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## Research Article

**Title:** *In vitro* antiherpetic activity of compounds extracted from *Sargassum fluitans* (Børgesen) Børgesen 1914. Identification and *in silico* evaluation of potential antiviral molecules in a hydroalcoholic extract.

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### Abstract:

Herpes viruses (HSV) are among the most common viruses that infect humans. They cause blisters and inflammation of the oral and genital mucosa as well as severe symptoms such as blindness, hearing impairment and fatal encephalitis. Although acyclovir (ACV) and its derivatives are highly effective in treating recurrent lytic infections, the emergence of resistant strains remains a concern. In this study, the *in vitro* anti-herpetic activity of a hydroalcoholic extract obtained from the brown seaweed *Sargassum fluitans*, collected from strandings in Cuba, was evaluated. The hydroalcoholic extract was not cytotoxic in Vero cells ( $CC_{50} > 5000 \mu\text{g}\cdot\text{mL}^{-1}$ ) and inhibited replication of HSV-1 (ACV-susceptible and -resistant) and HSV-2 (ACV-resistant) strains with  $EC_{50}$  of 271.66, 403.62 and  $14.99 \mu\text{g}\cdot\text{mL}^{-1}$  respectively. In addition, first studies about mechanism of action were evaluated with ACV-sensitive HSV-1 model. The extract showed strong inhibition when pre-incubated with the virus and when the extract and virus were added simultaneously on the cells. The protection of Vero cells during the post-infection assay was very effective up to 1 h. A high resolution UPLC/MS-MS analysis detected 139 compounds, of which 44 were identified. *In silico* analysis was performed to determine the putative interaction sites of two flavones of interest identified in the extract with the viral glycoprotein D (gD). Molecular docking analysis revealed that takakin and tectorigenin mainly interact with the gD protein active center through hydrophobic interactions (Leu44, Pro50, Thr174, Lys169, Tyr208, Gln209) while hydrogen bonding interactions are less abundant (Ser43, Asp46, Lys169, Tyr208). This research represents a contribution to the valorization of *S. fluitans* as a source of anti-herpetic compounds in the context of recurrent and abundant seaweed strandings.

**Keywords:** *Sargassum*, Phaeophyceae; Vero cells; Herpes simplex virus, flavones

## 1. Introduction

Herpes simplex viruses are among the most common viruses infecting humans, causing recurrent infections in over 3.7 billion people worldwide (Looker et al. 2015; Madavaraju et al. 2021). These viruses belong to the *Alphaherpesvirinae* subfamily, which includes herpes simplex virus type 1 (HSV-1), type 2 (HSV-2) and varicella zoster virus. Infection can remain asymptomatic or lead to life-threatening complications (Ramchandani et al. 2016). Transmission between humans occurs through physical contact (Akhtar J and Shukla, 2009) and both HSV-1 and HSV-2 can infect oral and genital sites (Agelidis and Shukla, 2015). Infections caused by HSV-1 are associated with blistering and inflammation of the oral and genital mucosa and, in the most severe cases, can lead to blindness, hearing impairment and fatal encephalitis. HSV-2 causes genital lesions and is linked to the acquisition and transmission of HIV infection (Looker et al. 2017). Recently, HSV infections have also been associated with the risk of developing cancer (Koujah et al. 2019; Jha and Verma, 2021).

Acyclovir (ACV) and its derivatives are highly effective for treating recurrent lytic infections of Herpesviruses. However, the emergence of resistant strains, mainly in immunocompromised patients, (Chowdhury et al. 2014; Frobert et al. 2014) and the relative neurotoxicity of the available antiviral drugs (D'Aiutoa et al. 2017) motivate the search for new anti-herpetic drugs, especially from natural sources (Tremel et al. 2020; Ruchawapol et al. 2021).

Seaweeds possess a great diversity of metabolites with bioactive potentialities (El-Beltagi et al. 2022; Jin et al. 2022; Wei et al. 2022). The brown algae genus *Sargassum* (*Phaeophyta*, *Sargassaceae*) includes more than 300 species distributed throughout the world, mainly in tropical and subtropical areas (Liu et al. 2012). Several *in vitro* and *in vivo* biological activities such as anti-inflammatory, antioxidant, antitumoral and antiviral, have been reported for *Sargassum* species (Liu et al. 2012; Rushdi et al. 2020; Chaturanga et al. 2021; Garcia-Ruiz et al. 2022).

The antiviral activity has been associated with the presence of terpenoids, flavonoids, sterols, sulfated polysaccharides, polyphenols, sargaquinoic acid, sargacromenol and pheophytin (Yende et al. 2014; Pliego-Cortés et al. 2022). Polyphenols compounds such as flavonoids are capable to inhibit replication of several viruses (Badshah et al. 2021). These compounds showed antiherpetic activity in Vero cells such as virucidal activity, inhibition of adsorption, virus penetration into cell (Garrett et al. 2012), proteins expression and viral genome synthesis (Ahmad et al. 2015; Musarra-Pizzo et al. 2020). Recent studies have shown that mixtures of different flavonoids or combination of flavonoids with antiviral synthetic drugs allow the enhancement of their antiviral effects (Ninfali et al. 2020).

The holopelagic species *Sargassum fluitans* Børgesen (Børgesen), occurs along the east coast of Florida and forms floating masses in the central gyre of the North Atlantic known as the Sargasso Sea. Since 2011, mass strandings of *Sargassum* have been reported in the Caribbean Sea, the western central Atlantic, the Gulf of Mexico (Johnsa et al. 2020, Skliris et al. 2022, Alleyne et al. 2023) and in West Africa (Oyesiku and Egunyomi 2014). Large rafts of *Sargassum fluitans* drift along the northern coast of Cuba during winter and during summer on the southern coast (Moreira et al. 2013). The valorization of this biomass could counteract the ecological and economic consequences, such as the negative impact on native flora and fauna due to the decomposition of algae and the impact on tourist beaches (Milledge et al. 2020). Although several studies have investigated processes to add value to pelagic seaweeds, such as using the biomass to produce biochemicals or biofuels (Milledge and Harvey 2016; Marx et al. 2021; Davis et al. 2021), its possible use for human health is less explored (Quintal-Novelo et al. 2018).

In this context, the aim of this research was to evaluate the *in vitro* antiviral activity of a *Sargassum fluitans* hydroalcoholic extract at different treatment schemes against HSV-1 and HSV-2. The biomass was collected from blooms and stranding events on the Cuban coast. Chemical analysis of the hydroalcoholic extract was undertaken to identify potential antiviral candidates. Since the herpes simplex viral envelope glycoprotein gD is essential for virus entry into host cells, molecular docking simulation analyses were performed to determine whether two flavones identified in the hydroalcoholic extract, tectorigenin and takakin, were able to interact with this protein.

## 2. Materials and Methods

### 2.1. Algal Material

*Sargassum fluitans* typically has long, strap-like crenellated fronds that can be several meters in length and spherical floats, without sting or hook. The color of the fronds can range from olive green to dark brown. One of the distinctive features of *Sargassum fluitans* is its ability to float on the ocean's surface. It forms large floating mats that can cover extensive areas and provide essential habitats for various marine organisms, including small fish, crabs, shrimp, and juvenile sea turtles. These floating mats serve as nurseries for many marine species, offering both shelter and a source of food. *Sargassum* species, including *Sargassum fluitans*, have complex life cycles that involve alternation between sexual and asexual reproduction. The seaweed release reproductive structures called "berry-like structures" that contain gas-filled bladders, aiding in buoyancy and keeping the algae afloat. Fresh wild samples of *Sargassum fluitans* were collected while still in the water in proximity to the shore in Playa de Guanabo (23°10'44 "N-82°07'01 "W) 2018, Havana, Cuba. The samples of *Sargassum fluitans* were identified from the other *Sargassum* species in the *Sargassum* rafts by Dr. Beatriz Martínez (Center of Marine Research, University of Havana, Cuba). Morphological characteristics such as absence of spines on the elongated air bladders, presence of thorns on stem, mean blade length (L), width (W) and L/W ratio (mm) were considered for sorting (Schell et al. 2015). A sample of the specimen was deposited in the collection of the National Aquarium of Cuba with the number ANC A.H.001. The algae were washed with potable water to remove salts and sand. Then they were dried in a forced-air oven at 50°C until a constant weight was obtained and ground in a hammer mill to a particle size of < 6 mm.

### 2.2. Preparation of the hydroalcoholic extracts of *S. fluitans*

Extraction was carried out by applying a hydroalcoholic solution (17% ethanol concentration) to the algae (10 mL per gram of dry algae). The extract was prepared with 10 g of dry and crushed seaweed, which was mixed with 100 mL of hydroalcoholic solution and placed on a water bath at 50°C, under constant stirring at 800 rpm for 120 min. The extract obtained was then cooled to 25°C and collected by filtration through a 180 mm diameter qualitative Whatman filter paper (Whatman, USA), and stored in amber vials at 4°C until analysis (Gutiérrez et al. 2017).

### 2.3. High-resolution UPLC/MS-MS analysis of *S. fluitans* hydroalcoholic extract

An ultra-high-performance liquid chromatography ("Acquity UPLC H-class", Waters, Milford, CT, USA) coupled to high resolution mass spectrometry equipped with an electrospray ionization source ("XEVO-G2-S Q-TOF", Waters, Manchester, UK) was used to determine the composition of the hydroalcoholic extract (Goh and Gledhill, 2012; Oesterle et al. 2022). The extract was centrifuged at 15.6 g for 10 minutes and injected at 10 mg.mL<sup>-1</sup> to enhance the MS signal. Because of suspicion of polar compounds, an HSS T3 column (2.1 x 150 mm, 1.8 µm, Waters) was used for a better separation. The UPLC parameters were: Flow: 0.3 mL.min<sup>-1</sup>; T = 40°C; Solvent A: H<sub>2</sub>O + 0.1 %

formic acid; Solvent B: ACN + 0.1 % formic acid. Separation of extracted compounds was realized with the following gradient: 0-2 min, 100% A; 2-10 min, 100-0% A; 10-12, 0% A; 12.1-17 min, 100% A. The MS parameters were: Acquisition polarities: negative and positive; m/z range: 100 to 1200; Scan time: 0.1 sec; Collision energies: 6V for low energy spectra (MS) and 15 to 50V ramping for high energy spectra (MS-MS). Injection volumes were 5  $\mu$ L for MS and 10  $\mu$ L for MS/MS analyses.

#### 2.4. Viruses and cells

The virus strains used in the study were the wild-type HSV-1 strain 17, sensitive to acyclovir, and two strains resistant to acyclovir (HSV-1r and HSV-2r), obtained from Pr. David Boutolleau (Virology Department, Hospital Pitié-Salpêtrière, France). Vero cells (African green monkey kidney cell line, ATCC CCL81) were used for replication of HSV. The cells were grown in MEM supplemented with 8% fetal calf serum (FCS, Eurobio) and 1% of antibiotics PCS (10,000 IU penicillin  $\text{mL}^{-1}$ , 25,000 IU colimycin  $\text{mL}^{-1}$ , 10 mg streptomycin  $\text{mL}^{-1}$ , Sigma). All the cells were incubated at 37°C in a humidified atmosphere supplied with 5%  $\text{CO}_2$  (Langlois et al. 1986; Boulho et al. 2017).

#### 2.5. Evaluation of the cytotoxicity of *S. fluitans* hydroalcoholic extract by cell viability

96-well flat-bottom microplates TC Falcon (Corning, France) were prepared by adding 100  $\mu$ L per well of a cellular suspension of Vero cells ( $3.5 \times 10^5$  cells. $\text{mL}^{-1}$ , 35,000 cells per well). Cytotoxicity was evaluated by the neutral red dye method (Langlois et al. 1986). *S. fluitans* hydroalcoholic extract was studied at final concentrations ranging from 250 to 5000  $\mu\text{g} \cdot \text{mL}^{-1}$  in the cell culture medium (6 wells per concentration). Cells were incubated for 72 hours at 37°C and 5%  $\text{CO}_2$  and examined under a phase contrast microscope to confirm the presence of the cell monolayers, evaluate the possible detachment of cells from the wells and observe possible alterations on cell morphology (swelling, shrinkage or granularity). Then, 50  $\mu$ L per well of neutral red were added for a cell viability assay, and the microplates were incubated for 45 min at 37°C. The cell culture supernatant containing the extracellular dye was eliminated, and the wells were washed out with 200  $\mu$ L PBS buffer (two times). Finally, 100  $\mu$ L of ethanol citrate was added to burst the living cells, and thus the intracellular neutral red was released and read by spectrophotometry at  $\lambda = 540$  nm (Pliego-Cortés et al. 2022). The OD is directly related to the percentage of viable cells, which is inversely related to the cytotoxic effect of the extract. Data were expressed in term of % cell viability after treatment with the extract, as compared to the untreated cells (100% cell viability).

$$\% \text{ of Cell Viability} = \text{OD}_{50} (\text{t}) / \text{OD}_{50} (\text{c}) * 100$$

$\text{OD}_{50}$  CC (t): media absorbance value of treated cells culture

$\text{OD}_{50}$  CC (c): media absorbance value of untreated cells

Using the same cell culture model, the cytotoxicity of acyclovir (ACV, [9-(2-hydroxyethoxymethyl)guanine], Thermo, France) was evaluated at concentrations ranging from 0.05 to 20  $\mu\text{g} \cdot \text{mL}^{-1}$ . This compound was employed as a reference inhibitor of HSV-1s, HSV-1r and HSV-2r.

#### 2.6. Evaluation of the antiviral activity of *S. fluitans* hydroalcoholic extract against HSV-1s, HSV-1r and HSV-2r

96-well flat-bottom microplates (Corning, France) were prepared by adding 100  $\mu$ L per well of a Vero cell suspension ( $3.5 \times 10^5$  cells. $\text{mL}^{-1}$ , 35,000 cells per well). Cells were infected with 50  $\mu$ L of viral suspension at a Multiplicity Of Infection (MOI) of 0.01; 0.001; 0.005  $\text{ID}_{50}/\text{cell}$  for HSV-1s, HSV-1r and HSV-2r respectively. Positive antiviral controls were performed by adding 50  $\mu$ L of acyclovir

per well at five concentrations (5.0, 1.0, 0.5, 0.1, 0.05  $\mu\text{g.mL}^{-1}$  for HSV-1 and 20, 10, 5, 1, 0.5  $\mu\text{g.mL}^{-1}$  for HSV1r and HSV-2r). For the algal extract antiviral assay, 50  $\mu\text{L}$  of the hydroalcoholic extract were added to the wells at the concentrations: 12.5, 25, 50, 100, 150, 200, 250, 500, 800, 1000  $\mu\text{g.mL}^{-1}$  for HSV-1s and 100, 200, 250, 300, 350, 400, 450, 500, 800 and 1000  $\mu\text{g.mL}^{-1}$  for HSV-1r and HSV-2r. A positive virus replication control and a positive cell control were also tested. The microplates were incubated for 72 hours at 37°C and 5%  $\text{CO}_2$ . After incubation, all microplates were examined under a phase contrast microscope to confirm the presence of cytopathic effect (CPE). Next, the neutral red test for cell viability was performed as previously described (Pliego-Cortés et al. 2022). The OD is related directly to the percentage of viable cells, which is inversely related to the cytopathic effect (CPE). The effective 50% antiviral concentration of the extract ( $\text{EC}_{50}$ , expressed as  $\mu\text{g.mL}^{-1}$ ) was determined using a linear regression based on cell control (0% CPE) and virus control (100% CPE). Antiviral data were expressed as percentage of cell viability. Selective Index (SI), was calculated in each case as follow:  $\text{SI} = \text{CC}_{50}/\text{CE}_{50}$  (Gunawan et al. 2021)

## 2.7. Antiviral activity at different treatment schemes

The antiviral activity of *S. fluitans* extract was evaluated at different treatment schemes (Pliego-Cortés et al. 2022) with HSV-1s/Vero model ( $\text{MOI } 0.001 \text{ ID}_{50}/\text{cells}$ ,  $3.5 \times 10^5 \text{ Vero cells.mL}^{-1}$ ).

### Pre-incubation of cells with the extract

This assay was made to assess if pre-incubation of cells with the extract could protect cells against viral infection. Microplates were prepared by adding to each well 100  $\mu\text{L}$  of cellular suspension ( $3.5 \times 10^5 \text{ cells.mL}^{-1}$ ), 50  $\mu\text{L}$  of acyclovir (5, 1, 0.5, 0.1 and 0.05  $\mu\text{g.mL}^{-1}$ ) or 50  $\mu\text{L}$  of the extract (250, 500 and 1000  $\mu\text{g.mL}^{-1}$ ) and 50  $\mu\text{L}$  of MEM. After 24 h of incubation at 37°C, 5%  $\text{CO}_2$ , microplates were washed with 100  $\mu\text{L}$  of PBS. Fifty  $\mu\text{L}$  of viral suspension and 150  $\mu\text{L}$  of MEM were added to cells and these were incubated for 72 h at 37°C, 5%  $\text{CO}_2$ .

### Pre-incubation of viruses with the extract

This assay was made to assess if pre-incubation of viruses with the extract could block or reduce viral infection. Microplates were prepared with 100  $\mu\text{L}$  of cellular suspension ( $3.5 \times 10^5 \text{ cells.mL}^{-1}$ ) and incubated for 24h (37°C, 5%  $\text{CO}_2$ ). Equal volumes of viral suspensions and the extract or acyclovir were incubated for one hour (37°C, 5%  $\text{CO}_2$ ). Afterward, 100  $\mu\text{L}$  of each mixed suspension were added to Vero cells and these were incubated for 72 h (37°C, 5%  $\text{CO}_2$ ).

### Influence of addition time of the extract on viral infection (Post-infection assay)

This assay was made to assess at which time of viral infection (virus adsorption to cells, intracellular replication) the extract was active. The confluent cell monolayers ( $3.5 \times 10^5 \text{ cells.mL}^{-1}$ ) were infected with 100  $\mu\text{L}$  of virus suspension and incubated (37°C, 5%  $\text{CO}_2$ ). The extract or acyclovir were added to cells at the same time than virus (0 h), or 1, 2, 3 or 5 h post-infection.

### Influence of the extract on viral adsorption to cells

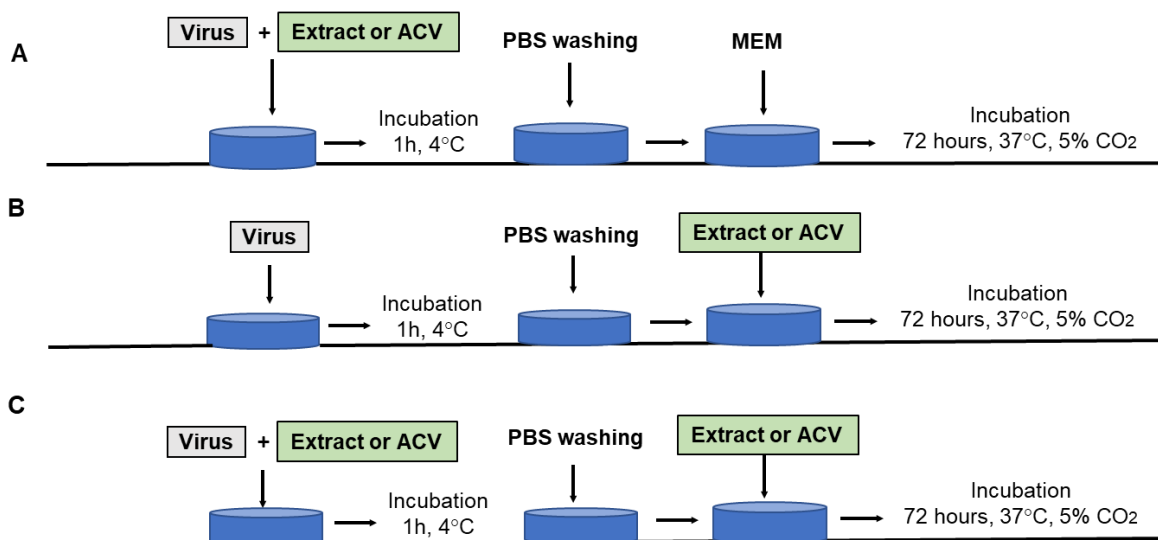
Vero cells ( $3.5 \times 10^5 \text{ cells.mL}^{-1}$ ) were seeded and incubated for 24 h at 37°C, 5%  $\text{CO}_2$  and then they were infected following three different treatments: treatment A (TA), treatment B (TB) and treatment C (TC) (Figure 1).

In TA, Vero cells were exposed to the virus (50  $\mu\text{L}$ ) and the extract or ACV (50  $\mu\text{L}$ ) simultaneously. Then cells were incubated for 1 h at 4°C to promote the viral adsorption. Later cell monolayers were washed with 100  $\mu\text{L}$  of PBS to remove the extract or ACV and unabsorbed virions. Then 200  $\mu\text{L}$  of

MEM with 8% of FCS were added, and the microplates were incubated for 72 h, at 37°C, 5% CO<sub>2</sub> (Figure 1.A).

In TB Vero cells were first exposed to the virus (50 µL) for 1 h at 4°C in absence of the extract or ACV. Then the unabsorbed virions were removed by rinsing the cell monolayers with PBS. Then 50 µL of the extract or ACV and 150 µL of MEM were added and cells were incubated (72 h, 37°C, 5% CO<sub>2</sub>) (Figure 1.B).

In TC Vero cells were exposed to the virus (50 µL) and the extract or ACV (50 µL) simultaneously and were incubated for 1 h at 4°C to allow viral adsorption. Then cell monolayers were washed with 100 µL of PBS, 50 µL of the extract or ACV and 150 µL of MEM were added and cells were incubated (72 h, 37°C, 5% CO<sub>2</sub>) (Figure 1.C).



**Figure 1.** Schematic representation of the different treatments of Viral adsorption assays. **A:** treatment A, **B:** treatment B and **C:** treatment C, ACV: acyclovir, PBS: phosphate buffer solution.

## 2.8. Molecular docking simulations

The structure of gD protein was found in PDB database (PDB: 4MYV; Lu et al. 2014). Simulations were performed with Autodock Vina software (Trott and Olson, 2010). The software AutoDock Tools v1.5.6 (ADT; Morris et al. 2009) was used to *in silico* prepare the protein and ligands (takakin and tectorigenin) molecules for simulations. All hydrogen atoms were added to the molecules, the Gasteiger charges were calculated, the non-polar hydrogens were eliminated, and the AD4 atom-type was assigned to each atom, following the methodology of (Verma et al. 2017). The LAPTc residues were considered rigid. The dimensions (26 Å) and the center of the molecular docking box were selected visually to contain all possible binding sites.

The 3D structures of takakin and tectorigenin were generated by the graphical drawing interface Avogadro version 1.2 (Hanwell et al. 2012). All rotatable torsion angles of both molecules were defined as flexible. The docking parameters set by default in the software were kept, except the following: energy\_range = 4 kcal, num\_modes = 20. The different conformers of takakin and tectorigenin were grouped using as criterion a RMSD value of 2Å. In each group, the conformation with the lowest free energy of binding, according to AutoDock Vina punctuation function, was selected. Results were analyzed with Ligplot+ v2.2.5 (Humphrey et al. 1996).

## 2.9. Statistical Analysis

All results were presented as mean of n replicated  $\pm$  standard deviation. According to the respective experiment (specified in each table or figure), the significant differences were analyzed by the *t*-Student test (two tails) or by one-way ANOVA. All analyses and graphics were performed and elaborated respectively using Microsoft Excel (Microsoft Office 2021) with a significance level  $\alpha$  established at 0.05 for all tests. Linear regression  $R^2$  coefficient was used to correlate the antiviral activity ( $EC_{50}$ ) with the *S. fluitans* extract concentration.

## 3. Results

### 3.1. Cytotoxicity of *S. fluitans* hydroalcoholic extract and ACV in Vero cells

After 3 days of treatment, no cytotoxic effect of the compounds on Vero cells was observed in the range of concentrations tested for the extract (up to 5000  $\mu\text{g.mL}^{-1}$ ). No visible microscopic alteration of normal cell morphology neither destruction or detachment were observed.

### 3.2. Antiherpetic activity of *S. fluitans* hydroalcoholic extract and ACV

The hydroalcoholic extract of *Sargassum fluitans* showed moderate inhibitory effect against HSV1-s and HSV1r while a high inhibitory effect against HSV-2r, with  $EC_{50}$  values of  $271.66 \pm 38.55 \mu\text{g.mL}^{-1}$ ,  $403.62 \pm 47.75 \mu\text{g.mL}^{-1}$  and  $14.99 \pm 0.88 \mu\text{g.mL}^{-1}$  respectively (Table 1). The highest SI extract was on the HSV-2r (333.35), which significantly differed from those obtained for HSV-1s and HSV-1r ( $p=0.0019$  and  $p=0.027$  respectively).

**Table 1.** Cytotoxicity and antiviral activity of *Sargassum fluitans* hydroalcoholic extract and Acyclovir against HSV-1s, HSV-1r and HSV-2r in Vero cells.

Virus strain	Treatment	$CC_{50}$ ( $\mu\text{g.mL}^{-1}$ )	$EC_{50}$ ( $\mu\text{g.mL}^{-1}$ )	SI
HSV-1 s	ACV	>5.00	<0.05	$\geq 100.00$
	extract	> 5000.00	$271.66 \pm 38.55$	$\geq 18.40$
HSV-1 r	ACV	>20.00	<1.00	$\geq 20.00$
	extract	>5000.00	$403.62 \pm 47.75$	$\geq 12.38$
HSV-2 r	ACV	>20.00	<0.05	$\geq 400.00$
	extract	>5000.00	$14.99 \pm 0.88$	<b><math>\geq 333.55^*</math></b>

Data are the mean  $\pm$  standard deviation ( $n = 3$ ). \* Significance difference ( $p < 0.05$ ) by row highlighted in bold, HSV-2 treatment vs. HSV-1s and HSV-1r treatments (*t*-test for independent samples, two tails).

### 3.3. Antiviral activity of the hydroalcoholic extract at different treatment schemes against HSV-1 sensitive

The antiviral modes of action of the extract were determined by performing different treatments on the HSV-1s/Vero model: pre-incubation of cells with the extract, pre-incubation of viruses with the extract, influence of extract addition time and influence on viral adsorption assay (Table 2).

#### Pre-incubation of cells with the extract



Vero cells were not protected from HSV-1 infection when the extract was added to cells, incubated and then washed before virus infection. The extract did not induce a durable antiviral activity in Vero cells. ACV conferred a significant cell protection ( $p=0.00035$ ) although cells were washed.

#### Pre-incubation of viruses with the extract

Vero cells were significantly protected when the virus was pre-incubated with the extract (250, 500 and 1000  $\mu\text{g.mL}^{-1}$ ) before infection ( $p=0.04$ ;  $p=0.0075$  and  $p=0.033$  respectively), suggesting a direct action of the extract on viral particles.

#### Influence of extract addition time on viral infection (Post-infection assay)

In the addition time assay three concentrations of the extract were evaluated (Table 2). At the lowest concentration (250  $\mu\text{g.mL}^{-1}$ ), the extract conferred the highest protection when added simultaneously with the virus to the cells (85 % of cell viability,  $p=0.01$ ). The antiviral activity diminished in the following post-infection times although cell viability was higher than 50%. Antiviral activity of ACV remained active up to 5 h post-infection (Table 3). At concentrations 500 and 1000  $\mu\text{g.mL}^{-1}$  the extract significantly protected cells ( $p=0.009$  and  $p=0.04$  respectively) even when added simultaneously with the virus. Besides, the extract conferred significant protection to cells at 500  $\mu\text{g.mL}^{-1}$  when added five hours after infection ( $p=0.009$ ). Cell viability values over 70% were observed at 500 and 1000  $\mu\text{g.mL}^{-1}$  for all post-infection times. In all cases the ACV remained active.

In summary, at the evaluated concentrations, the highest cell protection was conferred, when extract was added simultaneously to the cells with virus infection. On the other hand, results indicated that the decrease in antiviral activity from two hours post-infection onward at 250  $\mu\text{g.mL}^{-1}$ , was compensated by increasing the extract concentration.

**Table 2.** Antiviral activity of the hydroalcoholic extract of *S. fluitans* or ACV evaluated in parallel against HSV-1 in the Post-Infection Assay in Vero cells infected with HSV-1 sensitive to ACV. Antiviral activity expressed as percent of cell viability and viral suspension at MOI 0.001 ID<sub>50</sub>/cells. ACV: Acyclovir 5  $\mu\text{g.mL}^{-1}$ ; 0h: extract added simultaneously with virus infection to cells; 1h: after 1 hour; 2h: after 2 hours, 3h: after 3 hours; 5h: after 5 hours.

Post-infection time	Extract or ACV concentration		
	Extract: 250 $\mu\text{g.mL}^{-1}$ / ACV (5 $\mu\text{g.mL}^{-1}$ )	Extract: 500 $\mu\text{g.mL}^{-1}$ / ACV (5 $\mu\text{g.mL}^{-1}$ )	1000 $\mu\text{g.mL}^{-1}$ / ACV (5 $\mu\text{g.mL}^{-1}$ )
	Percent of cell viability		
0 h	<b>85.24</b> $\pm$ 0.03 */ 84.37 $\pm$ 0.05	<b>91.08</b> $\pm$ 0.006 */ 81.12 $\pm$ 0.001	<b>98.23</b> $\pm$ 0.09 */ 81.52 $\pm$ 0.12
1 h	65.99 $\pm$ 0.01 / 80.74 $\pm$ 0.06	<b>82.22</b> $\pm$ 0.014/ 82.45 $\pm$ 0.02	<b>85.74</b> $\pm$ 0.13/ 88.54 $\pm$ 0.19
2 h	55.89 $\pm$ 0.06/ 87.80 $\pm$ 0.08	<b>78.84</b> $\pm$ 0.034/ 76.81 $\pm$ 0.03	<b>90.96</b> $\pm$ 0.20/ 73.45 $\pm$ 0.18
3 h	59.39 $\pm$ 0.01/ 85.61 $\pm$ 0.08	<b>81.59</b> $\pm$ 0.044/ 79.44 $\pm$ 0.0007	<b>82.92</b> $\pm$ 0.08/ 76.03 $\pm$ 0.02
5 h	51.73 $\pm$ 0.09/ 83.17 $\pm$ 0.12	<b>74.86</b> $\pm$ 0.083/ 86.91 $\pm$ 0.06	<b>85.36</b> $\pm$ 0.02/ 89.42 $\pm$ 0.29

Data are the mean  $\pm$  standard deviation ( $n = 3$ ). \* Significance difference ( $p < 0.05$ ) by row, extract vs. virus control ( $t$ -test for independent samples, two tails). Values of cell viability higher than 70% are highlighted in bold.

#### Influence of the extract on viral adsorption to cells

When the extract and the virus were simultaneously added to the cells, adsorption was allowed to occur (1 h, 4°C) and then both were removed from cells monolayer, no cell protection was observed. However, when the extract was added to cells even after virus adsorption occurred, but remained in the cell culture during the 72 h of incubation, a significant inhibition of virus replication was observed (TB). The highest inhibition was observed in treatment C when both, virus and extract were added to cells simultaneously, then both removed but the extract was added again and remain during the 72 h of incubation. Similar results were obtained for ACV (Table 3).

**Table 3.** Effect of the hydroalcoholic extract of *S. fluitans* on HSV-1s (MOI 0.001 ID<sub>50</sub>/cells) adsorption to Vero cells. Antiviral activity is expressed as percent of cell viability, ACV: Acyclovir (5 µg.mL<sup>-1</sup>); TA: treatment A; TB: treatment B; C: Treatment C.

Concentration of the extract	TA	TB	TC
	Percent of cell viability		
250 µg.mL <sup>-1</sup>	54.70 ± 0.14	<b>93.11 ± 0.01 *</b>	<b>94.28 ± 0.25 *</b>
500 µg.mL <sup>-1</sup>	56.86 ± 0.004	<b>96.89 ± 0.07 *</b>	<b>100.00 ± 0.01 *</b>
1000 µg.mL <sup>-1</sup>	46.48 ± 0.33	<b>100.00 ± 0.12 *</b>	<b>100.00 ± 0.02 *</b>
Concentration of ACV	Percent of cell viability		
5 µg.mL <sup>-1</sup>	65.40 ± 0.24	<b>93.59 ± 0.02 *</b>	<b>95.41 ± 0.07 *</b>

Data are the mean ± standard deviation (n = 3). \* Significance difference (p < 0.05) by row, extract/ACV vs. virus control (t-test for independent samples, two tails). Values higher than 70% are highlighted in bold.

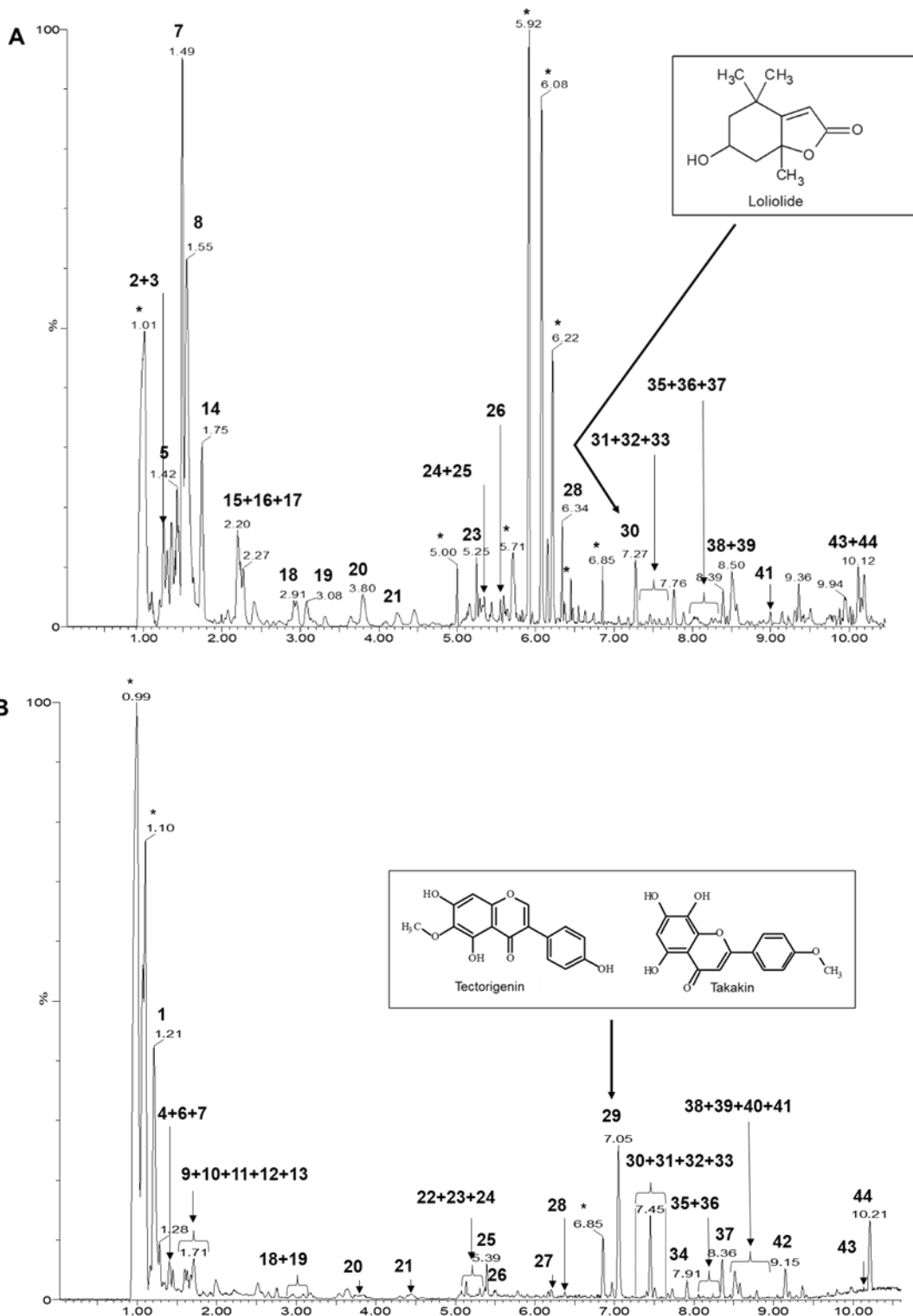
In summary the results previously described suggest that one of the modes of antiviral action of the extract is the inhibition of viral adsorption and/or penetration of the virus into cells, likely by the interaction of one or more compounds with viral envelope proteins instead of the cell receptors. To identify the potential candidate molecules responsible for the antiviral activity, a chemical characterization of the extract was made by high resolution UPLC/MS-MS.

### 3.3. UPLC/MS-MS of *S. fluitans* hydroalcoholic extract

Out of a total of 139 compounds, 44 compounds were detected as ions in both polarities and only 29 could be identified on the basis of their MS and MS/MS signatures (Table 4). For each of the secondary metabolites identified, a search for antiviral properties reported in the scientific literature was performed. Most of them are classical metabolites which haven't been reported until now as antiviral compounds. However, methanesulfonic acid and malic acid were previously reported effective on HIV (Patick et al. 1996; Natalini et al. 2011), (2-Deoxy-D-glucose) on SARS-CoV-2 (Pajak et al. 2022; Wali et al. 2022), Deazaguanine on Semliki virus (Girgis et al. 1990), Malic acid on HIV and rhinovirus infection (Honda et al. 2004; Hayden et al. 1985), fatty acid acetate as stimulating antiviral immunity (Antunes et al, 2023) and 2-Deoxy-D-glucose (2-DG), a Dual D-Glucose and D-Mannose Mimetic, against SARS-CoV-2 (Pajak et al. 2022). Three other compounds present in the extract attracted our attention as potential antiviral molecules never evaluated before. The first one, loliolide, is a commercially available carotenoid derivative already reported in seaweeds (Kimura and Maki, 2002). The second and third ones are non-commercially available flavones isomers, tectorigenin and takakin (corresponding to the same raw formula C<sub>16</sub>H<sub>12</sub>O<sub>6</sub>). These three molecules were selected for further *in vitro* and *in silico* antiviral evaluation (Figure 2, Table 5).

**Table 4.** Compounds identified by UPLC/MS-MS in the hydroalcoholic extract of *Sargassum fluitans*.

Compound	Mass formula	RT (min)	Adduct	Theoretical m/z	Mass error (mDa)
[(2S,3S,4S,5R)-6-(2,3-dihydroxypropoxy)-3,4,5-trihydroxy-tetrahydropyran-2-yl]methanesulfonic acid	C <sub>9</sub> H <sub>18</sub> O <sub>10</sub> S	1,21	[M-H] <sup>-</sup>	317,0542	0,2
Glyceric acid	C <sub>3</sub> H <sub>6</sub> O <sub>4</sub>	1,41	[M-H] <sup>-</sup>	105,0188	0,2
(3S,4R,5R)-1-(butylamino)-3,4,5,6-tetrahydroxy-hexan-2-one	C <sub>10</sub> H <sub>21</sub> NO <sub>5</sub>	1,42	[M+H] <sup>+</sup>	236,1498	0,3
Hexose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	1,44	[M-H] <sup>-</sup>	179,0556	0,4
Acetylcholine	C <sub>7</sub> H <sub>15</sub> NO <sub>2</sub>	1,55	[M+H] <sup>+</sup>	146,1181	1
<b>3-deoxy-d-glycero -d-galacto -2-nonulosonic acid (Kdn, family of deaminoneuraminic acid)/fructose lactate and galactose lactate also possible</b>	<b>C<sub>9</sub>H<sub>16</sub>O<sub>9</sub></b>	<b>1,6</b>	<b>[M-H]<sup>-</sup></b>	<b>267,0716</b>	<b>0,1</b>
Deoxy-Hexose + sulfate	C <sub>6</sub> H <sub>12</sub> O <sub>8</sub> S	1,62	[M-H] <sup>-</sup>	243,0175	0,1
dideoxy-hexose + sulfate	C <sub>6</sub> H <sub>10</sub> O <sub>8</sub> S	1,67	[M-H] <sup>-</sup>	225,0069	0,2
Malic acid	C <sub>4</sub> H <sub>6</sub> O <sub>5</sub>	1,71	[M-H] <sup>-</sup>	133,0137	0,3
Malic acid - H <sub>2</sub> O	C <sub>4</sub> H <sub>4</sub> O <sub>4</sub>	1,71	[M-H] <sup>-</sup>	115,0031	0,3
ethyl 2-[2-[(2-hydroxy-3-methoxy-propyl)amino]ethoxy]acetate	C <sub>10</sub> H <sub>21</sub> NO <sub>5</sub>	1,75	[M+H] <sup>+</sup>	236,14983	0,5
Adenin	C <sub>5</sub> H <sub>5</sub> N <sub>5</sub>	2,2	[M+H] <sup>+</sup>	136,0623	0,9
4-guanidinobutanoic acid	C <sub>5</sub> H <sub>11</sub> N <sub>3</sub> O <sub>2</sub>	2,22	[M+H] <sup>+</sup>	146,093	0,2
Guanin	C <sub>5</sub> H <sub>4</sub> N <sub>6</sub> O	2,23	[M+H] <sup>+</sup>	152,0572	0,9
6-hydroxynicotinic acid	C <sub>6</sub> H <sub>6</sub> NO <sub>3</sub>	2,95	[M+H] <sup>+</sup> /[M-H] <sup>-</sup>	140.0348/138.0191	0.7/0.8
Hypoxanthin	C <sub>5</sub> H <sub>4</sub> N <sub>4</sub> O	3,08	[M+H] <sup>+</sup> /[M-H] <sup>-</sup>	137.0463/135.0317	1/1
9-Deazaguanine	C <sub>6</sub> H <sub>6</sub> N <sub>4</sub> O	3,8	[M+H] <sup>+</sup> /[M-H] <sup>-</sup>	151.062/149.0463	0.5/0.6
Adenosine	C <sub>10</sub> H <sub>13</sub> N <sub>5</sub> O <sub>4</sub>	5,25	[M+H] <sup>+</sup> /[M-H] <sup>-</sup>	268.1046/266.0889	0.8/0.8
Guanine/Isoguanine	C <sub>5</sub> H <sub>4</sub> N <sub>6</sub> O	5,34	[M+H] <sup>+</sup> /[M-H] <sup>-</sup>	152.0572/150.0416	0.9/0.9
2-[3-[(2,4-dihydroxy-3,3-dimethyl-butanoyl)amino]propanoylamino]ethanesulfonic acid	C <sub>19</sub> H <sub>34</sub> O <sub>5</sub>	5,4	[M-H] <sup>-</sup>	325,1076	0,1
/L-/L peptide	C <sub>13</sub> H <sub>24</sub> N <sub>2</sub> O <sub>3</sub>	6,37	[M+H] <sup>+</sup> /[M-H] <sup>-</sup>	245.1865/243.1709	0.1/0.1
<b>Tectorigenine or takakin</b>	<b>C<sub>16</sub>H<sub>12</sub>O<sub>4</sub></b>	<b>7,05</b>	<b>[M-H]<sup>-</sup></b>	<b>299,0556</b>	<b>0,2</b>
Lolilolide	C <sub>11</sub> H <sub>16</sub> O <sub>3</sub>	7,27	[M+H] <sup>+</sup>	197,1178	0,6
Lolilolide	C <sub>11</sub> H <sub>16</sub> O <sub>3</sub>	7,42	[M+H] <sup>+</sup> /[M-H] <sup>-</sup>	197.1178/195.1021	0.6/0.7
Lolilolide	C <sub>11</sub> H <sub>16</sub> O <sub>3</sub>	7,58	[M+H] <sup>+</sup>	197,1178	0,6
9R/S,10R/S,13R/S-trihydroxyoctadec-10/11E-enoic acid (14 possible stereoisomers !)	C <sub>18</sub> H <sub>34</sub> O <sub>5</sub>	8,5	[M+Na] <sup>+</sup> /[M-H] <sup>-</sup>	353.2304/329.2328	0.7/0.7
9R/S,10R/S,13R/S-trihydroxyoctadec-10/11E-enoic acid (14 possible stereoisomers !)	C <sub>18</sub> H <sub>34</sub> O <sub>5</sub>	8,57	[M+Na] <sup>+</sup> /[M-H] <sup>-</sup>	353.2304/329.2329	0.7/0.7
9R/S,10R/S,13R/S-trihydroxyoctadec-10/11E-enoic acid (14 possible stereoisomers !)	C <sub>18</sub> H <sub>34</sub> O <sub>5</sub>	8,91	[M+Na] <sup>+</sup> /[M-H] <sup>-</sup>	353.2304/329.2330	0.7/0.7
<b>(2S)-2-[(E,1S)-1-hydroxydec-4-enyl]-2H-furan-5-one</b>	<b>C<sub>14</sub>H<sub>22</sub>O<sub>3</sub></b>	<b>10,19</b>	<b>[M+Na]<sup>+</sup>/[M-H]<sup>-</sup></b>	<b>261.1467/237.1459</b>	<b>3.2/3.2</b>



**Figure 2.** Chromatograms of the 29 identified compounds in *Sargassum fluitans*, in both positive (A) and negative (B) polarities. Loliolide and tectorigenin and/or takakin peaks are marked.

**Table 5.** Compounds of interest in the hydroalcoholic extract of *Sargassum fluitans* identified by UPLC/MS.

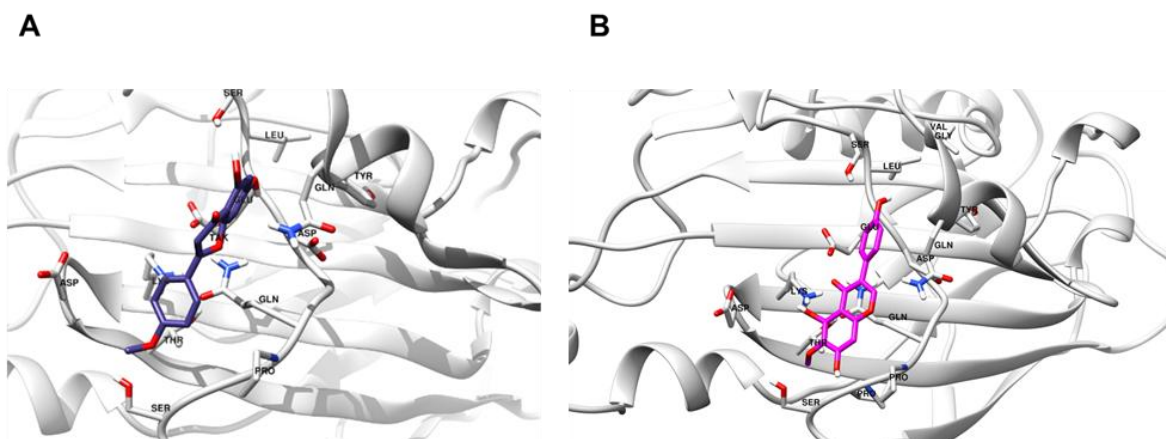
No.	Identified compound	Mass formula	Ion type	Polarity	RT(min)	Observed m/z	Theoretical m/z	$\Delta m/z$ (mDa)
29	Tectorigenin or takakin	C <sub>16</sub> H <sub>12</sub> O <sub>6</sub>	[M-H] <sup>-</sup>	-	7.05	299.0554	299.0561	0.7
30	Loliolide	C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	[M-H] <sup>+</sup>	+	7.27	197.1184	197.1172	1.2
31	Loliolide	C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	[M-H] <sup>+</sup>	+	7.42	197.1184	197.1172	1.2
33	Loliolide	C <sub>16</sub> H <sub>12</sub> O <sub>3</sub>	[M-H] <sup>+</sup>	+	7.58	197.1184	197.1172	1.2

**Legend.** RT: retention time; m/z: mass/charge ratio;  $\Delta m/z$ : variation of mass/charge ratio; mDa: milidalton.

Loliolide standard (Sigma-Aldrich, France) was bought and its antiviral activity was studied at 200  $\mu\text{g}\cdot\text{mL}^{-1}$ . This compound was not cytotoxic however had no antiviral effect in the Vero cell infection model against HSV-1s. Considering the suggested inhibition of viral adsorption by the extract and the antiviral activity reported in the literature for structurally related flavonoids, an *in silico* study of the interaction of takakin and tectorigenin with herpes simplex glycoprotein D was performed.

### 3.4. Modelling of the interaction between herpes simplex glycoprotein D (gD) with the flavones takakin and tectorigenin by Molecular Docking

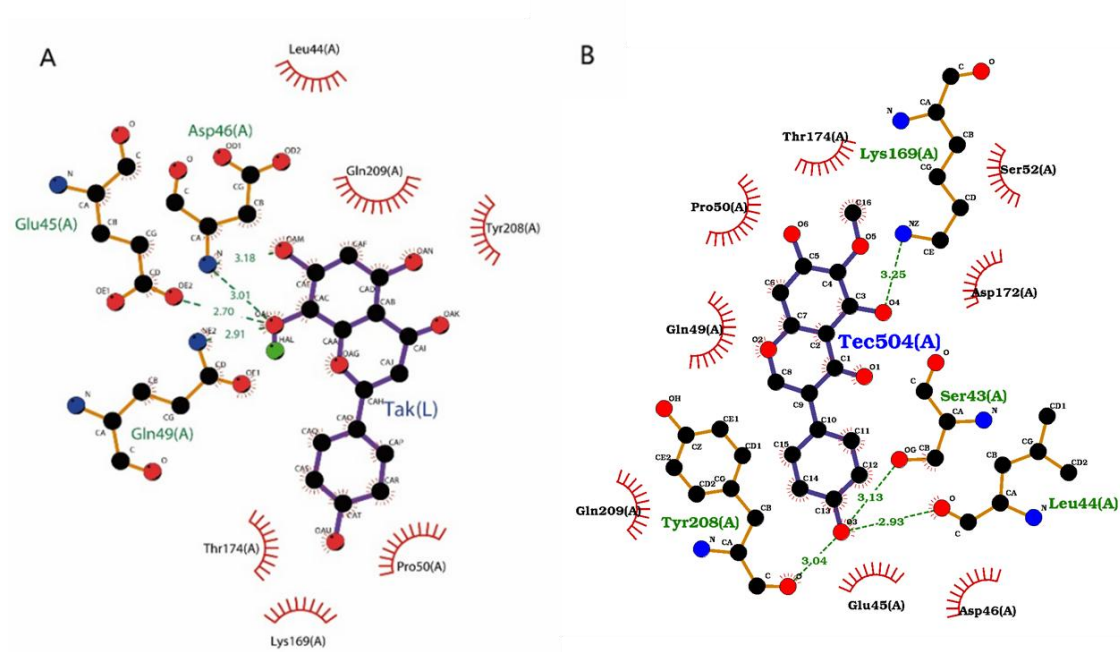
Takakin and tectorigenin compounds represent interesting structures to be studied as candidates for the antiviral activity, however they are not available as standards. No biological activity for takakin is described so far, while tectorigenin has one report of anti-herpetic activity against HSV-1 in Vero cells (Wang et al., 2022). In this view, we evaluated if these two flavones could interact with the HSV glycoprotein D, an essential envelope protein involved in adhesion and internalization of the virus in eukaryotic cells. The gD-takakin and gD-tectorigenin complexes were obtained by the AutoDock Vina v1.2 software (Eberhardt et al. 2021) and the conformations with the best energy values (-7.4 kcal/ moles and -5.971 kcal/moles respectively) were selected. Figure 3 reveals the insertion pattern in which both compounds can interact with the gD protein.



**Figure 3.** gD-takakin (A) and gD-tectorigenin (B) complexes in the interaction site of the protein (obtained by molecular docking, AutoDock Vina software). Takakin is represented in purple, tectorigenin in Magenta and gD protein in light grey.

The figure 4 shows that the stability observed for both takakin and tectorigenin is due to hydrophobic interactions with amino acid residues of the active center. In gD-takakin complex such residues are Leu44, Pro50, Thr174, Lys169, Tyr208 and Gln209. For gD-tectorigenin complex Glu45, Asp46, Gln49, Pro50, Asp172 and Gln209. On the other hand, the interactions through hydrogen bonding are less abundant and are established with hydroxyl groups in both cases. The amino acid residues that most contribute to the stabilization over time of the complexes through

hydrogen bonds are Glu45, Asp46, Gln49 (takakin) and Ser43, Leu44, Lys169 and Tyr208 (tectorigenin).



**Figure 4.** 2D representation of the interactions that occur between gD protein, takakin (A) and tectorigenin (B). Hydrophobic interactions between amino acid residues and both compounds are represented as red curved lines with black letters and hydrogen bonds with green dashed lines.

These data evidence the possible interaction of takakin and tectorigenin with the gD envelope glycoprotein, suggesting that they could take part to the antiviral activity of the *S. fluitans* hydroalcoholic extract by inhibiting adhesion and internalization of the HSV viruses in Vero cells. This *in silico* approach must be confirm *in vitro* by evaluating the anti-herpetic activity of this compounds in Vero cells.

## 4. Discussion

### ***Cytotoxicity and Antiviral Activity of S. fluitans***

Despite the existence of numerous studies on the antiherpetic activities of natural compounds (Hassan et al. 2019; van de Sand et al. 2021), the emergence of acyclovir and other nucleoside analogues resistant strains and its incidence in population, especially in immunocompromised patients, motivates the search of novel antiherpetic molecules in diverse natural sources. The antiherpetic activity of polysaccharides fractions (Bedoux et al. 2017) and the antiviral activity of crude extracts obtained from *Sargassum fluitans*, against human enteroviruses (Ponce et al. 2018; 2021) have been reported before by our group. In parallel to the hydroethanolic extract studied in this work, a global analysis of *S. fluitans* biomass composition revealed a rich content in monosaccharides (including fucose, rhamnose, arabinose, glucosamine, galactose, glucose, mannose, xylose, fructose, ribose, glucoheptose, and glucuronic acid), proteins, polyphenols and sulphated polysaccharides (data not shown), suggesting that many diverse metabolites can participate to the antiviral activity of extracts from this species.

The hydroalcoholic extract employed in the present study was obtained in the same conditions reported by Gutiérrez et al. 2017, under an optimized extraction process for total polyphenols. The extract was not cytotoxic in Vero cells, considering that at all concentrations evaluated (up to 5000  $\mu\text{g.mL}^{-1}$ ) the cell viability remained higher than 80-90%. This is similar to previous cytotoxicity evaluation in Vero cells which  $\text{CC}_{50}$  value of  $4315.94 \pm 168.10 \mu\text{g.mL}^{-1}$  (Ponce et al. 2021). In the current study the extract of *Sargassum fluitans* showed antiviral activity against ACV sensitive and resistant herpes virus strains in a dose-dependent manner. At concentrations above 200 and 400  $\mu\text{g.mL}^{-1}$  the fifty percent of cells were protected from HSV-1s and HSV-r infection with SI of 18.40 and 12.38 respectively. While the highest antiviral activity of the extract (SI = 333.55) was observed for HSV-2r.

### ***Activity at different treatment schemes against HSV-1 sensitive to Acyclovir***

The herpesvirus has a great capacity of infecting several human and non-human cells and a wide range of hosts (Karasneh et al. 2011). From a molecular point of view this capacity is due to the presence of a variety of glycoproteins in the virus envelope that mediate cell entry of the viral particle through a high diversity of cell receptors (Madavaraju et al. 2021). In the present study, the antiviral activity of the hydroalcoholic extract of *Sargassum fluitans* was evaluated at different treatment schemes against HSV-1s.

The pre-incubation of cells with the extract did not confer them protection against viral replication, while ACV protected them. Taking into account that ACV is a small nucleoside analogue, the most likely hypothesis is that it entered the cells during the incubation time and thus exerted its antiherpetic effect. When viruses were pre-incubated with the extract Vero cells were significantly protected. This clearly means a direct effect on viral particles that might be virustatic, virucidal or blockade of enveloped glycoproteins. Interestingly, although significant inhibition was observed when the extract was added simultaneously with the virus to the cells, when added 5 h after infection, more than 80 percent of the cells remained viable. From the viral adsorption inhibition assay, it was suggested that the extract inhibits this step of the replicative cycle and or penetration of the virus into the cells, by acting directly on the viral particle instead of any cellular receptor and the permanence of the extract in the cell culture significantly increased this observed protection.

In summary the results previously described suggest that the antiviral action of the extract is likely due to the interaction of one or more compounds with one or several herpes simplex viral envelope glycoproteins. The first step in HSV entry is the attachment through the envelope glycoproteins gB

and/or gC to heparan sulfate proteoglycans (HSPG) on the surface of the host cell. While gC protein is crucial in the heparan sulfate recognition during attachment of the HSV-1, gB is the most important glycoprotein during this step for HSV-2. Protein gC improves the interaction with heparan sulfate while gD and its interaction with the cellular gD receptor allows entry into the cell through membrane fusion (Cheshenko et al. 2002; Bacsa et al. 2011). Therefore, the target viral molecules might be any of the seven glycoproteins on the envelope of the herpesvirus, for instance, glycoproteins gB, gC, gD and gHgL, which are necessary to mediate entry of the virus in the cells, but gD can be considered as a critical target to inhibit viral entry in the cells. To identify the potential candidate molecules responsible for the antiviral activity, a chemical characterization of the extract was made by high resolution UPLC/MS-MS.

### **Chemical Characterization by UPLC/MS-MS of *S. fluitans* hydroalcoholic extract**

The brown seaweed genus *Sargassum* comprehends a great diversity of compounds (Biris-Dorhoi et al. 2020). In the present study, from 139 compounds detected by UPLC-MS/MS analysis, 44 were submitted to MS and finally 29 were clearly identified or few isomeric compounds were proposed. Among the proposed identified compounds, **loliolide**, **takakin** and **tectorigenin** resulted in interesting molecules due to few or no previous reported antiviral activity or first identified in *Sargassum fluitans*.

**Loliolide** has been found in *Sargassum horneri* (Dias et al. 2020; Han et al. 2021; Wang et al. 2021) and it has been reported for suppressing the oxidative stress and inflammation *in vitro* and *in vivo* (Dias et al. 2020; Han et al. 2021). **Loliolide** presents inhibition properties on the viral attachment, entry and fusion of hepatitis C viral particles. Induction of type I IFN host antiviral immune response has also been attributed (Chung et al. 2016). However, in our study, using a standard of loliolide, no antiviral activity against HSV-1s in Vero cells were observed at the evaluated concentrations.

On the other hand, **tectorigenin** has been reported for its antioxidant and anti-inflammatory activities by diminishing the levels of intracellular reactive oxygen species and increasing the expression of anti-oxidative enzymes, such as glutathione and catalase. A recent study reported the anti-herpetic activity of a tectorigenin derivative against HSV-1. The  $CC_{50}$  in Vero cells was 1 749.98  $\mu\text{g}\cdot\text{mL}^{-1}$  while the  $IC_{50}$  was 54.49  $\mu\text{g}\cdot\text{mL}^{-1}$ . This compound directly inactivated HSV-1 particles, modulated the expression of some Toll Like Receptors related pathways and suppressed the increase of inflammatory factor levels (Wang et al. 2022). So far, no biological activity is described for the compound **takakin**.

Considering that our extract is a phenolic-rich one (Gutiérrez et al. 2017), the extraction of the identified compounds represents an important step for further studies. The cell wall complexity with strong interactions between polysaccharides and proteins hinders bioactive compounds release during extraction. There are many physicochemical and biological methods to recover active compounds from algal biomass that guarantee increasing the yield and reducing extraction time. However, they need to be challenged to design eco-friendly process based on green and sustainable chemistry and engineering. Among those that can be used for the extraction of the compounds identified in *Sargassum fluitans* are the enzyme-assisted extraction, microwave-assisted extraction, ultrasound-assisted extraction, ultrasound–microwave-assisted extraction, hydrothermal-assisted extraction, high-pressure-assisted extraction, supercritical fluid extraction and pressurized liquid extraction (Grosso et al. 2015, Cotas et al. 2020; Garcia et al. 2021, Matos et al. 2021).



In view of the significant protection of Vero cells in its presence against HSV-2 ACV-resistant replication and inhibition of virus adsorption to cells observed in our study, an *in-silico* analysis was performed in order to preliminarily evaluate the capacity of flavones tectorigenin and takakin to interact with the viral glycoprotein gD of HSV-2.

### ***Modelling of the interaction between herpes simplex protein gD with flavones takakin and tectorigenin by Molecular Docking***

Tectorigenin and Takakin belongs to the phytochemical group of flavonoids, specifically flavones. Flavonoids are widely distributed as secondary metabolites produced by plants and seaweeds. They play important roles in plant and algal physiology and have been investigated for their *in vitro* and *in vivo* antiviral activities (Lin et al. 2000; Zakaryan et al. 2017). Some flavonoids are not toxic and their glycosides forms have higher antiviral activity than the non-glycoside ones (Ozçelik et al. 2011; Badshah et al. 2021). The proposed modes of action according to *in vitro*, *in vivo* and *in silico* results are the inhibitions of viral entrance, genome replication and proteins translation, as well as inhibition of virus assembly and release from cells. Immunomodulatory activities have been suggested as well (Ruchawapol et al. 2021; Šudomová et al. 2021). Recent studies have shown that mixtures of different flavonoids or combination of flavonoids with antiviral synthetic drugs allow the enhancement of their antiviral effects (Ninfali et al. 2020). On the other hand, the structural modification of these metabolites contributes in several cases to the improvement of their biological activities (Chatsumpun et al. 2016). Other studies have highlighted how the drug delivery systems may improve the aqueous solubility and bioavailability of flavonoids (Sims et al. 2020).

The antiherpetic mechanisms of action of flavonoids in Vero cells include virucidal activity, inhibition of adsorption, virus penetration into cell (Garrett et al. 2012), proteins expression and viral genome synthesis (Ahmad et al. 2015; Musarra-Pizzo et al. 2020). Some examples include the protocatechuic acid (Hassan et al. 2017), eachianone-G (Du et al. 2003; Tao et al. 2007), Galangin, Epicatechin, Epicatechin gallate, Quercetin and Genistein (Lyu et al. 2005).

*In silico* analysis have highlighted the antiviral potential of flavonoids such as quercetin and luteolin, (Sarwar et al. 2022; Cruz-Arreola et al. 2022), which have similar chemical structures with flavones takakin and tectorigenin identified in this study. Some flavonoids have been capable to block the catalytic triad of dengue virus protease NS2B/NS3 in molecular docking and simulation approaches (Qamar et al. 2017). Computational approaches showed that flavonoids such as luteolin, mundulinol, apigenin, quercetin and naringin exhibit binding affinity toward the protease and the spike protein of SARS-CoV-2 (Jain et al. 2021; El-Mageed et al. 2021), which make them interesting inhibitors (Rameshkumar et al. 2021).

In the present study the interactions of **takakin** and **tectorigenin** with the viral glycoprotein gD of HSV-2 was studied by *in silico* analysis. This protein is a type I membrane glycoprotein with 369 amino acid residues and 8-10 nm long approximately. It is organized into an ectodomain, a TMD and a short cytoplasmic tail (Cocchi et al. 2004; Madavaraju et al. 2021). The N-terminus is the receptor binding domain (RBD), the C-terminus is a pro-fusion domain (PFD) which interact with gH/gL and gB and also binds with the N-terminus region and forms an auto-inhibitory closed conformation. This domain is rich in proline residues and located proximal to the transmembrane segment (Fan et al. 2017). Some studies have shown that the infectivity of HSV-1 is affected when gD is absent. The RBD region is crucial to recognize the cell receptors, specially the first 22 aminoacids (Satoh et al. 2008; Gianni et al. 2009; Fan and Longnecker, 2010). Molecular docking results of our study revealed that takakin and tectorigenin compounds can interact with the gD protein. This interaction occurs at a superficial cavity of the protein where both compounds are mainly stabilized by hydrophobic interactions with residues such as Leu44, Glu45, Asp46, Gln49,

Pro50, Lys169, Thr174 and Gln209. In conjunction with these interactions, the formation of hydrogen bonds with residues Ser43, Leu44, Glu45, Asp46, Gln49; Lys169 and Tyr208, contributes to a lesser extent to the interaction.

The results obtained are consistent with those obtained for other compounds of similar structure, such as the flavone Myricetin (Wenmiao Li et al. 2020), that showed antiviral activity against HSV-1 and HSV-2 in Vero cells by inhibiting the CPE (Lyu et al. 2005). A Myricetin derivative has been described as an inhibitor of the reverse transcriptase of Human immunodeficiency virus type 1 in MT4 cells. Molecular docking approach suggested that the glycosylated moiety might enhance the antiviral activity, probably by favoring the internalization of the flavonoid into the cell (Ortega et al. 2017). Isaacs et al. demonstrated that the epigallocatechin gallate flavonoid interacts with and inhibits the gB and gD envelop proteins of HSV, which play essential roles during fusion and entry into the cell while quercetin inhibited all strains (Isaacs et al. 2008). Our study provides a first *in silico* approach to the possible mechanism of interaction of flavones takakin and tectorigenin with the HSV-2 viral protein gD. However the antiviral activity of these compounds must be evaluated *in vitro* in order to confirm the *in silico* results.

## 5. Conclusions

Natural renewable sources such as seaweeds, represent a promising source of bioactive compounds with pharmacological and cosmetic applications. Those with antiviral activities could be used in combination or as substitutes of synthetic drugs as long as their comprehends low-cytotoxicity and bioavailability. This study represents the first report of the antiherpetic activity of a crude extract of *Sargassum fluitans* against acyclovir resistant herpes simplex strains. Inhibition of virus adsorption to cells is supported by the *in vitro* and *in silico* results. The observed antiviral activity might be due to one compound or the synergic effect of several ones (Morán-Santibañez et al. 2018). The valorization of *Sargassum spp.* biomass for Human health applications such as treating infectious diseases requires a systematization in the study and characterization of its secondary metabolite composition (Oxenford et al. 2021) reason why a bio-guided fractioning of the extract will be necessary in order to support our findings. These results represent a contribution to the valorization of this species as a source of antiviral compounds.

## **6. Declarations**

### **6.1. Competing interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### **6.2. Authors' contributions**

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### **6.4. Availability of data and materials**

The data that support the findings of this study are available from the corresponding author, [NB].

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