

Antibacterial and anticancer activity of seaweeds and bacteria associated with their surface

Actividad anticancerígena y antibacteriana de macroalgas y bacterias asociadas a su superficie

Luis J. Villarreal-Gómez¹, Irma E. Soria-Mercado¹, Graciela Guerra-Rivas¹ and Nahara E. Ayala-Sánchez²

¹Facultad de Ciencias Marinas, Universidad Autónoma de Baja California, Km 107 Carretera Tijuana- Ensenada, Ensenada, Baja California, México. iesoria@uabc.edu.mx

²Facultad de Ciencias, Universidad Autónoma de Baja California, Km 107 Carretera Tijuana- Ensenada, Ensenada, Baja California, México

Resumen. - Las bacterias y algas marinas son una fuente inagotable de compuestos químicos que permiten producir una amplia variedad de metabolitos secundarios bioactivos. Las bacterias marinas se han convertido en objetivo importante para la industria de la biotecnología debido a la alta cantidad de compuestos bioactivos descubierto recientemente a partir de ellas. El objetivo de este estudio fue evaluar la actividad anticancerígena y antibacteriana de extractos de las algas marinas *Egrecia menziesii*, *Codium fragile*, *Sargassum muticum*, *Endarachne binghamiae*, *Centroceras clavulatum* y *Laurencia pacifica* recolectadas en Bahía Todos Santos, México. Se obtuvieron extractos orgánicos a partir de las algas libres de bacterias y de las bacterias asociadas a su superficie. Para probar la actividad antibacteriana se emplearon las cepas patógenas *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis* y *Pseudomonas aeruginosa* y para la actividad anticancerígena, células de carcinoma colorectal HCT-116. De la superficie de las algas marinas se aislaron 35 cepas bacterianas que se identificaron molecularmente y pertenecían a los phyla *Firmicutes*, *Proteobacteria*, y *Actinobacteria*. Las cepas Cc51 aislada de *Centroceras clavulatum*, Sm36 aislada de *Sargassum muticum*, y Eb46 aislada de *Endarachne binghamiae* mostraron actividad anticancerígena con un IC₅₀ de 6,492, 5,531 y 2,843 µg ml⁻¹ respectivamente. Asimismo, los extractos de las bacterias asociadas a las algas marinas inhibieron el crecimiento de la bacteria Gram negativa *Proteus mirabilis*. La actividad biológica observada en este estudio ofrece la oportunidad de un análisis químico posterior, tal como el aislamiento de los compuestos activos responsables, a fin de contribuir en el descubrimiento de nuevos fármacos.

Palabras clave: Compuestos bioactivos, bacterias simbiotes, algas bioactivas

Abstract. - Marine algae and bacteria are an inexhaustible source of chemical compounds that produce a wide variety of biologically active secondary metabolites. Marine bacteria have become an important target for the biotechnology industry because of the large number of bioactive compounds recently discovered from them. The aim of this study was to evaluate the antibacterial and anticancer activities of extracts from the seaweeds *Egrecia menziesii*, *Codium fragile*, *Sargassum muticum*, *Endarachne binghamiae*, *Centroceras clavulatum* and *Laurencia pacifica* collected from Todos Santos Bay, México. Organic extracts were obtained from bacteria-free algae and from surface-associated bacteria. Pathogen strains of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* were used to test antibacterial activity and HCT-116 colon cancer cells for anticancer activity. Thirty-five bacterial strains were isolated from the surface of seaweeds and molecular identified as belonging to the phyla *Firmicutes*, *Proteobacteria* and *Actinobacteria* by 16S rDNA sequencing. The strains Cc51 isolated from *Centroceras clavulatum*, Sm36 isolated from *Sargassum muticum*, and Eb46 isolated from *Endarachne binghamiae* showed anticancer activity, with IC₅₀ values of 6.492, 5.531, and 2.843 µg ml⁻¹ respectively. Likewise, the extracts from the seaweed-associated bacteria inhibited the growth of the Gram negative bacterium *Proteus mirabilis*. The biological activity observed in this study offers opportunities for further chemical analyses, such as the isolation of the active compounds responsible for this activity, in order to contribute to the discovery of new drugs.

Key words: Bioactive compounds, symbiotic bacteria, bioactive algae

INTRODUCTION

Natural products are a major resource for drug development. A large number of plants, microbes, and marine animals have been examined for bioactive secondary metabolites (Firakova *et al.* 2007). Marine algae such as harbor

endophytes, like their terrestrial counterparts, are a potential source of new secondary metabolites (Strobel *et al.* 2004). Despite this, the number of seaweed species studied and identified corresponds to only 2% of the 150,000 known

species worldwide (Harvey *et al.* 1988). From the species identified, approximately two thousand chemical compounds have been characterized (Gonzalez & Silva 2001). Bacteria are often found on the surface of many species of marine algae collected from natural habitats. These bacterial strains may be taken from the sea along with the algal cells, or may be found as a result of contamination in algal cultures (Jasti *et al.* 2005). Mixed bacterial populations observed on industrial crops and in natural ocean algal aggregations show that they use organic substances secreted by algal cells whether they are alive or dead. Nevertheless it has been noted that many algae present higher growth in the presence of bacteria than in their absence.

Several algal species need specific vitamins for their growth and bacteria may be partly responsible for the production of these substances. In addition, some seaweeds are capable of synthesizing antiviral, antibacterial, and/or antifungal compounds against several pathogens, and bacteria found on algae are believed to be the producers of these active compounds (Deig *et al.* 1974, Richards *et al.* 1978, Caccamese & Azzolina 1979, Selvaraj *et al.* 1989, Bhakuni *et al.* 1990, Pérez *et al.* 1990, Ballesteros *et al.* 1992, Bhakuni *et al.* 1992, Jasti *et al.* 2005). The production of bioactive metabolites is considered to be a response to ecological pressures such as competition, predation deterrence, and reproduction. Such chemicals are often common in sessile eukaryotic organisms like marine sponges and corals, seaweeds, and terrestrial plants (Rosenthal & Janzen 1979, Hay & Fenical 1988, Pawlik 1993).

Since the late 1980s, more than 50,000 natural products have been discovered from marine microorganisms. More than 10,000 had biological activity; among them, 8,000 had antibiotic and antitumor activities (Betina 1983, Berdy 1989). Marine bacteria often produce anticancer and antibacterial substances as a means of maintaining relationships between epiphytic micro-environments, inhibiting competing organisms and microbial pathogens (Avendaño-Herrera *et al.* 2005).

Some species of microorganisms show host specificity and specific antagonist activity against others; this specificity involves complex biochemical interactions between plants and microorganisms. Information is available that can provide direction in the selection of the type of organisms associated with medicinal plants when searching for new natural products (Strobel 2003). Microorganisms associated with plants produce necessary enzymes for colonization and like a symbiotic relation most of these microorganisms are able to use (at least *in vitro*) the cellular components

of the plant (Sieber *et al.* 1991, Leuchtman *et al.* 1992). Endophytic microorganisms are considered an outstanding source of bioactive natural products because many of them occupy literally millions of unique biological niches (higher plants) in many unusual environments. Thus, it would appear that a myriad of bio-typical factors associated with plants should be considered in the selection of a plant for study. It could be that these factors determine which microbes are present on plants as well as the biological activity of the products associated with these organisms. Despite the growing interest in chemical synthesis, natural bioactive products have a great impact on human health and are still out there waiting to be discovered and developed.

The objective of this study was to evaluate the antibacterial and anticancer activities of the organic extracts from six marine algae collected from Todos Santos Bay, Baja California, México, as well as the surface-associated bacteria.

MATERIAL AND METHODS

SAMPLE COLLECTION

The seaweeds *Egregia menziesii* (Turner) Areschoug (*Em*), *Codium fragile* (Suringar) Hariot (*Cf*), *Sargassum muticum* (Yendo) Fensholt (*Sm*), *Endarachne binghamiae* (*Petalonia binghamiae*) (J. Agardh) Vinogradova (*Eb*), *Centroceras clavulatum* (Agardh) Montagne (*Cc*), and *Laurencia pacifica* Kylin (*Lp*) were collected by hand at low tide in the coastal area off the Autonomous University of Baja California (UABC) (31°51'N; 116°40'W), Ensenada, BC, México. Collected samples (100 g) were transferred in zip-lock bags on ice. The algal surface was washed with fresh water, and a bacterial sample was taken from the surface with a sterile swab. Once the sample had been taken, the algal surface was rinsed again with sterile fresh water, dried with sterile paper, and stored in a sterile flask.

ISOLATION OF BACTERIAL STRAINS

A sterile swab was used to rub the seaweed surface and inoculating the removed bacteria on marine agar and marine broth (Difco) isolation plates. The inoculated plates were incubated for 24-48 h at 25°C until colonies were observed. Bacterial strains were purified through the third generation. Gram stain was used for initial bacterium classification; after staining the bacteria were stored in glycerol (-70°C).

PURIFICATION OF TOTAL DNA

Pure bacterial strains were cultured in marine broth at 25°C, and the total genomic DNA of each strain was extracted using the DNeasy Blood and Tissue Kit (Cat. No. 69506) according to the manufacturer's protocol (Qiagen Inc., CA, USA).

AMPLIFICATION OF THE 16S RIBOSOMAL DNA GENE

16S ribosomal DNA gene amplification was performed using PCR with the universal forward primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'; Galkiewicz & Kellogg 2008) and reverse primer 1387R (5'-GGGCGGWTGTACAAGGC-3'; Marchesi *et al.* 1998), using the following program: 5 min at 95°C, 1 min at 94°C, 1 min at 63°C, 1 min at 72°C for 35 cycles, 7 min at 72°C and held at 4°C.

SEQUENCING

The purified PCR products were commercially sequenced (Rebecca and John Moore Cancer Center Sequencing Facilities) using the universal primer 27F.

PHYLOGENIC ANALYSIS

Sequences were analyzed using the Basic Local Alignment Search Tool (BLAST, Altschul *et al.* 1990) for preliminary species identification. The sequences were initially aligned using the ClustalX program (Staley & Ta 1985), and then manually aligned in Bioedit (Hall & Brown 2001) using a segment of 623 base pairs to construct a neighbor-joining tree. A phylogenetic tree was constructed by a bootstrap test of 1000 replications, using the MEGA4 program (Tamura *et al.* 2007).

GROWTH CURVES AND CRUDE BACTERIAL EXTRACTS

Pure bacterial strains stored in glycerol were selected at a time for growth and incubated in a water bath (250 rpm) at 30°C. Cultures were left to grow for 2 to 4 days until reaching the exponential growth phase for each strain. Subsequently 20 g l⁻¹ of Amberlite XAD7 resin were added by stirring for 2 h, and then filtered through gauze and finally through Whatman Grade 802 filter paper (12.5 cm diameter). The solids were extracted exhaustively with acetone using 12.5 ml g⁻¹ of resin. Finally, crude acetone extracts were concentrated by distillation at reduced pressure (Buchi model 461 with a CVC2 vacuum pump). Each extract was given a code consisting of a capital letter and a lower-case letter, corresponding to the genus and species, respectively, of the alga from where the bacteria

were isolated, followed by a sequential number for each bacterial strain. For example, *Em1* refers to *Egregia menziesii*, bacterium 1; *Cc6* to *Centrocera clavulatum*, bacterium 6; and *Lp2* to *Laurencia pacifica*, bacterium 2.

PREPARATION OF ALGAL EXTRACTS

The clean algae stored in sterile flasks were covered with a mixture of methanol and dichloromethane (7:3) and stored for 2 weeks at room temperature. Subsequently, the extract was filtered and concentrated by distillation under reduced pressure.

CYTOTOXIC ACTIVITY

The algal and bacterial extracts were each tested against HCT-116 colon cancer cells (10 mg ml⁻¹ in DMSO), obtaining medium inhibitory concentration (IC₅₀) values in µg ml⁻¹, using a dilution method in a 96-well plate. After 72 h incubation, absorbance was read at 490 nm. DMSO was used as negative control and etoposide as positive control, both in the same concentrations as the compounds.

ANTIBACTERIAL ACTIVITY

The algal and bacterial extracts were tested against the pathogen bacteria *Staphylococcus aureus* (ATCC25923), *Klebsiella pneumoniae* (ATCC 13883), *Proteus mirabilis* (ATCC 35659), and *Pseudomonas aeruginosa* (ATCC 27853) at a concentration of 10 mg ml⁻¹ in DMSO. The percentage of cell survival was obtained using a dilution method during 12 h incubation at 37°C. Absorbance was read at 600 nm, using DMSO as negative control and vancomycin as positive control.

RESULTS

IDENTIFICATION OF BACTERIAL STRAINS

From the six species of marine algae collected, 35 bacterial strains were isolated. After Gram stain identification, 16 were classified as Gram positive and 19 as Gram negative.

Analysis of 16S rDNA sequences showed that only 33 bacteria could be classified within three main phyla (Fig. 1): *Actinobacteria* (*Cf2*, *Eb3*, *Sm2*, *Em6*, *Cc5*, and *Cc1*; Gram positive), *Proteobacteria* (*Cc3*, *Sm7*, *Cc7*, *Sm5*, *Cf4*, *Lp5*, *Lp2*, *Eb6*, *Eb7*, and *Cf5*; Gram negative), and *Firmicutes* (*Cf1*, *Eb2*, *Cc4*, *Em2*, *Em5*, *Lp3*, *Cf3*, *Eb5*, *Sm6*, *Cf6*, *Cc2*, *Sm4*, *Eb1*, *Cc6*, *Em4*, *Em1*, and *Eb4*; Gram positive).

Based on the genetic results, the phylogenetic tree could be divided into two main clusters: Gram positive and negative

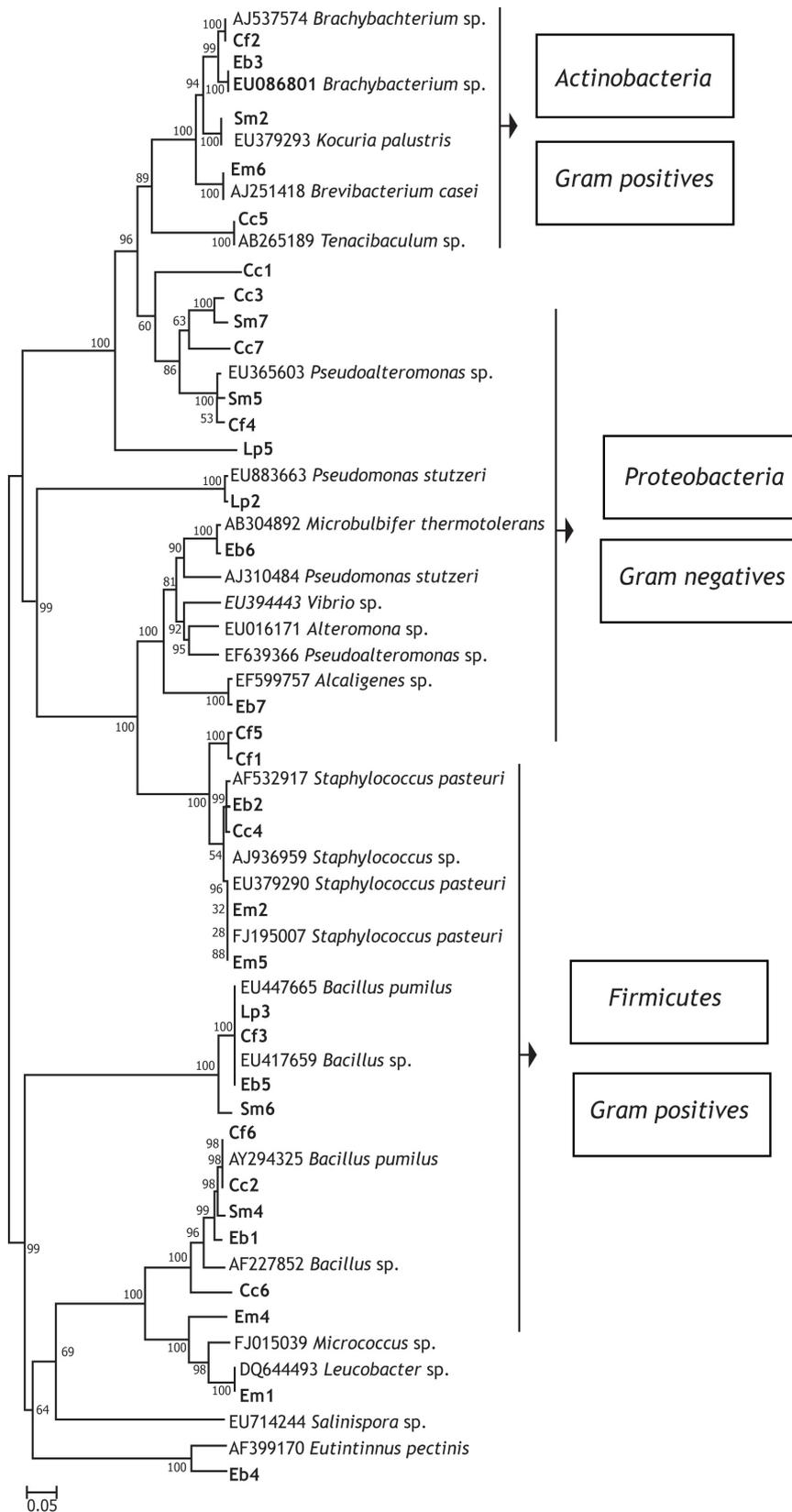


Figure 1. Phylogram of the evolutionary relationships of 58 taxa of marine bacteria with sequenced clones using universal primers 27F and 1387R for marine samples. The evolutionary history was assessed using the method of construction described by neighbor-joining (Saitou & Nei 1987), based on a total of 625 base pairs in MEGA4 (Tamura *et al.* 2007) / Filograma de las relaciones evolutivas de 58 taxas de bacterias marinas con las clonas secuenciadas usando los cebadores universales de bacterias 27F y 1387R para muestras marinas. La historia evolutiva fue determinada usando el método del vecino más cercano (Saitou & Nei 1987) con un total de 625 pares de bases en MEGA4 (Tamura *et al.* 2007)

bacteria. No particular association was found between any algal species and a specific type of bacteria. As shown in Fig. 1, all strains belonging to each different group of bacteria are evenly distributed with no species-relation pattern.

BIOLOGICAL ACTIVITY

The biological activity observed for the different organic extracts tested showed that the strains *Em6*, *Cc3*, and *Cc5* (isolated from *E. menziesii* and *C. clavulatum*) had low activity against colon cancer cells (HCT-116), with IC_{50} values of 58.0, 101.6, and 56.7 $\mu\text{g ml}^{-1}$, respectively. The most cytotoxic strains were *Cc1* (isolated from *C. clavulatum*) and *Sm6* (isolated from *S. muticum*), with IC_{50} values of 6.4 and 5.5 $\mu\text{g ml}^{-1}$, respectively, while strain *Eb6* (isolated from *E. binghamiae*) showed the highest anticancer activity, with an IC_{50} value of 2.8 $\mu\text{g ml}^{-1}$ (Table 1).

Fourteen bacterial strains and five algae (all except *E. menziesii*) showed antibacterial activity against *Proteus mirabilis*. None of the extracts from marine algae and bacteria were active against *S. aureus* and *P. aeruginosa*. Extracts of strains *Em2*, *Cf1*, *Cf6*, *Sm2*, *Sm6*, *Eb4*, *Eb5*, *Eb6*, *Cc1*, *Cc2*, *Cc3*, *Cc7*, *Lp2*, and *Lp3* presented weak antimicrobial activity against *P. mirabilis*, and bacterial growth greater than 50% was observed as shown by the LD_{50} values (Table 1). Likewise, extracts of the seaweeds *Cf*, *Sm*, *Eb*, *Cc*, and *Lp* showed LD_{50} values of 140.1, 140.1, 178.5, 181.9, and 107.1 $\mu\text{g ml}^{-1}$, respectively. The bacterium *K. pneumonia* showed low sensitivity against the extracts tested, and only two strains of *E. menziesii*, *Em1* and *Em4*, showed antibacterial activity, with LD_{50} values of 211.5 and 203.0 $\mu\text{g ml}^{-1}$, respectively (Table 1).

DISCUSSION

As a result of the Gram staining of the 35 bacteria analyzed in this study, 16 were classified as Gram positive and 19 as Gram negative. Previous bacterioplankton studies have shown that the majority of marine bacteria are Gram negative; however, recent studies on marine sediment microbial communities have found evidence that most bacteria from this environment appear to be Gram positive (Gontang *et al.* 2007). Strains isolated from the surface of *Laurencia pacifica* showed a trend towards Gram negative bacteria, but no trend was observed for the remaining seaweeds regarding Gram positive and Gram negative abundance. The Gram classification could be an interesting tool if the differences between the structure and chemical composition of the two main groups of bacteria are considered. These group-specific chemical characteristics could allow the interaction between bacteria and algae.

The abundance trend of specific bacterial groups in the marine algae observed in this study may be explained by these chemical interactions. Gallardo *et al.* (2004) found a predominance of Gram negative bacteria belonging to the following genera: *Vibrio* (20%), *Escherichia coli* inactive (18%), *Flavobacterium* (11%), *Flexibacter* (9%), *Moraxella* (9%), *Pseudomonas* (9%), *Aeromonas* (2%), *Acinetobacter* (2%), *Cotophaga* (2%), *Photobacterium* (2%) and *Alteromonas* (2%). Gram positive bacteria (*Staphylococcus*) were also found in the seaweed *Monostroma undulatum*.

Of the 35 bacterial strains isolated from the 6 seaweed, it was possible to phylogenetically identify 33, located within the phyla Firmicutes, Proteobacteria and Actinobacteria (the last is considered excellent producers of bioactive secondary metabolites) (Jensen *et al.* 2005, Macherla *et al.* 2005). The distribution of the 15 bacterial species identified showed no tendency toward the colonization of specific algae. However, it was noted that some bacteria grew only in very particular algae; for example, *Leucobacter* sp. was observed only in *Egrecia menziesii*. This bacteria class member of the Actinobacteria, were found linked to the nematode *Caenorhabditis elegans* (Muir & Tan 2008).

Industrial applications have been reported for some of the bacterial species found in this study. For example, *Alcaligenes* (*Eb7*), found exclusively on the alga *Endarachne binghamiae*, has been used for the industrial production of non-standard amino acids (Madigan *et al.* 2005). *Tenacibaculum* (*Cc5*), found only on the alga *Centroceras clavulatum*, has been found associated with sponges and seaweed producers of secondary metabolites in the Palau Islands (Suzuki *et al.* 2001). The strain *Sm2*, identified as *Kocuria palustris*, was exclusively found on *Sargassum muticum*; this alga is considered an invasive species in many countries, but the species of bacteria found on it (*Kocuria*) has industrial applications as the field gradient Organic (Kovacs *et al.* 1999). Finally, bacterial strains of the genus *Alteromonas* (*Lp5*) were found associated only with the alga *Laurencia pacifica*, usually isolated in seawater. The Proteobacteria species have industrial uses because they produce high molecular weight polysaccharides. These observations suggest that some bacteria can be industrially exploited.

Bacillus species proliferated on all algae, except *Egrecia menziesii*. A total of 9 strains (26%) of the 35 bacteria studied belonged to this species that is usually found in soil, seawater, and rivers. It has been reported that *Bacillus brevis*, *B. cereus*, *B. circulans*, *B. laterosporus*, *B. licheniformis*, *B. polymyxa*, *B. pumilus*, and *B. subtilis* produce antibiotics (Madigan *et al.* 2005). All entries are

Table 1. Anticancer and antibacterial activity values of seaweeds extracts: *Egregia menziesii* (Em), *Codium fragile* (Cf), *Sargassum muticum* (Sm), *Endarachne binghamiae* (Eb), *Centroceras clavulatum* (Cc) and *Laurencia pacifica* (Lp) and the extracts of the bacterial strains isolated from each algae, labeled with the abbreviation of the algae name and progressive numbers / Actividad anticancerígena y antibacteriana de los extractos de las algas: *Egregia menziesii* (Em), *Codium fragile* (Cf), *Sargassum muticum* (Sm), *Endarachne binghamiae* (Eb), *Centroceras clavulatum* (Cc) y *Laurencia pacifica* (Lp), y de los extractos de las bacterias aisladas de cada una de las algas, representadas con la abreviatura del nombre del alga y números progresivos

Seaweed and bacteria strains	HCT-116 Colorectal cancer cells IC ₅₀ (µg ml ⁻¹)	<i>Klebsiella</i> <i>pneumoniae</i> MIC (µg ml ⁻¹)	<i>Proteus</i> <i>mirabilis</i> MIC (µg ml ⁻¹)
<i>Egregia menziesii</i>	*	*	*
Em1	*	211.5	*
Em2	*	*	212.3
Em4	*	203.0	*
Em5	*	*	*
Em6	58.0	*	*
<i>Codium fragile</i>	*	*	140.1
Cf1	*	*	196.0
Cf2	*	*	*
Cf3	*	*	*
Cf4	*	*	*
Cf5	*	*	*
Cf6	*	*	213.9
<i>Sargassum muticum</i>	*	*	140.1
Sm2	*	*	165.7
Sm4	*	*	*
Sm5	*	*	*
Sm6	5.5	*	174.0
Sm7	*	*	*
<i>Endarachne binghamiae</i>	*	*	178.5
Eb1	*	*	*
Eb2	*	*	*
Eb3	*	*	*
Eb4	*	*	188.1
Eb5	*	*	209.7
Eb6	2.8	*	178.9
Eb7	*	*	*
<i>Centroceras clavulatum</i>	*	*	181.9
Cc1	6.4	*	180.5
Cc2	*	*	217.1
Cc3	101.6	*	153.1
Cc4	*	*	*
Cc5	56.7	*	*
Cc6	*	*	*
Cc7	*	*	159.1
<i>Laurencia pacifica</i>	*	*	107.1
Lp2	*	*	185.4
Lp3	*	*	288.1
Lp4	*	*	*
Lp5	*	*	*
Lp6	*	*	*

IC₅₀ : Medium inhibitory concentration
MIC : Minimum inhibitory concentration
* : No significant activity

important to mention, since several of the bacterial strains found are close to those used in the industry. Therefore, it is necessary to study the chemical interactions of algae and bacteria for a better understanding of the production process of secondary metabolites.

The biological activity results of this study revealed that a few strains have anticancer activity: *Eb6*, with an IC_{50} value of $2.8 \mu\text{g ml}^{-1}$, showed a sequence similarity of 98.03% to *Microbulbifer thermotolerans*, and *Cc1*, with an IC_{50} value of $6.4 \mu\text{g ml}^{-1}$, showing a sequence similarity of 79.02% to *Pseudoalteromonas* sp. In recent studies, these two species of Proteobacteria were found in biofilms in marine areas and were found to produce chemical compounds to protect themselves from predators. One of these compounds is tholacein, an alkaloid that has been shown to be synthesized predominantly in biofilms, the compound in nM concentrations inhibits protozoan cells and induce programmed cell death in eukaryotic cells. These biofilm-producing bacterial strains secrete chemicals that contribute to the specific defense for a successful survival in several environments and provide an ecological and evolutionary context for the discovery of bacterial metabolites to eukaryotic cells (Matz *et al.* 2008).

Another strain showing anticancer activity was *Sm6*, with an IC_{50} value of $5.5 \mu\text{g ml}^{-1}$ and a sequence similarity of 99.87% to *Bacillus*. Although this type of bacterium can be found in almost any substrate, it can be suggested that because of its association with *Sargassum muticum*, it seems to have acquired the ability to synthesize a compound able to inhibit colon cancer cells (HCT-116). *Sargassum muticum* has been found to show low antibacterial activity against some species of marine algae (Hellio *et al.* 2001).

The bacterial strains and marine algae showed no antibacterial activity against the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*. These results indicate that certain species have selective response mechanisms, as suggested by Matz *et al.* (2008). It is therefore possible that the algae used in this study, which were collected from the same coastal area, share similar defense mechanisms, creating an ecological and biotechnological interest in this natural marine natural product.

Finally, our findings indicate that the organisms, collected from the same area, show similar antibacterial activity against *Proteus mirabilis*. This bacterium is detrimental to human health causing wound infections, septicemia, pneumonia, and kidney stones, as well as other diseases. Despite the fact that *P. mirabilis* is susceptible to many antibiotics such as tetracycline, 10% to 20% of the strains are

resistant to ampicillin and first-generation cephalosporins, which is an interesting finding for ecology and bioactive chemical compounds.

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