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RESEARCH ARTICLE

Antiviral activity of *Sargassum fluitans* seaweed against echovirus 9, coxsackievirus A16 and coxsackievirus A24

Actividad antiviral del alga *Sargassum fluitans* (Børgesen) Børgesen 1914, frente al echovirus 9, el coxsackievirus A16 y el coxsackievirus A24

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Abstract

Enteroviruses cause human diseases such as Mouth, Hand and Foot Syndrome, hemorrhagic conjunctivitis, aseptic meningitis, and viral meningoencephalitis. As yet, there is not specific therapy for most enteroviruses infections. The brown seaweeds of *Sargassum* genus synthesize a great variety of metabolites, which make them potential sources of compounds with antiviral activities. This study aimed to evaluate the *in vitro* antiviral activity of a hydro-alcoholic extract of *Sargassum fluitans* against three human enteroviruses: coxsackievirus A16, echovirus 9 and coxsackievirus A24. Cytotoxicity of the extract was evaluated in Vero, RD and Hep-2 cells by MTT method. Antiviral activity (EC_{50}) was assessed by cytopathic effect inhibition in cells. Extracellular virucidal activity and reduction of viral yield were determined by endpoint viral titration assay. The antiviral activity was characterized by a time of addition assay. The extract showed antiviral inhibitory activity against all tested viruses. The extract also exhibited a virucidal effect against E9 and CVA16 and reduced the formation of infective particles in the cells in more than three logs. The extract was able to inhibit earlier and late stages in the enterovirus replication cycle. In conclusion, the present study demonstrated the effective *in vitro* antiviral activity of the brown seaweed *Sargassum fluitans* against clinically relevant enteroviruses, supporting their use as potential source of antiviral compounds.

Keywords: *Sargassum fluitans*, antiviral, enterovirus, echovirus, coxsackievirus

Resumen

Los enterovirus causan enfermedades humanas tales como el Síndrome de Mano, Pie y Boca, la conjuntivitis hemorrágica y la meningoencefalitis viral. Hasta el momento no existe una terapia específica para la mayoría de las infecciones por enterovirus. Las algas pardas del género *Sargassum* sintetizan una gran variedad de metabolitos, los cuales les convierten en fuentes potenciales de compuestos con actividad antiviral. Este estudio tuvo como objetivo evaluar la actividad antiviral *in vitro* de un extracto hidroalcohólico de *Sargassum fluitans* contra tres enterovirus humanos: coxsackievirus A16, echovirus 9 and coxsackievirus A24.

La citotoxicidad del extracto se evaluó en las células Vero, RD y Hep-2 mediante el método del MTT. La actividad antiviral (CE_{50}) se evaluó mediante la inhibición del efecto citopático en las células. La actividad virucida extracelular y la reducción del número de viriones se determinaron mediante ensayo de titulación viral de punto final. La actividad antiviral se caracterizó mediante un ensayo de tiempo de adición. El extracto mostró actividad antiviral inhibitoria contra todos los virus probados. El extracto también exhibió efecto virucida contra el echovirus 9 y el coxsackievirus A16 y redujo la formación de partículas virales infectivas en las células en más de tres logaritmos. El extracto fue capaz de inhibir las etapas tempranas y tardías del ciclo replicativo. Se concluye que el presente estudio demostró la efectiva actividad antiviral *in vitro* del alga parda *Sargassum fluitans* contra enterovirus clínicamente relevantes, apoyando su uso como fuente potencial de compuestos antivirales.

Palabras clave: *Sargassum fluitans*, antiviral, enterovirus, echovirus, coxsackievirus

Introduction

Human enteroviruses (EV) are members of the *Enterovirus* genus belonging to the *Picornaviridae* family (Zell *et al.,* 2017; Adams *et al.,* 2017). Some EV infections are asymptomatic; however, others could cause illness or be fatal (Kupila *et al.,* 2006; Pallansch *et al.,* 2013). These viruses are etiological agents of acute diseases such as poliomyelitis, Hand, Foot and Mouth Syndrome, haemorrhagic conjunctivitis. Remarkably, they represent the most common cause of aseptic meningitis and meningoencephalitis. In addition to acute diseases, EV have also been associated with chronic conditions, such as dilated cardiomyopathy and type 1 diabetes (Kupila *et al.,* 2006; Banerjee *et al.,* 2006; Pallansch *et al.,* 2013).

Up to date, only poliovirus and EV71 possess vaccines (Yi *et al.,* 2017; Baggen *et al*., 2018); however, no specific antiviral drugs or vaccines are available for the treatment of the rest of the EV (Shekerdemian y Desmond, 2006). In the last three years echovirus 9, coxsackievirus A16 and coxsackievirus A24 have been identified with high prevalence in clinical samples from Cuban patients with meningitis, Hand, Foot and Mouth Syndrome and hemorrhagic conjunctivitis, respectively (National Reference Laboratory of Enterovirus, Institute of Tropical Medicine: "Pedro Kourí"). Therefore, these viruses were selected in this study.

Natural products are an alternative for treating viral infections, considering its high content of biologically active metabolites. The brown seaweeds of *Sargassum* genus include more than 400 species and are employed traditionally because of their antioxidant, antinflamatory, antitumoral and antiviral properties (Liu *et al.,* 2012; Yende *et al.,* 2014; Ahmadi et al., 2015). Among the metabolites with antiviral activities synthesized by *Sargassum*, meroterpenoids, tannins, and fucoidans are the most extensively studied (Iwashima *et al.,* 2005; Hayashi *et al.,* 2006; Wei *et al.,* 2008; Lee y Seo, 2011; Sokolova *et al.,* 2011; Ho *et al.,* 2011). The species *Sargassum fluitans* (Børgesen) Børgesen 1914, *Sargassaceae*, floats in the Sargasso Sea, located in the North Atlantic Ocean. It beds to the Cuban coast in the winter and summer seasons (Moreira y Alfonso, 2013; Kim *et al.,* 2014). Despite the fact that *Sargassum'*s metabolites are a potential and valuable source for antivirus drug discovery, the antiviral activity of *Sargasum fluitans* has been poorly studied. However, previous studies in our group have shown the capacity of the hydroalcoholic extract of *Sargassum fluitans* to inhibit the replication of echovirus 9 (Ponce *et al.,* 2018). Therefore, this study aimed to investigate the *in vitro* antiviral activity and mode of action of *Sargassum fluitans* against enterovirus strains of clinical and epidemiological relevance.

Materials and methods

Algal material

The hydroalcoholic extract of *Sargassum fluitans* was kindly provided by Dr. Olga Valdés, from the Institute

of Marine Science, Cuba. The seaweed samples were frozen and ground previous extract preparation. The hydroalcoholic extract was prepared with the wet seaweed in proportion 1:3 (seaweed: hydroalcoholic solution 30% ethanol-water) by five days with shaking at intervals and kept in 10°C. Then the extract was centrifugated at 2000 rpm for 10 minutes. It was roto-evaporated at 50°C for two hours and then it was freeze-dried at -20°C in 5 ml tubes. The lyophilized material was diluted in the maintenance medium corresponding to each cell culture at a final concentration of 5 mg/ml, kept at 4°C temperature and protected from sunlight.

Viruses and cells

The Hill reference strain of echovirus 9 (E9), G10 reference strain of coxsackievirus A16, and a clinical isolate of coxsackievirus A24 (CVA24) were obtained from the stock of the National Reference Enterovirus Laboratory, Virology Department, Tropical Medicine Institute "Pedro Kourí".

African green monkey kidney cells (Vero, ATCC CCL-81), Human rhabdomyosarcoma cells (RD, ATCC® CCL-136™), and human epidermoid carcinoma cells (Hep-2, ATCC®CCL-45™) were provided by the Cell Culture Laboratory of the Tropical Medicine Institute: "Pedro Kourí". These cell lines were selected according to the viruses' capacity to replicate and show cytopathic effect (CPE). Vero cells were grown in 199 modified Eagle medium (GibcoTM). RD and Hep-2 cells were grown in Eagle's Minimal Essential Medium (MEM). All cells were supplemented with 5% FBS (Invitrogen), 0.22% sodium bicarbonate (Sigma) and 50 mg/L gentamicin (Invitrogen). Cells were seeded in 96-well plates at an initial density of $2.5_{*}10^{5}$ cells per well.

Cytotoxicity Assay

Cytotoxicity assays were made in order to determine the subtoxic concentrations to employ in the antiviral assays. Cytotoxicity of the hydroalcoholic extract of *Sargassum fluitans* on Vero, RD and Hep-2 cells was evaluated *in vitro* by MTT assay as described by Mosmann (Mosmann, 1983). The cells were seeded in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere for 72 hours. Then, 100 µL of various concentrations (5-0.125 mg/ml) of the extract were added to the cells by using sextuplicate wells for each concentration and further incubated for 48 hours. MTT solution (10 µL, final concentration 0.5 mg/ml) were added to each well, which were further incubated for 4 hours at 37°C. The supernatant was removed and isopropanol was added to solubilize the formazan crystals. The optical density (OD) was measured in a microplate reader at 520 nm with a reference filter of 630 nm using a plate spectrophotometer MRX Revelation (Dynex Technologies®, United Estate) and the program Dynex Revelation 4.02. The cytotoxicity was calculated by regression analysis and expressed as 50% cytotoxic concentration (CC_{50}), as follows:

$CC_{50}= OD_{50}$ CC (t)/OD_{50} CC(c)x100

OD_{50} CC (t): media absorbance value of treated cells culture.

OD_{50} CC (c): media absorbance value of control cells culture considered as 100% of cell viability.

Antiviral Activity Assays

Preliminary screening

The antiviral activity of the extract was evaluated by CPE inhibition assay (Álvarez *et al.,* 2009). In brief, cells were seeded in 96-well plates at a density of $2.5_{*}10^{5}$ cells per well. The confluent cell monolayer was treated with serial sub-toxic concentrations of the extract and an equal volume of virus suspension (100 $TCID_{50}$) in six replicas. Thereafter, the cells were incubated at 37°C in a 5% CO_2 atmosphere and observed daily for CPE under a light microscope. Wells were read when all viral controls showed at least a focus of CPE. The 50 percent effective antiviral concentration (EC_{50}), defined as the concentration that reduced CPE by 50 percent with respect to the virus control (100 percent of CPE), was calculated by regression analysis. *Selectivity index* (*SI*), as a

marker of antiviral activity, was calculated by dividing the CC_{50} by the $CE_{50.}$ (del Barrio and Parra, 2000).

Assay of viral titer reduction intracellularly

This assay was performed to determine if the extract could inhibit the formation of infective viral particles of E9 and CVA16 in Vero and RD cells. The cells were incubated with the extract for 1 hour at a concentration of 0.125, 0.250 and 0.5 mg/ml. Thereafter, the cells were infected with 100 $TCID_{50}$ of the virus. When all virus control showed CPEs, cultures were collected for three freeze– thaw cycles. The content of each well was centrifuged at 2 000 rpm, 4°C for 10 minutes and, subsequently, the supernatant was collected. The reduction of the infective viral titre was calculated according to the Spearman- Karber formula (Poliovirus Laboratory Manual, WHO 2004).

Karber formula: Log $TCID_{50}$ = - L – d (S – 0.5)

L = logarithm of minor virus dilution

d = difference among the dilution's logarithms

S = addition of the number of positive cytopathic effect wells

Determination of extracellular virucidal activity

The direct effect extracellular virucidal activity of the extract on E9, CVA24 and CVA16, was performed according to the method described previously by del Barrio and Parra, 2000. Equal volumes (1ml) of 100 $TCID_{50}$ of the different strains and 125, 250 and 500 μg/ml of the extract were mixed and incubated at 37°C for 2 hours. The samples were then diluted and added to confluent Vero, Hep-2, and RD cells, respectively. After incubation at 37°C for five days, the viral titer in cell culture was determined by Karber formula. The positive extracellular virucidal activity was considered by reducing the infective titer in 1 log_{10} or more (Hu and Hsiung, 1989).

Time of addition experiment

Time of addition assays allows predicting the inhibited stage by the extract considering a decrease of the viral productivity in relation to the virus control (Chiang *et al.,* 2002; Thibaut *et al.* 2014). Vero and RD cells grown in 24-well plates were infected with 100 $TCID_{50}$ of the E9 and CVA16 strains, respectively, and treated with 500 μg/ml of the extract. The extract was added to the cells at different time points with respect to the virus inoculation. The times of addition were one hour before inoculation (time -1), at the same time of inoculation (time 0), and 2, 3, 4, and 6 hours after inoculation. After incubation at 37°C for 24 h, the supernatants of each time of addition were collected, the infective titer in cell culture was calculated, and the percentage of inhibition with respect to untreated control cultures was calculated for each treatment.

Statistical

Cytotoxicity and antiviral activity of the extract in each cell line were expressed as the media cytotoxic concentration (CC_{50}) and the median effective concentration (EC_{50}), respectively of each experiment and its standard deviation. The CC_{50} and EC_{50} values were determined by linear regression analysis using the Microsoft Office Excel 2013 program, considering a regression coefficient value (R^2) equal and higher than 0.85 (Chiang *et al.,* 2002).

An ANOVA test was made for determination of statistical differences between the $TCID_{50}$ value of each extract concentration and the $TCID_{50}$ value of virus control, considering $p \leq 0.05$.

Results

Cytotoxicity of the hydroalcoholic extract of Sargassum fluitans

In general, the hydroalcoholic extract of *Sargassum fluitans* showed a low cytotoxic effect on the tested cell lines. The RD and Vero cells showed similar cytotoxicity values (4569.037 ± 173 μg/ml and 4315.94 ± 168 μg/ml respectively). By contrast, the extract became more toxic for the Hep-2 cells, backed by a lower CC_{50} value (1263.117 ± 72 μg/ml).

Antiviral in vitro assay

The extract inhibited the replication of CVA24, E9, and CVA16, resulting in the protection of the cells from the CPE induced during enterovirus infection (Fig. 1). The SI for CVA24, E9, and CVA16 are shown in Table I. In each case, the SI values are higher than two, CVA24 (2.36), E9 (95.05) and CVA16 (137.02).

Virucidal activity of Sargassum fluitans extract

The hydroalcoholic extract of *Sargassum fluitans* had no virucidal activity against CVA24 while it did against CVA16 and E9 (Fig. 2). The positive virucidal activity was considered according to Hu and Hsiung, (1989) criteria. These authors reported that reducing

Table I. Antiviral activity of the hydroalcoholic extract of *Sargassum fluitans* against coxsackievirus A16, coxsackievirus A24 and echovirus 9.

Virus/Cells	CC_{50} (µg/mL)	CE_{50} (µg/mL)	SI (CC_{50}/ CE_{50})
CVA24/Hep-2	1263,117 ± 72,22	534,838 ± 36,24	2,36
CVA16/RD	4569,037 ± 173,71	33,345 ± 3,33	137,02
E9/Vero	4315,94 ± 168,10	45,404 ± 0,18	95,05

Symbology: CC_{50}: media cytotoxic concentration, CE_{50}: media effective concentration, SI: selective index

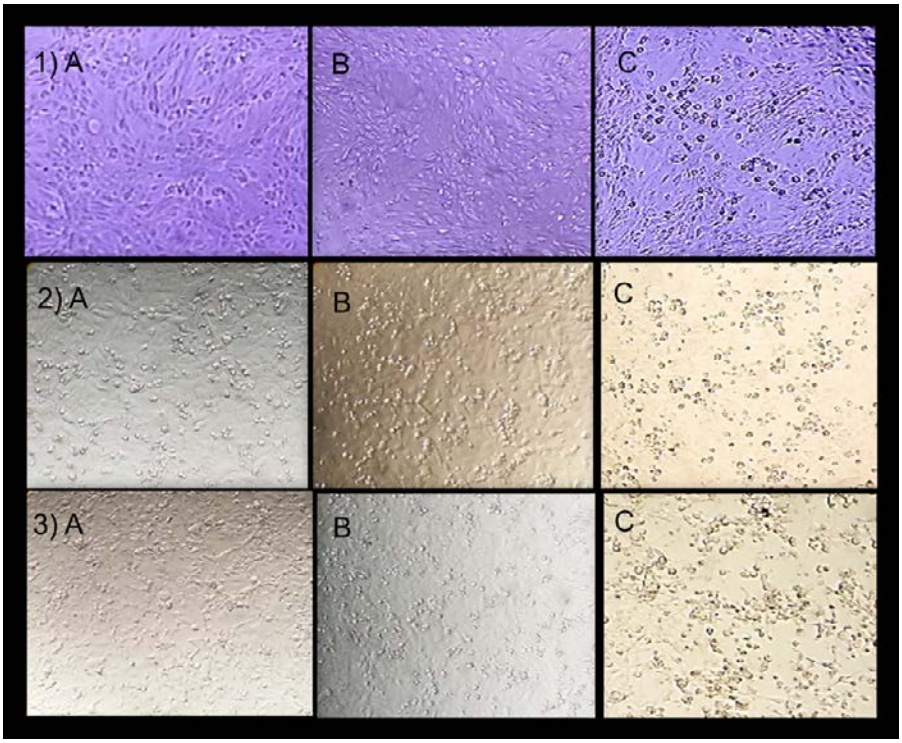

Fig. 1. Effect of the hydroalcoholic extract of *Sargassum fluitans* on CPE in cells infected with EV. 1) E9 in Vero cells, (A) Cell control; (B) Treatment (500 µg/ml), (C) Virus control. 2) CVA16 RD cells (A) Cell control; (B) Treatment (240 µg/ml), (C) Virus control. 3) CVA24 Hep-2 cells. (A) Cell control; (B) Treatment (700 µg/ml); (C) Virus control (100x).

the infective titer by more than one and two $\log_{10}$ in relation to the virus control means the diminution of the infectivity in more than 90 and 99 percent, respectively. The viral titer reduction for E9 was 2.4 and 2.5 $\log_{10}$ with 125 and 250 µg/ml, respectively, suggesting an almost total loss of the infectivity. In both concentrations, the infective titer reduction value was similar. This suggests that the virucidal activity does not change considerably with increasing concentrations of the metabolites that carry on this action. For CVA16, there was a reduction of the infective titer in 1.06 $\log_{10}$ with 250 µg/ml.

Effect of the hydroalcoholic extract of Sargassum fluitans on the infective titer of E9 and CVA16

As part of the secondary characterization assays of the antiviral activity, viral titer reduction assay was performed in order to know whether the extract was able to diminish the infective virus titer of E9 and CVA16 in the cells. In both cases, there was a reduction in more than 2 $\log_{10}$ of the infective titer at the evaluated concentrations of the extract (Fig. 3). The reduction values were 4.13 $\log_{10}$ for E9 with 500 µg/ml and 4.33 $\log_{10}$ for CVA16 with 240 µg/ml.

Effect of the time of addition of the hydroalcoholic extract of Sargassum fluitans on E9 and CVA16 replication

To determine which stage of the enterovirus replicative cycle was targeted by the extract, "time of addition" experiment was performed in cells infected with the viruses and exposed to hydroalcoholic extract of *Sargassum fluitans* at different time of infection. As shown in Fig. 4, there was a reduction of the E9 infective titer in more than 6 $\log_{10}$ when the extract was added an hour before

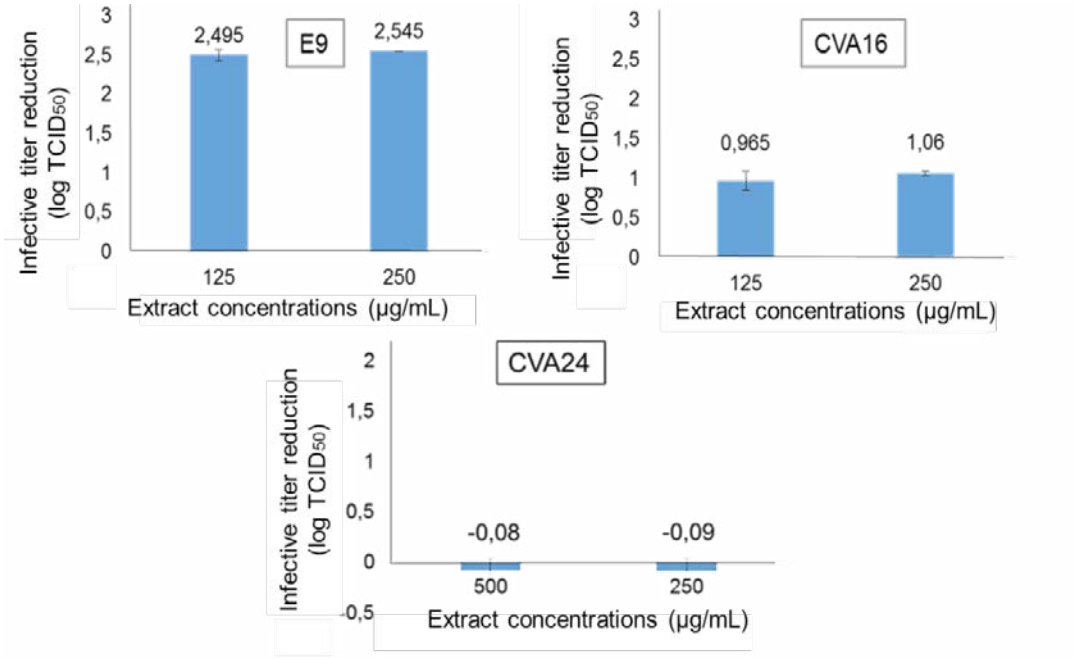

Fig. 2. Virucidal assay of the hydroalcoholic extract of *Sargassum fluitans* against echovirus 9 (E9) in Vero cells, coxsackievirus A16 (CVA16) in RD cells, and coxsackievirus A24 (CVA24) in Hep-2 cells, for 2 hours, 37ºC. Each point represents the media of two independent experiments and its standard deviation. There are significant differences between the $TCID_{50}$ of virus control and the $TCID_{50}$ of each extract concentration for E9 and CVA16 (ANOVA, p≤0,01).

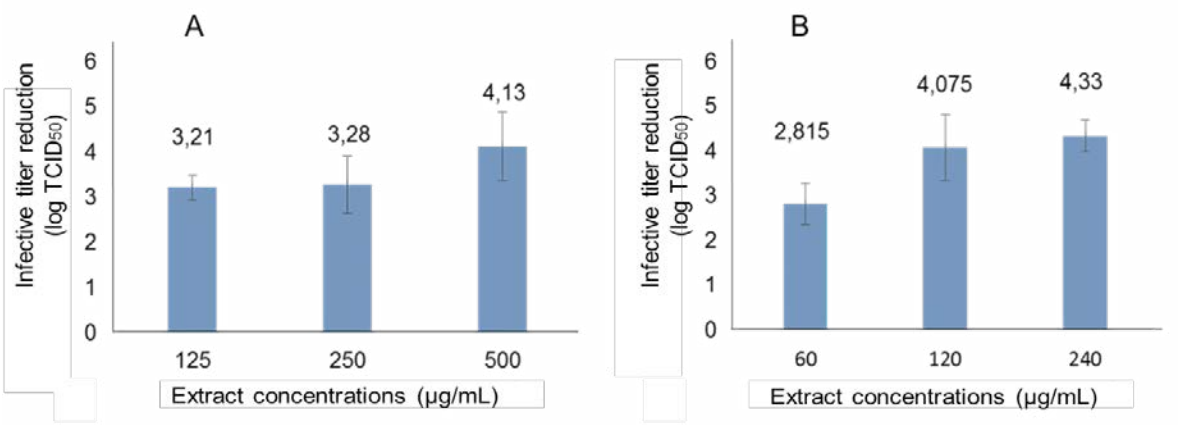

Fig. 3. Effect of the hydroalcoholic extract of *Sargassum fluitans* on the formation of infective viral particles in Vero cells inoculated with echovirus 9 (A) and RD cells inoculated with coxsackievirus A16 (B). Each point represents the media of three experiments and its standard deviation. There are significant differences between the $TCID_{50}$ of virus control and the $TCID_{50}$ of each extract concentration (ANOVA, $p \leq 0,05$).

the viral inoculation and 2.13 log_{10} when the extract was added simultaneously. The highest reduction of the CVA16 infectious titer (1.69 log_{10}) was observed by adding the extract six hours after the inoculation.

Discussion

The first step in evaluating a product as an antiviral candidate consists of determining its harmful effects on the cells (Álvarez *et al.,* 2009). The extract concentrations are selected to calculate the media cytotoxic concentration (CC_{50}) or the value that causes cytotoxicity in the 50 percent of the test units. Though enteroviruses present similarities in terms of structure and antigens, there is no unique cell line in which all EV strains can infect and replicate efficiently. Indeed, several susceptible and permissive cell lines for EV (e.g. Vero, RD, L20B, A-549

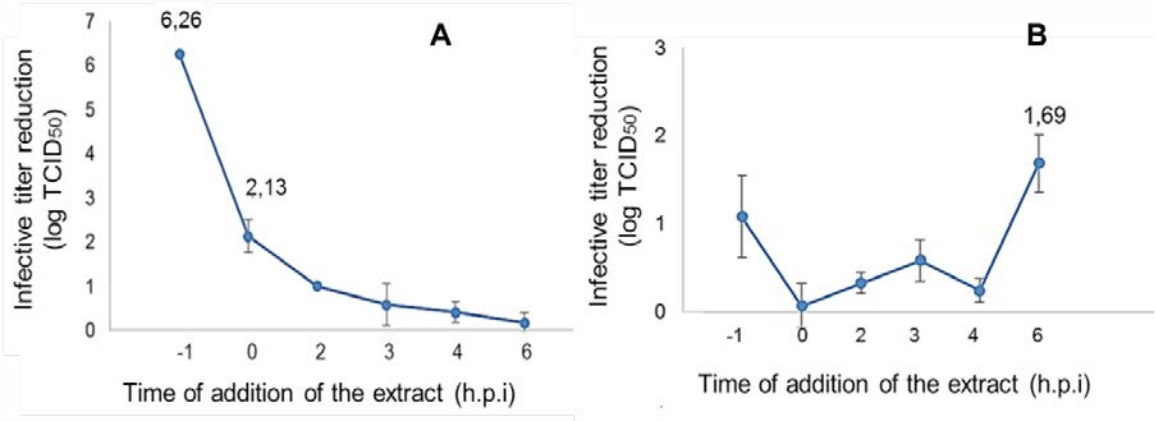

Fig. 4. Effect of the time of addition of the hydroalcoholic extract of *Sargassum fluitans* on echovirus 9 (A) and coxsackievirus A16 (B) replication in Vero and RD cells, respectively. The extract was added 1 hour before viral inoculation (-1), at the same time (0) and 2, 3, 4 and 6 hours after viral inoculation. Each point represents the average of the infective titer reduction of two experiments in relation to the control and its standard deviation.

and MRC-5) have been thoroughly characterized over the years (Sarmiento *et al.,* 2007; Adekunle y Cephas, 2014; Adeniji *et al.,* 2017). In this study, different CC_{50} values of the extract were obtained for each cell line, which could be related to their susceptibility to the toxic metabolites (Table 1). Cytotoxicity assays were useful to establish the concentrations to be used in antiviral assays, meaning sub-toxic concentrations of the extract (del Barrio and Parra, 2000).

One of the main characteristics of the method used to determine the antiviral activity of a compound is that it should be able to detect inhibition in any viral replicative cycle step. CPE-based antiviral assay measures a decreased CPE of the infected cell treated with different extract concentrations (del Barrio and Parra, 2000). Since CPE is a morphological change in cell monolayer caused by virus replication, the diminished CPE observed in our study indicates that the extract inhibits the replication of CVA24, E9 and CVA16 (Fig. 1). To know the magnitude of the antiviral activity of the extract the Selective index was calculated. The higher the SI value, the stronger the antiviral activity (del Barrio and Parra, 2000). Given that fractionation of the extract should increase the value of the SI if the active molecules are not toxic to the cells (Semple *et al.,* 2001; Fortin *et al.,* 2002) a value equal two or higher is accepted for natural products (Chiang *et al.,* 2002; Zhoua *et al.,* 2006).

Despite CVA24, E9 and CVA16 belonging to the same genus, they have distinct genetic and structural characteristics (Norder *et al.,* 2003; Oberste *et al.,* 2014). This could explain the differences in the antiviral activity of the extract for each virus. It is worth noting that the evaluation of the extract was performed in different cell lines, and therefore the mode of action could be specific in each case. Even though the CVA24 has the lower SI value, the presence of antiviral activity of the extract against this clinical isolate suggest the putative susceptibility of the circulating strains of this virus. Clinical isolates might be less susceptible than laboratory or reference strains to the antiviral action of a compound or approved drug. This phenomenon is observed in some RNA viruses such as influenza virus and coronavirus. Several studies demonstrated the antigenic differences among reference strains and clinical isolate, affecting the antibody recognition as well as the pathogenicity and virulence in humans (Dittmann *et al.,* 2008; Kazuya *et al.,* 2012). In this study the lower SI value corresponds to the clinical isolate of CVA24, this virus was isolated in Hep-2 cells from a patient with hemorrhagic conjunctivitis. One of the mechanisms of action of the extract might be the blockade of the viral antirreceptor, which in the case of enterovirus consist in a canyon or depression. Several compounds as Pleconaril interact with this region and imped the cellular receptor recognition. Mutations in this region might result in an unsuccessful antiviral action. Comparative sequence studies will help to elucidate the genetic differences between the clinical isolate and the reference strains in order to know if the SI difference values cause resistance to the antiviral compound, which need to be identified. Besides a CVA24 reference strain might be used. On the other hand, it is important to take into account that the antiviral action could be related to the blockade of the cellular receptor, which might be different in the cell line employed.

The extract showed an extracellular virucidal activity against E9 and CVA16, which suggests that *Sargassum*-derived metabolites can inactivate the viral particle by destroying or blocking the viral sites of adhesion to cells. In addition, the extract diminished the infective titer of both viruses in the cell in more than two logarithms, which is agree with previously reported data indicating a reduction of the 99 percent of infective particles (Hu and Hsiung, 1898). These results support the potential uses of this natural product as a disinfectant solution and further studies will be required for their use as a drug to treat enterovirus disease.

Previous preliminary characterization of the hydroalcoholic extract of *Sargassum fluitans* (Ponce *et al.,* 2018) showed compounds such as quinones,

triterpenes, proanthocyanidins, and catechins, tannins, reducing polysaccharides, proteins, and non-reducing polysaccharides. In general, the most abundant metabolites were tannins, amine groups, and reducing and non-reducing polysaccharides. Other authors have found these metabolites in plants and Sargassum seaweeds (Jassim *et al.,* 2003, Casas *et al.,* 2006; Ghada *et al.,* 2011; García *et al.,* 2016; Gutiérrez *et al.,* 2018), which have been associated with antiviral activities. Consequently, all tested enterovirus could be targeted by more than one antiviral mechanism.

It is noteworthy that the antiviral activity of some Sargassum genus's species has been evaluated mostly against HIV and herpesvirus (Ahmadi *et al.,* 2015). The active molecules are polyphenols (e.g. tannins, flavonoids, lignans, proanthocyanidins, quinones), glycosides, thiophenes, polycetones, alkaloids, terpenoids, polysaccharides, proteins and peptides (Zhu *et al.,* 2006; Behbahani *et al.,* 2013). Several metabolites such as anthraquinones, triterpenes, catechins derivatives and polysaccharides have shown antiviral activity against HIV, herpes simplex and cytomegalovirus (Zhu *et al.,* 2005). Of note, the plastoquinones from *Sargassum micracanthum* were also able to inhibit the replication of cytomegalovirus and measles (Iwashima *et al.,* 2005). Some authors have reported the antiviral activity of some flavonoids and tannins against EV (Yang *et al.,* 2013; Wang *et al.,* 2016). The antiviral activity of some species of Sargassum genus is attributed to its high composition of sulfated polysaccharides such as carrageenans, alginates, fucoidans and laminarans. Interestingly in this context, in a prior study we have identified a large amount of reducing and non-reducing polysaccharides (Ponce *et al.,* 2018). These molecules inhibit the virus adsorption and penetration into the cell as well as genome replication and protein translation (Zhu *et al.,* 2005; Lopes *et al.,* 2013; Ahmadi *et al.,* 2015). Thus, the antiviral activity of the hydroalcoholic extract of *Sargassum fluitans* demonstrated in this study may be related to proanthocyanidins, terpenoids, polysaccharides and amine groups associated with the presence of amino acids, proteins, or peptides. Further studies are necessary to investigate this thoroughly.

Likely, the putative active compound (s) present in the extract used in this study could be specific for each virus. In the case of being shared, the activity varies according to their molecular target affinity (Cos *et al.,* 2006). Specific inhibition against enteroviruses such as the crisopanic acid, an anthraquinone from *D. longifolia* has been reported in a previous study (Semple *et al.,* 2001). Interestingly, Zhang *et al.* reported in 2006 the antiviral activity of a watery extract of *Sargentodoxa cuneata* against PV and EV from group B (Zhang *et al.,* 2006). Other authors have reported the antiviral activity of flavonoids and tannins from medicinal plants against EV (Yang *et al.,* 2015; Wang *et al.,* 2016).

Considering that EV are non-enveloped viruses, the presence of virucidal activity of the hydroalcoholic extract of *Sargassum fluitans* against E9 and CVA16 can be caused by the ability of one or more compounds interacting with the capsid proteins. This interaction could contribute to the protein denaturalization and destruction of the capsid, allowing the loss of virions infectivity (Koch, 1985; Lopes *et al.,* 2013). It is also likely that the viral anti receptor may have an affinity for any of the extract compounds resulting in the blockade of its adsorption to the cell.

The results of the time of addition experiments suggest that antiviral action takes place on early and late replication stages of the replicative cycle for E9 and CVA16, respectively. According to the replicative cycle of poliovirus, intact virus particles are observed in the cytoplasm (parental virions) directly below the plasma membrane in micro pinocytic vesicles within the period of 0 and 1 hour after inoculation (Koch, 1985). Considering the poliovirus replication cycle is similar to those found in most of the EV, it is tempting to speculate that inhibition of E9 replication occurs at early stages, such as adsorption and penetration of the virus

into cells. Indeed, no viral titer reduction was seen when the extract was added at two hours post-infection, suggesting that the intermediate and late stages of the replicative cycle are not inhibited. According to Koch and Koch (1985) the release of progeny virions begins within six hours. It is therefore conceivable that inhibition of CVA16 replication by the extract is related to the impediment of the virus's release or perhaps because of an indirect antiviral action on the cells by preventing their lysis. It is important to remark that even when poliovirus represents a model of replication, differences in the duration of each stage of the replicative cycle might exist for each species. The time required for a single replication cycle varies from five to ten hours, depending on many factors, such as the host cells, the virus, pH, temperature and the number or viral particles (Oberste and Gerber, 2014). Although the EV have been widely studied, some aspects of the morphogenesis, remain still to be elucidated (Jiang *et al.,* 2014). Therefore, further studies are required to gain more comprehensive knowledge about the kinetics of the replicative cycle of studied viruses, which will help to elucidate most accurately the stage of the replication cycle inhibited by the extract. Moreover, a bio-guided fractionation of the extract must be performed to elucidate whether the several multiple modes of actions are due to several active compounds.

Molecules with antiviral activity could have an affinity for the same receptor or two different cell receptors present in the cell lines employed in this study. Bostina *et al.,* reported that the E9 receptor is the α5-β3 integrin (Bostina *et al.,* 2011). The CVA16 uses as cellular receptors the α5β6 and α5β1 integrins, the transmembrane protein type III, SCARB2 and the heparan sulfate molecule. As adsorption receptors, CVA16 uses sialic acid glycans (Upla, 2008; Yang *et al.,* 2009; Yamayoshi *et al.,* 2014); while others CVA share the ICAM-1 molecule as cellular receptor (Yang *et al*., 2009). In general, this assay was performed in different cell lines for each virus. Whether the antiviral mechanism involved could be specific in each case also needs to be addressed.

Conclusions

This study represents the first preliminary study that establishes the antiviral activity of *Sargassum fluitans* against echovirus 9, coxsackievirus A16 and coxsackievirus A24.

Futures studies are warranted to identify the bioactive compounds and elucidate their mechanisms of action.

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Abbreviations

CVA16: coxsackievirus A16

CVA24: coxsackievirus A24

E9: echovirus 9

CPE: cytopathic effect

EV: enteroviruses

Hep-2: Human epithelial type 2 cells

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

WHO: Word Health Organization

Vero: African green monkey kidney cells

HIV: human immunodeficiency virus

FBS: Fetal Bovine Serum

PV: poliovirus

Competing Interests

The authors declare there are no competing interests.

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