



Antiviral activity of a crude extract of *Thalassia testudinum* Banks ex König against the human enteroviruses echovirus 9 and coxsackievirus A16

[Actividad antiviral de un extracto crudo de *Thalassia testudinum* Banks ex König contra los enterovirus humanos echovirus 9 y coxsackievirus A16]

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Abstract

Context: Echovirus 9 (E9) and coxsackievirus A16 (CVA16) are etiological agents of aseptic meningitis and hand, foot, and mouth disease, respectively. Currently, there are no approved antiviral drugs against these viruses. The marine plant *Thalassia testudinum* represents a potential source of bioactive compounds for the treatment of viral diseases with clinical and epidemiological importance.

Aims: To evaluate the *in vitro* antiviral activity of a hydroalcoholic extract of the marine plant *T. testudinum* against clinical isolates of echovirus 9 and coxsackievirus A16.

Methods: The cytotoxicity of the extract was evaluated in Vero and RD cells via the MTT method. The antiviral activity (EC₅₀) was determined by the inhibition of cytopathic effects. Extracellular virucidal activity and reduction in viral yield were determined via a viral titration assay. The viral genome was quantified after manual extraction and amplified using semi-nested RT-PCR.

Results: The extract was non-cytotoxic to Vero or RD cells (CC₅₀ = 3840.96 ± 116.12 µg/mL and 3822 ± 220.5 µg/mL, respectively) and inhibited the virus replication (CE₅₀ = 512.82 ± 22.32 µg/mL and 1367.1 ± 58.66 µg/mL, respectively), verified by the selective index values of 7.48 and 2.79 against echovirus 9 and coxsackievirus A16, respectively. The extract showed extracellular virucidal activity against E9 and reduced the infective titer by more than two logarithms. The CVA16 genome concentration was reduced when the cells were treated with increasing concentrations of the extract, although no significant differences were detected.

Conclusions: *Thalassia testudinum* crude hydroalcoholic extract inhibited the replication of the human enteroviruses E9 and CVA16. These results provide preliminary evidence of the potential of this plant as a source of antiviral compounds.

Keywords: antiviral; coxsackievirus; echovirus; marine plant; *Thalassia testudinum*.

Resumen

Contexto: El echovirus 9 (E9) y el coxsackievirus A16 (CVA16) son agentes etiológicos de la meningitis aséptica y la enfermedad de la mano, el pie y la boca, respectivamente. Actualmente, no hay medicamentos antivirales aprobados contra estos virus. La planta marina *Thalassia testudinum* representa una fuente potencial de compuestos bioactivos para el tratamiento de enfermedades virales con importancia clínica y epidemiológica.

Objetivos: Evaluar la actividad antiviral *in vitro* de un extracto hidroalcohólico de *T. testudinum* contra los aislados clínicos del echovirus 9 y el coxsackievirus A16.

Métodos: La citotoxicidad del extracto se evaluó en células Vero y RD a través del método MTT. La actividad antiviral (EC₅₀) se determinó mediante la inhibición de los efectos citopáticos. La actividad virucidal extracelular y la reducción en el rendimiento viral se determinaron mediante un ensayo de titulación viral. El genoma viral se cuantificó después de la extracción manual y se amplificó usando RT-PCR semi-anidada.

Resultados: El extracto no fue citotóxico a las células Vero o RD (CC₅₀ = 3840.96 ± 116.12 µg/mL y 3822 ± 220.5 µg/mL, respectivamente) e inhibió la replicación del virus (CE₅₀ = 512.82 ± 22.32 µg/mL y 1367.1 ± 58.66 µg/mL, respectivamente), verificado por los valores de índice selectivo de 7.48 y 2.79 contra echovirus 9 y coxsackievirus A16, respectivamente. El extracto mostró actividad virucidal extracelular contra E9 y redujo el título infeccioso en más de dos logaritmos. La concentración del genoma CVA16 se redujo cuando las células se trataron con concentraciones crecientes del extracto, aunque no se detectaron diferencias significativas.

Conclusiones: El extracto hidroalcohólico crudo de *Thalassia testudinum* inhibió la replicación de los enterovirus humanos E9 y CVA16. Estos resultados proporcionan evidencia preliminar del potencial de esta planta como fuente de compuestos antivirales.

Palabras Clave: antiviral; coxsackievirus; echovirus; planta marina; *Thalassia testudinum*.

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INTRODUCTION

Human enteroviruses, belonging to the *Picornaviridae* family, include enteroviruses, coxsackieviruses, rhinoviruses, polioviruses, and echoviruses (ICTV 2024; Sinclair and Omar, 2023), which cause severe and deadly epidemic diseases (Nikonov et al., 2017). Echovirus 9 (E9) is associated with viral pneumonia, encephalitis (Wang et al., 2020), nonpoliovirus-related flaccid paralysis (Danthanarayana et al., 2015; Rao et al., 2012), hepatitis (Bajema et al., 2017) and severe infections in infants, neonates and young children worldwide. Coxsackievirus A16 (CVA16) is one of the causative agents of hand, foot, and mouth disease (HFMD) in pediatric patients (Fang et al., 2018). HFMD is a disease characterized by fever and vesicles that appear on the hands, feet and buttocks, or genitals. In addition, infection with CVA16 is associated with the development of acute myopericarditis and herpangina (Mao et al., 2014; Yuan et al., 2023). To date, vaccines have been developed against the poliovirus group, which has limited the circulation of wild-type strains. Additionally, monovalent and polyvalent vaccines have been developed against enterovirus 71 (Zhu et al., 2023). However, no prophylactic or therapeutic approved treatments are available for the remaining enteroviruses (Baggen et al., 2018; Hwu et al., 2020; Yi et al., 2017).

Currently, terrestrial plants represent the most studied source of phytopharmaceuticals; however, marine pastures, which are composed of marine plants with flowers (Enerstvedt, 2018), have been less explored. These latter synthesize diverse chemical molecules in response to their unique ecological conditions (Hernández et al., 2016). *Thalassia testudinum* is the most abundant marine angiosperm inhabiting the Caribbean Sea, and some studies have reported the bioactive properties of extracts, fractions, and compounds obtained from this plant (de la Torre-Núñez et al., 2012; Hernández-Balmaseda et al., 2021; Regalado et al., 2009, 2011). These compounds include coumarins, reducing glucosides, phenols, tannins, α -amins, flavonoids, carbohydrates, catechins, and alkaloids (Regalado et al., 2009). Flavones thalassiolins A, B, and C have shown *in vitro* antiviral activity against the HIV cDNA integrase enzyme (Rowley et al., 2002), whereas thalassiolin D, a new flavone O-glucoside sulfate isolated from *Thalassia hemprichii*, displays moderate *in vitro* antiviral HCV protease activity (Hawas et al., 2017). However, no studies to date have shown antiviral activity against human enteroviruses.

In this context, we hypothesized that *T. testudinum*, a Caribbean marine angiosperm known for its bioac-

tive flavonoids, may exhibit antiviral activity against E9 and CVA16.

MATERIAL AND METHODS

Virus

In the present study, clinical strains of echovirus 9 (Hill reference strain) and coxsackievirus A16 (G10 reference strain) were used. Both belong to the National Reference Laboratory of Enterovirus of the Institute of Tropical Medicine "Pedro Kourí", Havana, Cuba.

Cell culture

Vero cells (ATCC® CCL-81™) were cultured in Earle-based MEM (Gibco™) supplemented with 2 mmol/L; glutamine supplemented with 1% HEPES solution, 1% sodium bicarbonate (7.5%), 2 mmol/L glutamine, and 5% fetal bovine serum (SIGMA®) and 1% neomycin antibiotic (Gibco BRL) and were used for the replication of echovirus 9. Human rhabdomyosarcoma cells (RD, ATCC® CCL-136™) were cultured in Earle-based MEM (Gibco™) supplemented with 1% HEPES solution, 1% 7.5% sodium bicarbonate, 2 mmol/L glutamine, 5% fetal bovine serum (SIGMA®) and 1% neomycin antibiotic (Gibco BRL) and were used for the replication of coxsackievirus A16. The cells were seeded in 96-well plates (Costar) at a density of 2.5×10^5 cells per well and were maintained in a 5% CO₂ atmosphere at 37°C.

Virus titration

Enterovirus titration was performed according to the Poliovirus Laboratory Manual titration protocol (WHO, 2004). The cells were seeded in 96-well plates, and when the cell monolayers were completely confluent, the growth media was removed, and 100 μ L of each decimal serial virus dilution was added in six replicates to the wells. One hundred microliters of medium was added to control cells (noninfected cells). The cells were checked daily through a microscope, at 40 $\times$ magnification (OLYMPUS®), and after five days, they were fixed and dyed with crystal violet. The infective titer was expressed as the mean infective dose in cell culture per unit volume (DICC₅₀/mL), and the Spearman-Kärber endpoint method was used as in equation [1].

$$\text{Log TCID}_{50} = -L - d(S - 0.5) \quad [1]$$

where L is the logarithm of minor virus dilution, d is the difference among the dilution logarithms, and S is the addition of the number of positive cytopathic effect wells.

Plant material collection and hydroalcoholic extract preparation

Thalassia testudinum Banks and Soland ex. K. D. Koenig, 1805 (*Hydrocharitaceae*) was collected in July 2018 in the Protected Natural Landscape Rincón de Guanabo, with coordinates ("23°10'44" N - 82°07'01" W). The specimen was taxonomically classified by Dr. Beatriz Martínez Daranas of the Marine Research Center of the University of Havana. A voucher sample was deposited in the herbarium of the Cuban National Aquarium, Havana, Cuba, with catalog number IdO39. Whole dry and ground *T. testudinum* leaves (840 g) were continuously extracted with EtOH-H₂O (50:50, v/v) for 24 h at room temperature. The extract was filtered and concentrated under reduced pressure at 40°C to yield 54 g of crude extract.

Chemical characterization of the hydroalcoholic extract

The batch used in the present study contained a percentage of phenols and thalassiolin B, the main component, in accordance with the quality criteria previously established. Qualitative and quantitative analyses of the extract were performed via standard phytochemical screening techniques, including colorimetric quantification, TLC, and HPLC analyses. The structures of the compounds were established by analysis of their spectroscopic (1D and 2D NMR) and spectrometric (HRMS) data, as well as by comparison of these data with those reported in the literature (Regalado et al., 2009). For the biological assay, the dried extract was dissolved in cell culture medium at a concentration of 10,000 µg/mL and subsequently filtered with a cellulose filter (0.45 µm, Minisart® Sartorius, Germany).

Cytotoxicity of *Thalassia testudinum* extract in Vero and RD cells

Cytotoxicity was evaluated via the MTT assay (Mosmann, 1983). The cells were seeded in 96-well plates and incubated at 37°C in a 5% CO₂ atmosphere for 48 h. Once the monolayers were completely confluent, the growth media was removed, 100 µL of increasing concentrations of the extract was added to the wells, and the cells were incubated for 48 h in a 5% CO₂ atmosphere at 37°C. Afterwards, the extract was removed, and the plates were washed three times with PBS. Then, 100 µL was added to each well, 10 µL of MTT (5 mg/mL, Sigma-Aldrich) was added, and the plates were incubated for 4 h under the same conditions described above. After this time, the supernatant was removed, and 100 µL of isopropanol was added to solubilize the formazan crystals. The optical density (OD) was subsequently measured using a microplate reader at 520 nm with a reference filter of

630 nm, as determined by a spectrophotometer MRX Revelation (Dynex Technologies, USA). Cytotoxicity was expressed as the median cytotoxic concentration (CC₅₀) and was determined via linear regression analysis. The percentage of cell viability was calculated as in equation [2].

$$CC_{50} = \frac{DO_{50} CC(t)}{DO_{50} CC(c)} \times 100 \quad [2]$$

Where DO₅₀ CC (t): absorbance media value of cells treated with the extract; DO₅₀ CC (c): absorbance media value of nontreated cells (considered 100% of cell viability).

Antiviral assays

Preliminary screening for the inhibition of cytopathic effects

The antiviral activity of the extract was evaluated via a CPE inhibition assay (Ponce et al., 2021). Completely confluent cell monolayers were treated with 100 µL of serial nontoxic concentrations of the extract and incubated for 1 hour at 37°C in a 5% CO₂ atmosphere. After this time, 100 µL of the virus (100 TCID₅₀) was added to the cells in sextuplicate, and the plates were incubated. The cells were observed daily under a light microscope for CPE. When all the wells corresponding to infected nontreated cells presented at least one CPE focus, infected treated cells were also read for CPE. The media effective concentration (EC₅₀) and the selective index (SI = CC₅₀/EC₅₀) were calculated (Ponce et al., 2021).

Determination of extracellular virucidal activity

The extracellular virucidal activity of the extract was determined, for which equal volumes of virus (100 or 10 TCID₅₀) and extract (250, 2500, or 5000 µg/mL) were mixed and incubated for 2 h at 26°C and 37°C, respectively. Then, 100 µL of each sample was added to cells seeded in 96-well plates, and the plates were incubated at 37°C and 5% CO₂ for five days. After incubation, the viral titer was determined as described previously. A titer reduction of 1 log₁₀ or more was considered positive for virucidal activity (Hu and Hsiung, 1989).

Determination of the infective titer reduction

This assay was performed to determine whether the extract could inhibit the formation of infective viral particles. Different concentrations of the extract were added to the cells, which were incubated for 1 h at 37°C and 5% CO₂. The cells were subsequently inoculated with 100 TCID₅₀ of the virus, and once all infected and untreated cells presented a CPE, the plates were frozen and thawed three times. The content of each well was centrifuged at 2,000 rpm, and 4°C, for 10 minutes (centrifuge Eppendorf 5615,

Table 1. Semi-nested RT-PCR of human enteroviruses.

Volume (μL)	Reaction 1	Reaction 2
5	Buffer (Tris HCl 67 mM and pH 8.8, NH ₄ SO ₄ 17 mM; EDTA 6 μM, MgCl ₂ 2 mM, 2-mercaptoetanol, 1 mM)	
2	dNTP	
1	Primer EV-PCR 2	
1	Primer EV-PCR 1	Primer EV-PCR 3
1	reverse transcriptase enzyme	Taq-polymerase enzyme
5/2	Extracted RNA	DNA from Reaction 1
29	RNAase-free Water	

Spain). The supernatants were subsequently collected, and the virus titer was determined as described above.

Detection of the viral genome by semi-nested RT-PCR in cells treated with the extract

Manual extraction of the viral nucleic acid from the supernatant of infected cells treated with the extract was performed according to the manufacturer's instructions (Qiagen). Viral nucleic acid was amplified via semi-nested reverse transcription-polymerase chain reaction (RT-PCR) (Table 1) via the following primers: EV-PCR1 (antisense) 5'-CATTGTCACCA TAA-GCAGCA-3', EV-PCR2 (sense) 5'-TCCTCCGG CCCCCCTGAATGCG-3', and EV-PCR 3 (antisense) 5'-ACACACGGACACCACCCAAAGTAGTCGGTTC C-3' for amplification of a 114 bp noncoding region of the 5' end of the genome (5'NTR) (Tapparel et al., 2009). Thirty cycles of denaturation (95°C), primer binding (55°C for normal and 42°C for degenerate), and extension (70°C for normal or 60°C for degenerate) were performed. The supernatant of noninfected nontreated cells was considered the negative control, whereas that from infected nontreated cells represented the positive control.

The amplification products were tested on a 2% agarose gel. For this purpose, 7 μL of each reaction product or the corresponding control was previously mixed with 3 μL of run buffer (Coral load of 10x). The samples were run at 110 V and 400 mA for 25 minutes in an electrophoresis chamber. After this time, the gel was observed in a transilluminator with ultraviolet light (UVT-02S, Biobase, China). The positive result for the presence of the viral genome in the sample was verified by the observation of a band with a size of 100 base pairs.

Quantification of viral nucleic acid

Quantification of previously amplified viral nucleic acid was performed using the commercial QIAx-

pert kit (Qiagen, 2014) according to the manufacturer's instructions. It allows the quantification of macromolecules (nucleic acids, proteins) in small sample volumes (Unger et al., 2019).

Statistical analysis

The cytotoxicity of the extract and antiviral concentration were expressed as the median value of the cytotoxic concentration (CC₅₀) and effective concentration (EC₅₀), respectively, along with their standard deviation. Both values were determined via regression analysis (Chiang et al., 2002). The statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparison test ($p < 0.05$), two-way ANOVA with two-sample t-tests ($p < 0.05$), and one-sample t-test via GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, Massachusetts, USA). The values are expressed as the mean $\pm$ standard deviation (SD).

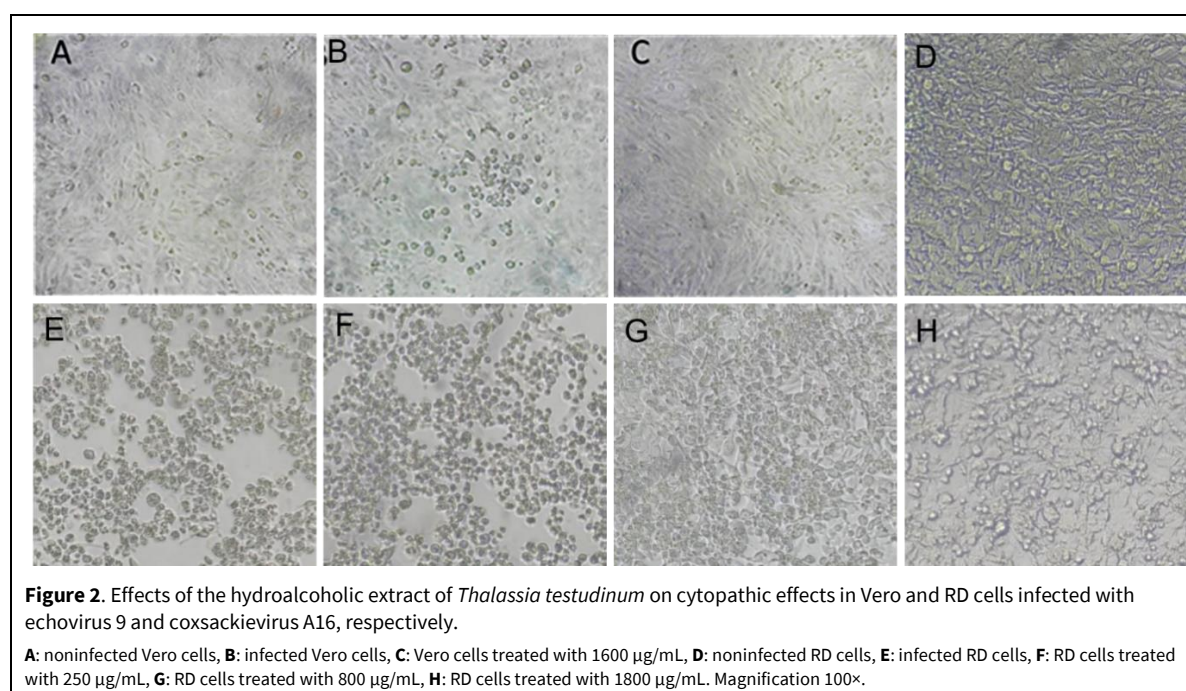
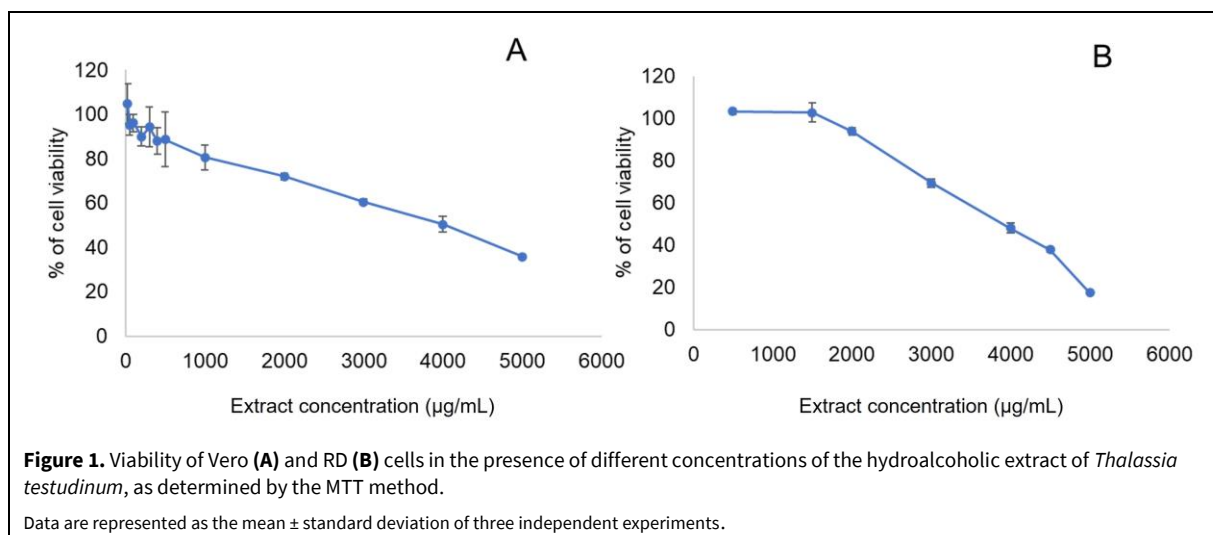
RESULTS

Cytotoxicity of *Thalassia testudinum* extract to Vero and RD cells

The cytotoxicity of the hydroalcoholic extract of *Thalassia testudinum* to Vero and RD cells was evaluated at concentrations ranging from 500 to 5000 μg/mL. In both cases, cell viability decreased with increasing concentrations of the extract (Fig. 1). Linear regression analysis revealed CC₅₀ values of 3840.96 ± 116.12 μg/mL and 3822 ± 220.5 μg/mL for Vero and RD cells, respectively (Table 1).

Antiviral activity

Antiviral activity was evaluated by CPE inhibition with nontoxic concentrations of the extract (200 to 2000 μg/mL). The extract inhibited E9 and CVA16 replication, as verified by a decrease in CPE with increasing concentrations (Fig. 2). In the presence of 1,600 μg/mL of the extract, the Vero cells preserved



their epithelial morphology similar to that of the non-infected cells, in contrast to the infected and nontreated cells, which were characterized by refringent and rounded cells. RD cells exhibited a characteristic spindle-shaped morphology and large multinucleated cells (Kok et al., 1998) when treated with 1800 µg/mL, whereas infected, nontreated cells displayed a CPE characterized by rounded and detached cells.

With increasing concentrations of the extract, a reduction in the CPE was observed, as shown in Fig. 3, for both viruses, with effective media concentrations (EC_{50}) of 512.82 ± 22.32 µg/mL and 1367.1 ± 58.66 µg/mL, respectively. For E9 at 1000 µg/mL total protection of cells against infection was observed, whereas for CVA16, concentrations above 2000 µg/mL were needed.

The *Thalassia testudinum* hydroalcoholic extract presented SI values above 2 for both E9 and CVA16, which corroborates its antiviral activity; however, this activity was greater against echovirus 9 (7.48) than against CVA16 (2.79), as summarized in Table 2.

Antiviral modes of action

Extracellular virucidal activity against E9

The extracellular virucidal activity of the extract was evaluated against E9. Virucidal activity was considered positive when the infective titer was reduced by 1 log₁₀ or more (Hu and Hsiung, 1989). The assay was performed by mixing different equal volumes of different concentrations of the extract (1250, 2500, and 5000 µg/mL) with an equal volume of two virus doses.

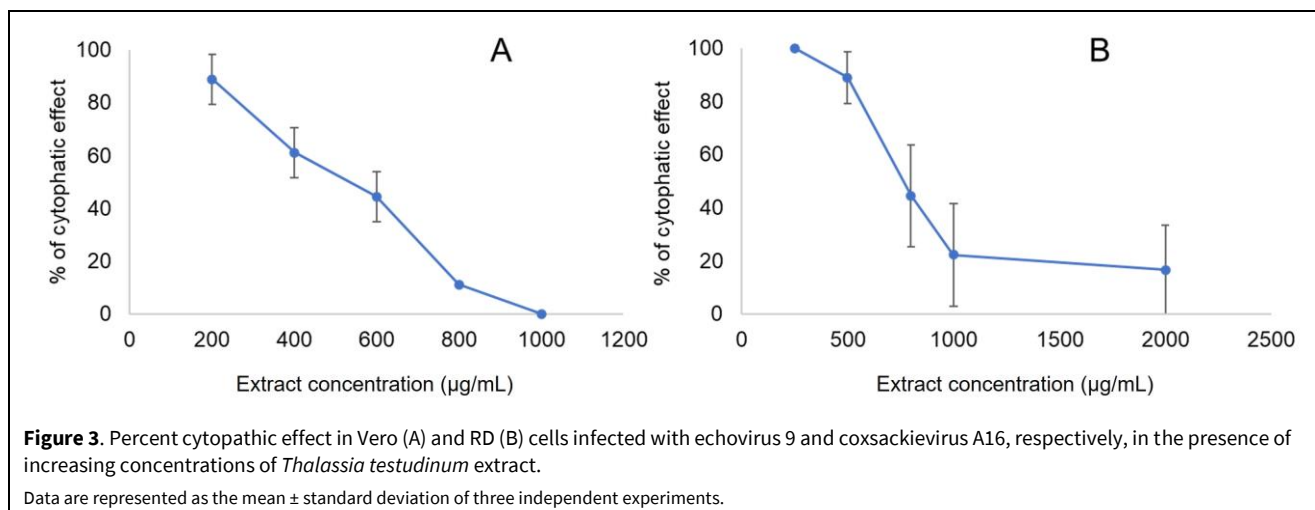
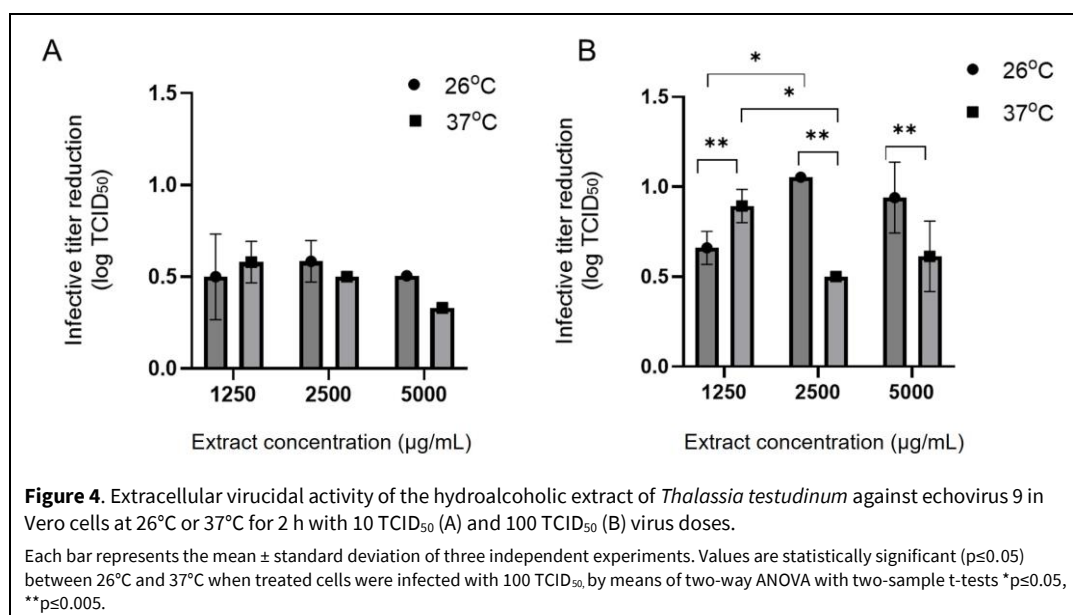


Table 2. Antiviral activity of the hydroalcoholic extract of *Thalassia testudinum* against echovirus 9 and coxsackievirus A16.

Virus	Cells	CC ₅₀ (µg/mL)	CE ₅₀ (µg/mL)	SI (CC ₅₀ /CE ₅₀)
E9	Vero	3840.96 $\pm$ 116.12	512.82 $\pm$ 22.32	7.48
CVA16	RD	3822 $\pm$ 220.5	1367.1 $\pm$ 58.66	2.79

Data represent the mean $\pm$ standard deviation of three independent experiments. CC₅₀: median cytotoxic concentration, EC₅₀: media effective concentration, SI: selective index.



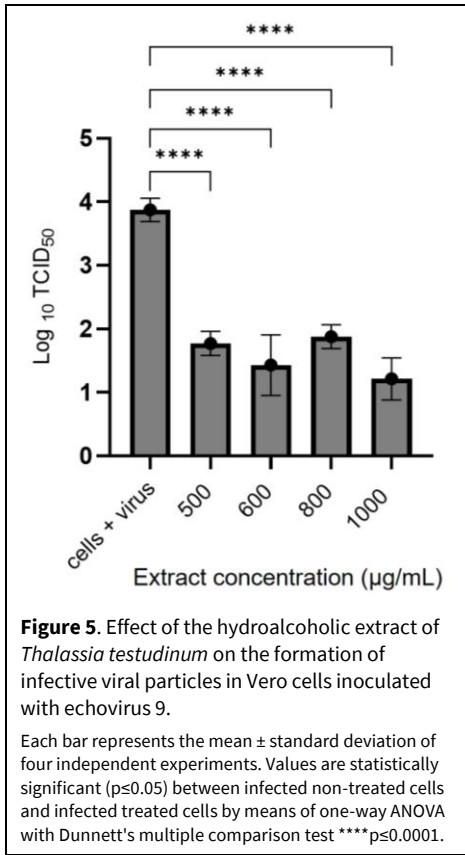
These mixtures were incubated for two hours at 26°C and 37°C. As shown in Fig. 4, under these conditions, the extract reduced the infective titer to less than 1 log₁₀ when incubated with the highest virus dose (10 TCID₅₀). However, the infective titer was reduced by 1.05 log₁₀ and 0.95 log₁₀ with 2500 µg/mL and 5000 µg/mL, respectively.

Two-way ANOVA was performed to analyze the effects of temperature and extract concentration on

reducing the infective titer. When a virus dose of 100 TCID₅₀ was used, there was a statistically significant interaction effect between temperature and the concentration of the extract ($p = 0.0004$). Simple main effects analysis revealed that only temperature had a statistically significant effect on the reduction in the infective titer ($p = 0.0033$). No significant differences were found when higher virus doses were used (10 TCID₅₀) (Fig. 4).

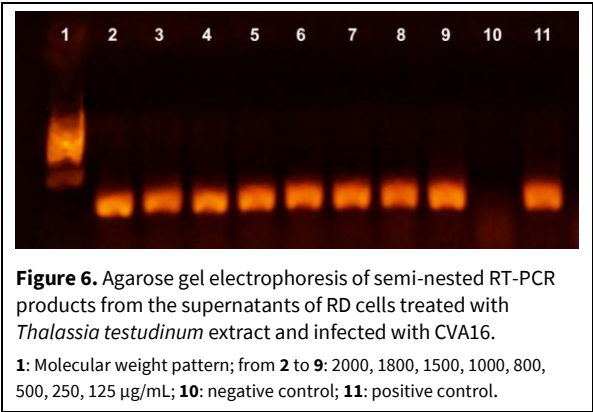
Reduction in the E9 infection titer in Vero cells

As part of the secondary antiviral assays, a reduction in the infective titer was performed to determine whether the extract inhibits the formation of infective viruses during replication. The results are shown in Fig. 5. At all evaluated concentrations, there was a significant reduction in the infective titer in treated cells, which was greater than 2 log₁₀ greater than that in infected nontreated cells. A one-way ANOVA with Dunnett's multiple comparison test was performed to compare the infective titers between infected, nontreated cells and infected, treated cells (p<0.05). There was a significant difference in the infective titer between cells infected with the extract and those that were not treated (p<0.0001). Therefore, it inhibits the formation of infective particles.



RT-PCR detection and quantification of the viral genome in cell culture in the presence of *Thalassia testudinum* hydroalcoholic extract

To determine whether the antiviral activity against CVA16 is related to the inhibition of viral genome synthesis, the viral nucleic acid concentration at 48 hours post-infection was determined. For all the concentrations of the extract, bands with similar intensities to those of infected nontreated cells (positive control) were observed (Fig. 6).



The viral genome was quantified in each case, and a decrease in the nucleic acid concentration was observed with increasing concentrations of the extract (Fig. 7). Considering that one nanogram of the enterovirus genome might contain approximately 1.82 × 10¹² RNA molecules, our results revealed that at the highest concentration of the extract (1,668,212 × 10⁹ RNA molecules), compared with infected nontreated cells (1,749,566 × 10⁹ RNA molecules), a reduction of 4% in the number of molecules was observed (Table 3). A one-sample t-test showed significant differences (p<0.05, p=0.0217) between the means of the viral nucleic acid concentrations of the infected and extract-treated cells compared to the concentration value in the infected and untreated cells, taken as a reference value. This means that a significant reduction of viral nucleic acid is observed when cells are treated with the extract.

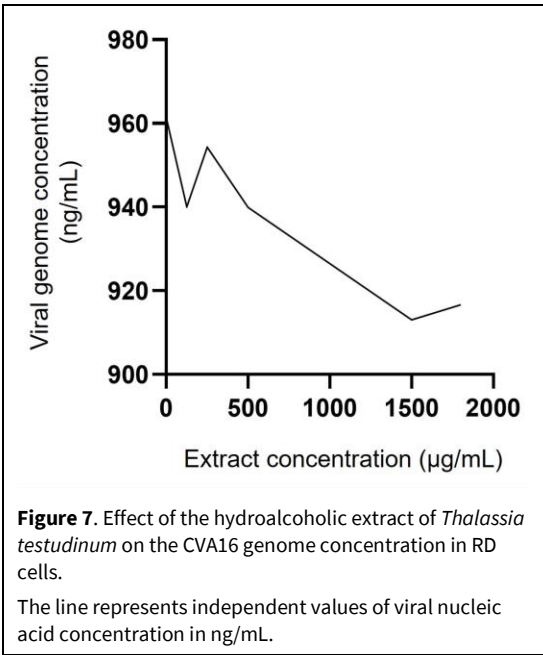


Table 3. Quantification of CVA16 viral nucleic acid in the presence of increasing concentrations of *Thalassia testudinum* hydroalcoholic extract.

Extract concentration (µg/mL)	Viral nucleic acid concentration (ng/mL)
1800	916.6
1500	913
500	939.9
250	954.3
125	940
Non-infected nontreated cells	No viral genome
Infected non-treated cells	961.3

DISCUSSION

Most human enteroviruses lack approved therapeutic or prophylactic treatment but still cause important diseases, especially in children and immunosuppressed patients (Anjum et al., 2024). Owing to their structural diversity and multiple mechanisms of action, natural products obtained from marine plants, seaweeds, animals, and microorganisms are promising sources of drugs (Haque et al., 2022). Although the number of clinically approved marine drugs has increased, to date, few of them are aimed at treating viral infectious diseases (Nagahawatta et al., 2024). Extracts obtained from *Thalassia testudinum* leaves have shown promising biological activities (Regalado et al., 2012; Rodeiro et al., 2008), making it an interesting source for studying its antiviral properties.

Cytotoxicity of *Thalassia testudinum* extract to Vero and RD cells

Before evaluating the bioactive potential of a new product, it is essential to understand the potential toxic effects it may cause in the experimental model selected for the biological assay. Cytotoxicity refers to the ability of a compound or product to disrupt fundamental cellular functions, resulting in damage that can be observed. Various methods can be used to assess the cytotoxic potential of the compound being evaluated, including the neutral red uptake assay, the Kenacid blue binding assay, and the MTT reduction assay, among others (Arencibia, 2003). These findings provide information about the effects of the product on growth, membrane permeability, protein, hormone, and enzyme synthesis capacity, energy metabolism, cell transformation, replication, and ribonucleic acid transcription, among other processes (Freshney, 2011). The MTT reduction assay has been widely used for these purposes (Arencibia et al., 2003). A colorimetric assay is used to measure the metabolic activity of cells based on the reduction of MTT by the mitochondrial enzyme succinate dehydrogenase. As a result, formazan crystals (blue-violet-colored compounds) are formed, and the amount of product formed is proportional to the number of viable cells.

The cytotoxicity of *Thalassia testudinum* extract was evaluated via this method in Vero and RD cells.

For both cell lines, a dose-dependent effect was observed, characterized by a decrease in cell viability with increasing concentrations of the extract (Fig. 1). However, the extract was non-cytotoxic and showed similar median cytotoxic concentration (CC₅₀) in both cases, with values above 3000 µg/mL. These results are consistent with those obtained by Rodeiro et al. (2018), who reported that an aqueous-ethanolic extract of *Thalassia testudinum* had a CC₅₀ in Vero cells above 1000 µg/mL. Interestingly, no toxicity was observed in RD cells, which are tumor cells derived from muscle tissue, in accordance with a previous report on the potential antitumoral activity of this extract (Rodeiro et al., 2018).

Antiviral activity

Human enteroviruses can replicate in different cell lines (Adekunle et al., 2014; Adeniji et al., 2017; Sarmiento et al., 2007); however, there is not a single cell line sensitive to all enteroviruses (Hamza et al., 2011). Both Vero and RD cell lines are sensitive to enterovirus replication because they possess receptors for virus entry into cells and are therefore extensively used in diagnostic and antiviral research (Liu et al., 2023; Rai et al., 2024; Zhang et al., 2024). Additionally, Vero cells are interferon-deficient, making them highly suitable for isolating a wide range of viruses (Sène et al., 2022). Due to the absence of a reference compound or drug against enteroviruses, the inclusion in our study of an antiviral activity control group was limited. Although there are compounds that have shown *in vitro* activity, so far there are no antivirals approved by the FDA due to drug interactions, drug resistance, and safety concerns (Anasir et al., 2021).

After the CC₅₀ value was determined, the antiviral activity of the hydroalcoholic extract of *Thalassia testudinum* was evaluated against the different strains. Several methods exist for determining the antiviral activity of a product or compound. One of the most important elements is that no step of the viral replication cycle that can be inhibited is discarded, and that it is performed with subtoxic concentrations of the

product to be evaluated against a fixed viral dose. There are quantitative methods that evaluate several viral markers, including the determination and quantification of nucleic acids or viral proteins, quantification of the number of viral particles (electron microscopy), quantification by fluorescence (marking of a viral protein), and qualitative methods such as inhibition of the cytopathic effect (CPE).

The antiviral activity of *Thalassia testudinum* extract was evaluated as a cytopathic effect (CPE) inhibition assay. Antiviral activity is observed when, in the presence of any product or compound, there is a decrease in the CPE of treated cells compared with that of nontreated cells. This assay has been widely used for evaluating the antiviral activity of products and compounds (Hayashi et al., 2024; Jackman et al., 2024; Shi et al., 2024). CPE consists of a cell morphology modification or alteration that can be observed by an optical microscope and is caused by virus replication. For both E9 and CVA16, a decrease in the CPE was observed with increasing concentrations of the extract. Infected Vero cells treated with concentrations above 1000 µg/mL maintained their morphology, whereas RDs presented a spindle-shaped morphology. At lower concentrations, rounded and detached cells were observed. This phenomenon is consistent with enterovirus CPE, which may include autophagy, apoptosis, cell shrinkage, size reduction, and rounding (Han et al., 2024; Mao et al., 2024).

The selectivity index (SI) is defined as the ratio of the toxic concentration (CC₅₀) of the evaluated product to its effective bioactive concentration (EC₅₀). Ideally, the evaluated product should have a high cytotoxic concentration and a low active concentration. Determination of the SI value for any herbal drug and/or isolated compound is the next evaluation stage (Indrayanto et al., 2021). The higher the SI value is, the stronger the antiviral activity (Clarysse et al., 2000; Hu and Hsiung, 1989; Sidwell and Smee, 2000). In the present study, extract SI values of 7.48 and 2.79 were obtained for echovirus 9 and CVA16, respectively. Some authors suggest that for natural products, an SI equal to or greater than 2 indicates antiviral activity (Pérez et al., 2012), whereas others accept values between 5 and 10 (Indrayanto G., 2022), considering that a fractionation of the extract should increase this value if the active molecule is not associated with toxicity (Cos et al., 2006; Fortin et al., 2002; Semple et al., 2001; Zhoua et al., 2006).

Although echovirus 9 and coxsackievirus A16 belong to the same genus, they exhibit distinct genetic and structural characteristics (Dábilla and Dolan, 2024), which may influence the differences observed in the inhibition of viral replication and therefore in the selective index values. It is essential to note that

the evaluation of antiviral activity for each virus was conducted in different cell lines, indicating that the extract's mode of action may vary in each case. Another factor to consider is that active molecules or metabolites may be virus-specific, and if they are common to both, the effectiveness of the antiviral action could depend on their specificity or affinity for molecular targets (Cos et al., 2006).

Previous studies reported the antiviral activity of compounds isolated from *Thalassia testudinum* against HSV, porcine herpesvirus 1, hepatitis C virus (Calland et al., 2012), adenovirus 3 and 8 (Chiang et al., 2002), and HIV (Rowley et al., 2002). The compounds thalassiolins A, B, and C inhibited HIV integrase. Molecular modeling suggests a binding mode at the catalytic core domain of the viral enzyme (Rowley et al., 2002). Thalassiolin A was evaluated in silico against the spike protein of SARS-CoV-2 as an entry inhibitor with interesting docking scores (Gentile et al., 2022). On the other hand, Regalado et al. (2012) identified apigenin in the hydroalcoholic extract of *Thalassia testudinum*, a flavone with antiviral activity against the enteroviruses PV2 (Visintini et al., 2014) and EV71 (Li et al., 2015; Zhang et al., 2014).

Antiviral modes of action

The extracellular virucidal activity observed when a lower virus dose was used might be related to the fact that the number of infective particles did not exceed the number of virucidal molecules in the extract. Considering that human enteroviruses are nonenveloped, the virucidal activity of the hydroalcoholic extract of *Thalassia testudinum* against echovirus 9 might be related to the capacity of one or more compounds to interact with the capsid proteins, causing their denaturation, decrease in capsid stability and, consequently, a loss of infectivity (Lopes et al., 2013).

On the other hand, the reduction in the infective titer might be associated with additional mechanisms, such as the inhibition of adsorption, penetration, macromolecular synthesis, maturation or release of virus particles (Ahmadi et al., 2015; Wang et al., 2015; 2016). To determine the specific antiviral mechanisms of action, it is necessary to conduct targeted antiviral assays.

The hydroalcoholic extract of *Thalassia testudinum* is composed mainly of polyphenols, proanthocyanidins, and soluble proteins and contains relatively few flavonoids and polysaccharides (Regalado et al., 2012). These metabolites include ethyl4-(sulfoxy) benzoate, 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, chrysoeriol-7-O-D-glucopyranosyl-2-sulphate (thalassiolin B), apigenin 7-O-D-glucopyranosyl-2-sulphate (thalassiolin C), chrysoeriol 7-O-

D-glucopyranoside, apigenin 7-O-D-glucopyranoside, 5,7-dihydroxy-30,40-dimethoxyflavone 7-O-D-glucopyranoside, luteolin-30-sulphate, chrysoeriol and apigenin (Rodeiro et al., 2018). Compounds such as phenols, tannins, flavonoids, triterpenes, proanthocyanins, and catechins are capable of inhibiting virus adsorption and the synthesis of viral nucleic acids and proteins (Li et al., 2015; Zhang et al., 2014). Oxidative stress is generated during virus infections (Eltayeb et al., 2024; Shokeen et al., 2024); therefore, the strong antioxidant activity reported for the aqueous ethanol extract of *Thalassia testudinum* leaves (Rodeiro et al., 2018) might contribute to the antiviral activity observed against E9 and CVA16. In addition, antiviral activity can be determined by the synergistic or additive effects (Chouhan and Singh, 2011) of the different compounds present in the extract (Hernández-Balmaseda et al., 2021).

In the present study, semi-nested RT-PCR was used for the detection of the enterovirus genome (Giulieri et al., 2015; Koishi et al., 2010). The CVA16 genome was quantified in those cells treated with the extract. A significant difference was detected between infected, nontreated cells and treated cells, as evidenced by a decrease in the number of genome molecules. These findings suggest that antiviral activity might also be related to the inhibition of viral nucleic acid synthesis, through the inhibition of the viral enzyme RNA-dependent RNA polymerase (dRNAPRNA). In addition, the inhibition of viral adsorption, entry, assembly, or release is probable (Ahmadi et al., 2015; Wang et al., 2015, 2016).

To date, there are no FDA-approved antivirals for treating non-polio enterovirus infections. Antivirals tested in clinical trial phases have failed due to drug resistance and safety concerns (Ulferts et al., 2016). Vaccine development against these viruses faces limitations due to the existence of several species, limited cross-reactivity, and difficulty in predicting serotype-specific epidemiological patterns. Although the compound pleconaril acts as an inhibitor of enterovirus entry, its clinical use is limited due to toxicity issues (Fleischer and Laessig, 2003), while the compound fluoxetine demonstrated in vitro activity but requires further clinical investigation. Many inhibitors targeting the surface capsid of enteroviruses have been identified. However, despite showing potent in vitro activity in the nM to pM range, most of them caused undesirable side effects and failed during their clinical trials (Anasir et al., 2021).

This limited the inclusion of a positive antiviral control in our study, despite the fact that compounds such as pleconaril and broad-spectrum ribavirin exert *in vitro* antiviral activity against enteroviruses (Krogvold et al., 2023). Although this practical aspect re-

stricted the comparative evaluation of extract efficacy, several studies describe the *in vitro* and *in vivo* antiviral activity of crude extracts and compounds against non-polio enteroviruses relative to untreated/uninfected and untreated/infected control groups, respectively (Dai et al., 2019; Ogbale et al., 2018; 2021), such as those described in our study. However, as part of further studies to elucidate the mechanisms of action of the bioactive compounds identified in the extract, both synthetic and natural antiviral positive control compounds will be included.

CONCLUSION

This work represents the first preliminary study reporting the antiviral activity of the marine plant *Thalassia testudinum* against human enteroviruses. Future research should focus on bio-guided fractionation, mechanistic assays, and *in vivo* validation to elucidate active constituents and confirm efficacy.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Contribution	Ponce Rey LR	Frómata Román R	Casanova Nodarse E	González García K	Morier Díaz LM	Resik S	Rodeiro Guerra I
Concepts or ideas	x					x	x
Design	x				x	x	x
Definition of intellectual content	x					x	x
Literature search	x	x	x		x		
Experimental studies	x	x	x	x	x		
Data acquisition	x	x	x	x			
Data analysis	x	x	x	x		x	x
Statistical analysis	x	x	x				
Manuscript preparation	x					x	x
Manuscript editing	x	x	x	x	x	x	x
Manuscript review	x	x	x	x	x	x	x

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