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Research Article

Antiviral activity of red algae phycocolloids against herpes simplex virus type 2 *in vitro*

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ABSTRACT

Herpes simplex virus type 2 (HSV-2) is a human infectious agent with significant impact on public health due to its high prevalence in the population and its ability to elicit a wide range of diseases, from mild to severe. Although several antiviral drugs, such as acyclovir, are currently available to treat HSV-2-related clinical manifestations, their effectiveness is poor. Therefore, the identification and development of new antiviral drugs against HSV-2 is necessary. Seaweeds are attractive candidates for such purposes because they are a vast source of natural products due to their highly diverse compounds, many with demonstrated biological activity. In this study, we evaluated the *in vitro* antiviral potential of red algae extracts obtained from *Agarophyton chilense, Mazzaella laminarioides, Porphyridium cruentum,* and *Porphyridium purpureum* against HSV-2. The phycocolloids agar and carrageenan obtained from the macroalgae dry biomass of *A. chilense* and *M. laminarioides* and the exopolysaccharides from *P. cruentum* and *P. purpureum* were evaluated. The cytotoxicity of these extracts and the surpluses obtained in the extraction process of the agar and carrageenans were evaluated in human epithelial cells (HeLa cells) in addition to their antiviral activity against HSV-2, which were used to calculate selectivity indexes (SIs). Several compounds displayed antiviral activity against HSV-2, but carrageenans were not considered as a potential antiviral therapeutic agent when compared to the other algae extracts with a SI of 23.3. Future assays *in vivo* models for HSV-2 infection should reveal the therapeutic potential of these algae compounds as new antivirals against this virus.

1. Introduction

Algae are heterogeneous and large groups of unicellular or multicellular organisms that can be either prokaryotic or eukaryotic. Microalgae have been widely studied because they are an important source of natural products, such as extracellular sulfated polysaccharides [1,2]. Huheihel et al. 2001 [3] reported that sulfated polysaccharides extracted from algae of the genus Porphyridium exhibit antiviral potential against Herpes simplex virus type 2 (HSV-2). HSV-2 is highly prevalent in humans, affecting more than 400 million people worldwide as of 2012 according to the World Health Organization (WHO), which represented 11% of the global population at the time [4]. More importantly, approximately 20 million new cases are estimated to occur each year [5]. Although in most cases HSV-2 infection is asymptomatic, this virus can produce varied clinical manifestations that range from mild to severe disease that may sporadically reactivate symptomatically throughout the lifespan of infected individuals [5,6]. The most common clinical manifestations produced by HSV-2 are genital lesions and neonatal encephalitis, but the virus may also spread to other tissues in immunosuppressed individuals, such as those experiencing acquired immunodeficiency syndrome (AIDS) due to infection with the human immunodeficiency virus (HIV) [5]. Genital skin lesions are initially characterized by the formation of a prodrome phase or erythema that are followed by the formation of vesicles that, when ruptured, give way

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to painful ulcerative lesions that can last up to 12 days [7]. This clinical condition may be more extensive, aggressive, and painful in immunocompromised patients [8].

Currently, several antiviral drugs are being used as palliative and preventive methods against the cutaneous effects of HSV-2 [9–11]. Topical creams with acyclic nucleoside analogs, such as acyclovir (ACV), can be used locally to treat HSV-2 lesions because they selectively inhibit the viral DNA polymerase [12–14]. Although ACV is one of the most common drugs used for the treatment of symptomatic HSV-2, it only reduces the duration of the skin lesions by 1–2 days from a total of 8–10 days [15–17].

HSV-2 is a neurotropic virus that can establish lifelong latency in neurons that enervate skin areas related to the primary infection, particularly neurons of the dorsal root ganglia of the sacral area. In these cells, HSV-2 evades the host's immune response by limiting viral protein expression through the silencing of viral mRNAs [5,6], this feature makes HSV-2 challenging in terms of identifying solutions that can either resolve or prevent infections [18]. As a strategy in the last decades, numerous efforts have been made at characterizing natural products with antiviral activity, including algae extracts, in order to identify new compounds that inhibit virus replication and can potentially be used to treat viral infections [11,19,20]. These studies have shown that marine algae are exceptionally rich sources of bioactive compounds [21–23]. As a result, several bioactive compounds with antiviral activity have been characterized that include organic antivirals, such as lipids, proteins, and carbohydrates [11,24]. In rhodophyte algae, carbohydrates are sulfated, such as polygalactan macromolecules present in the amorphous matrix of the cell wall, which are highly valued in the food industry as gelling, viscosifying and emulsifying agents [25,26]. Fig. 1 shows the chemical structure of the monomers of the main carbohydrates found in rhodophyte algae.

Carrageenans are one of the most valued sulfated polysaccharides obtained from algae. They are formed by alternative subunits of D-galactose and 3,6-anhydrous-galactose that are linked through β−1,4-glycosidic bonds, presenting 15% to 40% ester-sulfate content [25]. These compounds have been reported to have antiviral potential against a broad spectrum of viruses, including pathogens such as HIV, cytomegalovirus (CMV), herpes simplex viruses (HSVs), and other viruses, such as vesicular stomatitis virus (VSV) [28–33]. Previous investigations have reported *in vitro* antiviral effects of sulfated polysaccharides from various red algae against HSV-2 that had low cytotoxicity and potent antiviral activity, blocking up to 100% infection [11,34–39].

Chile has a rich diversity of seaweed species, some of which have been traditionally extracted from natural beds for the production of phycocolloids [40]. Among them, *Agarophyton chilense (Gurgel, Norris & Fredericq, 2018) (ex Gracilaria chilensis (Bird, McLachlan & Oliveira, 1986))* and *Mazzaella laminarioides (Fredericq, 1993),* two rhodophyta macroalgae species, are extensively used for the extraction of agar-agar and carrageenans, respectively, which are two phycocolloids that are highly demanded by the food, pharmaceutical, and nutraceutical industries [41].

On the other hand, *Porphyridium cruentum (Naegeli, 1849)* and *Porphyridium purpureum (Drew & Ross, 1965)* are red microalgaes. These microalgaes are bound in mucilage, which is a compound constantly excreted by the cell. This forms a capsule surrounding the cell that contains polysaccharide sulfate, known as sPS [42,43]. Raposo et al. 2014 [44] evaluated the antiviral activity of sulfated polysaccharides from two strains of *P. cruentum*, one from Spain and one from Israel. This

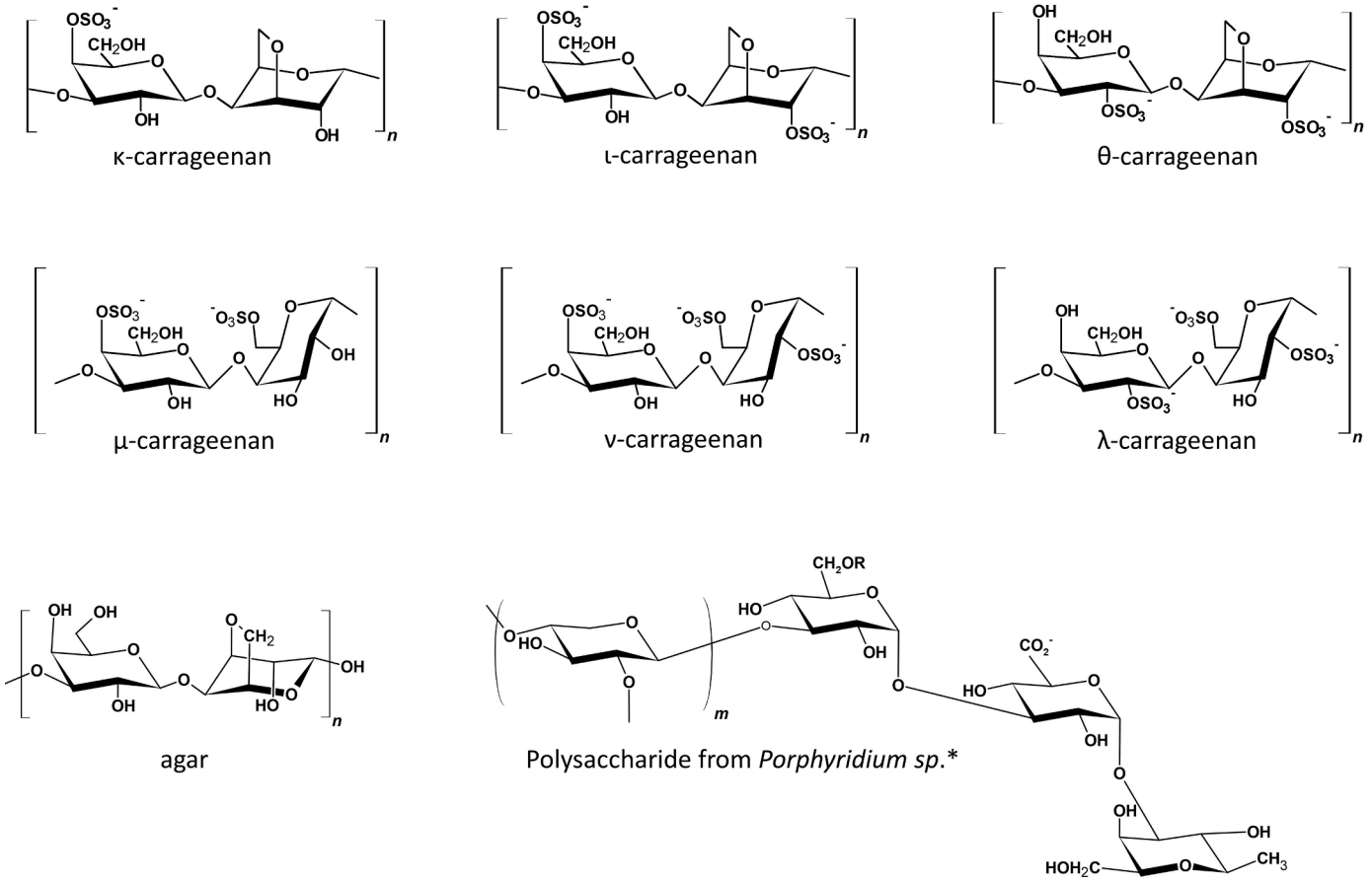

Fig. 1. Chemical structure of red algal phycocolloids. *Proposed structure ($m = 2$ or 3) of a backbone of polysaccharide from *Porphyridium* sp. according to the data of partial acid hydrolysis [27]. Molecular structures were drawn using ACD/ChemSketch™, (version *2019.2.2, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2020)*.

found that the strain from Spain showed higher antiviral activity against HSV-1, HSV-2, Vaccinia virus (VV) and Vesicular stomatitis virus (VSV).

However, to our knowledge, there are no studies related to the potential activity of compounds extracted from *A. chilense* or *M. laminariodes* against HSV-2. In this work, we evaluated the antiviral activity of phycocolloids obtained from two species of rhodophyta macroalgae, namely *A. chilense* and *M. laminarioides*, and from two species of red microalgae, specifically *P. cruentum*, and *P. purpureum*, against HSV-2 *in vitro*.

2. Materials and methods

2.1. Collection and purification of red algae phycocolloids

Microalgae cultures were started with 10 mL of suspension of *P. cruentum* (UTEX 161) and *P. purpureum* (CCAP 1380/3) strains in Walne medium. Each 10 mL of microalgae culture was added to 140 mL of Walne medium supplemented with vitamins. The preparation was incubated for 13 days in a 16 h light/8 h dark alternating regime with a light intensity of 200 μmol photons m^{-2} s^{-1} and an agitation of 150 RPM. Then, each 150 mL of culture was inoculated into 850 mL of Walne's medium supplemented with vitamins under the conditions mentioned above. Exopolysaccharides from *P. cruentum* and *P. purpureum* were obtained according to a modified extractive procedure described by Arias et al. 2016 [45]. The microalgal cultures were centrifuged at 4000 RPM for 6 min in order to separate the precipitated biomass from the supernatant. Then, 100 g (10% w/v) of sodium acetate ($NaCH_3COO$) was added to each culture. The pH was adjusted to 7.0 by adding glacial acetic acid (HCH_3COO) (Merck, Germany). Then, the preparation was incubated with ethanol 96% (Droguería Diprolab, Chile) at a 2:5 proportion to precipitate phycocolloids present in the extract and centrifuged at 4000 RPM for 10 min. The exopolysaccharides (pellet) were diluted in deionized water and dialyzed with a 3.5 kDa MWCO dialysis membrane (Spectra/Por® 3, Spectrum Laboratories) using 2 L of deionized water, which was replaced three times in 6 h. Finally, exopolysaccharides were frozen at −20 °C and lyophilized for five days at −70 °C ± 1 °C. The yield was determined by using the following equation (Eq. (1)).

$$yield\ (\%) = \left(\frac{dry\ hydrocolloid\ (g)}{dry\ algae\ (g)}\right) \times 100\% \quad (1)$$

On the other hand, phycocolloids from *A. chilense* and *M. laminarioides* were obtained according to a modified extractive procedure described by Souza et al. 2012 [46] and Arias *et al.* 2016 [45], respectively. The red macroalgae *A. chilense* and *M. laminarioides* were collected at Caleta Lenga (Latitude −36.764330°; Longitude −73.173123°) and Caleta Cocholgüe (Latitude −36.590200°; Longitude −72.980159°) respectively, in Biobío Region, Chile. The collection of specimens of both *A. chilense* and *M. laminariorides* was carried during the month of June in winter. The red macroalgae were washed with water in order to eliminate salts, sand, and epiphyte organisms.

Then, the macroalgae were frozen at −20 °C and lyophilized for five days at −70 °C ± 1 °C using an FDU-7006 Freeze Dryer (Operon Co. Ltd., Korea), ground with a cast iron corn grinder, and filtered through a 0.5 mm mesh (U.S. Standard Sieve Series, Dual Manufacturing Co). For *A. chilense,* 15 g of lyophilized alga powder was resuspended in 1000 mL of deionized water (1.5% w/v) and maintained under agitation for 15 h at 200 RPM and 25 °C. The residual biomass recovered was incubated at 90 °C for 45 min in 1000 mL of deionized water. The preparation was then centrifuged at 4000 RPM for 7 min (Rotofix 32 A, Hettich, Germany), and subsequently the supernatant was recovered and incubated with ethanol 96% (Droguería Diprolab, Chile) at a 1:3 proportion to precipitate the agar in the extract. Finally, both the supernatant and agar were frozen at −20 °C and lyophilized for five days at −70 °C ± 1 °C.

The procedure for obtaining agar from the freeze-dried algal biomass of *A. chilense* is shown in Supplementary Figure 1.

Regarding *M. laminarioides,* 10 g of lyophilized alga powder was resuspended in 1000 mL of deionized water (1.0% w/v) and maintained under agitation for 2 h at 90 °C under magnetic agitation. The preparation was then centrifuged at 4000 RPM for 10 min. The supernatant was then recovered and dialyzed with a 3.5 kDa MWCO dialysis membrane (Spectra/Por® 3, Spectrum Laboratories) using 2 L of deionized water, which was replaced three times in a 6 h timeframe. The preparation was concentrated by solvent evaporation (Multivapor™ P-6 / P-12, Büchi). The concentrated preparation was incubated with ethanol 96% (Droguería Diprolab, Chile) at a 1:5 proportion to precipitate the carrageenan present in the extract. Finally, the carrageenan was frozen at −20 °C and lyophilized for five days at −70 °C ± 1 °C. The procedure for obtaining carrageenan from the freeze-dried algal biomass of *M. laminarioides* is shown in Supplementary Figure 2.

2.2. Fourier transform infrared spectroscopy analysis of phycolloids obtained from red microalgae and red macroalgae

The Fourier Transform infrared spectroscopy (FTIR) spectra of dried exopolysaccharides extracted from both *P. cruentum* and *P. purpureum*, agar and supernatant extracted from *A. chilense*, and carrageenan and supernatant extracted from *M. laminarioides* were recorded using the Spotligh 400 FT-IR system (Perkin Elmer Instrument). This was equipped with an attenuated total reflectance (ATR) sampling device in the wavenumber range of 4000–399 cm^{-1}, and 16 accumulations at a spectral resolution of 8 cm^{-1}.

2.3. Evaluation of cytotoxicity and antiherpetic activity of red algae phycocolloids

2.3.1. HeLa cell cultures

Monolayers of HeLa cells (ATCC® CCL-2™) were grown in culture dishes in Dulbecco's modified Eagle Medium DMEM (Thermo Fisher Scientific Inc., MA, USA) with 5% Fetal Bovine Serum (FBS) Gibco® (Thermo Fisher Scientific Inc., MA, USA) supplemented with 0.1% pyruvate (Thermo Fisher Scientific Inc., MA, USA), 0.1% non-essential amino acids (Thermo Fisher Scientific Inc., MA, USA), and 0.1% penicillin/streptomycin (Thermo Fisher Scientific Inc., MA, USA) with pH 7.2. These cells were incubated at 37 °C in a 5% CO_2 atmosphere.

2.3.2. Virus preparation

Vero cells (ATCC® CRL-1586) were used to propagate HSV-2 (333) ZAG, which is a recombinant virus that encodes the gene for the green fluorescent protein (GFP) in its genome under the control of a constitutive human cytomegalovirus-CMV promoter [47]. In order to determine the titer of this virus, supernatants of infected cells DARGAN were collected and serially diluted over Vero cells cultured in flat-bottom 96-well plates with Opti-MEM™ Reduced-Serum Medium (Thermo Fisher Scientific Inc., MA, USA).

2.4. Cell viability assessment

HeLa cells were cultured in black wall 96-well clear-bottom plates and treated with algae carrageenans at decreasing concentrations for 24 h and then incubated with AlamarBlue® (Thermo Fisher Scientific) reagent at a 1:10 v/v ratio for 1 hour at 37°C in a 5% CO_2 atmosphere. Cell viability was determined by measuring fluorescence intensity at 595 nm (Ex564 nm), which is proportional to the metabolic rate of the culture and thus its viability. Fluorescence measurements were carried out using a Synergy Neo HTS Multi-Mode Reader (Biotek Instruments, WI, USA). As a control for cell death, 70% ethanol (EtOH) was added to the cells for 10 min. prior to the addition of the AlamarBlue® reagent. The ratios of the 50% cytotoxic concentration (CC_{50}) were obtained by interpolating in log(dose) vs. response curves. All the experiments were done in triplicates.

2.5. Treatments with the algal phycocolloids and HSV-2 infections

HeLa cells were cultured in black wall 96-well clear-bottom plates and treated with algae phycocolloids at decreasing concentrations for 1 hour at 37 °C in a 5% CO_2 atmosphere. Then, the supernatants were removed and the cells were infected for one hour with HSV-2 (333) ZAG at a multiplicity of infection (MOI) of 1, at 37 °C in a 5% CO_2 atmosphere. Infected untreated cells (UT) and acyclovir treatment (ACV, 50 μg/mL) were used as positive and negative controls respectively, and uninfected cells were also used as a negative control (UI) [6]. A Synergy Neo HTS Multi-Mode Reader (Biotek Instruments, WI, USA) was used to quantify GFP fluorescence derived from algae-carrageenan-treated cells infected with HSV-2. Using these data, the EC_{50} was determined using non-linear regression. All the experiments were done in triplicates. The selectivity index (SI) was determined using the following equation (Eq. (2)) [48].

$$SI = \frac{CC_{50}}{EC_{50}} \quad (2)$$

2.6. Statistical analysis

The experiments carried out with red macroalgae were performed in triplicate, and the values were expressed as the arithmetic means with the respective uncertainty measure expressed as Standard Error of the Mean (SEM). The differences between samples were determined by a one-way ANOVA with a Dunnet's test for multiple comparison using the GraphPad Prism® software version 6.01.

3. RESULTS

3.1. Red algae phycocolloids extraction

For the red microalgae, the yield of exopolysaccharides (EPS) extraction was 0.267 g L^{-1} in *P. cruentum,* and 0.553 g L^{-1} in *P. purpureum.* The yield of phycocolloids extraction was 7.1 ± 1.77% [w/w] agar and 24.4 ± 5.94% [w/w] carrageenan from the macroalgae dry biomass of *A. chilense* and *M. laminarioides,* respectively.

3.1.1. Algae phycocolloids hamper the expression of HSV-2-encoded genes

In order to assess any potential toxic effects of algae phycocolloids over the cells evaluated in this study, culture viability was determined 24 h after treating the cultures with varying concentrations of the alga extracts. As a positive control for cell death, ethanol (EtOH) was used, which significantly reduces resazurin reduction by the cells and subsequently indicates diminished cell viability, as indicated by the AlamarBlue® assay (Fig. 2). For *P. cruentum,* no significant differences were observed in cell viability in response to exopolysaccharide concentrations between 0.078 and 1.125 g L^{-1} when compared to the negative control consisting of untreated cells ($p < 0.0001$) (Fig. 2A). Similarly for *P. purpureum,* no significant differences were observed in cell viability for exopolysaccharide concentrations between 0.078 and 2.500 g L^{-1} when compared to the negative control ($p < 0.0001$) (Fig. 2B). For *M. laminarioides,* no significant differences were observed in cell viability for the supernatant at concentrations between 0.078 and 1.125 g L^{-1} when compared to the negative control ($p < 0.0001$) (Fig. 2C). Also, no significant differences were observed for cell viability with carrageenan concentrations between 0.078 and 0.625 g L^{-1} when compared to the negative control ($p < 0.0001$) (Fig. 2D). For *A. chilense,* no significant differences were observed in cell viability for both supernatants at concentrations between 0.078 and 0.312 g L^{-1} when compared to the negative control ($p < 0.0001$) (Fig. 2E). Finally, no significant differences were observed in cell viability for the agar at concentrations between 0.078 and 0.312 g L^{-1} when compared to the negative control ($p < 0.0001$) (Fig. 2F).

50% cytotoxic concentrations (CC_{50}) for these compounds were 3.84, 2.48, 2.12, 2.10, 2.06, and 1.68 g L^{-1} in *P. purpureum* exopolysaccharides, *A. chilense* agar, *P. cruentum* exopolysaccharides, *M. laminarioides* carrageenan, *A. chilense* supernatant, and *M. laminarioides* supernatant, respectively (Table 1).

To assess whether the algae phycocolloids interfere with the replication cycle of HSV-2, different concentrations of phycocolloids were evaluated over the cells. Fig. 3 shows representative images of the virus GFP-derived fluorescence in HeLa cells infected with HSV-2 and different hydrocolloid concentrations. In this figure, it is possible to observe that the phycocolloids from the red macroalgae *A. chilense* and *M. laminarioides* reduced the virus GFP-derived fluorescence at lower concentrations when compared with phycocolloids obtained from the red microalgae *P. cruentum* and *P purpureum.*

In *P cruentum,* significant differences were observed in the antiviral activity of the exopolysaccharide at concentrations between 1.250 and 5.000 g L^{-1} when compared to the negative control consisting of untreated cells ($p < 0.0001$) (Fig. 4A). On the other hand, significant differences were observed with *P. purpureum* regarding the antiviral activity of exopolysaccharide at concentrations between 0.625 and 5.000 g L^{-1}, as compared to the negative control ($p < 0.0001$) (Fig. 4B). The EC_{50} values in *P cruentum* and *P purpureum* extracts were 0.93 and 0.44 g L^{-1}, respectively (Table 1). These results indicate that *P purpureum* extracts reduce the GFP fluorescence associated to HSV-2(333) ZAG with a lower concentration than *P cruentum* exopolysaccharides.

For *M. laminarioides,* significant differences were observed for the antiviral activity of the supernatant at concentrations between 0.156 and 5.000 g L^{-1} when compared to the negative control ($p < 0.05$) (Fig. 4C). Additionally, significant differences were observed regarding the antiviral activity of carrageenan at concentrations between 0.078 and 5.000 g L^{-1} compared to the negative control ($p < 0.05$) (Fig. 4D). These results are in agreement with the EC_{50} values obtained, where high differences between *Mazaella laminaroides* supernatant and *Mazaella laminaroides* carrageenan were observed. The determined EC_{50} values for *M. laminarioides* supernatant and *M. laminarioides* carrageenan. were 0.27 and 0.09 g L^{-1}, respectively (Table 1). These results suggest that a lower concentration of *M. laminarioides* carrageenan is required to decrease the GFP fluorescence associated with HSV-2 in order to interfere with the replication cycle of this virus.

For *A. chilense*, significant differences were observed for cells infected and treated with the supernatant at concentrations between 0.312 and 5.000 g L^{-1} compared to the negative control ($p < 0.001$) (Fig. 4E). On the other hand, significant differences were observed for cells infected with HSV-2 and treated with concentrations between 0.312 and 5.000 g L^{-1} ($p < 0.0001$) of *A. chilense*'s agar (Fig. 4F). A slight difference was found between the supernatant and agar of *A. chilense* regarding the EC_{50} with values of 0.22 and 0.20 g L^{-1}, respectively (Table 1). Currently, there are no reports in the literature regarding the antiviral activity of *A. chilense* over HSV-2. Nevertheless, there are reports on algae from the genus *Gracilaria* that showed antiviral activity against HSV-2 [57]. To determine the algae extracts' therapeutic potential, the selective index (SI) for each extract and isolated compound was determined. The SIs obtained are presented in Table 1.

3.2. Fourier transform infrared spectrum of isolated phycocolloids from red algae

To characterize the functional organic groups present in the agar from *A. chilense*, an infrared absorption spectrum was measured, and it was later adjusted according to an algorithm based on Fourier transformations (Fourier Transformed InfraRed, FTIR) [58].

FTIR spectra of algae exopolysaccharides obtained from red microalgae *P cruentum* and *P purpureum* in the range 4000 – 500 cm^{-1} are presented in Fig. 5A and 5B, respectively. The FTIR of sulphated exopolysaccharides from *P. cruentum* show a broad band at 3413 cm^{-1} assigned to O—H stretching vibration [59], the band at 2927 cm^{-1} was assigned to C—H stretching vibrations [59], the bands at 1736 cm^{-1} and

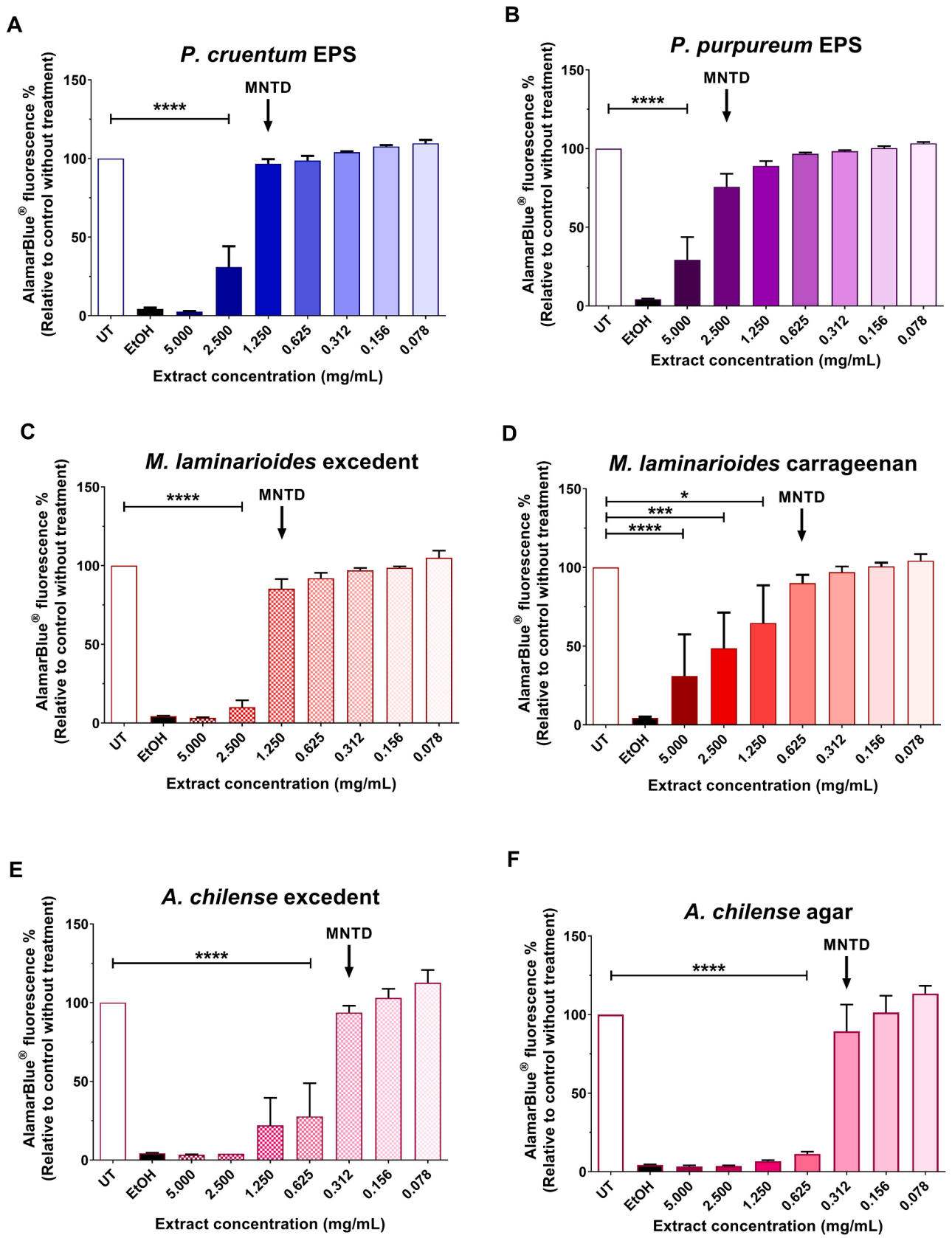

Fig. 2. Maximum nontoxic dose (MNTD) of the extracted algae phycocolloids. HeLa cells were incubated with serially diluted algae phycocolloids for 24 h, starting with 5 mg/ml. Cell viability was evaluated at 24 h post-treatment using AlamarBlue®. MNTD for HeLa cells treated with (A) *P. cruentum* exopolysaccharides. (B) *P. purpureum* exopolysaccharides. (C) *M. laminarioides* supernatant. (D) *M. laminarioides* carrageenan. (E) *A. chilense* supernatant. (F) *A. chilense* agar. UT corresponds to Untreated cells, and EtOH represents cells treated with 70% ethanol. Data shown are means ± SEM (Standard Error of the Mean) of three independent experiments. The data were analyzed using one-way ANOVA test with a Dunnet's test for multiple comparisons. $^{****}p < 0.0001$; $^{***}p < 0.001$; $^{**}p < 0.01$; and $^{*}p < 0.05$.

Table 1
Phycocolloids with antiviral activity extracted from marine algae.

Specie	Origin	Type of extraction	Composition	Reference
Porphyridium cruentum	Switzerland (UTEX 161)	Microfiltration	Xylose, galactose, glucose and glucuronic acids in the molar ratios of 1.5/1.3/0.6/0.5	Balti et al. 2018 [49]
Porphyridium purpureum	Switzerland (SAG Culture Collection)	Alcohol precipitation at room temperature	46 ± 1.4% (207 ± 9.1 mg $L-1$) xylose, 30% ± 0.5 (132 ± 3.3 mg $L-1$) galactose, 20 ± 0.7% (92 ± 2.2 mg $L-1$) of glucose and 3.8 ± 0.22% (17 ± 0.86 mg $L-1$) glucuronic acid.	Medina-Cabrera et al. 2020 [50]
Mazaella laminarioides	Chile	Hot extraction	K-carrageenans in cystocarpic and vegetative individuals λ-carrageenans in tetrasporic individuals	Arias et al. 2016 [45]
Gracilaria birdiae	Brazil	Hot extraction	Galactose (65.4%), 3,6-anhydrogalactose (25.1%), 6-O-methylgalactose (9.2%) and 3-O- and 4-O-methyl-galactose (0.33%)	Souza et al. 2012
Enteromorpha compresa	India	Extraction at 90 °C for 8 h in N,N-dimethylformamide	Rhamnose, galactose, glucose, xylose and glucuronic acid in the molar ratios 49/9/15/13/14	Lopes et al. 2017 [51]
Undaria pinnatifida	Korea	Extraction with 0.1 N HCl and 75% ethanol precipitation	Molecular weight molecules 3749–2569 g/mol fucose:galactose:mannose in the molar ratios 8:7:4 2122–2059 g/mol fucose:galactose:mannose in the molar ratios 3:5:5 1644 g/mol fucose:galactose:mannose in the molar ratios 3:5:2 1389 g/mol fucose:galactose:mannose in the molar ratios 3:3:1	Kim et al. 2017 [52]
Soliería filiformis	Mexico	Enzymatic assisted extraction	Monosaccharide composition (μg mg-1 dry weight) Arabinose 0.09 ± 0.02, galactose 10.62 ± 0.9, glucuronic acid 0.24 ± 0.05, glucosamine 0.38 ± 0.03, glucose 6.35 ± 0.7, mannose galactose 2.52 ± 0.5, rhamnose 0.28 ± 0.02, ribose 0.87 ± 0.006	Peñuela et al. 2021 [53]
Nostoc muscorum	Egypt	Extraction with 20 mM potassium phosphate buffer assisted by ultrasound	Lectin	Saad et al. 2022 [54]
Undaria pinnatifida	New Zealand	Extraction for 6 h with 1% (w/v) $H2SO_4$ at 20 °C, 0.2 M HCl at 20 °C, or 2% $CaCl_2$ at 75 °C.	Sulfated galactofucan	Harden et al. 2019 [55]
Splachnidium rugosum			Sulfated fucan	
Gigartina atropurpurea			Sulfated galactan	
Plocamium cartilagineum			Complex sulfated galactan	
Sargassum patens	China	Extraction with boiling water and ethanol precipitation	Sulphated polysaccharide	Zhu et al. 2004 [56]
Specie	Origin	Type of extraction	Composition	Reference
Porphyridium cruentum	Switzerland (UTEX 161)	Microfiltration	Xylose, galactose, glucose and glucuronic acids in the molar ratios of 1.5/1.3/0.6/0.5	Balti et al. 2018 [49]
Porphyridium purpureum	Switzerland (SAG Culture Collection)	Alcohol precipitation at room temperature	46 ± 1.4% (207 ± 9.1 mg L^{-1}) xylose, 30% ± 0.5 (132 ± 3.3 mg L^{-1}) galactose, 20 ± 0.7% (92 ± 2.2 mg L^{-1}) of glucose and 3.8 ± 0.22% (17 ± 0.86 mg L^{-1}) glucuronic acid.	Medina-Cabrera et al. 2020 [50]
Mazaella laminarioides	Chile	Hot extraction	K-carrageenans in cystocarpic and vegetative individuals λ-carrageenans in tetrasporic individuals	Arias et al. 2016 [45]
Gracilaria birdiae	Brazil	Hot extraction	Galactose (65.4%), 3,6-anhydrogalactose (25.1%), 6-O-methylgalactose (9.2%) and 3-O- and 4-O-methyl-galactose (0.33%)	Souza et al. 2012
Enteromorpha compresa	India	Extraction at 90 °C for 8 h in N,N-dimethylformamide	Rhamnose, galactose, glucose, xylose and glucuronic acid in the molar ratios 49/9/15/13/14	Lopes et al. 2017 [51]
Undaria pinnatifida	Korea	Extraction with 0.1 N HCl and 75% ethanol precipitation	Molecular weight molecules 3749–2569 g/mol fucose:galactose:mannose in the molar ratios 8:7:4 2122–2059 g/mol fucose:galactose:mannose in the molar ratios 3:5:5 1644 g/mol fucose:galactose:mannose in the molar ratios 3:5:2 1389 g/mol fucose:galactose:mannose in the molar ratios 3:3:1	Kim et al. 2017 [52]
Soliería filiformis	Mexico	Enzymatic assisted extraction	Monosaccharide composition (μg mg^{-1} dry weight) Arabinose 0.09 ± 0.02, galactose 10.62 ± 0.9, glucuronic acid 0.24 ± 0.05, glucosamine 0.38 ± 0.03, glucose 6.35 ± 0.7, mannose galactose 2.52 ± 0.5, rhamnose 0.28 ± 0.02, ribose 0.87 ± 0.006	Peñuela et al. 2021 [53]
Nostoc muscorum	Egypt	Extraction with 20 mM potassium phosphate buffer assisted by ultrasound	Lectin	Saad et al. 2022 [54]
Undaria pinnatifida	New Zealand	Extraction for 6 h with 1% (w/v) H_2SO_4 at 20 °C, 0.2 M HCl at 20 °C, or 2% $CaCl_2$ at 75 °C.	Sulfated galactofucan	Harden et al. 2019 [55]
Splachnidium rugosum			Sulfated fucan	
Gigartina atropurpurea			Sulfated galactan	
Plocamium cartilagineum			Complex sulfated galactan	
Sargassum patens	China	Extraction with boiling water and ethanol precipitation	Sulphated polysaccharide	Zhu et al. 2004 [56]

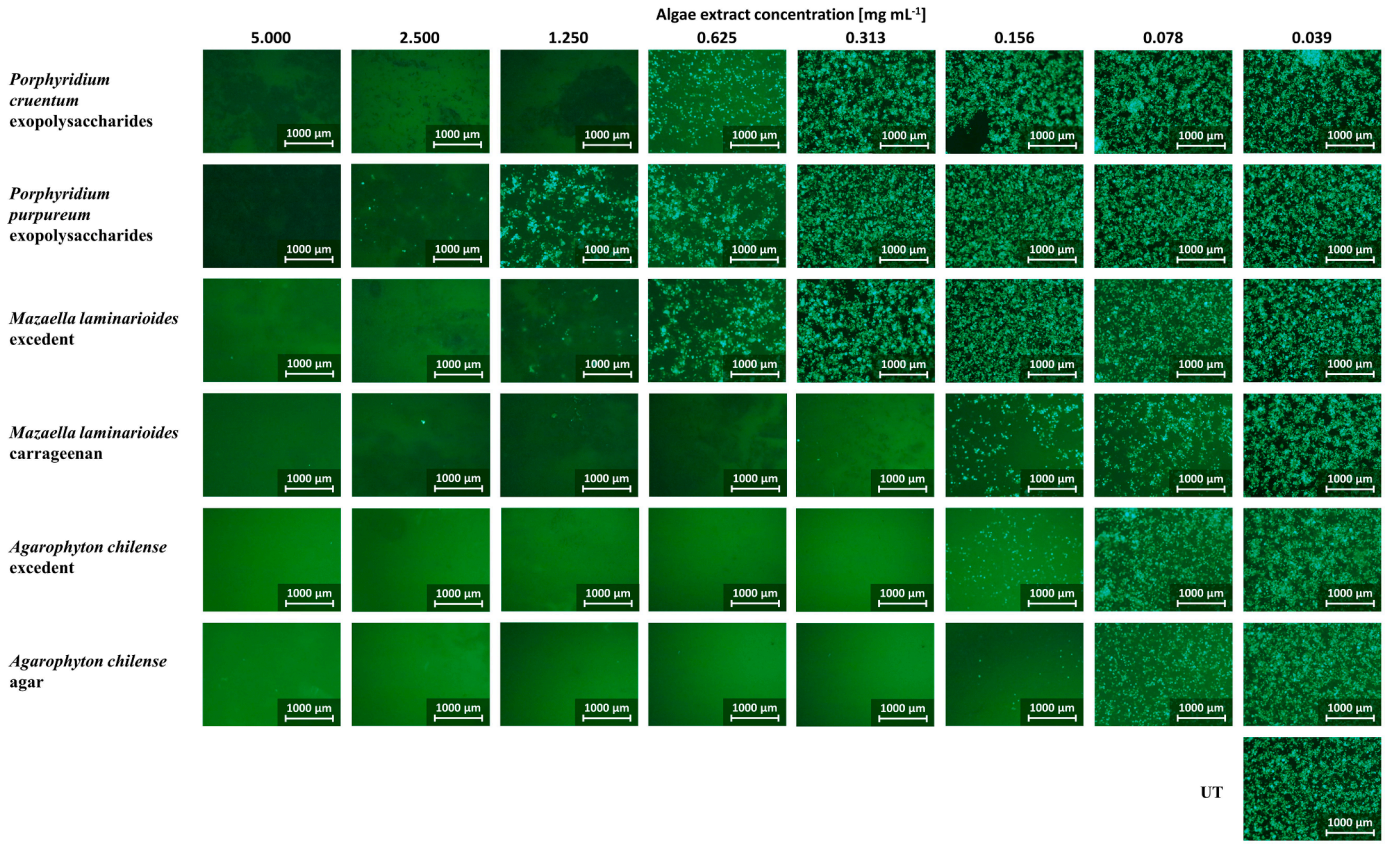

Fig. 3. Antiviral activity of the algae hydrocolloid extracts added to HSV-2-infected HeLa cells. The algae phycocolloids were added at decreasing concentrations 1 h after infection with HSV-2 and left in the media until the measurement at 24 h after infection. Representative images of virus-encoded GFP-derived fluorescence in cells treated either with *P. cruentum* exopolysaccharides, *P. purpureum* exopolysaccharides, *M. laminarioides* supernatant, *M. laminarioides* carrageenan, *A. chilense* supernatant or *A. chilense* agar. UT, Untreated.

1651 cm^{-1} were assigned to stretching vibration of C = O [60] , the band at 1257 cm^{-1} was assigned to asymmetric vibration of *S = O* group [60], and the band at 1151 cm^{-1} was assigned to C—O glycosidic band vibration [61]. The band at 1043 cm^{-1} was assigned to C—O stretching vibration [62], and the band at 579 cm^{-1} was assigned to SO_2 sulfate group. On the other hand, the FTIR of sulphated exopolysaccharides from *P. purpureum* show a broad band at 3419 cm^{-1} assigned to O—H stretching vibration [59], the band at 2925 cm^{-1} was assigned to C—H stretching vibrations [59], the bands at 1733 cm^{-1} and 1651 cm^{-1} were assigned to stretching vibration of C = O [60], the band at 1257 cm^{-1} was assigned to asymmetric vibration of *S = O* group [60], and the band at 1157 cm^{-1} was assigned to C—O glycosidic band vibration [61]. The band at 1041 cm^{-1} was assigned to C—O stretching vibration [62].

FTIR spectra of algae lyophilized carrageenan and supernatant obtained from red algae *M. laminarioides* in the range 4000–500 cm^{-1} are presented in Fig. 6A and 6B, respectively. The FTIR of carrageenan from *M. laminariorides* shows a broad band at 3439 cm^{-1} assigned to O—H stretching vibration, and the band at 2956 cm^{-1} was assigned to C—H stretching vibrations. The band at 1664 cm^{-1} was assigned to stretching vibrations of the –C = O group [63]. The band at 1261 cm^{-1} were assigned to asymmetric vibration of *S = O* group. The band at 1155 cm^{-1} were assigned to C—O and C—C stretching vibrations of pyranose rings [63]. The band at 933 cm^{-1} was assigned to stretching vibration of C—H from 3,6-anhidro-galactopyranosyl residue. The band at 840 cm^{-1} was assigned to stretching vibration of S-O from C-2 equatorial sulfate residue. The band at 773 cm^{-1} was assigned to the skeleton bending of pyranose ring [64]. The band at 704 cm^{-1} was assigned to stretching vibration -C = O group, and the band at 580 cm^{-1} was assigned to vibration sulfate group (Table 2).

On the other hand, the FTIR of supernatant from *M. laminariorides* shows a broad band at 3444 cm^{-1} assigned to O—H stretching vibration, and the band at 2951 cm^{-1} was assigned to C—H stretching vibrations. The band at 1655 cm^{-1} was assigned to stretching vibrations of the –C = O group [63]. The band at 1259 cm^{-1} were assigned to asymmetric vibration of *S = O* group. The band at 1151 cm^{-1} were assigned to C—O and C—C stretching vibrations of pyranose rings [63]. The band at 931 cm^{-1} was assigned to stretching vibration of C—H from 3,6-anhidro-galactopyranosyl residue. The band at 845 cm^{-1} was assigned to stretching vibration of S-O from C-2 equatorial sulfate residue. The band at 771 cm^{-1} was assigned to the skeleton bending of pyranose ring [64]. The band at 708 cm^{-1} was assigned to stretching vibration -C = O group. Finally, the band at 582 cm^{-1} was assigned to vibration sulfate group. The coincidence in the absorption bands found in the carrageenan with those found in the supernatant indicates that the lyophilized product obtained from the supernatant corresponds to non-precipitated carrageenan.

FTIR spectra of algae lyophilized agar and supernatant obtained from red algae *A. chilense* in the range 4000–500 cm^{-1} are presented in Fig. 7A and 7B, respectively. The FTIR of agar from *A. chilense* shows a broad band at 3429 cm^{-1} assigned to O—H stretching vibration, and the band at 2929 cm^{-1} was assigned to C—H stretching vibrations. The band at 1655 cm^{-1} was assigned to stretching vibrations of the –C = O group [63]. The band at 1240 cm^{-1} is assigned to the sulfate ester groups of sulfated polysaccharides [64]. The band at 1159 cm^{-1} were assigned to C—O and C—C stretching vibrations of pyranose rings [63]. The band at 933 cm^{-1} was assigned to vibration of the C—O-C bridge of 3,6-anhidro--galactopyranosyl residue [64,65]. The band at 773 cm^{-1} was assigned to the skeleton bending of pyranose ring [64]. The band at 700 cm^{-1} was assigned to stretching vibration -C = O group and the band at 575 cm^{-1} was assigned to vibration sulfate group. On the other hand, the FTIR of

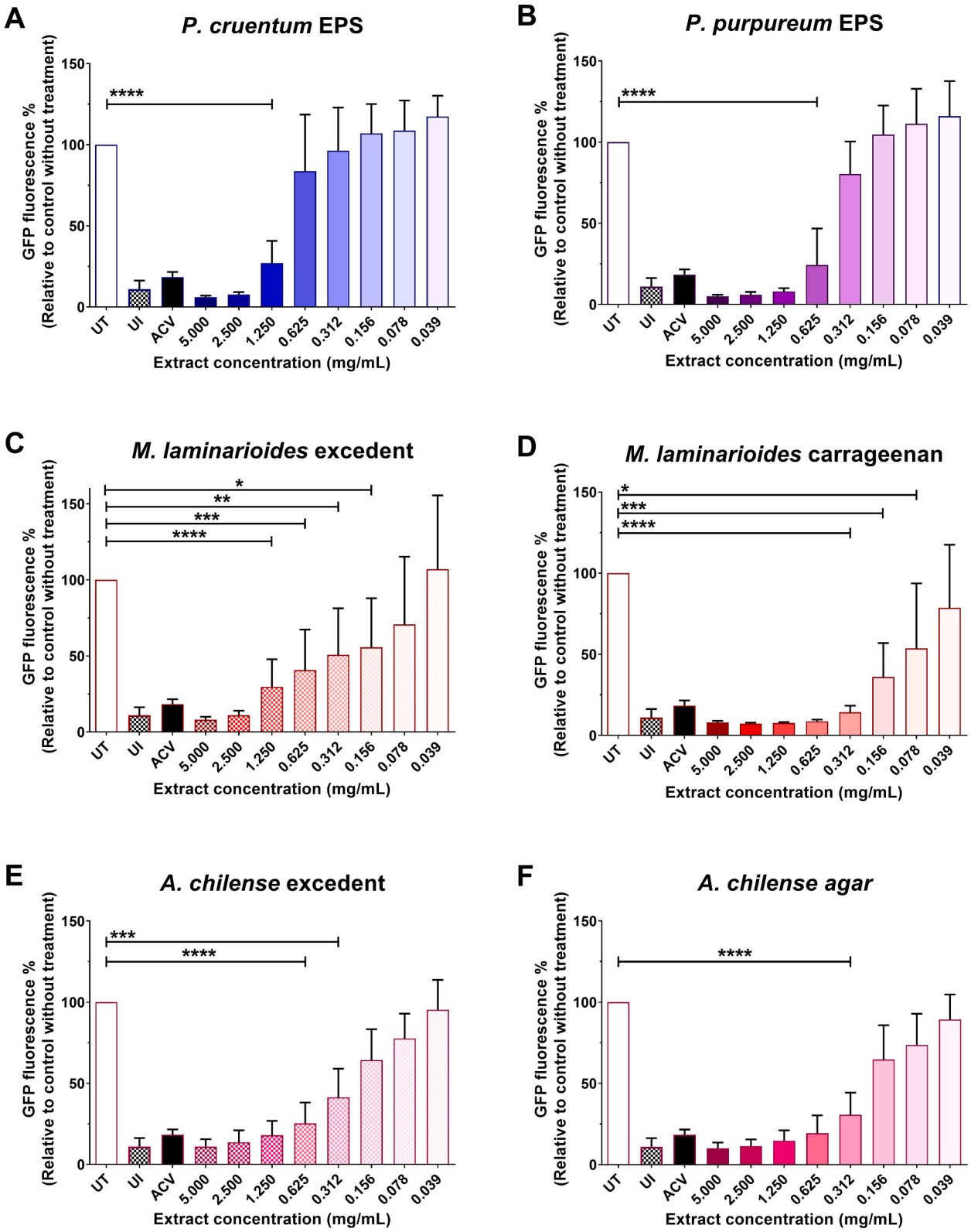

Fig. 4. *In vitro* algae hydrocolloid antiviral activity against herpes simplex virus type 2. HeLa cells were infected with a GFP-encoding HSV-2 at a multiplicity of infection (MOI) of 1. Then, 1 h after infection cells were treated with decreasing concentrations of the algae phycocolloids for the remaining time of the assay, or acyclovir (ACV) at 50 μg/ml. UT: Untreated cells. UI: Uninfected cells. GFP fluorescence of HSV-2-infected cells (determined with a multi-mode fluorescence reader at 24 hpi) treated with the serially diluted hydrocolloid extracts of (A) *P. cruentum*. (B) *P. purpureum* (C), *M. laminarioides* supernatant (D), *M. laminarioides* carrageenan (E), *A. chilense* supernatant or, (F) *A. chilense* agar. Data shown are means ± SEM (Standard Error of the Mean) of three independent experiments. The data were analyzed using one-way ANOVA test with a Dunnet's test for multiple comparisons. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; and *$p < 0.05$.

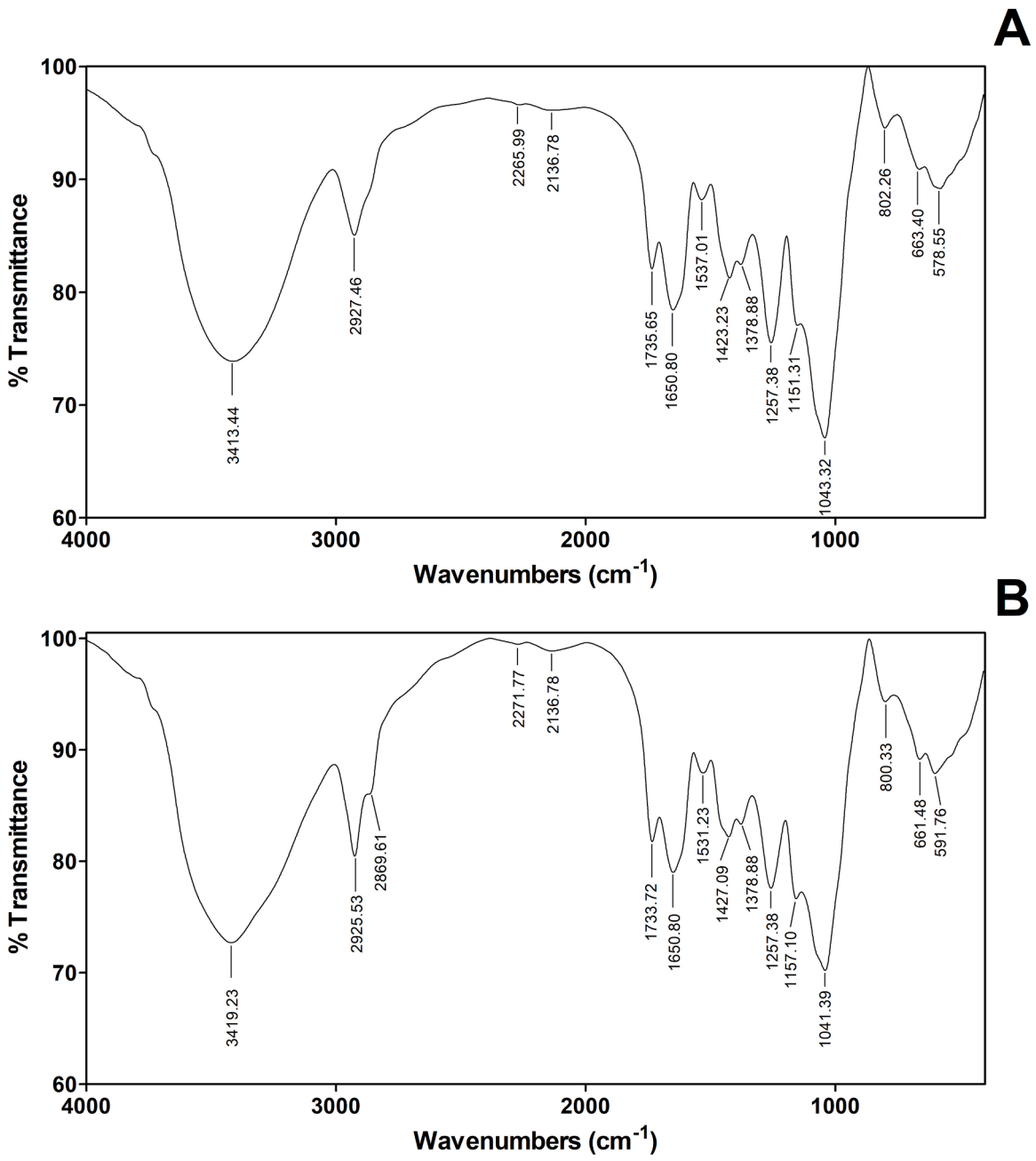

Fig. 5. Fourier transform infrared (FTIR) spectrum of lyophilized red microalgae phycocolloids (A) *P. cruentum* exopolysaccharides (B) *P. purpureum* exopolysaccharides.

supernatant from *A. chilense* shows a broad band at 3417 cm^{-1} assigned to O—H stretching vibration, and the band at 2929 cm^{-1} was assigned to C—H stretching vibrations. The band at 1649 cm^{-1} was assigned to stretching vibrations of the –C = O group [63]. The broad band at 1227 cm^{-1} was assigned to the sulfate ester groups of sulfated polysaccharides [64]. The band at 1155 cm^{-1} was assigned to C—O and C—C stretching vibrations of pyranose rings [63]. The band at 935 cm^{-1} was assigned to vibration of the C—O-C bridge of 3,6-anhidro-galactopyranosyl residue [64,65]. The band at 773 cm^{-1} was assigned to the skeleton bending of pyranose ring [64]. The band at 696 cm^{-1} was assigned to stretching vibration -C = O group. Finally, the band at 579 cm^{-1} was assigned to vibration sulfate group. The coincidence in the absorption bands found in the agar with those found in the supernatant indicates that the lyophilized product obtained from the supernatant corresponds to non-precipitated agar.

4. Discussion and conclusions

In this work, phycocolloids were extracted from two red microalgae, *P. cruentum* and *P. purpureum*, and from two red macroalgae, *A. chilense* and *M. laminarioides* collected from the coasts of Biobío Region, Chile. The cytotoxicity of the phytocolloids was evaluated by measuring 50% cytotoxic concentrations from the extracted phycocolloids. In addition, their antiviral activity against HSV-2 was determined by measuring the 50% effective concentration. The CC_{50} of *P. cruentum* exopolysaccharides was determined to be 2.12 g L^{-1} while the CC_{50} for *P. purpureum* was 3.84 g L^{-1}. These results agree with those reported by Huheihel et al. 2002 [34], who found that a *Porphyridium* sp. polysaccharide concentration of 1 g L^{-1} causes cell growth to stop after 3 days of initiating treatment of Vero cells. Moreover, the EC_{50} for *P. cruentum* and *P. purpureum* were found to be 0.93 g L^{-1} and 0.44 g L^{-1},

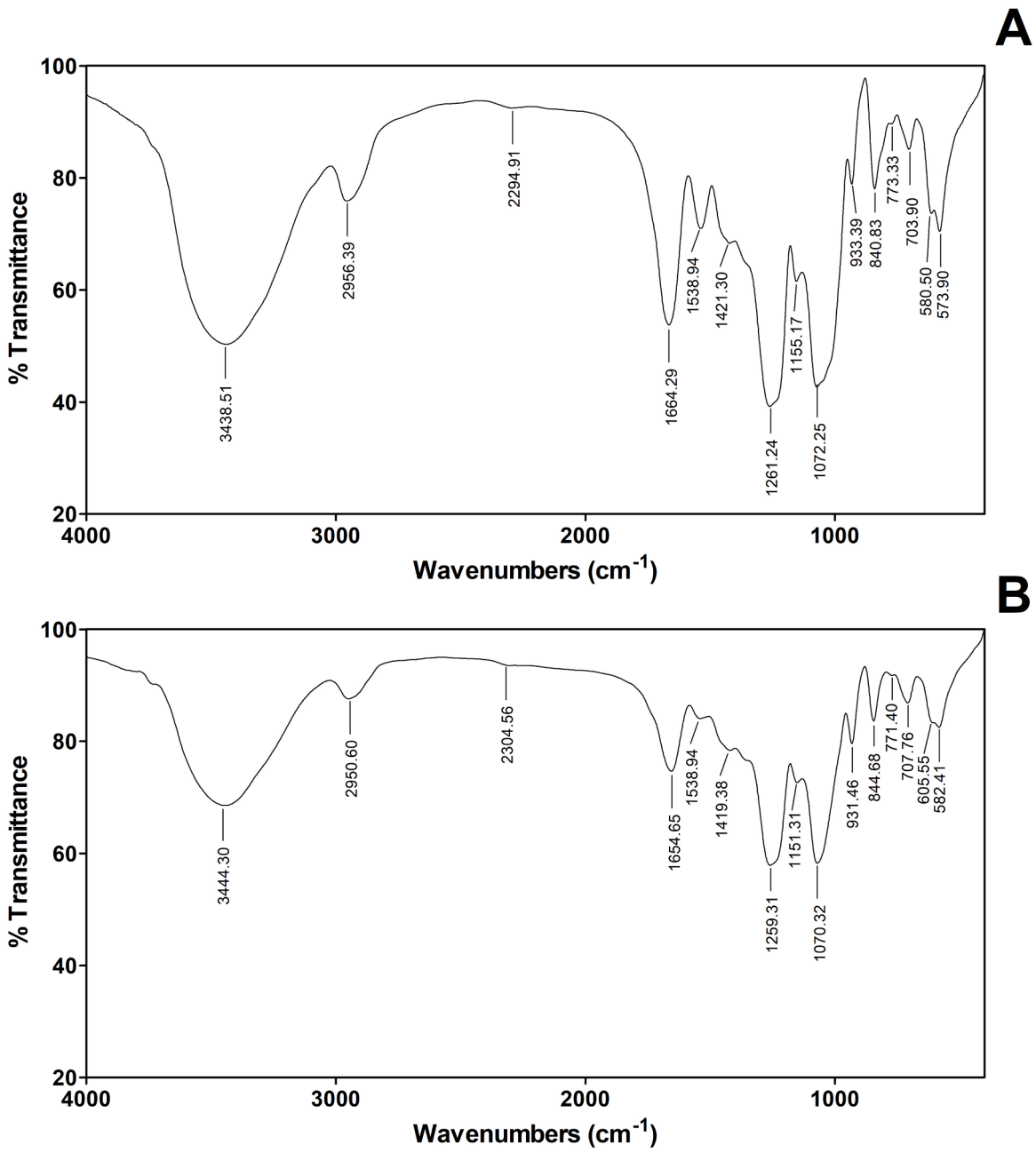

Fig. 6. Fourier transform infrared (FTIR) spectrum of lyophilized algae phycocolloids (A) *M. laminarioides* carrageenan (B) *M. laminarioides* supernatant.

Table 2
Cytotoxic concentrations (CC_{50}) of algae phycocolloids. The 50% cytotoxic concentration (CC_{50}) was determined by quantifying the resazurin-derived fluorescence in the cell cultures, which is proportional to the culture's metabolic rate and viability. The 50% effective concentration (EC_{50}) was determined by quantifying virus-derived fluorescence and a non-linear regression was performed using the log of the concentrations of phycocolloids used.

Algae phycocolloids	CC_{50} [g L^{-1}]	EC_{50} [g L^{-1}]
P. cruentum exopolysaccharides	2.12	0.93
P. purpureum exopolysaccharides	3.84	0.44
M. laminarioides supernatant	1.68	0.27
M. laminarioides carrageenan	2.10	0.09
A. chilense supernatant	2.06	0.22
A. chilense agar	2.48	0.20

respectively. The above results demonstrate that the exopolysaccharides of these microalgae exhibit antiherpetic activity with HSV-2. However, according to the SI values obtained, it is not assured that these exopolysaccharides satisfy the minimum potential as antiviral drug.

The phycocolloid yields obtained in our work for *A. chilense* (7.1%) are lower than those obtained by Souza *et al.* 2012 [46] for *A. birdiae*, who obtained an extraction yield of 27.2%. A possible cause for the lower yield obtained in the extraction of phycocolloids from *A. chilense* is the seasonality of algal harvesting. It is worth mentioning that *A. chilense* specimens were harvested in June, which corresponds to winter in Chile. On the other hand, the yield obtained in the extraction of phycocolloids from *M. laminarioides* was 24.4%, which was higher than those obtained by Arias et al. 2016 [45], who obtained yields of 18%, 14%, and 13% for *M. laminarioides* for the cystocarpic, vegetative and tetrasporic, respectively. It is important to indicate that the phycocolloid extractions of both *A. chilense* and *M. laminarioides* were carried out from vegetative

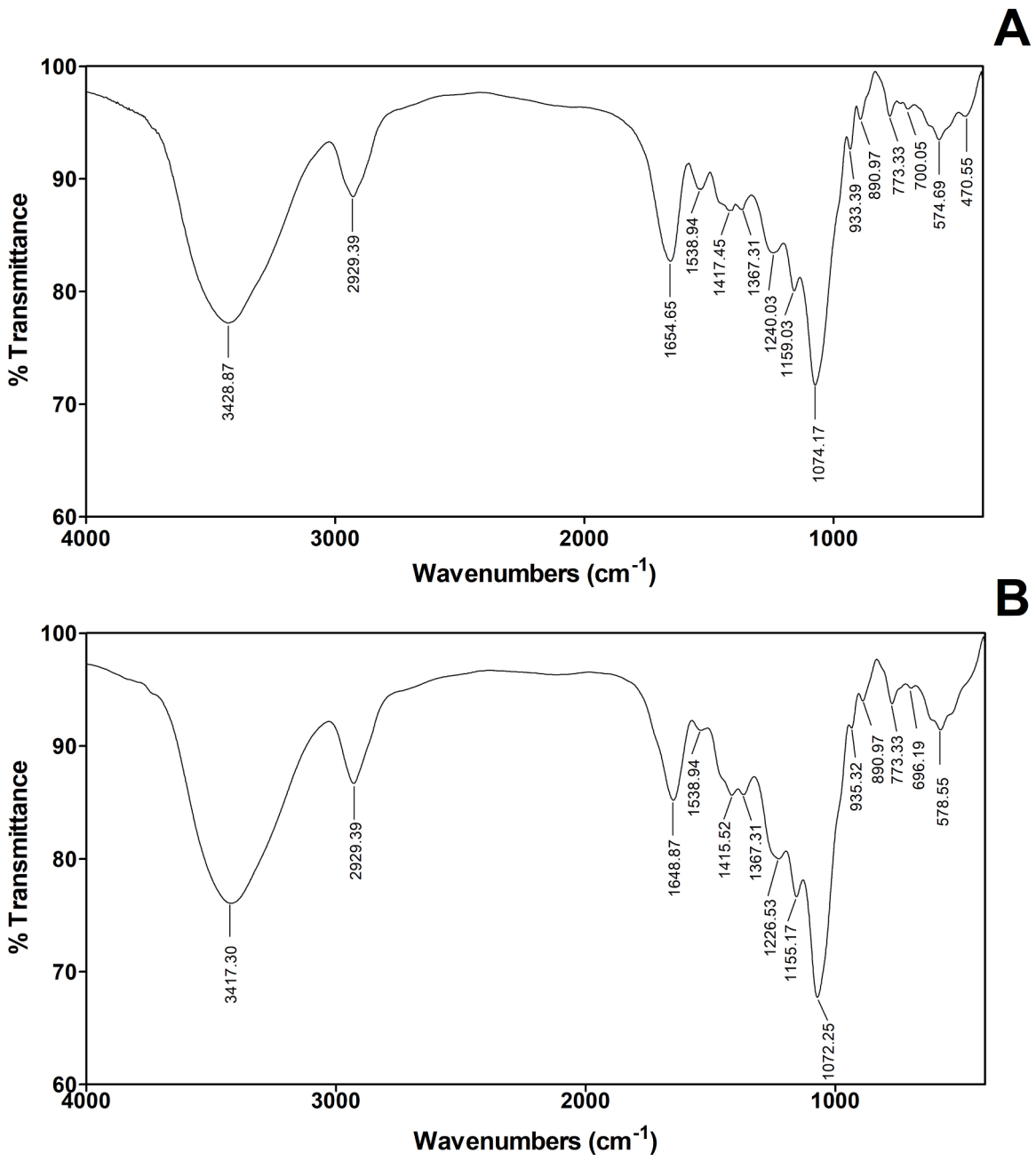

Fig. 7. Fourier transform infrared (FTIR) spectrum of lyophilized algae phycocolloids (A) *A. chilense* agar (B) *A. chilense* supernatant.

fronds to avoid altering the reproductive viability of the natural grassland. To the best of our knowledge, there are no other studies that have determined the antiviral activity of *A. chilense* extracts against HSV-2. Nevertheless, there are studies with other *Gracilaria* species, namely those performed by Mazumder et al. 2002 [66] and Zandi et al. 2007 [67]. Studies with *G. corticate* have reported EC_{50} values for three fractions obtained from this alga, 2.4×10^{-1}, 3.84×10^{1}, and 4.59×10^{1} μg mL^{-1}. On the other hand, Zandi et al. 2007 [67] reported EC_{50} values for extracts of *G. corticata* and *G. salicornia* of 8.20×10^{1} and 9.93×10^{1} μg mL^{-1}, respectively. Considering these EC_{50} values, both *G. corticata* and *G. salicornia* extracts seem to be biologically more active against HSV-2 than *A. chilense* extracts. In this study, EC_{50} values from red macroalgae phycocolloids were lower than the EC_{50} values obtained for the red microalgae exopolysaccharides extracts. These results indicate that the concentrations of the phycocolloids from *M. laminarioides* and *A. chilense* required to obtain the same antiviral effect against HSV-2 are lower than those from *P cruentum* and *P purpureum*. It is noteworthy to mention that Dargan 1998 [68] and Berezin *et al.* 2019 [69] established that any antimicrobial compound with a selective index higher than 10 (SI > 10) ensures a minimum potential as an antiviral drug, although higher values are usually expected *in vitro* tests [11]. According to this criterion, both the carrageenan from *M. laminarioides* (SI = 23.3) and the agar

Table 3
Selectivity Index (SI) of algae phycocolloids.

Algae extract	SI (CC_{50}/EC_{50})
P. cruentum exopolysaccharides	2.3
P. purpureum exopolysaccharides	8.7
M. laminarioides supernatant	6.2
M. laminarioides carrageenan	23.3
A. chilense supernatant	9.4
A. chilense agar	12.4

from *A. chilense* (SI = 12.4) display antiviral therapeutic potential against HSV-2 (Table 3).

The supernatants obtained from the precipitation of ficocoloides from *A. chilense* and *M. laminarioides* were analyzed by FTIR. These were used in the antiviral activity tests in order to evaluate the antiherpetic activity of fractions of phycocolloids with lower molecular weight, which may not have precipitated with ethanol. Analyzing the FTIR spectra of the supernatants, a pattern of bands similar to the respective precipitates can be seen, indicating that both precipitate and supernatant have a similar chemical nature. However, considering the selectivity index values, the supernatants have no potential to be used as antiviral drugs (Table 3).

It is also important to mention that the potential cytotoxicity of macroalgae phycocolloids does not necessarily represent a negative factor for using these compounds as phytopharmaceuticals for the treatment of herpetic lesions. This effect may contribute to killing infected cells, thus reducing the duration of herpetic lesions. On the other hand, it is noteworthy mentioning that the antiviral potential of the extracts assessed herein may be higher than determined because these extracts were only applied to the cells post-infection with HSV-2. Also, it is important to recall that virus fitness in the cell cultures after infection and treatment was assessed by detecting the virus-derived fluorescence of GFP encoded in the viral genome and not directly plaque-forming units (PFUs). Thus, future experiments should corroborate that reduced GFP expression in the infected cells directly relates to lesser virus yield from these cells. Additionally, the assessment of the antiviral activity of the phycocolloids against HSV-2 in an animal model should provide valuable information on the therapeutic potential of these compounds. Nevertheless, our results provide a preliminary indicator of the antiviral potential of these red algae phycocolloids, which in the future could eventually be formulated in phytopharmaceuticals for the treatment of herpetic lesions caused by HSV-2.

Finally, it is worth mentioning that the bands obtained with the Fourier transform infrared spectrum of the phycocolloids extracted from *A. chilense* coincide with the expected functional groups in agars. In addition, a previous study by Arias *et al.* 2016 [45] determined that the sulfated galactans of phycocolloids from *M. laminarioides* were composed by κ/μ and ι/ν-carrageenans, which the phycocolloids were extracted by a similar procedure as the ones used in this work and characterized by infrared spectroscopy technique.

In this first stage of this research, the evaluation of the antiviral activity of total phycocolloids was carried out with the purpose of assessing whether these phycocolloids exhibit anti-herpetic activity. Currently used as stabilizing, thickening and gelling agents in the food industry, these compounds would have additional value with these potential anti-herpetic traits, thus satisfying another goal of this research. At a later stage of this research, it is intended to fractionate the obtained phycocolloids and perform a more exhaustive chemical analysis to identify those fractions that exhibit greater bioactivity.

Supplementary Figure 1: Procedure for obtaining agar from freeze-dried algal biomass of *Agarophyton chilense.*

Supplementary Figure 2: Procedure for obtaining carrageenan from freeze-dried algal biomass of *Mazaella laminarioides.*

Declaration of Competing Interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants participation in speaker's bureaus membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2023.e00798.

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