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PRELIMINARY SCREENING OF PHYTOCHEMICAL AND BIOLOGICAL ACTIVITY OF *Sargassum lapazeanum*

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ABSTRACT. Algae are exposed to substantial stress, in response, these organisms have developed efficient defense systems, such as protective secondary metabolite synthesis, making algae a primary source of bioactive compounds with a wide spectrum of biological activities. Thus, algae show potential for use in treatments of thrombotic, infectious, and chronic degenerative diseases. Therefore, the objective of this study was to evaluate the phytochemical compounds and pharmacological activity of an extract obtained from *Sargassum lapazeanum,* an alga endemic to the Gulf of California. Algae were collected in the intertidal zone of Tarabillas beach (Bahía de La Paz, BCS). The biological activities of the ethanolic extract and its fractions were evaluated using chromatographic techniques. In addition, a bioautographic assay of hemolytic activity was conducted, and phytochemical profiles and acute toxicity in *Artemia franciscana* were evaluated. The relationships among the main extract components were also determined. The ethanolic extract exhibited significant antioxidant and hemolytic activity, which was mainly attributed to its content of anthrones, anthraquinones, and unsaturated triterpenes. Its toxicological activity reached an LC_{50} value of 225.1 μ g mL⁻¹, which was mainly attributed to alkaloids, flavonoids, anthrones, and sap suggest that *Sargassum lapazeanum* has great pharmacological potential with biomedical applications.

Key Words: antioxidant, flavonoids, hemolytic, toxicity, triterpenes.

Estudio preliminar de la actividad fitoquímica y biológica de *Sargassum lapazeanum*

RESUMEN. Las algas están expuestas a un gran estrés, en respuesta, estos organismos han desarrollado eficientes sistemas de defensa, como la síntesis de metabolitos secundarios protectores, lo que las convierte en una de las principales fuentes de compuestos bioactivos con un amplio espectro de actividades biológicas. Así, las algas tienen potencial para su uso en tratamientos de enfermedades trombóticas, infecciosas y crónico degenerativas. Por lo tanto, el objetivo de este estudio, fue evaluar los compuestos fitoquímicos y la actividad farmacológica de un extracto obtenido de *Sargassum lapazeanum*, un alga endémica del Golfo de California. Las algas fueron recolectadas en la zona intermareal de la playa Tarabillas (Bahía de La Paz, BCS). Se evaluó la actividad biológica del extracto etanólico y sus fracciones mediante técnicas cromatográficas, se realizó un ensayo bioautográfico de la actividad hemolítica y se evaluaron los perfiles fitoquímicos y la toxicidad aguda en *Artemia franciscana*. Así también se determinó la relación entre los componentes fitoquímicos y la actividad biológica. El extracto etanólico mostró una importante actividad antioxidante y hemolítica, atribuida principalmente a su contenido de antronas, antraquinonas y triterpenos insaturados. Su actividad toxicológica alcanzó un valor LC_{so} de 225.1 µg mL⁻¹, atribuida
principalmente a compuestos alcaloides, flavonoides, anonas y saponinas. Los resultados sugieren que *lapazeanum*, tiene un gran potencial farmacológico con aplicación biomédica.

Palabras clave: antioxidante, flavonoides, hemolítico, toxicidad, triterpenos

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INTRODUCTION

Some marine organisms produce secondary me- tabolites, which increase the biological efficiency of species and generate interspecific and intraspecific changes that affect community dynamics (Lobban & Harrison, 1994; Pelletreau & Targett, 2008). In particular, photosynthetically active macroalgae serve as food sources for other organisms and possess various carbon acquisition mechanisms while playing important ecological roles that help maintain environmental quality (Raven & Hurd, 2012). Currently, the number of studies aimed at isolating novel biologically active compounds from macroalgae has increased worldwide (Frikha *et al*., 2011; Hakim & Patel, 2020; Yang *et al*., 2023). By in large, these studies have searched for bioactive compounds via bioassayguided fractionation (Murillo-Álvarez, 2001; Akbari *et al*., 2020).

Multiple biotic and abiotic factors affect the production of secondary metabolites and the structural chemistry of the seaweed, including herbivory, substrate availability, temperature, irradiance, nutrient concentrations, physiological conditions, and the reproductive stage of the alga (Lobban & Harrison, 1994; Pelletreau & Targett, 2008). Among secondary metabolites, terpenoids, phlorotannins, polyphenols, volatile hydrocarbons, and products of mixed biogenetic origin are of particular interest. These secondary metabolites exhibit chemical compositions, which are responsible for the potential use of algae as fertilizers, fodder, and food supplements (Rindi *et al*., 2012; Kim & Chojnacka, 2015).

Sargassum lapazeanum is a subtropical species son, 1944; Rocha-Ramírez & Siqueiros-Beltrones, 1990; Rivera & Scrosati, 2006), and like other macroalgae, creates microhabitats that provide refuge for

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other organisms, however, this alga has not been stud- ied very much, so it is necessary carry out research fo- cused on evaluation the potential of *S*. *lapazeanum* as a source of secondary metabolites, which is essential for better understanding of its biology and ecological interactions with the environment and other Sargassum species (Pelletreau & Targett, 2008). This study intends to expands the current knowledge of the phytochemistry and the biological activity (antioxidant, hemolytic, and cytotoxic) of *S*. *lapazeanum*.

MATERIALS AND METHODS

Biological material

Sargassum lapazeanum was manually collected in the intertidal zone of Tarabillas beach (24° 27' 55.1" N, 110° 41' 20.1" W) in Bahía de La Paz, BCS, Mexico. Algae were collected at a maximum depth of 1 m in the intertidal zone, washed with tap water to remove epiphytes or sand, and dried in the sun. Once dry, the samples were ground to a particle size of 1 mm with a manual mill and stored in plastic bags.

Ethanolic extract (EE)

Dried algae (300 g) were macerated with etha-
nol (96%) at room temperature for three days. Af-
ter which, the extract was filtered with filter paper (Whatman No. 4), and the algal residue was extracted five times under the same conditions. All obtained filtrates were pooled and concentrated to dryness under reduced pressure at 40 °C in a rotary evaporator (Yamato, Orangeburg, NY, USA). The obtained ethanolic extract (EE) was stored at -18 °C.

Ethanolic extract fractionation

Three grams of EE were adsorbed in 60 g of normal-phase silica gel and subjected to solid-liquid separation by sequential washing with n-hexane, dichloromethane, acetone, methanol, and water. Thinlayer chromatography (TLC) was used to combined the eluates, and six primary fractions were obtained (F0-F5). Each fraction was analyzed by TLC using normal-phase silica gel plates developed in dichloromethane-methanol eluting solution (9:1). The F4 fraction was selected for chromatographic column fractionation (CCF) with normal-phase silica gel and partitioned in a polarity gradient with n-hexane, dichloromethane, methanol, and water. Nine secondary fractions were obtained (FF0-FF8). The antioxidant and hemolytic activity, cytotoxicity, and phytochemical profiles of the nine fractions were evaluated.

Free radical scavenging activity bioautographic assay

The bioautographic assay was conducted based on the methodology described by Kannan *et al*. (2010), which uses the CCF technique. For this, glass plates were covered with normal-phase silica gel 60. A total of 20 μg mL^{-1} of the secondary fractions (FF0-FF8) and EE previously diluted in methanol were applied to the plates. The chromatographic plate was further developed in the solvent system (dichloromethane:methanol, 9:1) and dried at room temperature. The TLC plate was sprayed with 0.4% 2,2-diphenyl-1-picrylhydrazil (DPPH) in methanol and left in the dark for 30 min at room temperature. The presence of discolored (white) areas that contrast with the violet background of the developer solution indicated the presence of scavenging activity.

Hemolytic activity

The EE and FF0, FF2, FF3, FF4, FF6, FF7, and FF8 fractions were evaluated in TLC plates that were developed in the same way as those mentioned in the previous section. Hemolysis was evaluated by bioautographic TLC according to the method of Singh *et al*. (2022). Blood was obtained from an apparently healthy individual and suspended (9:1) in anticoagulant solution 3.5% sodium citrate. The blood was centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. The erythrocytic pellet was washed with a phosphate-saline solution and centrifuged at 3000 rpm for 10 min, and the supernatant was discarded. This process was repeated three times. The obtained sediment was suspended in phosphate-buffered saline (PBS) at pH 7 for a final concentration of 3% (v/v). The TLC plate was covered by this saline solution and allowed to stand vertically for 30 min at room temperature. At the end of the reaction, the discolored areas (white) were evaluated in contrast to the pink background of the developing solution, as these indicate erythrocyte hemolysis.

Acute toxicity in *Artemia franciscana*

The cytotoxicity of the EE and secondary fractions (FF0-FF8, except FF5) were evaluated in *A*. *franciscana* Linnaeus, following the methodology of McLaughlin *et al*. (1998). For each extract, three concentrations were analyzed (by triplicate) to deter- mine the dose-response relationship. The assay was performed in flat-bottom 96-well polystyrene mi-
croplates. The extracts were prepared in concentra-
tions of 10, 100, and 1000 μg mL⁻¹ dissolved in 5% dimethylsulfoxide (DSF) and filtered seawater. Then, 15 larvae of *A*. *franciscana* were added to each well. Sodium dodecylsulphate (DSD) and DSF were used as positive and negative controls, respectively. The plates were incubated for 24 h at 35 °C. After which, live larvae were counted, and the LC_{50} value was esti- mated using the statistical method of Probits.

Phytochemical screening

A preliminary phytochemical screening of EE and secondary fractions (FF0-FF8) was conducted following the methods of Harborne (1990). Chromatographic plates were prepared as previously described. After which, they were exposed to the following developing solutions: Dragendorff reagent (Sigma-Aldrich, St. Louis, MO, USA) for alkaloids; Lieberman-Burchard reagent for unsaturated sterols and unsaturated and saturated triterpenes; 5% FeCl, in n-butanol and 5% HCl for phenols, pyrogallic tan-

nins, and catecholic tannins; 1% AlCl₃ in ethanol for flavonoids; 10% KOH in ethanol for coumarins, anthraquinones, and anthrones; and 10% H_2SO_4 in ethanol for saponins. Subsequently, the plates were analyzed with ultraviolet light at 250 and 360 nm. The colorimetric reactions were interpreted as the pres- ence or absence of the chemical groups mentioned above.

Phytochemistry and biological activity

The retention factor (Rf) was used to assess the relationship between phytochemistry and pharmacological activity. This value expresses the position of a substance on the chromatographic layer. Rf values were calculated by dividing the distance traveled by the compound of interest by the distance traveled by the eluent (Kenndler, 2004). The compounds present in the fractions were observed in a TLC plate developed with a 10% H₂SO₄. Finally, pharmacological activities were related based on the similarity of the Rf values and those of the chemical compounds of the phytochemical analysis.

RESULTS

The fractionation process of *S*. *lapazeanum* is shown in Figure 1. The F2 and F3 fractions obtained from solid-liquid extraction were pooled due to their chromatographic similarity (Fig. 1a). Fraction F4 was chosen for further fractionation on a chromatographic column (Fig. 1b) because it had the highest number of compounds with high polarity.

DDPH scavenging activity

The autographic assay revealed relevant activity in the EE and its fractions. The FF4 and FF8 fractions exhibited the highest activity within the solvent system used for CCF (dichloromethane:methanol, 9:1;

Fig. 2), which was reflected in the high intensity of decolorization of the DPPH solution.

Hemolytic activity

Notably, fractions FF3, FF4 and FF6 showed the highest hemolytic activity according to the bioautographic assay. Most of these compounds were weakly polar within the solvent system (dichloromethane:methanol, 9:1). However, compounds of high polarity were also found in fraction FF6. Also of note, fraction FF7 contained an isolated compound of medium polarity, although compounds with higher hemolytic activity were present in the FF3 fraction, which was reflected in the discoloration intensity (Fig. 3).

Toxicity test in *Artemia franciscana*

Figure 4 shows the results of the mean lethal concentration (LC_{50}) . These values were inversely proportional to the toxicities shown by the fractions. With the exception of FF6 from the EE of *S. lapazeanum*, all fractions were found to have high acute toxicity against *A*. *franciscana*. The EE presented a mortality of 85% with a CL_{50} of 225.1 μ g mL⁻¹. After fractionation, maximum mortality (81%) was presented by the FF3 fraction (CL_{50} of 443.7 μ g mL⁻¹).

Phytochemical screening

The main compounds in the *S*. *lapazeanum* fractions were flavonoids and alkaloids, which were present in almost all fractions. Phenols, tannins, and saturated sterols or triterpenes were only present in FF2, FF3, and FF4. The FF3 fraction was shown to have the highest amount of phytochemical compounds tested. The fractions that showed the best fractionation, which was reflected in superior isolation between compounds, were FF1 with alkaloids and flavonoids,

Figure 1. Chromatographies of: a) crude extract (EE) and primary fractions; b)secondary fractions. The analysis was conducted on normal phase silica gel chromatographic plates, developed in the dichloromethane:methanol (9:1) eluent system and sprayed with a 0.25% vanillin solution in $10\% \, \text{H}_2\text{SO}_4$.

Figure 2. Autographic assay showing the antioxidant activity of the secondary fractions obtained from column fractionation (FF0-FF8) and the crude extract (EE) of *Sargassum lapazeanum*. The analysis was performed on a normal-phase silica gel thin layer chromatographic plate, developed in the dichloromethane:methanol (9:1) eluent system, and sprayed with the 0.04% DPPH solution.

FF5 with alkaloids and a minimal presence of unsaturated sterols, and FF7 with unsaturated triterpenes and a minimal presence of alkaloids (Table 1).

Evaluation of biological activity

The relationship between antioxidant and hemolytic activity and the results of the phytochemical assay were analyzed by means of the Rf value. It was observed that this relationship is inversely proportional to the polarity of the compound. Thus, it can be inferred that the antioxidant activity of *S*. *lapazeanum* is attributable to alkaloid compounds, triterpenes, unsaturated sterols, catechol tannins, anthrones, and anthraquinones, while its hemolytic activity is attributable to alkaloids, triterpenes, unsaturated sterols, phenols, pyrogallic tannins, and flavonoids. Among

the compounds with antioxidant activity, the alkaloids with an Rf value of 0.82 in the FF1 fraction, anthrones with an Rf value of 0.95 in the FF0 fraction, and anthraquinones with an Rf value of 0.96 in the FF2 fraction were noteworthy. Of the compounds with hemolytic activity, flavonoids with an Rf value of 0.76 in the FF2 fraction and the unsaturated triterpenes with an Rf value of 0.87 in the FF3 fraction stand out. Unsaturated sterols with Rf values of 0.70 and 0.72 in the FF5 and FF2 fractions, respectively, are also noteworthy (Table 2).

DISCUSSION

Seaweeds are known to be major sources of antioxidant compounds, including sulfated polysaccharides, terpenes, phlorotannins, carotenoid pigments

Figure 3. Bioautographic assay showing the hemolytic activity of the secondary fractions obtained from column fractionation (FF0-FF8) with the exception of FF1 and FF5 and the crude extract (EE) OF *Sargassum lapazeanum*. The analysis was performed on a normal-phase silica gel thin layer chromatographic plate, developed in the dichloromethane:methanol (9:1) eluent system, and sprayed with the 3% red blood cell suspension in PBS.

Figure 4. Percent mortality (bars) and LC50 (●) against *Artemia salina* nauplii, of fractions FF0-FF8, except FF5, of the ethanolic extract (EE) obtained from *Sargassum lapazeanum*.

(e.g., astaxanthin and fucoxanthin), sterols, catechins, and proteins (Indu & Seenivasan, 2013) . In *Sargassum* species, compounds, such as meroterpenoids, phlorotannins, fucoidans, sterols, and glycolipids, have been isolated and found to demonstrate antibacterial, antifungal, antiviral, anti-inflammatory, anticoagulant, antioxidant, hypoglycemic, lipid-low- ering, protective of the nervous system, and antime- lanogenic activity, suggesting that these species are rich sources of agents with the potential to support and improve human health (Liu *et al*., 2012; Indu & Seenivasan, 2013; Srivastava, 2013; Subramanian *et al*., 2014; Yende *et al*., 2014; Park *et al*., 2015; Meh- dinezhad *et al*., 2016).

In the present study, the DPPH free radical scavenging activity of the polar compounds present in the fractions of *S*. *lapazeanum* was demonstrated. This

algal species exhibits high potential as a source of antioxidant compounds. Based on the phytochemi- cal screening results, these compounds could include alkaloids, triterpenes, unsaturated sterols, catecholic tannins, and/or coumarins such as anthraquinones and anthrones. When observing the relevant scavenging activity of DPPH by coumarins (anthrones and an- thraquinones), it may be asserted that this group of polar compounds contributes to the important antioxi- dant activity observed in *S*. *lapazeanum*.

In recent years, progress has been made regarding the pharmaceutical applications of coumarins, especially those based on their antioxidant properties (Kostova *et al*., 2006). Studies that have sought to develop innovative agents by evaluating the interactions among coumarins and ROS have concluded that various coumarins are able to capture free radi-

Fraction Table 1. Phytochemical screening of the ethanolic extract fractions from *Sargassum lapazeanum*.

* Minimal presence

	Biological activity	Rf	Chemical compound	Fraction
		0.48	Anthrones	FF ₆
		0.49	Flavonoids	FF ₂
		0.52	Flavonoids and Anthraquinones	FF4, FF6
		0.53	Coumarins and Anthraquinones	FF3, FF4
		0.54	Flavonoids	FF3
		0.55	Alkaloids, Flavonoids and Anthraquinones	FF1, FF2, FF4
		0.56	Alkaloids, Unsaturated triterpenes, Flavonoids and Anthraquinones	FF6, FF7
		0.57	Unsaturated triterpenes	FF ₂
		0.59	Alkaloids	FF ₆
	Hemolytic	0.60	Alkaloids, Pyrogallic tannins and Flavonoids	FF1, FF2, FF3
		0.61	Anthraquinones	FF ₂
	Antioxidant	0.62	Unsaturated triterpenes, Catecholic tannins and Anthraquinones	FF2, FF3, FF6
		0.63	Alkaloids, Unsaturated triterpenes, Unsaturated sterols, Pyrogallic FF1, FF2, FF3, tannins and Flavonoids	FF4, FF6
	Hemolytic	0.64	Pyrogallic tannins and Flavonoids	FF3, FF4
		0.65	Sterols	FF4
		0.66	Alkaloids, Sterols and Flavonoids	FF1, FF2, FF3, FF4
Polarity	Hemolytic	0.67	Unsaturated triterpenes and Phenols	FF2, FF4
		0.68	Sterols	FF ₂
		0.69	Anthraquinones and Saponins	FF2, FF3
	Antioxidant and He- 0.70 molytic		Unsaturated sterols	FF5
	Antioxidant and He-0.71 molytic		Unsaturated sterols and Unsaturated triterpenes	FF2, FF3, FF4, FF5, FF6, FF7
	Antioxidant and He- 0.72 molytic		Unsaturated sterols	FF ₂
		0.74	Saponins	FF3, FF4
	Hemolytic	0.76	Flavonoids	FF ₂
	Antioxidant	0.78	Alkaloids and Anthraquinones	FF1, FF2
	Antioxidant	0.82	Alkaloids	FF1
		0.85	Alkaloids	FF1
	Hemolytic	0.87	Unsaturated triterpene	FF3
	Hemolytic	0.88	Alkaloids and Unsaturated triterpene	FF1, FF2
		0.93	Unsaturated triterpene	FF ₂
	Antioxidant	0.95	Anthrones	FF ₀
	Antioxidant	0.96	Anthraquinones	FF ₂
		0.97	Alkaloids and Flavonoids	FF0, FF1
		0.98	Alkaloids and Unsaturated triterpene	FF0, FF2
		0.99	Unsaturated triterpene and Flavonoids	FF2, FF3

Table 2. Rf values of the chemical compound and the biological activity of the FF0-FF8 fractions obtained from *Sargassum lapazeanum*.

cals and may protect against tissue damage caused by the degeneration of oxidants, including oxidative stress associated with aging (Martín-Aragón, 1994). Hydroxycoumarins are typical phenolic compounds that act as potent metal chelators and free radical scavengers. For example, coumarin 7-hydroxyl-3- (1,1-dimethylprop-2-enyl), which was isolated from *Sargassum wightii* and *Sargassum polycystum*, may be involved in the antioxidant and antitumor activities shown by this species (Yuvaraj & Arul, 2014). It is highly likely that *S*. *lapazeanum* produces similar coumarins to those reported in other *Sargassum* species.

Erythrocytes provide a simple way to study the protective or toxic effects of a wide variety of substances and processes associated with oxidative stress (Mole & Sabale, 2014). In the present study, the hemolytic activity of polar *S*. *lapazeanum* compounds could be related to alkaloid compounds, triterpenes, unsaturated sterols, phenols, pyrogallic tannins, and flavonoids. Triterpenes exhibited the most evident hemolytic activity; however, there is no evidence of hemolytic terpenes in *Sargassum* species. Given that antibacterial activity is the main biological activity attributed to *Sargassum* species, it is probable that these types of compounds have both antibacterial and hemolytic potential (Devi *et al*., 2013; Kannan *et al*., 2013; Asha-Kanimozhi *et al*., 2015). Triterpenes glycosides or terpenoid saponins are also included in this group. These compounds are mainly known for their ability to interact with cell membranes by modifying structural and functional properties, although they have also been shown to possess a large pharmacological spectrum of compounds with remarkable hemolytic and cytotoxic activities (Popov, 2002). It is probable that the compounds with hemolytic activity present in *S*. *lapazeanum* are glycosidic (i.e., triterpenoid saponins) due to the great hemolytic potential that has been reported for this phytochemical group (Devi *et al*., 2013; Asha-Kanimozhi *et al*., 2015).

The highest toxicity of *S*. *lapazeanum* against *A*. *franciscana* was observed in samples that mainly shared alkaloid compounds, flavonoids, anthrones, and saponins. Some authors mention that notable le-
thality was evident in A. franciscana based on LC_{50} values <1000 μg mL⁻¹, indicating the presence of a³ potent cytotoxic agent or probable insecticide, although additional studies are needed (Ara *et al.*, 1999). Within the *Sargassum* genus, LC₅₀ values have been reported for different compounds and species, including the hexanic extract of \bar{S} . *myriocystum* (LC₅₀ of 273.28 μg mL⁻¹) and the methanolic extracts of \bar{S} . *wightii* (LC₅₀ of 161 μg mL⁻¹), *S. polycystum* (LC₅₀ of 250 to 500 μg mL⁻¹ and 502 μg mL⁻¹ to 617 μg mL⁻¹), *S. swartzii* LC₅₀ of 928 μg mL⁻¹ to 61 μg mL⁻¹), and *S. binderi* (735 μg mL⁻¹ to 121 μg mL⁻¹). The different LC_{50} values of the last three species are due to the type of extract examined (Ara *et al.*, 1999; Orhan *et al*., 2003; Iyapparaj *et al*., 2012; Daud *et al*., 2015; Kurniatanty *et al*., 2015; Asha-Kanimozhi *et* value of 225.1 μg mL⁻¹ in the case of the crude extract, followed by an LC₅₀ value of 443.7 μg mL⁻¹ in the FF3 fraction. This reflects moderate toxicological activity, which may be the result of the synergistic effect of several compounds present in the EE. Thus, fractionation could enhance toxicity to match or exceed the toxicity of the EE.

The flavonoids present in *S*. *lapazeanum* showed relevant hemolytic activity. Sulfated flavonoids are a group of conjugated metabolites in which the sulfate component represents an adaptation to the marine environment (Jensen *et al*., 1998). These compounds have important effects on plant biochemistry and physiology because they act as antioxidants, enzyme inhibitors, and precursors of pigments that protect against light damage and toxic substances. In addition, these compounds are involved in defense moids isolated from an aqueous extract of *S*. *poly-cystum* were found to be responsible for important toxicological activities (Arsianti *et al*., 2020). Toxic- ity results with *A*. *franciscana* were similar to those obtained from assays that have evaluated anticancer, antitumor, antiproliferative, and larvicidal activities against mosquito species such as *Aedes aegypti* and

Culex quinquefasciatus (Ara *et al*., 1999; Ali *et al*., 2013; Kurniatanty *et al*. 2015). It is very probable that the flavonoids present in *S*. *lapazeanum* that exhibited relevant toxicological and hemolytic activity are of a sulfated nature, which suggests that they may perform well as anticancer agents.

Preliminary analysis of bioactive compounds present in *Sargassum lapazeanum* showed that this alga has a high potential as a source of antioxidant compounds, hemolytic and cytotoxic activity, although further research is needed to isolate bioactive compounds and their possible application in pharmaceuticals.

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