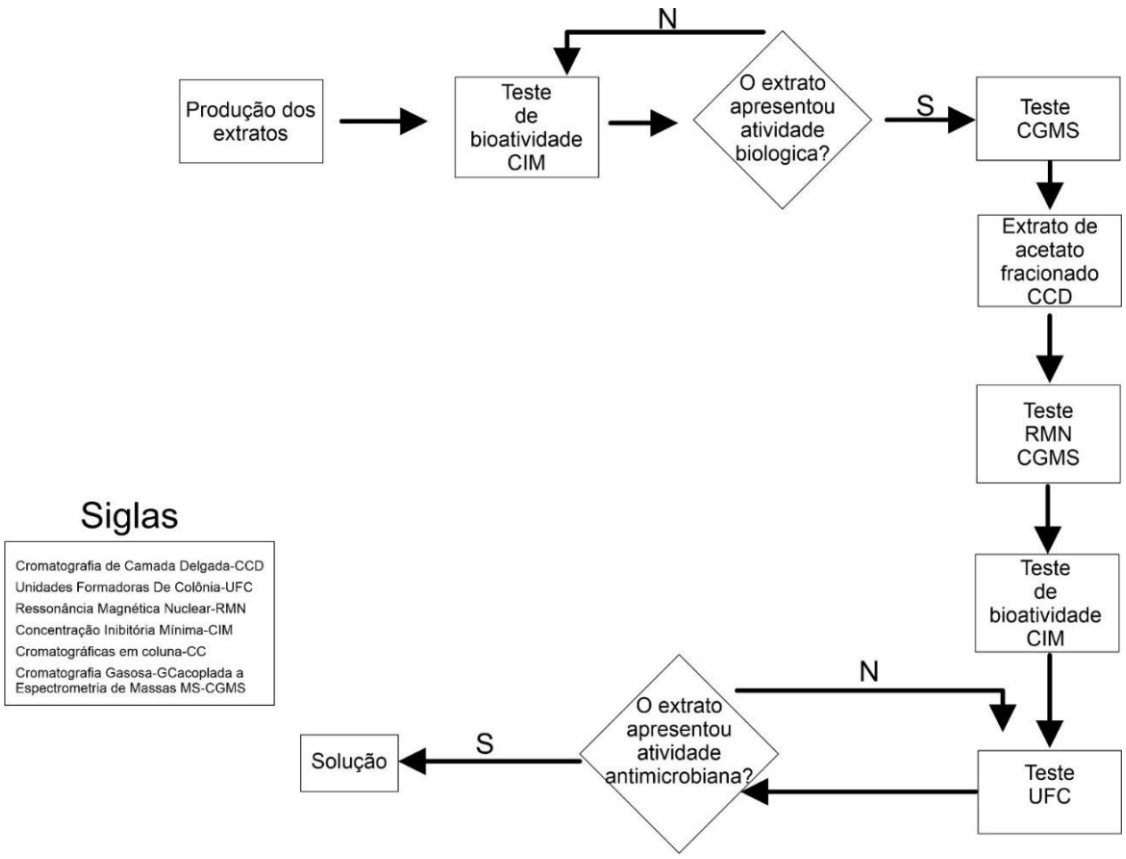


Figure 3: Concept map. Source: Author, 2022.

Thus, following the conceptual map, the processes were presented below in the methodology adopted to produce the extracts, the bioactivity tests and the characterization of the phytochemical aspects.

3.1 COLLECTION AND IDENTIFICATION OF BOTANICAL MATERIAL

The algae was collected at Villas do Atlântico beach (figure 04), located in the municipality of Lauro de Freitas (-12.948604507261612, -38.341121775692315). The city of Lauro de Freitas, with its privileged geographical location in the Metropolitan Region of Salvador, is an example of a demographic explosion and "disorderly" urban growth. (CARIGÉ, 2007) Summers are hot and partly cloudy, while winters are muggy or what we call warm. Praia de Vilas do Atlântico is marked by the presence of sandstone banks exposed on the beach during periods of low tide. The width of the beach face varies from 15 to 30m, with slopes between 7° and 11° and medium-grained sand. Waves predominate in the surf zone with heights of over 1.0 m, which spread their energy over at least three surf lines. The beaches are generally clean. There are no fixed constructions on the seafront after the beach or in the adjacent coastal zone. There are beach huts, with an average of one hut every 250m. The beaches partially preserve the natural landscape, with the occurrence of vegetated terraces and dunes on the post-beach. (SILVA *et al.*, 2005)

Identification at a specific level was carried out using works commonly used in marine phycology.

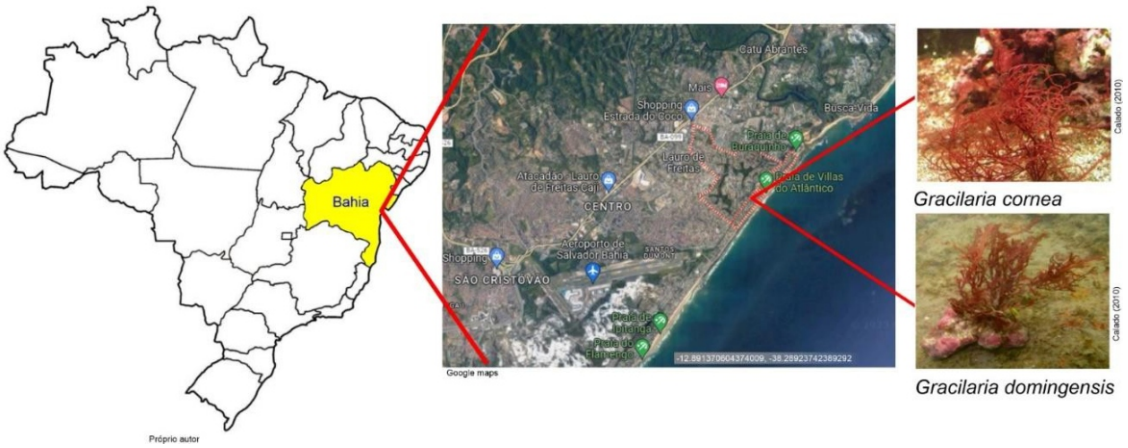


Figure 04. Place where the botanical material was collected and identified. Source: author's own assemblage.

3.2 EXTRACTION PROCESS

The collected material was washed with the seawater itself to remove impurities impurities, then then was put to drying, in

The plant material was macerated at room temperature and then placed in an oven (40ºC) until it lost its moisture. The plant material was macerated and placed in contact with the solvents Hexane, Ethyl Acetate and then Ethanol, in closed containers for three consecutive extractions at intervals of at least 72 hours for each solvent. Once the extraction stage was complete, the material was filtered and then, after the solvent was evaporated, the extract was stored in sterilized glass jars at room temperature to complete drying and stored at -4ºC until it was used during the tests.

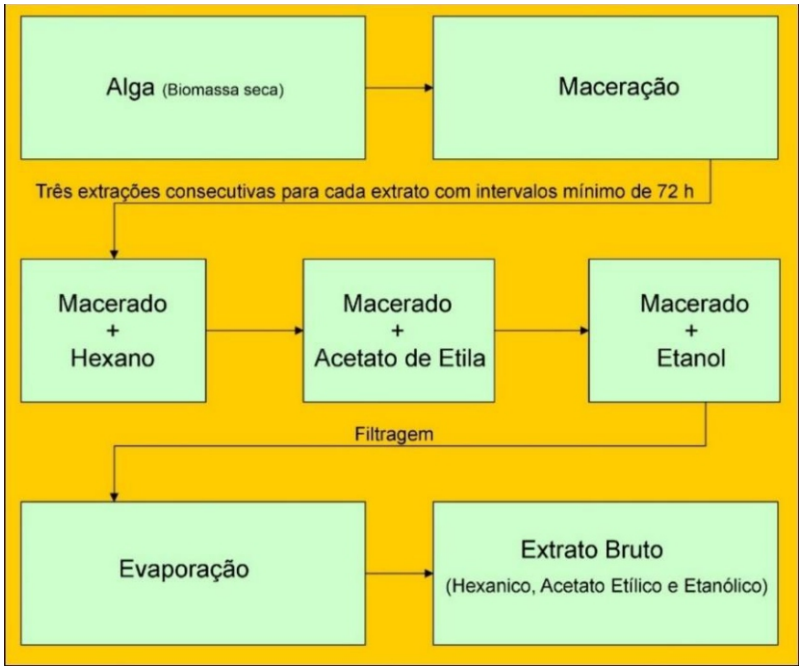


Figure 05. - Process of Obtaining Extracts (Source: Author)

3.3 DETERMINATION OF ANTIMICROBIAL ACTIVITY

The antibacterial activity was verified at the Experimental Laboratory, located at the State University of Bahia, campus II (UNEB), during the months of April and May 2022. The method used to carry out the tests was the broth microdilution technique in 96-well Elisa plates, based on the CLSI/NCCLS M7-A6 document (NCCLS, 2017).

The bacterial strains were initially seeded on Petri dishes containing nutrient agar, using the plate depletion technique.

(HENRY, 1995). In the experiment, the culture medium used for the bacteria was Nutrient Broth (Caldo Nutriente) from the manufacturer HIMEDIA. The following microorganisms were used for this test: *Bacillus subtilis* (ATCC6633), *Bacillus cereus (ATCC 0096), Micrococcus luteus (ATCC* 10240) and *Escherichia coli* (ATCC 94863) (Figure 6).

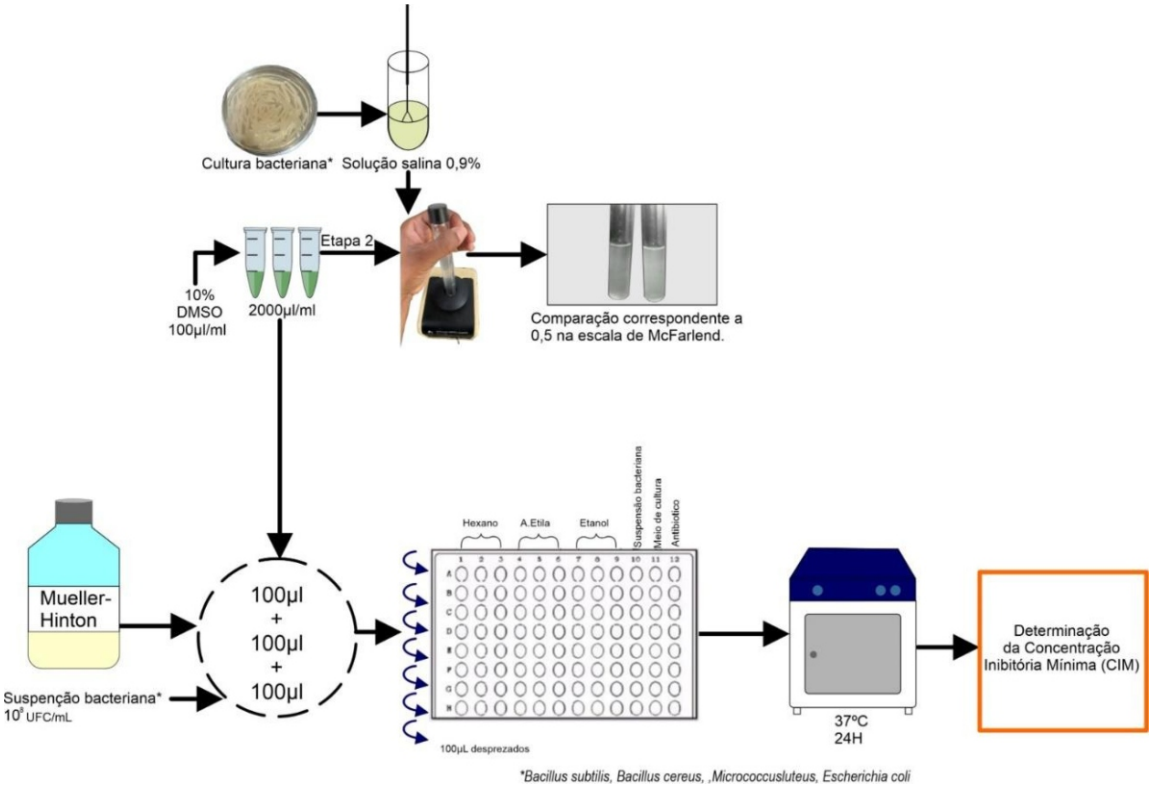


Figure 6 Schematic of the methodology used to evaluate antimicrobial activity Source: Author

3.3.1 Preparation of the sample stock solution

The stock solution of the extracts was prepared at a concentration of 2000µg/mL. The diluent used was 100uL of 10% v/v Dimethyl Sulfoxide (DMSO) in water and 900uL of Nutrient Broth medium. Homogenization was carried out using a tube shaker.

3.3.2 Preparation of the microorganism suspension

The suspension of each microorganism tested was prepared by removing an aliquot of the biomass of the newly cultivated microorganism using a platinum loop and transferring it to a test tube containing 0.9% saline solution. After homogenization, a comparison was made between this

suspension of microorganisms in saline solution with the turbidity of a solution of Barium Sulphate (BaSO4), corresponding to 0.5 on the McFarlend scale.

3.3.3 Minimum Inhibitory Concentration (MIC)

Microdilutions were carried out by distributing 100 µL of Mueller-Hinton broth medium into the wells of the plate using a multichannel micropipette. The sample stock solution of each extract was tested in triplicate, with 100 µL of it at a concentration of 1000 µg/mL being dispensed into wells A1 to A9 (4 mg/ml).

As a negative control, 100 µL of Mueller-Hinton broth culture medium and 100uL of the bacterial suspension were used in the wells of column A10. 100 µL of Mueller-Hinton broth culture medium and 100 µL of DMSO were added to the wells of column A11. The wells of column A12 received the antibiotic chloramphenicol to confirm the sterility of the plate.

Serial dilutions were carried out with a dilution factor of two, obtaining the following sample concentrations: 1000, 500; 250; 125; 62.5; 31.25; 15.62; 7.81 and 3.9 µg/mL. Serial dilutions were made using a multichannel micropipette, initially homogenizing (3x) the solutions from the wells in row A and then transferring 100µL to row B and from there to C, successively until the last row (H), with 100µL being discarded at the end of the dilution.

In the final stage of serial dilution, 100 µL of the microorganism suspension was distributed in all the wells of columns A1 to A10, giving a final volume of 200 µL in each well, after which the Elisa plate was covered and the bacteria incubated for 24 hours at 37°C. After this period, the results were read.

3.3.4 Determination of the Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) was interpreted as the lowest concentration of the antimicrobial agent that completely inhibited the growth of the microorganisms in the microdilution wells, as per

detected with t h e naked eye by observing the turbidity of the wells when bacteria grow compared to the control (NCCLS, 2003).

3.3.5 MTT assay protocol for cell viability and proliferation

In order to confirm the observed cell viability data by the turgidity or transparency of the wells when determining the minimum inhibitory concentration (MIC), the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to measure cell metabolic activity as an indicator of cell viability and proliferation. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-[4,5- dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide or MTT) to purple-colored formation crystals by metabolically active cells.

3.4 DETERMINATION OF PHYTOCHEMICAL COMPOSITION

3.4.1 Gas Chromatography Coupled with Mass Spectrometry (GCMS)

In the Gas Chromatography (GC) coupled with Mass Spectrometry (MS) experiment, a volume of 1.0 µL of the crude extract sample was injected into an Agilent HP 6890 gas chromatograph to separate the fragments and molecular components. The oven temperature varied under the following conditions: initial temperature of 40ºC and final temperature of 280ºC, corresponding to a total analysis time of 25 min. The results obtained were compared with the National Institute of Standards and Technology (NIST) library.

3.4.2 Fractionation

The fractions of the crude extract were obtained through column chromatographic (CC) separations on silica gel 60 and elution took place by gradient with solvents of increasing polarity. Subsequently

Chromatographic analysis by thin layer chromatography (DLC), the plates were activated in an oven at 100ºC for a minimum of 1 hour before each test, comparative chromatographic run. The chromatographic plates were developed by irradiating them with ultraviolet light at wavelengths of 254 and 366 nm, as well as using iodine vapors. The fractions were grouped by degree of similarity of the chromatographic profile on the CCD, named by letter (F) followed by number, where the letter (F) represents the name "fraction" and the number the time interval of collection of this fraction in the container, after which they were sent to the Bahia Nuclear Magnetic Resonance Laboratory (LABAREMN) for hydrogen nuclear magnetic resonance spectroscopy. The chemical shifts (δ) were expressed in parts per million (ppm). Tetramethylsilane (TMS) was used as an internal reference and deuterated chloroform (CDCl3) from Cambridge Isotope Laboratories, Inc. (CIL)® was used to solubilize the samples. The NMR spectra were processed using the free ACD Labs software version 12.0.

Figure 10 shows a flowchart summarizing the chromatographic procedures carried out on the extract of the *C. cornea* species which led to the isolated compounds.

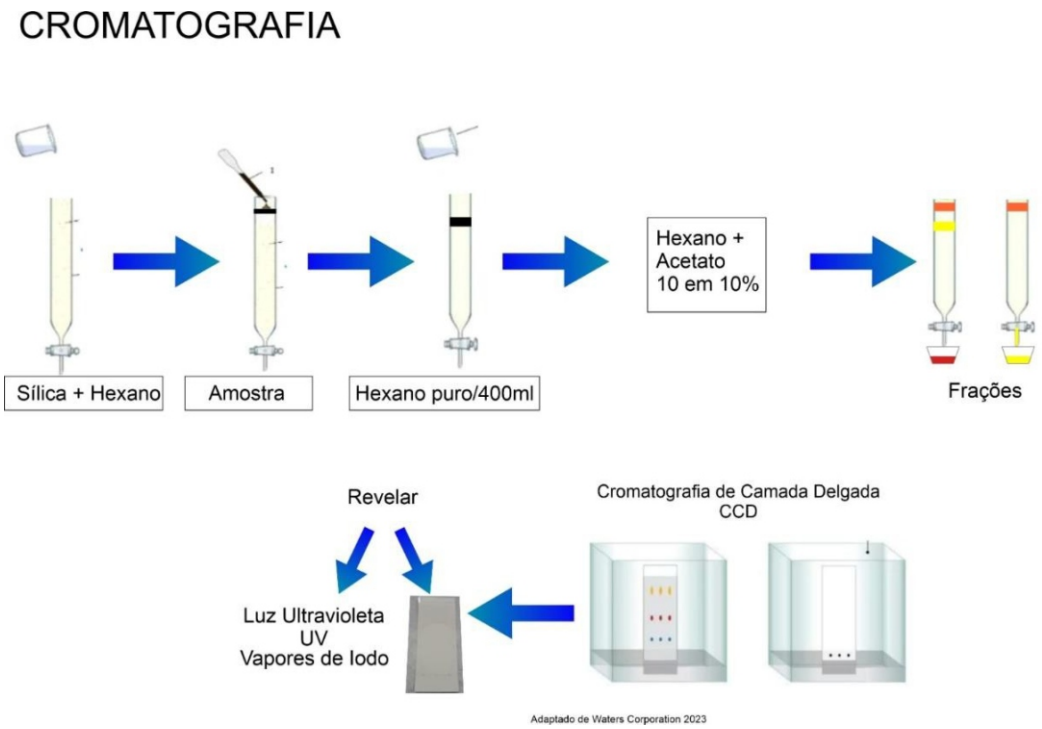


Figure 07. Flowchart of the chromatographic procedures with the C. cornea extract. Cornea extract. Source: Author

3.5 Microdilution Assay and Bacterial Kinetics with Quantification of Colony Forming Units (CFU)

3.5.1 Microbial suspension (A)

Four (4) test tubes were prepared for the *Bacillus subtilis* microorganism, then 9990 µL of saline solution was added to the first test tube and 9900 µL to the other three test tubes. 10 µL of the suspension of the *Bacillus subtilis* microorganism was removed and inoculated into the first tube. After homogenization, 100 µL was transferred to the second tube and so on to the third and fourth test tubes.

3.5.2 Count at Plate

100 µL of each of the dilution tubes **2, 3 and 4 were** used and the spread plate technique was applied to Petri dishes already containing Mueller Hinton Agar. After solidifying, the Petri dishes were placed in an inverted position in the oven for 24 hours and then the colonies in each one were counted.

3.5.3 Preparation of the 96-well plate to evaluate growth (same suspension A)

2.0 mg of the *Gracilaria cornea* extract sample was used and solubilized in 500 µL of DMSO (4.0mg/mL)**.** Three (3) eppendorfs were numbered:

- 200 µL of the culture medium (Mueller-Hintonbroth) and 200 µL of the *Gracilaria cornea* extract sample (2.0 mg/ml) were added to **eppendorf 1;**
- 320 µL of the culture medium (Mueller-Hintonbroth) and 80 µL of the *Gracilaria cornea* extract sample (0, 2 mg/mL) were added to **eppendorf** 2;
- In **eppendorf 3,** 392 µL of the culture medium (Mueller-Hinton broth) and 8 µL of the *Gracilaria cornea* extract sample (0.02mg/mL) were added.

With the aid of a multichannel micropipette, 100 µL of the sample was taken from each eppendorf and distributed in triplicate in each well of columns 1, 2 and 3 and 100 µL of the suspension of the microorganism *Bacillus subtilis was* added to each well (the same as at the beginning: suspension A).

As a negative control, 100 µL of Mueller-Hinton broth culture medium and 100 uL of the bacterial suspension (A) were used in triplicate in the wells of column A5 and the wells of column A6 received only Mueller-Hinton broth culture medium (200µL), to confirm the sterility of the plate. The Elisa plate was then covered and the bacteria incubated for 24 hours at 37°C. After this period they were removed from each well and placed in an eppendorf tube (1) containing 990 µL of saline solution and homogenized, a second dilution was made by removing 100 µL from the eppendorf (1) and adding the eppendorf (2) containing 900 µL of saline solution and then a third dilution by removing 100 µL from the eppendorf (2) and adding the eppendorf (3) containing 900 µL of saline solution, after homogenization this third dilution was used to make the plate count.

3.5.4 Count at Plate

100 µL of the eppendorf (3) were used and the spread plate technique was applied to the Petri dishes in triplicate, already containing the Mueller Hinton Agar culture medium. After solidifying, the Petri dishes were placed in an inverted position in the oven for 24 hours and then the colonies were counted using a mirrored magnifying glass.

3.6 Data processing

To generate the experimental graph, the data obtained from the laboratory tests was processed using the non-linear regression option in the GrahPad Prism software version 6. The data was processed after the kinetic test had been carried out.

4. RESULTS AND DISCUSSION

Three extracts of each alga were obtained (*Gracilarias domingensis:* hexane, ethyl acetate and ethanol and *Gracilarias cornea*: hexane, ethyl acetate and ethanol) from Villas do Atlântico beach. All the materials were collected on the same expedition in December 2021, processed in the same methodological way and the MIC test carried out, except for the ethanolic extract of *Gracilaria domingensis,* which added fungi during the evaporation stage and was discarded. Due to all the atypical events in the years 2021 and 2022, the limitations of the COVID-19 pandemic on campus and in the experimental laboratories and the delay in acquiring the solvents, plus the short period of the course, the need to choose one of the extracts to continue the tests was assessed. The extract was selected for the remaining stages in order of the most active (AcEOt - GC**)** in the MIC test. The results of the experimental research, with laboratory tests, are presented in the following topics.

4.1 Determination of antimicrobial activity

4.1.1 Determination of the Minimum Inhibitory Concentration (MIC)

An antimicrobial susceptibility test is carried out to find out which drugs (in this case the extract) have a high probability of therapeutic success against an infection according to previously defined parameters and recommendations (NCCLS, 2017).

Determining the minimum inhibitory concentration (MIC) of an antimicrobial against a particular strain means knowing the lowest concentration of the drug capable of inhibiting the growth of the bacterial population in question.

The present study therefore looked at the effectiveness of the plant extracts of *G. domingensis and G. cornea* on the bacterial growth of standard strains of Escherichia coli, Micrococcus luteus and Bacillus subtilis: *Escherichia coli, Micrococcus luteus and Bacillus subtilis.* The lowest concentration of the antimicrobial agent that completely prevented the growth of the microorganisms in the microdilution wells was interpreted and detected with the naked eye by observing the clouding of the wells when the microdilution agent occurred.

growth of the bacteria (NCCLS, 2017). The viability of the microorganisms was determined by the MTT reduction method, which determines the functionality of cellular dehydrogenases (SILVA, 2009). Images 11 and 12 show that 24-hour incubation with the extracts (Hexanic, Ethyl Acetate and Ethanolic) of both algae (*G. domingensis and G. cornea)* did not alter the cell viability of the bacterial strains of *Escherichia coli.* However, incubation with the ethyl acetate extract of the *G. cornea algae* showed a drop in cell viability in proportion to the increase in concentration, indicating the bioactivity of this extract.

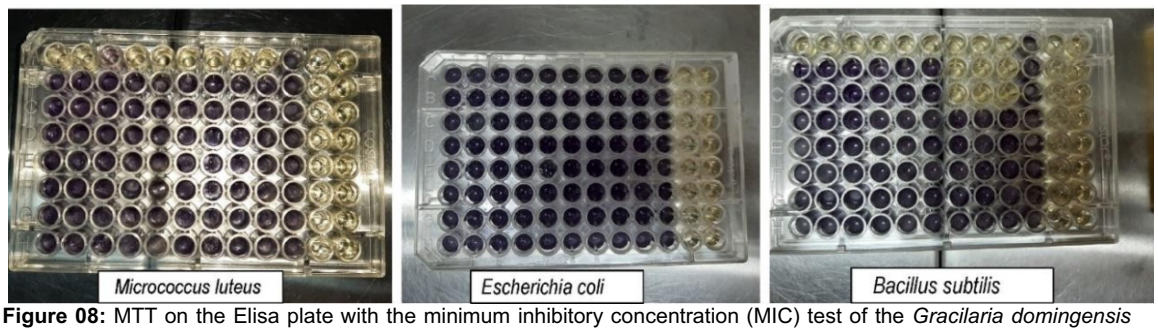


Figure 08: MTT on the Elisa plate with the minimum inhibitory concentration (MIC) test of the *Gracilaria domingensis* and *Gracilaria cornea* extracts. **Legend**: Wells: 1,2,3: Hex. G.d; 4,5,6: Acet. G.d; 7,8,9: Hex.G.c; 10,11,12 controls

The plant extract from the *Gracilaria cornea* seaweed under study inhibited the growth of the Gram-positive bacterium *Bacillus subtilis (Table 1), with* the ethyl acetate extract showing inhibition at a concentration of 250µg/mL and the ethanol extract showing inhibition at a concentration of 500 µg/mL. In a study carried out by Afonso *et al* (2021) using the species of the genus *Gracilaria gracilis against Vibrio anguillarumao*, its ethanolic extract showed efficiency at a lower concentration (250µg/ML) in the Microdilution test. It is possible that the active antimicrobial metabolites present in *Gracilaria gracilis* are mostly extracted by ethanol and hexane, when compared to acetate. On the other hand, the active antimicrobial metabolites present in Gracilaria cornea are mostly extracted by ethyl acetate, which may be due to the different affinities of the solvents with the secondary metabolites of the species in question. Some solvents have chemical properties that allow for better extraction of specific compounds, such as polarity, solubility and molecular interactions. This is why

he choice of solvent depends on the characteristics of the metabolites to be extracted and the chemical properties of the species under study.

Tabela 1: Concentrações inibitórias minimas(CIM) dos extratos de *Gracilaria domingensis* e *Gracilaria cornea*, frente ás

Bactérias	***Espécies***	***Extrato em Hexano***		***Extrato em Acetato***		***Extrato em Etanol***	***Controle***
		Gracilaria domingensis	*Gracilaria cornea*	*Gracilaria domingensis*	*Gracilaria cornea*	*Gracilaria cornea*	Cloranfenicol
Gram-positivas	*Bacillus subtilis*	1000µg/ml	500µg/ml	1000µg/ml	250µg/ml	500µg/ml	18,20±1,8
	Micrococcus luteus	1000µg/ml	1000µg/ml	1000µg/ml	1000µg/ml	1000µg/ml	24,00±2,5
Gram-negativas	*Escherichia coli*	-	-	-	-	-	20,07±0,9

(Fonte Próprio autor)

However, the inhibitory activity observed was lower than that of the conventional antibiotics recommended by the NCCLS, which were tested under similar methodological conditions. It is possible that these differences have to do with the fact that the antibiotics used are substances with a high degree of purity. In contrast to the crude extracts investigated, which have chemically distinct constituents in their composition and variations in the concentrations of their compounds, causing them to have different biological actions. This is one of the reasons why researchers have not yet reached a consensus on the acceptable levels of inhibition for plant compounds compared to the antibiotics in use (DUARTE, 2006).

Table 1 shows that both extracts (hexane and acetate) of the *G. domingensis* algae had a minimum inhibitory concentration of 1000 µg/ml*. domingensis*, there was a minimum inhibitory concentration of 1000 µg/ml, suggesting that the compounds extracted from this species in terms of antibacterial potential did not influence the inhibition of the Gram-positive microorganisms *Bacillus subtilis* and Micrococcus luteus, while for the Gram-negative strain (Escherichia coli) in both extracts of *G. domingensis* and *Gracilaria cornea* there was no inhibition of growth, as can be seen in image 11.

According to Silva et al. (2020), the cause of this phenomenon can be attributed not only to the techniques and solvents used, but also to the

mode of action of bioactive compounds. These substances alter the permeability and integrity of bacterial cells. However, when dealing with the complexity of cell walls, some groups of bacteria have an outer membrane, as in the case of Gram-negative bacteria, resulting in a hydrophilic characteristic capable of limiting the diffusion of hydrophobic compounds through their lipopolysaccharide coating.

Considering the activity of the different solvents studied (Hexane, Ethyl Acetate and Ethanol), it was possible to see that, in general, the best activity was of the *Gracilaria Cornea* extract in Ethyl Acetate (Graph 01), inhibiting the microorganism *Bacillus subtilus* at the lowest concentration in the MIC test, with a significant influence, as can be seen in Image 09 interpreted in Table 1.

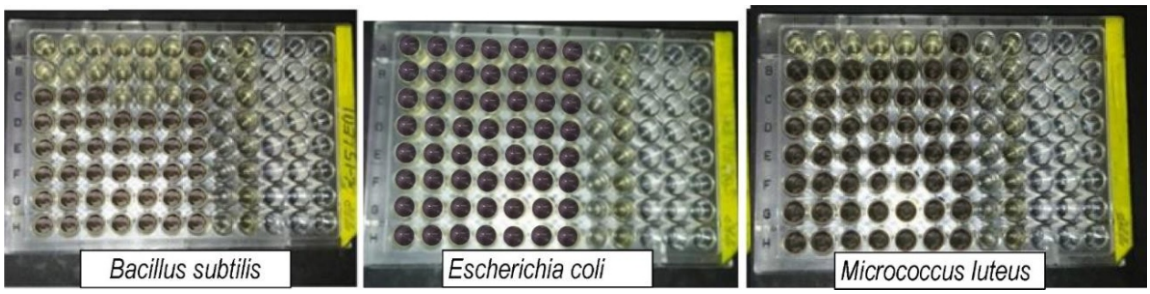
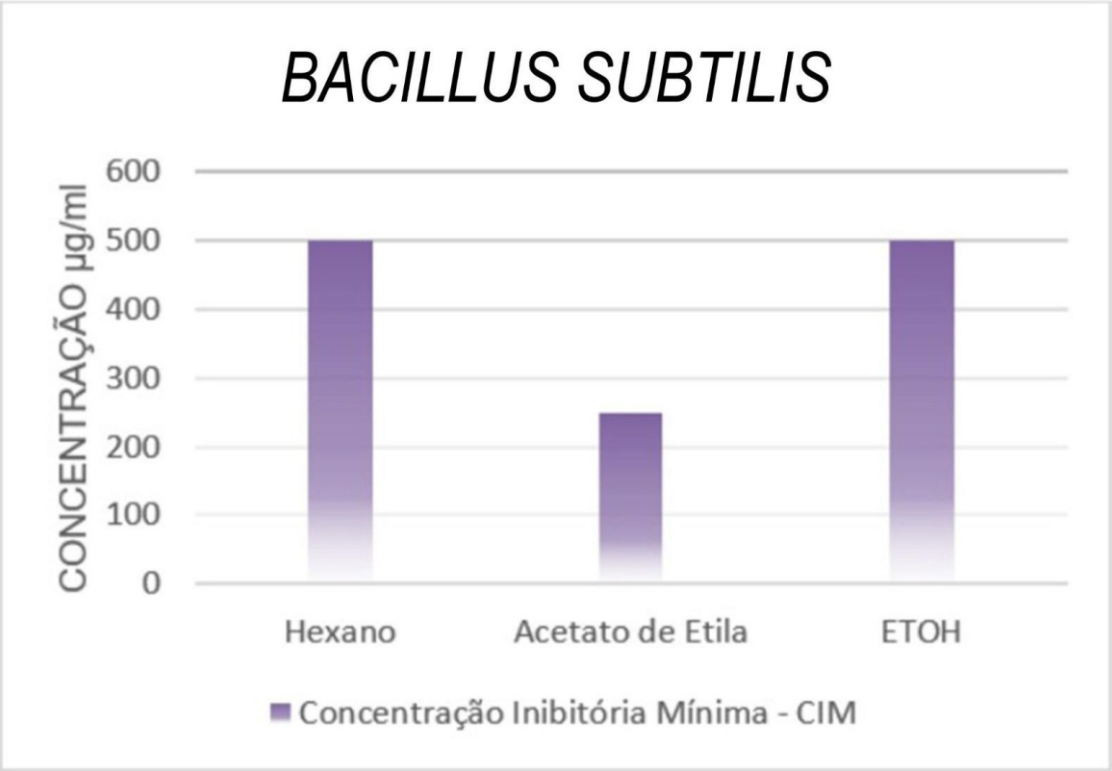


Figure 09. Elisa plate with the minimum inhibitory concentration (MIC) test of *Gracilaria domingensis* extracts and *Gracilaria cornea.* **Legend**: Wells:1,2,3, ETOH G.c; 4,5,6: Ethyl Acetate G.c; 7,8,9: controls.

It is possible to say that *Gracilaria cornea* has more bioactive substances in its composition that are responsible for the antimicrobial potential for the *Bacillus subtilis* strain than *Gracilaria domingensis*.



Graph 1: Minimum Inhibitory Concentration (MIC) of *Gracilaria cornea* extracts

4.2 Determination of phytochemical composition

4.2.1 Gas Chromatography Coupled with Mass Spectrometry - CGEM

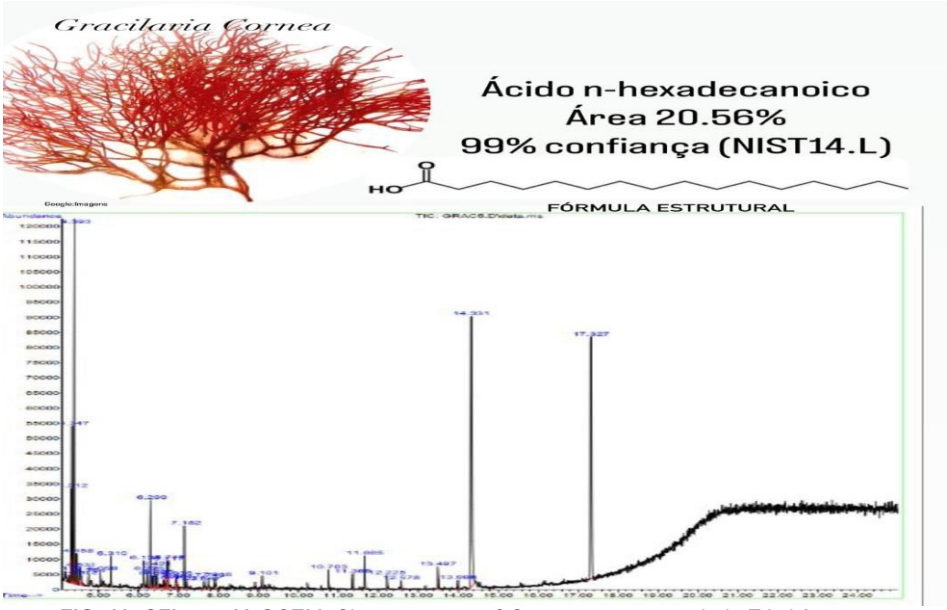


FIG. 10. CFigure.13 GCEM: Chromatogram of *G. cornea* compounds *in Ethyl Acetate*

The chromatogram of the compounds (Fig. 13) showed mentionable scores of 20% area, identifying the component, n-Hexadecanoic Acid, with a 99% degree of confidence compared to the most widely used mass spectral reference library by a team of experienced mass spectrometers at the National Institute of Standards and Technology (NIST) (Table 02).

Table 02 CGMS: Constituents of the Ethyl Acetate extract of *G. cornea* from Praia de Vilas do Atlântico compared to the National Institute of Standards and Technology (NIST) data library (RT- Retention Time, M/Z-Mass)

PICO TR	TR	Area (%)	M/Z	COMPONENT	NIST14.L (% TRUST WITH THE LIBRARY)
14.331	**14.329**	**20.56**	**73.00**	**n-Hexadecanoic acid**	**99**
17.327	17.327	15.99	185.00	Tributyl acetylcitrate	86
6.299	6.301	4.50	58.00	Benzoic acid, 2 (dimethylamino)	37
7.152	7.154	3.04	200.80	Ethane, hexachlor	91
4.393	4.396	16.54	69.10	4,5-Octanediol, 3,6-dimethyl	53
4.347	4.344	7.54	59.00	1,1-Dimethyl-3-chloropropanol	38
4.312	4.310	4.06	69.00	2-Hexene, 3,5-dimethyl	47
11.665	11.663	1.87	57.00	Heptadecane	91
5.310	5.311	1.67	89.90	2-Butene, 2-chloro	59
10.763	10.764	1.09	162.90	1,4 Benzenedicarboxylic acid	90
12.225	12.223	0.90	73.00	Tetradecanoic acid	87

Source: Own the Author

In studies such as those by Manilal *et al* 2010 and Arulkumar *et al* 2018, they report that n-hexadecanoic acid has antibacterial activity and proves to be effective in preventing infections created by bacteria. The n-hexadecanoic component is significantly effective against gram-positive and gram-negative organisms (Krishnan and Mohan, 2016).

4.2.2 Fractionation

Fractionation was carried out using chromatographic separation on a column with silica gel 60, using 18g of the crude ethyl acetate extract of *Gracilaria cornea.* Different proportions of eluents of increasing polarity (100% n-hexane, EtOAc and MeOH) were used as the mobile phase, resulting in 25 fractions, table 03 (approximately 8 ml), each of which was collected in a separate vial and then waited for the evaporation time.

Table 3. Fractionation of the ethyl acetate extract of *Gracilaria cornea*

Hexane (%)	Ethyl Acetate (%)	Collected Fractions
100	00	1-3
90	10	4-6
80	20	7-11
70	30	12-15
60	40	16-17
50	50	18-19
00	100	20-25

Source: Author

The 25 fractions were grouped according to the degree of similarity of the chromatographic profile on the CCD, giving a total of 9 fractions. The fractionation of the extract is shown in Table 4, as well as the yield values of the fractions obtained, expressed in milligrams (mg). The fractions were then sent to the Bahia Nuclear Magnetic Resonance Laboratory (LABAREMN) for hydrogen H NMR spectroscopy.

Table 4: Fractions of *Gracilaria cornea* in Acetate

FRACTIONS	**BIOMASS**
F2	31.0mg
F4	32.0mg
F5	38.7mg
F6-F7	29.3mg
F12-F14	28.0mg
F19	11.7mg
F20-F21	17.7mg
F24	91.0mg
F25	32.7mg

Source: Author

The spectrum of the fraction (F5) shows a typical set of H signals from a fatty component, characterized by the signals at Δ 0.803 ppm (a triplet), from the fatty acid terminal methyl, Δ 1.25 ppm (from CH2 - chain), Δ 1.63 ppm q (7.5 Hz) and a triplet at Δ 2.34 ppm. The spectrum of the sample (F6-7) shows the presence of the same component as F5, plus a second component, in a smaller proportion in the mixture, also suggestive of being of a greasy nature with signals between Δ 2.4 ppm and 2.09 ppm. In addition to the components of F5 and F6 - 7 (Figure 14.a), the spectrum of F12-14 shows signals indicative of the presence of an aromatic component in the extract, with signals ranging from Δ 2 ppm to Δ 2.09 ppm.

range between Δ 7.30 ppm and 7.33 ppm and signals from other impurities, with a range between Δ 2.0 ppm and 6.2 ppm, in small concentrations (Figure 14.b). F19; 20-21, F24 and F25 show the same pattern of signals in the spectra as the previous samples, which also suggests the presence of fatty acid, except for the presence of a signal at Δ 1.31 ppm (Figures 14b and 14.c)

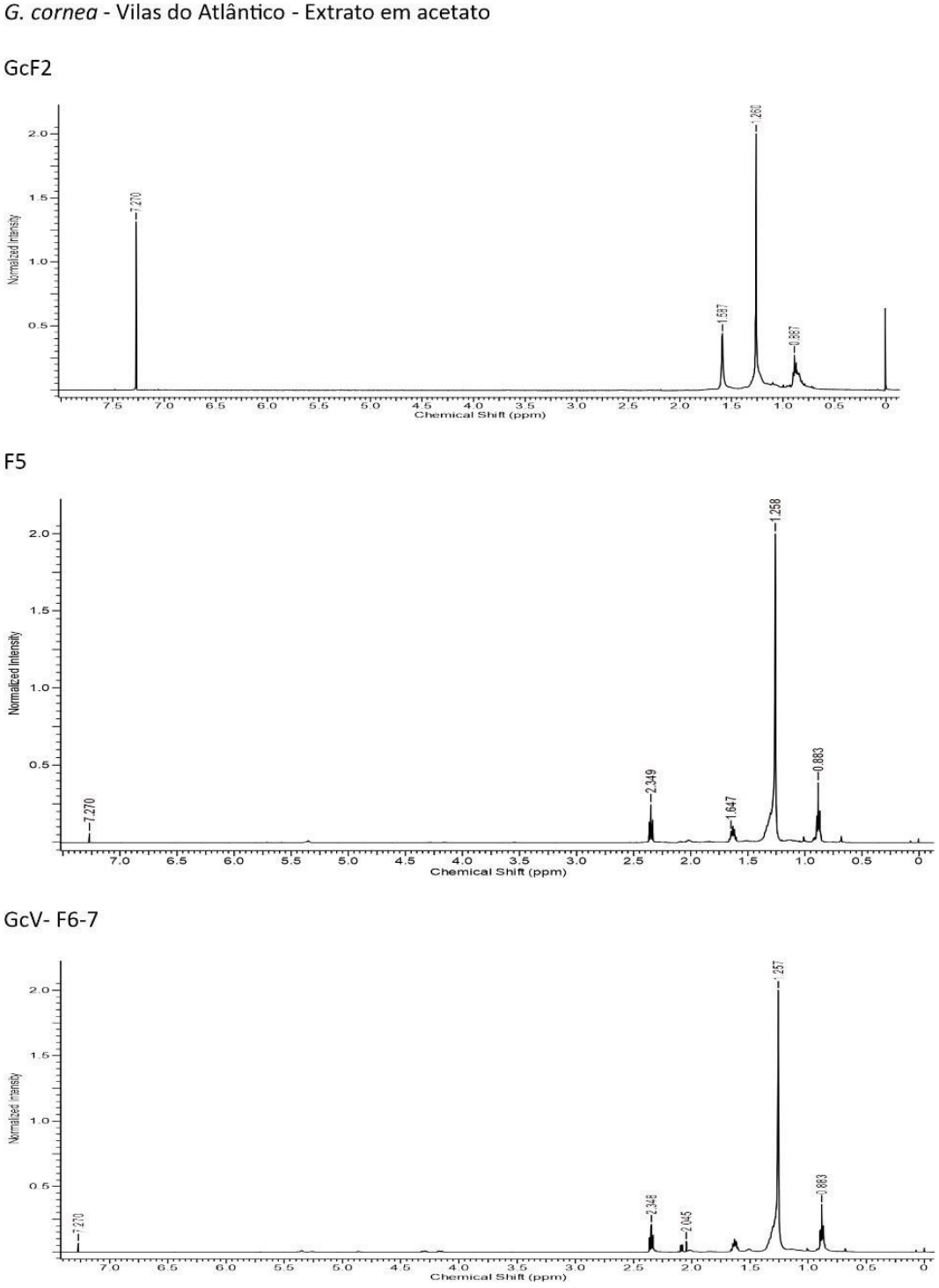


Figure 14.a NMR spectra of the active fractions (F2 to F7) of the ethyl acetate extract of *Gracilaria cornea*

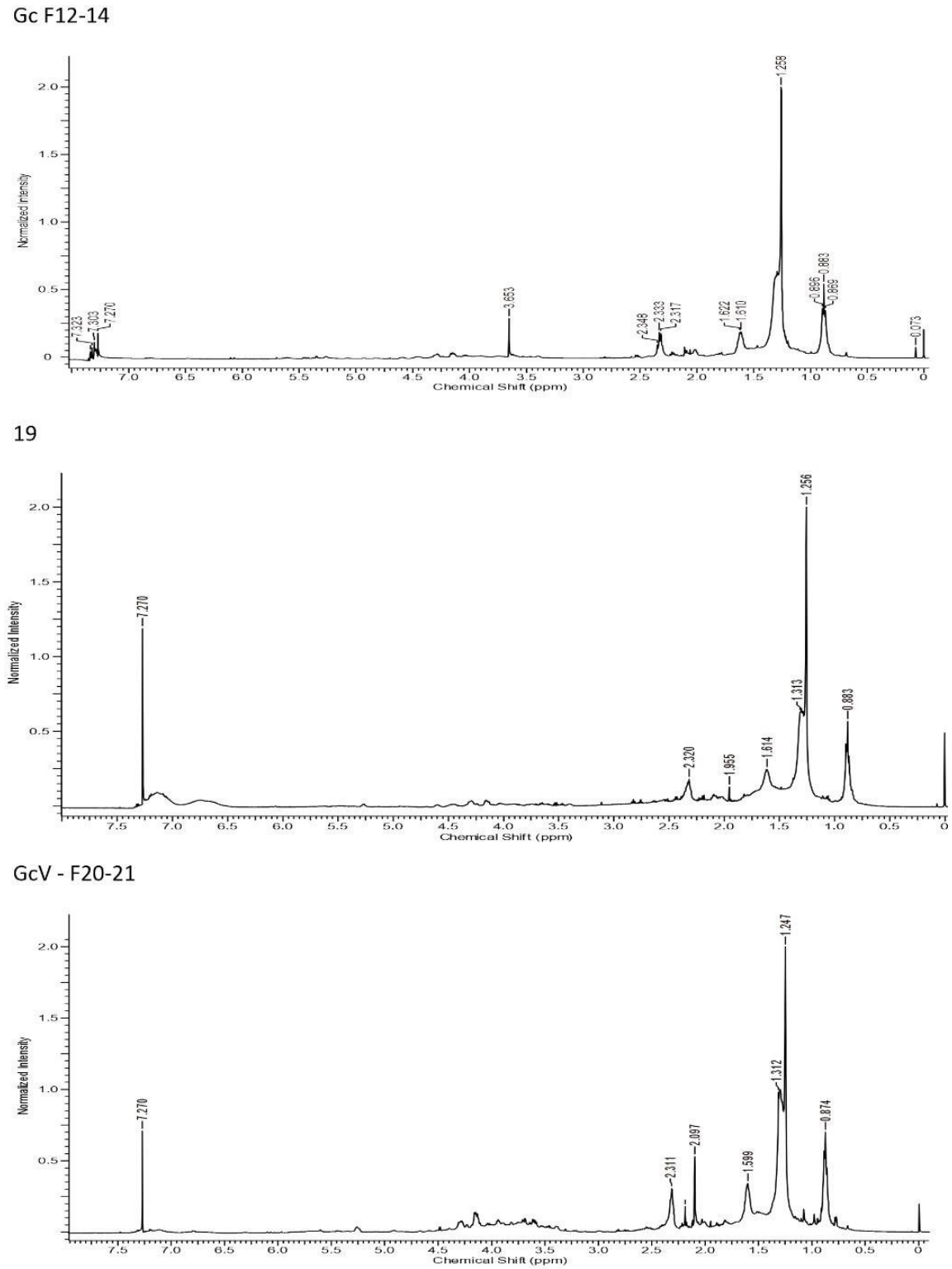
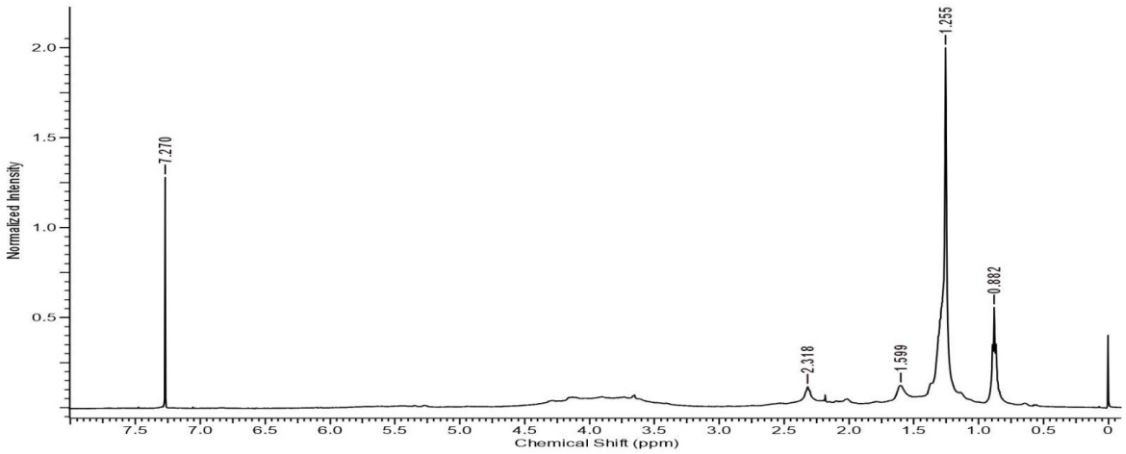


Figure 14.b NMR spectra of the active fractions (F12 to F21) of the ethyl acetate extract of *Gracilaria cornea*

F24



GcV F25

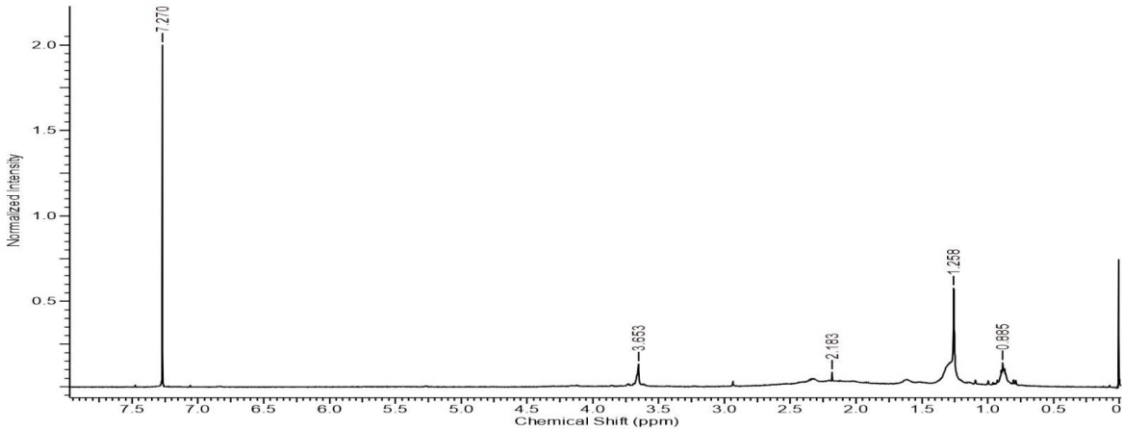
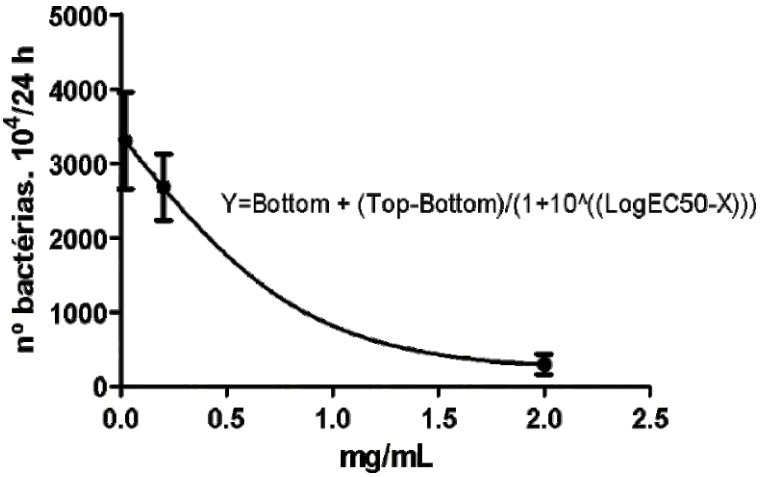


Figure 14.c NMR spectra of the active fractions (F24 to F25) of the ethyl acetate extract of *Gracilaria cornea*

Fractions F5 and F24 had the highest biomass and were the purest. When tested on the microorganisms *Bacillus subtilis* and *Bacillus cereus,* they proved to be active at a concentration of 1000ug/ml.

4.3 Bacterial Kinetics with Quantification of Colony Forming Units (CFU)



Graph 02. Effect of *G. cornea* acetate extract on inhibition of bacterial growth. Where: Y = number of bacteria/10^4/24h, X=concentrations of Extract, Bottom= concentration of maximum inhibition response Top = concentration of response from 50% to maximum response

When evaluating the results of antimicrobial activity using the Minimum Inhibitory Concentration (MIC) test and then counting the CFU in the Petri dish, in the presence of the ethyl acetate extract of *Gracilaria Cornea the* bacterial growth rate (CFU) fell as the concentration of the extract increased.

The bacterial strains grown only in culture medium were quantified at 416,^{109} CFU, after the presence of the ethyl acetate extract of *G. cornea* at a concentration of 2.0mg/ml 292,^{104} colony-forming units were counted, at a concentration of 0.2mg/ml 2,675,^{104} and at a concentration of 0.02mg/ml 3,299,104 colony-forming units.

The CGMS data of the acetate extract of *G. cornea* were suggestive of the presence of n-Hexadecanoic acid as the major component of the ethyl acetate extract of *G. cornea,* which may be the agent that reduces the growth of colony-forming units. In studies such as

A. Mohan et al (2016), n-Hexadecanoic acid (palmitic acid) isolated from the ethanolic extract of the leaves of *Canthium parviflorum, has been* shown to be a potential source of antimicrobial compounds (flavonoids, glycosides, phenols and tannins), with the action of inhibiting the growth of different pathogens (Gram-negative (Escherichia coli), Gram-positive bacteria (Staphylococcus

aureus and Bacillus subtilis) and fungi (*Candida albicans)*, leading to new options for the treatment of infectious diseases. This leaves new lines of study to discover the main constituents of this leaf and the origin of this activity. The comparison between the control and the extract at different concentrations showed a reduction in bacterial growth for the *Bacillus subtilis* strain tested.

Some studies have explored the potential of bioactive substances extracted from algae, as they may contain compounds with antimicrobial properties, such as sulphated polysaccharides, peptides and terpenoids, with the ability to negatively affect the growth and survival of certain bacteria.

5. FINAL CONSIDERATIONS

The purpose of this study was to investigate the activity and chemical profile of the ethyl acetate extract of *Gracilaria cornea,* from Villas do Atlântico beach, in order to identify potentially active compounds to inhibit the activity of the following microorganisms: Bacillus subtilis (ATCC6633), Bacillus cereus (ATCC 0096), Micrococcus luteus (ATCC 10240) and *Escherichia coli (ATCC* 94863). The GCMS test indicates that n-Hexadecanoic acid is the main component of the Ethyl Acetate extract of *Gracilaria cornea.*

The immense coastline that covers the Brazilian coast, especially that of the state of Bahia, presents a diversity of habitats, with unique ecological niches and a wide distribution of different species of algae. To date, no work on the algae *G. cornea* from Villas do Atlântico beach has been published, so this chemical study can be considered unprecedented. Preliminary analyses were carried out on the extracts of *Gracilarias Domingensis* and *Gracilarias cornea,* all of which were collected at the same time, in December 2021, and subjected to the same maceration and extraction process, using Hexane, Ethyl Acetate and Ethanol as solvents.

Comparative NMR analysis of the extract fractions (AcEOt - GC) evaluated the set of signals and their chemical hydrogen shifts. The spectrum of the fraction (F5) shows a set of fatty component signals, while F24 shows the same pattern of signals, which also suggests the presence of a fatty acid. F5 and F24 had greater mass and different and more interesting signals to work with. These fractions were then used to test the Minimum Inhibitory Concentration and bacterial kinetics. When tested on the microorganisms *Bacillus subtilis* and *Bacillus cereus,* they proved to be active at a concentration of 1000ug/ml.

6. CONCLUSION

In view of the results found, it was possible to conclude that the extracts of *Gracilaria cornea had an* efficient effect on the bacterial activity of *Bacillus subtilis,* inhibiting its growth at a concentration of 250ug/ml, which suggests that these extracts have bioactive components and that n-Hexadecanoic acid may be the main component that exerts bioactivity according to the chromatographic fractionation. The antimicrobial activity kinetics test showed that in the presence of the ethyl acetate extract of *Gracilaria Cornea, the* bacterial growth rate of the microorganism *Bacillus subtilis* (CFU) was reduced compared to the growth of colony-forming units without the presence of this extract.

As for *Gracilaria domingensis, there* was no significant influence from the extracts, which may be due to the small amount of substances present in this species. No studies were found in the literature that evaluated the antimicrobial activity of both species, thus highlighting the need for more studies involving these two macroalgae.

6.1 Future steps

This study has the potential for further research. The data obtained suggests that antimicrobial activity against other microorganisms should be evaluated. It is possible to try to collect algae from other regions, establish possible interfering factors and compare them, as well as using new methodologies to delimit and explore the potential of the secondary metabolites of the algae studied and their applications.

In addition, other isolated components may be discovered, which could contribute to our scientific studies.

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