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*Full Length Research Paper*

# *In vitro* antiviral activity of diterpenes isolated from the Brazilian brown alga *Canistrocarpus cervicornis*

**Magui Aparecida Vallim[1], Juliana Eymara Barbosa[1], Diana Negrão Cavalcanti[1], Joel Campos De-Paula[2], Viveca Antonia Giongo Galvão da Silva[1], Valéria Laneuville Teixeira[1]* and Izabel Christina Nunes de Palmer Paixão[1,3]**

[1]Programa de Pós-Graduação em Biologia Marinha, Instituto de Biologia, Universidade Federal Fluminense, P. O.Box 100.644, CEP 24001-970, Niterói, RJ, Brazil.
[2]Instituto de Biociências, Universidade Federal do Estado do Rio de Janeiro, Avenida Pasteur 458, CEP 22290-240, Urca, Rio de Janeiro, RJ, Brazil.
[3]Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brazil.

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**They describe in this paper that the dolastane diterpenes 4-hydroxy-9,14-dihydroxydolasta-1(15),7-diene (1) and 4,7,14-trihydroxydolasta-1(15),8-diene (2), isolated from the marine brown alga *Canistrocarpus cervicornis* inhibited HSV-1 infection in Vero cells. The concentration required to inhibit HSV-1 replication was not cytotoxic. Their results suggest that the structures of diterpenes 1 and 2, dolastane diterpenes from Brazilian brown algae, are promising for future antiviral drugs.**

**Key words:** *Canistrocarpus cervicornis*, dictyotaceae, phaeophyceae, dolastane diterpene, cytotoxicity, antiviral against HSV-1.

## INTRODUCTION

Species of the family Dictyotaceae produce a large array of bioactive secondary metabolites and have a broad defensive action against herbivores in the marine environment. These species have been shown to exhibit antifungal, antibacterial (Zanfardino and Gavagnin, 2009) and antiviral activities (Abrantes et al., 2009; Barbosa et al., 2004; Cirne-Santos et al., 2006; 2008; Pereira et al., 2004; 2005). Phycochemical studies have been undertaken on the family Dictyotaceae resulting in the isolation of more than 300 diterpenes from at least 35 species collected all over the world (Vallim et al., 2005).

*Canistrocarpus cervicornis* (Kützing) De Paula and De Clerck (De Clerck et al., 2006) is an important and abundant alga from the Brazilian coast (Falcão and Menezes de Széchy 2005). Although, many diterpenes have been isolated from Brazilian *C. cervicornis* (e.g. Fleury et al., 1994; Kelecom and Teixeira 1988; Teixeira et al., 1986a, b), the antiviral activity of this type of compound is unknown. The Figure 1 presents the structure of dolastane diterpenes 1 and 2 and the previously reported the antiviral activity against HIV-1 (Human Immunodeficiency virus type 1) and HSV-1 (Herpes simplex virus type 1) of dolabellane (3-5) and dichotomane (6-7) diterpenes (Abrantes et al., 2009; Barbosa et al., 2004; Cirne-Santos et al., 2006; 2008; Pereira et al., 2004; 2005).

The HSV-1 infections may cause oral disorders, keratoconjutivitis and encephalitis infection that varies in severity from sub-clinical infections to fatal ones. Most of clinical anti-herpes virus compounds are nucleoside analogues, such as acyclovir (ACV), the most common drug used on the treatment of HSV infections (Brady and Bernstein, 2004). Nonetheless, drug-resistant HSV strains frequently emerge during a long ACV-based treatment due to the reduced expression or the not functioning of timidine kinase. Therefore, the search for new antiviral agents, especially with different mechanisms

*Corresponding author. E-mail: valerialaneuville@gmail.com. Tel: +55 021 21 26292296. Fax: +55 021 21 2629 2292.

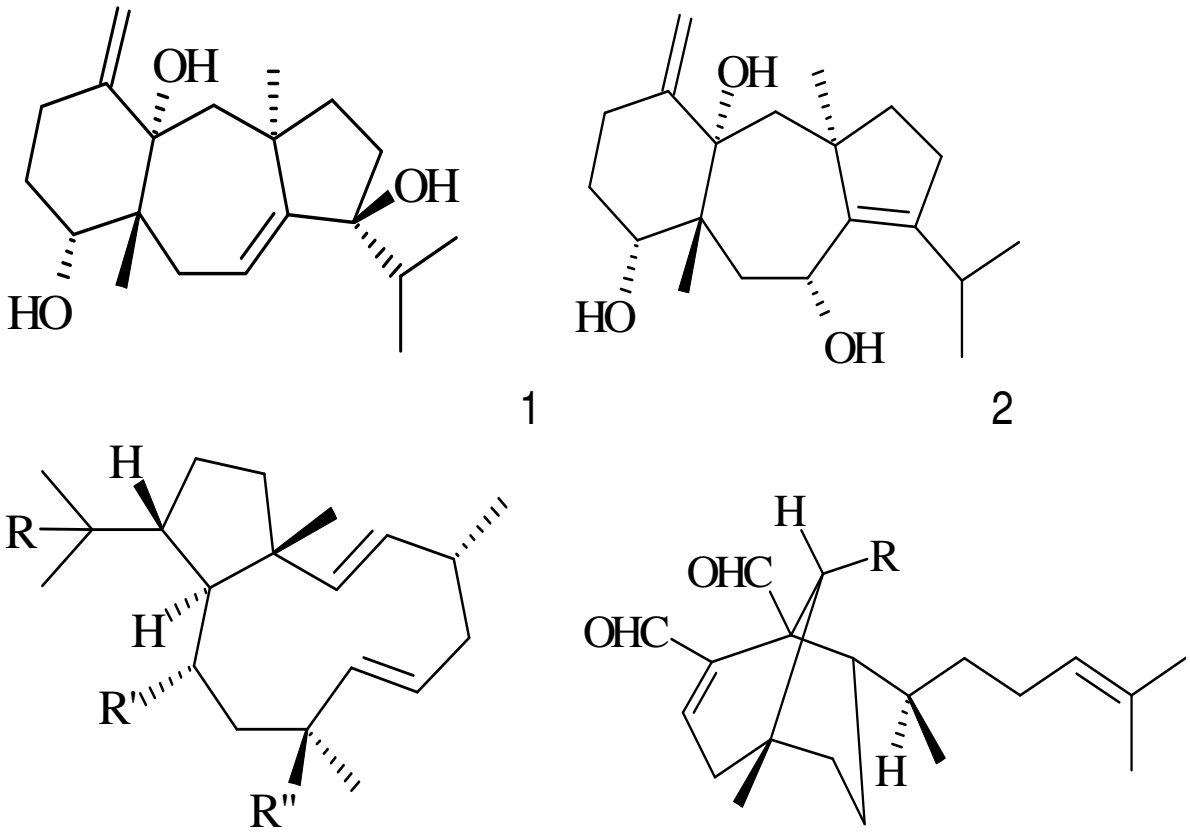


**Figure 1.** Dolastane diterpenes 1 and 2 isolated from Brazilian *C. cervicornis*.
3 R = R' = OAc, R" = OH 6 R = OH
4 R = R' = OH, R" = OAc 7 R = OAc
5 R = R' = R" = OH.

of actions, is a crucial goal (De Clerq, 2004; Morfin and Thouvenot, 2003; Newman et al., 2003). The present study, we evaluate for the first time the antiviral activity against HSV-1 of dolastane diterpenes isolated from species *C. cervicornis*.

## MATERIALS AND METHODS

Specimens of *C. cervicornis* were collected at Búzios, Rio de Janeiro, Brazil, and they were identified by De-Paula, one of the authors of this paper. The seaweeds were washed with local sea water, separated from sediments, epiphytes and other associated organisms. Voucher specimens was deposited in the Herbarium of the Universidade do Estado do Rio de Janeiro (HRJ 10754). The diterpene 4-hydroxy-9,14-dihydroxydolasta-1(15),7-diene (1) was isolated from the $CH_2Cl_2$ crude extract of *C. cervicornis* after column chromatography on silica gel. The fractions eluted in $CH_2Cl_2$ 100% contained the dolastane diterpene 1. The fractions eluted with $CH_2Cl_2$/EtOAc 7:3 to $CH_2Cl_2$/EtOAc 1:1 yielded crude diterpene 4,7,14-trihydroxydolasta-1(15),8-diene (2). The combined fractions were subjected to silica gel column chromatography. The fractions eluted with *n*-hexane/ EtOAc (3:7) yielded the diterpene 2. The compounds 1 and 2 were identified by comparison of physical and spectroscopic data with reported values (Crews et al., 1982; González et al., 1983; Teixeira et al., 1986a). The diterpenes, at over 99% purity, were diluted in 100% dimethyl sulfoxide (DMSO) and stored at -20˚C. The resulting DMSO concentrations during the assays were below 0.1%, a level that is not significantly cytotoxic. African green monkeys kidneys cells (Vero cells; ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 5% fetal bovine serum (FBS; HyClone), 100 U/mL of penicillin, 100 µg/mL of streptomycin and 2.5 µg / mL of amphotericin B (Fungizon) and incubated at 37˚C in 5% $CO_2$. The Vero cells were treated with PBS-1X/EDTA (Reagen) and 0.25% trypsin (GIBCO) so as to achieve the experimental conditions. HSV-1 was kindly provided by Dra. Marcia Wigg (Universidade Federal do Rio de Janeiro, Brazil). Virus stocks were stored at -70°C until use. Monolayers of Vero cells in 96-multiwell plates ($1 \times 10^4$/well) were treated with several concentrations (50, 250, 500 and 1000 µM) of diterpenes 1 and 2 of the *C. cervicornis* for 72 h at 37˚C in atmosphere of 5% $CO_2$. Then, 50 µL of a 1 mg/mL solution of 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) was added to evaluate the cell viability according to procedures described elsewhere (Gilbert et al., 2002). The cytotoxic concentration in 50% ($CC_{50}$) was calculated by linear regression analysis of the dose-response curves generated from the data using Microsoft Excel. All experiments were performed in triplicate at least three times.

The antiviral activities of 1 and 2 were investigated according to Reed and Munch 50% end point method (Reed and Munch, 1938). At first, Vero cells grown to confluence in 96-well plate were infected with HSV-1 (AR-29) at 1 multiplicity of infection for 1 h at

**Table 1.** Dose-dependent cytotoxicity and percentage of inhibition of viral replication observed in Vero cells in the presence of the diterpenes 1 and 2 from the brown alga *C. cervicornis.*

| Sample | $CC_{50}$ (µM) | % Inhibition(*) |
|---|---|---|
| Diterpene 1 | 1423 (± 83) | 90 |
| Diterpene 2 | 706 (± 100) | 99 |
| Acyclovir | 860 (± 32) | 99 |

(*) Base on $TCID_{50}$ which means could determine a median tissue culture infective dose that amount of a pathogenic agent that will produce pathological change in 50% of Vero cell cultures incubated with 50µM of the substances.

37˚C. After that, infected cells were washed with PBS to remove residual viruses and a complete culture medium, with or without diterpenes, were added. 72 h after infection, the virus titer of each sample was determined in terms of the 50% tissue culture dose ($TCID_{50}$/mL) by end point dilution. In addition we used mock-treated HSV1-infected cells as positive control. To evaluate the virucidal activity of the diterpenes 1 and 2 from *C. cervicornis*, we conducted cell-free tests with viral suspension in a medium without FCS at a concentration of $10^7$ - $10^5$ particles/mL in the presence and in the absence of the substances (50 µM). The samples were incubated with viral suspension for periods of 1, 2 and 4 h at 4˚C.

## RESULTS AND DISCUSSION

We observed that both compounds were not cytotoxic for Vero cell lineage (Table 1). We also observed that the $CC_{50}$ of the diterpene 1 was 48% higher than acyclovir's. Acyclovir is more selective for viral DNA polymerase than for cellular enzyme, having minimal toxicity (Brady and Bernstein, 2004). This finding is very promising for future antiviral trials. A reduction of 3 logs of infectivity was evident for diterpene 2 of *C. cervicornis* when compared to the values obtained with HSV-1 control (Table 1) according to $TCID_{50}$ method. Although, both compounds have shown viral inhibition rates above 90% we observed specifically for diterpene 2 the same inhibitory rate of acyclovir. For this reason, our future studies are concerned about the kinetic activity of polymerase from HSV-1 in the presence of these compounds. In the virucidal assay, we discovered that only after 4 h of incubation did the diterpene 2 show virucidal activity at the dilution of $10^6$ (78%) and for this reason we cannot say that these compounds may be presented as possible virucidal molecules (data not showed).

Many investigators have reported the inhibitory effects of algal extracts and their compounds on the replication of HSV-1, with special emphasis in sulfated polysaccharides (Damonte et al., 2004; Harden et al., 2009; Witvrouw and De Clercq, 1997). In this study, we showed the antiviral activity of diterpenes obtained from *C. cervicornis*. Both diterpenes 1 and 2 extracted from *C. cerviconis* were able to inhibit *in vitro* replication of herpes simplex virus type 1. However, the viral inhibition rate obtained with diterpene 2 was higher than that of diterpene 1. The presence of the 7-hydroxyl group can have a positive influence on the inhibition of HSV-1. Since the same profile of antiviral diterpenes could not be observed in the absence of cells, that is, these substances will not be considered virucidal. Thus, we considered these substances promising as anti-HSV-1 and therefore able to advance to the *in vivo* antiviral studies.

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