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Anti-HSV activity of *Hypnea musciformis* cultured with different phytohormones

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Abstract: Four extracts from the seaweed *Hypnea musciformis* (Wulfen in Jacq.) J.V. Lamour. (Rhodophyta), collected directly from its natural habitat or cultivated in the presence of phytohormones, were evaluated for their ability to inhibit the replication of acyclovir-resistant herpes simplex viruses types 1 (HSV-1) and 2 (HSV-2) strains. The main purpose was to determinate whether these growth conditions would affect the antiviral activity. Our results showed the possibility of improving the anti-HSV activity by using extracts from algae cultured in the presence of phytohormones.

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Introduction

Herpes simplex virus (HSV) infections are among the most common human diseases with a worldwide distribution. It is estimated that 60 to 95% of the adult population is infected by these viruses. Generally, HSV-1 has been associated with orolabial disease, with most infections occurring during childhood, and HSV-2 with genital disease following infection from sexual onset (Auslander et al., 2005). Most of the drugs used in the treatment of the infections caused by these viruses are synthetic and usually without selectivity among the infected cells, therefore causing some toxic reactions. In spite of the availability of an effective antiviral agent ([9-(2-hydroxyethoxymethyl)] acyclovir) to treat these infections, resistant strains have already been isolated, most of them from immunocompromised patients (Schaeffer et al., 1978). Therefore, the search for new anti-HSV drugs is critical. Some marine algae have been shown to inhibit the propagation of different microorganisms, including bacteria (Mayer et al., 2009), fungi (Genovese et al., 2009) and viruses (Santos et al., 1999; Romanos et al., 2002a,b; Tziveleka et al., 2003; Mendes et al., 2010; Mendes et al., 2011). *Hypnea musciformis* (Wulfen) J.V. Lamour has shown anti-HSV-1 activity (Santos et al., 1999), and, in this work, the anti-HSV activity was evaluated of this red alga cultivated in the presence of three different phytohormones: indoleacetic acid (IAA), gibberellic acid (GA_3) and 6-benzylaminopurine (6-BAP). The main purpose was to determinate whether these growth conditions would affect the antiviral activity.

Materials and Methods

Algal extracts from calluses

The red alga *Hypnea musciformis* (Wulfen in Jacq.) J.V. Lamour. (Rhodophyta, Gigartinales) was collected by hand from the sublittoral fringe of the rocky shores at Prainha, Arraial do Cabo, state of Rio de Janeiro. The apical fragments were excised from the thalli and submitted to incubation at 22+1 ºC with a photon flux density of 30+5µmol photons.m^{-2}.s^{-1} in Provasoli's enriched seawater (Provasoli, 1968) with 1% added agar (Bravin et al., 2006).

Liquid media with 100 µM of indoleacetic acid (IAA), gibberellic acid (GA_3) or 6-benzylaminopurine (6-BAP) were tested in order to determine whether these apical fragments were able to produce calluses. Natural seawater was used as control. After sixty days, old cultures of these apices developed calluses (Bravin et al., unpublished data). These experiments were conducted in the Macroalgae Culture Laboratory, Department of Botany, of the Biology Institute. These calluses were employed in

the extraction process, performed by decoction (10% wt/v) in boiling water for 10 min followed by lyophilization.

Four different extracts were prepared by percolation at 100 µM: indoleacetic acid (A), gibberellic acid (B), 6-benzylaminopurine (C) and one of the alga collected from its natural habitat (D).

Cells and viruses

Vero cells were grown in Eagle's minimum essential medium (MEM) supplemented with 2 mM L-glutamine, 50 µg/mL gentamicin, 2.5 µg/mL fungizon, plus 10% of heat-inactivated fetal bovine serum (FBS) (Schmidt, 1979) and maintained at 37 ºC in an atmosphere of 5% CO_2. Herpes simplex acyclovir-resistant virus types 1 and 2 isolated from typical oral and genital lesions, respectively, were used.

Cytotoxicity

The lyophilized algal extracts were solubilized in dimethyl sulphoxide (final concentration 1%) and diluted in water to a concentration of 400 µg/mL, sterilized by filtration through a Millipore membrane (0.22 µm) and frozen at -20 ºC until use. The cytotoxicity assay was performed by incubating triplicate Vero cell monolayers cultivated in 96-well microplates with two-fold serial dilutions of the extracts for 48 h at 37 ºC. Morphological alterations of the treated cells were observed in an inverted optical microscope (Leitz) and the maximum non-toxic concentrations (MNTC) were determined (Walker et al., 1971). The cellular viability was further evaluated by the neutral red dye-uptake method (Borenfreund & Puerner, 1985). The 50% cytotoxic concentration (CC50) was defined as the dilution that caused a reduction of 50% in the number of viable cells.

Antiviral assay

Anti-HSV activity was evaluated by reduction of the virus titers using TCID50 (50% tissue culture infective dose) determinations. Vero cell monolayers cultivated in 96 well-microplates were treated with the algal extracts at the MNTC. Immediately after, logarithmic dilutions of HSV-1 and HSV-2 were added to treated and untreated cell cultures and incubated in a 5% CO_2 atmosphere at 37 ºC. Following incubation, the virus titers were calculated using the Reed & Muench (1938) statistical method and expressed as TCID50 values. Results were expressed as percentage of inhibition (PI) (Nishimura et al., 1977) using antilogarithmic values of the TCID50 values as follow: PI = [1-(antilogarithm of the test value/antilogarithm of the control value)] x 100 and the viral inhibition index (VII) calculated using the formula VII = B - A, where B is the virus titer in the virus-infected control (no extract), and A is the virus titer in the test sample. The dose-response curve was established starting from the MNTC. The 50% inhibitory concentration (IC50) was determined, as well as the selectivity indices (SI).

Mechanism of action assays

Virucidal assay

Aliquots of 100 µL of HSV-1 or HSV-2 were added to either 900 µL of each extract at its MNTC or of MEM-Eagle without serum (control), according to Chen et al. (1988). All samples were incubated at 37 ºC for two hours and, immediately after, were diluted and inoculated in Vero cell monolayers, incubated for 48 h at 37 °C in an atmosphere of 5% CO_2. Additionally, residual titers of the treated and untreated viruses were expressed as percentage of inhibition.

Cellular receptors assay

Extracts were added to the Vero cell monolayers before infection. In the pre-treatment to evaluate the effect on the cell receptors, each extract was added to Vero cell monolayers, incubated at 4 ºC for 1 h, washed three times with cold MEM-Eagle and logarithmic dilutions of HSV-1 and HSV-2 were added to treated and untreated cell cultures and incubated at 37 ºC for 48 h. Virus titers in treated and untreated cells were determined. Activity was expressed as percentage of inhibition.

Penetration assay

Vero cell monolayers were inoculated with logarithmical dilutions of HSV-1 or HSV-2 and incubated for 1 h at 4 ºC. After adsorption, the monolayers were washed, treated with the extracts at the MNTC, and incubated for 1 h at 37 ºC. Afterwards, monolayers were washed, MEM-Eagle was added and the cells were incubated at 37 ºC for 48 h in an atmosphere of 5% CO_2. Virus titers in treated and untreated cells were determined and the activity was expressed as percentage of inhibition.

Results

Cytotoxicity

Experiments to evaluate the cytotoxic concentration of the extracts were performed before the antiviral activity assay. The three extracts obtained from the algae cultivated with phytohormones did not change the cellular morphology at the highest concentration used (200 µg/mL), although the crude extract showed cytotoxic effects at 100 µg/mL (MNTC = 50 µg/mL). The CC50

(50% cytotoxic concentration) was higher than 200 µg/mL for all extracts (Table 2).

Antiviral activity

The 50% inhibitory concentrations (IC50) and selectivity index (SI) are listed in Table 2. All extracts presented inhibitory activity for both viruses. For HSV-1, two of the extracts obtained from cultivated algae (in the presence of GA3 and 6-BAP) showed great improvement in the antiviral activity when compared to the control algal extract. As to HSV-2, even though the extracts obtained from the algae cultivated in the presence of phytohormones presented a significant antiviral activity, none showed a selectivity index higher than that of the natural algae (>140.84).

Mechanisms of antiviral action

In order to determine the mechanism by which the *H. musciformis* extracts inhibited the viral infection, a number of experiments were performed. For HSV-1, all extracts showed virucidal activity and were capable of interacting with viral receptors, preventing the binding of viral particles to their receptor molecule. During the penetration step, only the extracts obtained from the treated algae showed activity (68.4% inhibition), while the crude extract of *H. musciformis* showed almost none (20.6% inhibition) (Figure 1). For HSV-2, a great improvement in the virucidal activity was observed with the extracts of algae treated with IAA and GA3. The ability to bind with cellular receptors was observed in all extracts, except the one treated with 6-BAP. The inhibition of viral penetration was maintained in all extracts (Figure 2).

Discussion

Phytohormones, or plant hormones, are naturally occurring chemicals that influence and control a majority of the plant growth and development processes. Plant hormones also regulate the reproduction cycles exhibited by plants and allow plants to respond to environmental changes (Bradely, 1991).

Auxins, like indoleacetic acid, are a class of phytohormones that primarily cause elongation in plants. Auxins are a common class of phytohormones for land-based plants, but are also found in algae (Li et al., 2007). Their presence in algae indicates that auxins also have an effect on algal growth and further investigation may prove beneficial.

Gibberellins (gibberellic acid) are another class of plant hormones that plays an important role in the elongation of plant cells and various other metabolic functions. If a gibberellin treatment is administered to algae, an increase in overall growth is expected. In one study, it was noted that Gibberellin 7 caused cells to appear to be larger and healthier than in the control group of cells (Johnston, 2004).

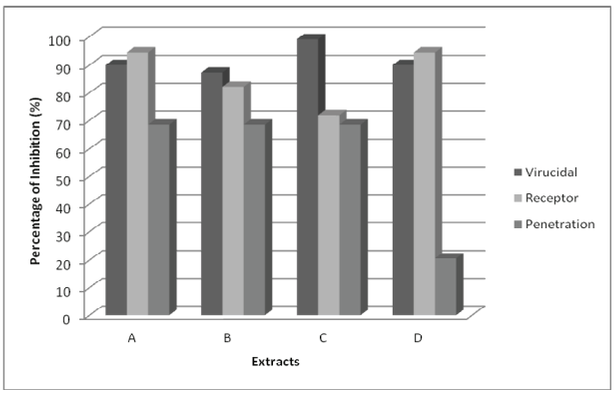

Figure 1. Mechanism of action against HSV-1. *H. musciformis* cultivated in the presence of indolylacetic acid (A), gibberellic acid (B), 6-benzylaminopurine (C) and from its natural habitat (D). Virucidal effect-virus particles were treated with extracts. The samples were incubated at 37 ºC for 2 h, diluted, and then inoculated in Vero cell monolayers. Receptor-extracts were added to Vero cells for one hour before infection. The monolayers were washed and inoculated with HSV-1. Penetration Vero cell monolayers were inoculated with HSV-1, incubated for 1 h at 4 ºC, washed, treated with the extracts at the MNTC and incubated for 1 h at 37 ºC. The monolayers were washed and MEM-Eagle was added. After 48 h of incubation at 37 ºC, the residual titers in the presence and absence of the extracts were determined and expressed as percentage of inhibition (PI).

Table 2. Cytotoxicity and antiviral activity of *H. musciformis* algal extracts cultivated in the presence of different phytohormones.

Code	Condition	[1]MNTC (µg/mL)	[2]CC50 (µg/mL)	HSV-1		HSV-2	
				[3]IC50 (µg/mL)	[4]SI	[3]IC50 (µg/mL)	[4]SI
A	IAA/100 µM	200	>200	14.4	>13.88	4.9	>40.81
B	GA_3/100 µM	200	>200	0.67	>298.5	10.3	>19.41
C	6-BAP/100 µM	200	>200	5.45	>36.69	63.4	>3.15
D	natural	50	>200	11.9	>16.8	1.42	>140.84

[1]MNTC: Maximum Non-Toxic Concentration, [2]CC50: 50% Cytotoxic Concentration, [3]IC50: 50% Inhibitory Concentration, [4]SI-Selectivity Index, Virus control titer = $10^{7.5}$ TCID50/mL.

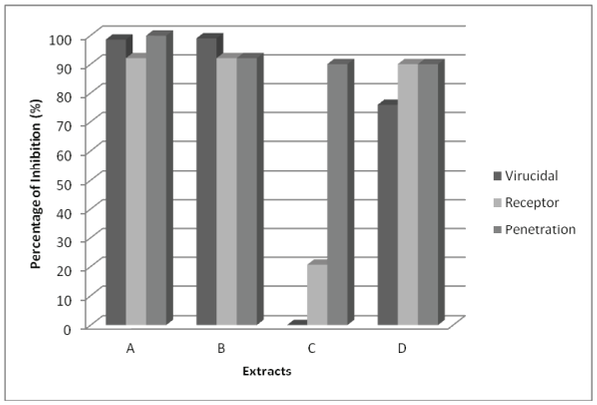

Figure 2. Mechanism of Action against HSV-2. *H. musciformis* cultivated in the presence of indolylacetic acid (A), gibberellic acid (B), 6-benzylaminopurine (C) and from its natural habitat (D). Virucidal effect - virus particles were treated with extracts. The samples were incubated at 37 ºC for 2 h, diluted, and then inoculated in Vero cell monolayers. Receptor - extracts were added to Vero cells for 1 h before infection. The monolayers were washed and inoculated with HSV-2. Penetration - Vero cell monolayers were inoculated with HSV-2, incubated for 1 h at 4 ºC, washed, treated with the extracts at the MNTC and incubated for 1 h at 37 ºC. The monolayers were washed and MEM-Eagle was added. After 48 h of incubation at 37 ºC, the residual titers in the presence and absence of the extracts were determined and expressed as percentage of inhibition (PI).

Cytokinins, including 6-BAP, are plant growth substances that promote cell division. Cytokinin-like structures have been found in extracts of brown, green and red algae and are believed to play the same role as in vascular plants: promotion of cell division (Stirk et al., 2003; Tarakhovskaya et al., 2007).

In this work, extracts were obtained from *Hypnea musciformis* alga cultured in the presence of three distinct phytohormones: IAA, GA3 and 6-BAP at 100 µM each. Cultivated in the presence of these hormones, this species possibly presented alterations in its chemical constitution, as indicated in this work by the differences in the ability to inhibit HSV-1 and HSV-2 replication.

Typically, marine algal extracts have low toxicity to cell cultures (Romanos et al., 2002b; Adhikari et al., 2006; Mandal et al., 2007; Wang et al., 2007; Mendes et al., 2010). Our findings point to similar results. No cellular morphological alterations were observed when cells were exposed to extracts of algae cultivated with hormones at concentrations as high as 200 µg/mL. The crude extract, however, showed only limited toxicity, evidence in the determination of the MNTC, since cellular viability was not affected by the addition of extracts at the concentration of 200 µg/mL.

The crude extract of *H. musciformis* showed an inhibition of 90% against HSV-1 at the MNTC, while the extracts obtained from the same algae treated with the phytohormones showed over 99% inhibition at the MNTC. Comparing the selectivity indices (SI), the extract obtained from the algae treated with GA3 was more than 10 times higher than the crude extract (298.5 vs. 16.8, respectively). The algae treated with 6-BAP also showed a higher SI, but the same was not found with IAA (36.69 and 13.88, respectively). As for HSV-2, at the MNTC the extracts of algae treated with any one of the hormones inhibited more than 98% of viral replication, while the crude algal extract inhibited 90%. In terms of the SI, none of the alga treated with hormones exceeded the SI value of the crude algal extract (140.84).

The algal extracts evaluated in this paper were obtained by decoction. With this extraction method, the substances present in these extracts are predominantly polar molecules, in the form of polysaccharides and proteins.

Some algal-derived chemical compounds have already had their antiviral activity demonstrated (Witvrouw & De Clercq, 1997; Romanos et al., 2002b). Among these are the sulfated polysaccharides. A very important chemical compound of *H. musciformis* is carragenan, which is a sulfated polysaccharide used in the food industry in the production of cheeses, beer and fruit juices. It is also used in the pharmaceutical, textile, paper, leather and cosmetic industries (Hoppe, 1969) and in clinical medicine, where is used to treat peptic ulcers (South & Whittick 1987).

The results of our study show that the inhibitory activity of the marine algal extracts occurs primarily in the initial periods of the virus-cell interaction through interaction with virus particles and/or cellular receptors, avoiding the adsorption of the particles, in agreement with available literature data (Witvrouw & De Clercq, 1997; Romanos et al., 2002a,b; Ghosh et al., 2004; Serkedjieva, 2004; Harden et al., 2009; Mendes et al., 2010; Bouhlal et al., 2011; Mendes et al., 2011).

Our interest in the search for drugs produced by algae that can be used in the treatment of HSV infections assumes even grater importance in view of the emergence of acyclovir-resistant strains The selectivity and restricted mode of action of the extracts produced here contrasts with the toxicity and collateral effects of other antiviral drugs. In addition, infection is much more aggressive in immunocompromised patients (Schimidtke et al., 2001; Witt et al., 2009), who can develop extensive mucocutaneous HSV lesions during the course of antiviral therapy and fail to respond to high-dose acyclovir therapy (Chatis & Crumpacker, 1992).

This work indicates that is possible, through laboratory cultivation, to improve the antiviral activity of natural products without the influence of environmental factors. Another favorable aspect is the lack of intervention in the ecosystem, avoiding preoccupation with the

exhaustion of the algae because of its high economic value (Bravin & Yoneshigue-Valentin, 2002).

Further studies are being performed to determine the bioactive substances in these extracts and to correlate them with higher or lower antiviral activity in comparison with the algae in its natural habitat.

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