Potential antiviral activity of *Plexaurella regia* on the replication of Herpes Simplex Virus type 1

Potencial atividade antiviral de Plexaurella regia no ciclo replicativo do vírus Herpes Simples tipo 1

Viveca Giongo[1], Camilly Pestana Pires de Mello[1], Felipe Mateini[1], Juliana Barbosa[2], Jussara Pinheiro Barbosa[3], Beatriz Grosso Fleury[3], Clovis Barreira e Castro[4], Angelo da Cunha Pinto[3], Izabel Christina Nunes de Palmer Paixão[1]

ABSTRACT

Introduction: Latency and resistance of acyclovir-resistant strains of Herpes simplex virus type 1 (HSV-1) have been associated with serious sequelae in immunocompromised individuals, such as AIDS patients. Consequently, the search for new substances with anti-HSV activity is both necessary and urgent. **Objective:** To investigate whether extracts obtained from *Plexaurella spp* can be used in preclinical studies of drugs against herpes simplex virus type 1. **Methods:** Cell viability and inhibitory drug concentrations as screening tests were used to investigate ethyl acetate and dichloromethane extracts from *Plexaurella spp* as antivirals. **Results:** The results of viability assays demonstrated that extracts from *Plexaurella regia* and *Plexaurella grandiflora* showed less cytotoxicity, but only *Plexaurella regia* reached a very expressive CC_{50} value. In antiviral assays, *Plexaurella regia* showed an even more significant result of effective concentration (EC_{50}) and therapeutic index (<2.5 µg/mL and 51.6 µg/mL, respectively) compared with acyclovir (ACV). **Conclusion:** These results demonstrated that extracts from corals have anti-herpetic activities and could contribute towards new strategies to stop the increasing incidence of resistance in herpes-related diseases.

Keywords: natural products; antivirals; drug resistance; HSV-1.

RESUMO

Introdução: Latência e resistência de cepas de Herpes simples tipo 1 (HSV-1) ao aciclovir têm sido associados a sequelas graves em pacientes imunocomprometidos, como pacientes com AIDS. Por essa razão, a pesquisa por novas substâncias com atividade anti-HSV-1 é uma necessidade urgente. Objetivo: Investigar se os extratos obtidos de *Plexaurella spp* poderiam ser usados em estudos pré-clínicos de drogas contra o vírus herpes simples tipo 1. **Métodos:** A viabilidade celular e concentrações inibitórias das drogas foram utilizados como testes de triagem para investigar os extratos etil acetato e diclorometano de *Plexaurella spp* como antivirais. **Resultados:** Os resultados de viabilidade demonstraram que os extratos de *Plexaurella regia* e *Plexaurella grandflora* não foram citotóxicas, mas somente *Plexaurella regia* alcançou um valor de CC_{50} expressivo. Nos ensaios antivirais, *Plexaurella regia* mostraram um resultado ainda mais significante de concentração efetiva (EC_{50}) e índice terapêutico (<2.5 µg/mL e 51.6 µg/mL, respectivamente) comparado com aciclovir (ACV). **Conclusão:** Estes resultados mostram que os extratos de corais têm atividade anti-herpética e podem contribuir para novas estratégias de redução da incidência de resistência de doenças relacionadas aos herpes vírus.

Palavras-chave: produtos naturais; antiviral; resistência a medicamentos; HSV-1.

INTRODUCTION

Marine organisms comprise over half a million species. Due to their unusual living environment in comparison with terrestrial organisms, marine organisms produce a variety of substances, which quite often have various unprecedented chemical structures. Issues such as competition for space and predation have originated new biochemical pathways for these organisms, providing essential metabolites for their adaptation[1]. Over 40 novel natural product compounds are now commercially available, including antiviral products isolated from various marine organisms, which provide alternative therapeutic drugs[2,3].

A literature survey revealed that Gorgonian corals have proven to be a prolific source of a variety of biologically active compounds with cytotoxic effects on human leukaemia[4], such as being inhibitors of acetylcholine receptors[5-7]. The species of corals in tropical or temperate waters of the western North Atlantic are relatively well known, but those found at the south of the Amazon River are not, despite having common elements with the fauna of the Caribbean octocorals[8]. Silva & Pérez[7], in 2002, reported 59 species of octocorals on the Brazilian coast based on the biosynthetic origin of bioactive terpenes. This classification could provide a system to produce *ex situ* compounds[9].

Almost 90% of people worldwide have one or both HSV-1 and HSV-2 viruses. In developed countries, the acquisition of HSV-1 is delayed from early childhood to adolescence or young adulthood[10]. Herpes simplex virus (HSV) belongs to *Herpesviridae*, subfamily *Alphaherpesvirinae*, and contamination occurs through direct contact with infected secretions, mostly during infancy, with clinical manifestations varying from labial lesions to gingivostomatitis, keratoconjunctivitis, and genital infections[11]. The virus persists for life in local sensory ganglia and reactivation depends on the status of the patient's immune system. The primary infection or virus reactivation

[1]Department of Cell and Molecular Biology, Institute of Biology, *Universidade Federal Fluminense* (UFF) – Niterói (RJ), Brazil
[2]Department of Marine Biology, Institute of Biology, UFF – Niterói (RJ), Brazil.
[3] Department of Chemistry, Institute of Chemistry, *Universidade Federal do Rio de Janeiro* – Rio de Janeiro (RJ), Brazil.
[4]Department of Invertebrates, National Museum, UFRJ – Rio de Janeiro (RJ), Brazil.

is the cause of herpes encephalitis (HSE), and both demonstrated the need for new drugs due to the increasing resistance to acyclovir, penciclovir, ganciclovir, foscarnet, and cidofovir[12]. Resistant viral isolates can be observed especially in immunocompromised patients, in patients with HIV, and in recipients of solid organ or bone marrow transplants who are treated with antivirals for long intervals. Presently, the main focus is to circumvent this problem through the development of broad-spectrum antivirals, in particular those targeting common cellular pathways[13]. For HSV-1, the growing resistance makes the search for innovative antivirals both necessary and urgent[14,15].

This study involved an evaluation of the fractions obtained from *Plexaurella regia, Plexaurella grandiflora* and *Muriceopsis sulphurea,* all endemic on the Brazilian coast, as antivirals against HSV-1.

METHODS

Collection and preparation of crude extracts

The octocorals were collected by scuba divers in Parque Municipal Marinho de Recife de Fora, Porto Seguro, Bahia, and stored in ethanol to obtain the crude extracts.

The extracts were fixed in organic solvents of different polarities (hexane, dichloromethane, and ethyl acetate) and evaporated separately under reduced pressure.

Analysis by Gas Chromatography coupled to mass spectrometry (GCMS)

The mass spectra of low resolution (70 ev) was obtained on a Hewlett Packard 5987A. The fragments were described by the ratio mass/charge (m/z) and their intensities expressed as a percentage of base peak (100%). The chromatographic column used was a fused silica capillary column with stationary phase HP-5 MS (5% phenyl methyl siloxane) measuring 30 m long with an internal diameter of 0.25 mm and a thickness of 0.25 µm. The samples were injected using 1 µl of the fractions, previously diluted in CH_2Cl_2. The chromatographic conditions were: initial column temperature: 50°C (4 min), gradient: 100C/min, final temperature of the column: 290°C (20 min), injector temperature: 270°C, detector temperature: 290°C, carrier gas: hydrogen, injection mode: flow division split 1:20.

Cells and viruses

Vero cells (African green monkey *Cercopithecus aethiops* kidney cells; ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified medium, supplemented with 5% of fetal bovine serum (FBS; HyClone, Logan, UT, USA), 0.1 µM HEPES, and 2.5 µg/mL gentamycin, at 37°C in 5% CO_2. Vero cells were subconfluent in all assays and were used prior to passage 20. Stock of HSV-1 was obtained with HSV-1 (AR- 29)[16], KOS[17] strain at a multiplicity of infection (MOI) equal to 0.1 for 1 h at 37°C. Briefly after the incubation, the monolayer was washed out with phosphate-buffered saline (PBS) and cells were cultured for an additional 48 h. After this period, cells were lysed through three cycles of freezing and thawing, centrifuged at 1500×*g* at 4°C for 20 min to remove cellular debris, and the supernatants were collected, titered by plaque assay, and stored at -70°C for further studies.

Cytotoxicity assays

The MTT cytotoxic assay was performed in Vero cells in 96-multiwell plates (10^5/well) treated with different concentrations of the crude extracts from *Plexaurella regia*, *Plexaurella grandiflora,* and *Muriceopsis sulphurea* at 37ºC with atmosphere of 5% CO_2 for 72 h. Afterwards, 50 µL of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT; Sigma) and 1mg/mL of stock, were added to each well for 3 h. After incubation and discard, each well received 50 µL of acid-isopropanol (0.04N HCl in isopropanol). The concentration was determined by an automatic plate reader with a 570 nm test wavelength and a 690 nm reference wavelength[18,19]. The 50% cytotoxic concentration (CC_{50}) was calculated by linear regression analysis of the dose-response curves generated from the data.

Plaque assay

Monolayers of Vero cells in six-well plates were exposed to different dilutions of the supernatant from yield-reduction assays for 1 h at 37°C. Next, cells were washed with PBS and DMEM medium containing 5% FBS and then 1% methylcellulose (Fluka) (overlay medium) was added to cells. After 72 h at 37°C, the monolayers were fixed with 10% formaldehyde in PBS and stained with a 0.1% solution of crystal violet in 70% methanol, and the virus titers were calculated by scoring the plaque-forming units (PFU). Additionally, other experiments were performed in the format of a plaque-reduction assay. In those particular cases, various concentrations of the compounds were added in the overlay medium and after 72 h, cells were fixed and plaques counted.

Plaque reduction assay

Monolayers of Vero cells (10^5) in 24-well plates were infected with HSV-1 (AR-29 or KOS strain) at an MOI equal to 1 for 1 h at 37°C. Cells were washed with PBS to remove residual viruses and various concentrations of the extract in Medium 199 with 2,5% FBS were added. After 20 h, cells were lysed, cellular debris were cleared by centrifugation, and virus titers in the supernatant were determined by the plaque-forming assay using Vero cells, as described in the previous item. For comparison, linear regression of the dose response curves for ACV was also performed to calculate EC_{50} values.

RESULTS

The octocorals were collected by scuba divers in the Marine Park of Recife de Fora, Porto Seguro, Bahia, and stored in ethanol to obtain the crude extracts. Specimens were identified at Museu Nacional, UFRJ[20,21]. The extracts were separately prepared in organic solvents of different polarities (hexane, dichloromethane

(DCM), and ethyl acetate (EtOAc)), and evaporated under reduced pressure except *P. grandiflora*, which was extracted with DCM and EtOAc (**Table 1**).

Analysis by thin layer chromatography (TLC) of crude extracts of each coral revealed that hexane and DCM extracts of *P. regia* and *M. sulphurea* showed the same chromatographic profile while *P. grandiflora* corresponded to that observed in other species, i.e., DCM extract provided a separate chromatogram of the EtOAc extract.

For a detailed analysis, each crude extract (15 mg) was filtered on a column by adsorption on silica gel 60–70 to 230 mesh, using DCM (30 mL). After evaporation of solvents, an aliquot of each sample (1 mg/mL) was analyzed by gas chromatography-mass spectrometry (GC-MS), showing that *P. regia* and *M. sulphurea* crude extracts have a similar chemical composition with predominance of sterols, fatty acids, and fatty acid esters. While *P. grandiflora* produces a variety of sesquiterpenes, *P. region* produces valencene and sesquiterpene. However, for *M. sulphurea,* such metabolite was not detected in the fractions analyzed by GC-MS (**Tables 2**, **3** and **4**).

Viability and antiviral activities of the octocorals fractions were identified according to the species and the solvent used for purification. Thus, P-1 corresponds to *Plexaurella regia*, P-2 to *Plexaurella grandiflora,* M-1 to *Muriceopsis sulphurea* and each one adds 1 for hexane, 2 for dichloromethane and 3 for ethyl acetate.

Table 1 – Crude extracts of *P. grandiflora, P. regia,* and *M. sulphurea*, extracted successively with hexane, dichloromethane (DCM), and ethyl acetate (EtOAc).

Octocoral	Hexane	DCM	AcOEt
Plexaurella grandiflora	—	14.3 g	0.38 g
Plexaurella regia	4.5 g	0.3 g	0.06 g
Muriceops sulphurea	4.6 g	0.4 g	0.13 g

Table 2 – Secondary metabolites from *Plexaurella grandiflora*, analysed by gas chromatography coupled with mass spectrometry (GC-MS).

Fraction	Molecular formula	M+*	%	Substance
1	$C_{15}H_{24}$	204	3	β-cubebene
2	$C_{15}H_{24}$	204	2	β-cariofilene
3	$C_{15}H_{24}$	204	14	γ-muurolene
4	$C_{15}H_{24}$	204	28	α-amorfene
5	$C_{15}H_{24}$	204	4	α-muurolene
6	$C_{18}H_{36}O_2$	284	6	Ethyl palmitate
7	$C_{20}H_{38}O_2$	310	3	Ethyl oleate
8	$C_{20}H_{40}O_2$	312	2	Ethyl stearate
9	$C_{30}H_{60}O_2$	452	6	Cetyl myristate
10	$C_{32}H_{64}O_2$	480	35	Cetyl palmitate
11	$C_{34}H_{66}O_2$	506	20	Stearate octadecenyl
12	$C_{27}H_{46}O$	386	15	Cholest-5-en-3-ol (cholesterol)
13	$C_{28}H_{36}O$	398	16	Ergost -5,22-dien-3-ol
14	$C_{28}H_{48}O$	400	33	23*S*-methyl cholesterol

*Molecular ion.

Tests of cell viability performed in Vero cells from kidneys of African green monkeys (*Cercopithecus aethiops*) showed that all extracts from *Muriceopsis sulphurea* provided very low CC_{50} values and consequently have not been investigated in antiviral assays (**Figure 1**). The same was observed for hexane and dichloromethane extracts from *P. grandiflora*. Moreover, the results obtained with the ethyl acetate were the most promising, reaching a CC_{50} value similar to the reference, the LCA[22], reaching 132 µg/ mL (**Figure 1**). From analyzing the results separately for each fraction, we identified the fractions P-1.3, P-2.2 and P-2.3 as a dose dependent toxicity ensuring that the toxicity of the substance is in accordance to the administered dose.

The antiviral assay was performed with the fractions that showed the highest values of CC50. It has been also identified the fraction P-1.3

Table 3 – Secondary metabolites obtained from *Plexaurella regia*, analysed by gas chromatography coupled with mass spectrometry (GC-MS).

Fraction	Molecular formula	M+	%	Substance
1	$C_{15}H_{24}$	204	50	Valencene
2	$C_{30}H_{60}O_2$	452	6	Cetyl myristate
3	$C_{32}H_{64}O2$	480	46	Cetyl palmitate
4	$C_{32}H_{66}O_2$	482	10	Palmitate octadecenyl
5	$C_{34}H_{68}O_2$	508	7	Cetyl stearate
6	$C_{27}H_{46}O$	386	11	Cholest-5-en-3-ol (cholesterol)
7	$C_{28}H_{36}O$	398	16	(22E,24S)crinosterol
8	$C_{28}H_{48}O$	400	31	Ergost -5- en-3-ol
9	$C_{29}H_{48}O$	412	36	Stigmast -5-en-3-ol
10	$C_{32}H_{64}O_2$	256	50	Palmitic acid
11	$C_{20}H_{42}$	282	17	n-Eicoseno
12	$C_{24}H_{50}$	338	6	Tetracosane
13	$C_{25}H_{52}$	352	12	Pentacosane
14	$C_{26}H_{54}$	366	13	Hexacosane
15	$C_{27}H_{56}$	380	13	Heptacosane
16	$C_{28}H_{58}$	394	11	Octacosane
17	$C_{29}H_{60}$	408	9	Nonacosane

Table 4 – Secondary metabolites obtained from *Muriceops sulphurea*, analysed by gas chromatography coupled with mass spectrometry (GC-MS).

Fraction	Molecular formula	M+	%	Substance
1	$C_{18}H_{36}O_2$	284	7	Ethyl palmitate
2	$C_{20}H_{32}O_2$	304	4	Arachidonic acid
3	$C_{30}H_{60}O_2$	452	5	Cetyl myristate
4	$C_{32}H_{64}O_2$	480	23	Cetyl palmitate
5	$C_{34}H_{66}O_2$	506	12	Cetyl oleate
6	$C_{34}H_{68}O_2$	508	5	Cetyl stearate
7	$C_{17}H_{34}$	238	41	1-Heptadecene
8	$C_{31}H_{54}O$	442	15	4 α-methylgorgostanol
9	$C_{27}H_{46}O$	386	25	Cholest-5-en-3-ol (cholesterol)
10	$C_{28}H_{36}O$	398	14	Ergost -5,22-dien-3-ol
11	$C_{28}H_{48}O$	400	19	23*S*-methyl cholesterol
12	$C_{30}H_{50}O$	426	22	Gorgosterol

as the compound with antiviral activity (**Figure 2**). Concentrations were 100% effective in inhibiting viral production, even at a concentration of 2.5 µg /mL (**Figure 2**). These data were used for the determination of TI (Therapeutic Index), which indicates the safety of the substance as an antiviral drug. TI is determined by the ratio CC_{50}/ EC_{50} (**Table 5**).

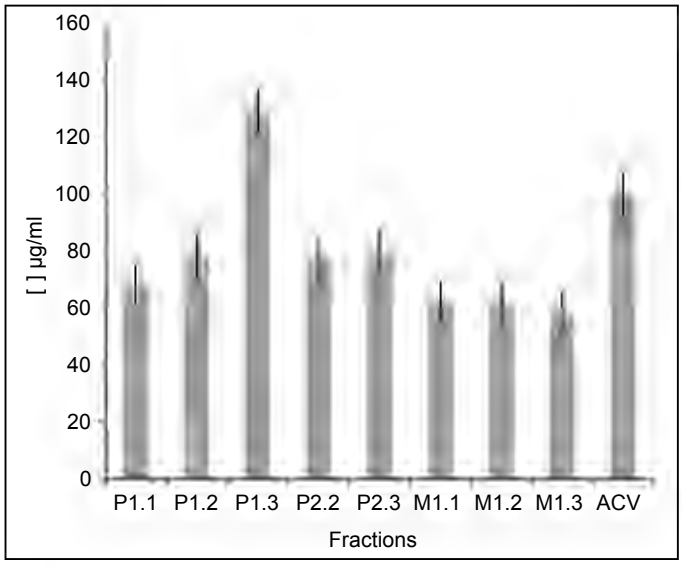

Figure 1 – Effect of coral extracts on Vero cell viability. The cytotoxic concentration that causes 50% lysis and cell death (CC_{50}) were obtained by linear regression of measurements obtained from the average of triplicates of four different concentrations (25, 50, 75, and 100 µg/ml). P-1 refers to *Plexaurella regia*, P-2 to *Plexaurella grandiflora,* and M-1 to *Muriceopsis sulphurea.* According to the solvent used for purification, 1 - hexane, 2 - dichloromethane and 3 - ethyl acetate.

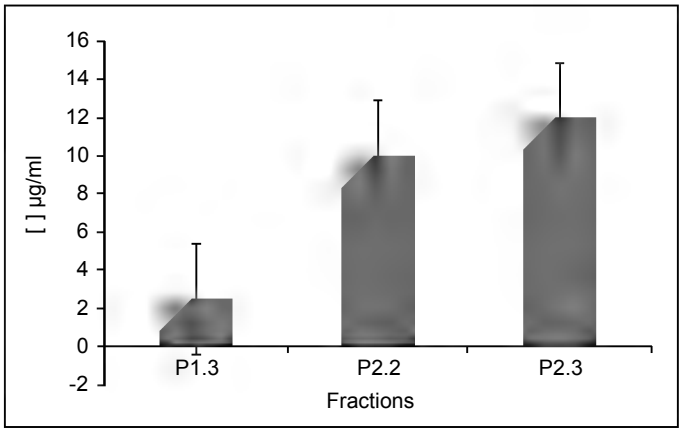

Figure 2 – Concentrations of coral fractions that inhibit 50 % of viral production. The antiviral effect that causes 50 % of inhibition (EC_{50}) was obtained by plaque assay with 2,5, 5, 10, 12, 15 and 20 µg/ml of hexane fraction from *Plexaurella regia* (P1.3), dichloromethane (P 2.2), and ethyl acetate (P 2.3) from *Plexaurella grandiflora* in Vero cells infected with HSV-1 (MOI 1).

Table 5 – Antiviral activities of extracts from *Plexaurella regia*, *Plexaurella grandiflora,* and *Muriceopsis sulphurea* on HSV-1 replication.

Fraction	CC50[a]	EC50[b]	TI[c]
P-1.1	68		
P-1.2	78	-	-
P-1.3	129	<2.5	51.6
P-2.2	77	12	6,4
P-2.3	80	10	8
M-1.1	62	-	-
M-1.2	61	-	-
M-1.3	59	-	-
ACV*	>100**	0.91**	110***

*Acyclovir; **data obtained by Castro[17]; ***CC50=100mg/mL; a: In this assay, Vero cells were cultured in the presence of the extracts for 72 hours at 37°C; b: Antiviral assay that determined the concentration of the extract that reduces the titer of HSV-1 by 50% in Vero cell culture; c: Selectivity Index means how safe some fractions could be as antivirals as reference EC_{50} = 2.5 µg/mL.

DISCUSSION

Recently, the great challenge for HSV infections lies in the search for drugs that could control the development of resistance and latency, especially in AIDS patients and individuals after hematopoietic stem cell transplantation (HSCT)[23]. Resistance to ACV is mediated in 95% of the cases by mutations in the TK gene and in 5% of the cases by mutations in the DNA pol gene, resulting in the alteration of enzyme activity[15,24].

Genotyping findings confirmed that the UL23 TK gene of HSV-1 has an uncommonly high polymorphism[15,25]. The recently discovered inhibitors of the HSV helicase-primase are the most potent development candidates today, but they depend on long-term studies[26].

Marine sponges are considered notable sources of bioactive compounds found in the marine environment. The most important antiviral reported so far is the nucleoside Ara-A (vidarabine), isolated from the sponge Tethya crypta. It inhibits viral DNA polymerase and DNA synthesis of herpes, vaccinia, and varicella zoster viruses[27]. In our study we analysed crude extracts, believing that natural products have great relevance in pharmacology due to their high chemical diversity. The purification of P. regia and P. grandiflora extracts revealed fatty acid esters as common substances for both algae. Additionally, it is consistent with previous antiviral studies that showed that fatty acid esters, specially C14 and C15 isoforms, are able to inactivate enveloped viruses like herpes[28]. These bioactive molecules are often secondary metabolites, whose main function is to enable and/or modulate cellular communication and defence.

CONCLUSION

The highest antiviral activity for HSV-1 was obtained by *P. regia* extract isolated by ethyl acetate. The majority compound is valencene and its molecular formula is C15H24. In conclusion, we suggest Plexaurella spp as a source for anti-HSV compounds.

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Address for correspondence:
IZABEL CHRISTINA NUNES DE PALMER PAIXÃO
Universidade Federal Fluminense, Instituto de Biologia
Rua Outeiro de São João s/n – Campus do Valonguinho, Centro
Niterói (RJ), Brazil
CEP: 24020-141
E-mail: izabeluff@gmail.com

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