Cytotoxic and antiviral activity of extracts and compounds isolated from *Clusia fluminensis* Planch. & Triana (Clusiaceae)

Atividade citotóxica e antiviral de extratos e compostos isolados de Clusia fluminensis Planch. & Triana (Clusiaceae)

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ABSTRACT

Introduction: The worldwide distribution of herpes simplex virus type 1 (HSV-1) allied to the emergence of resistant strains makes necessary and urgent the search and development of new substances capable of preventing and treating HSV-1 infections. Studies demonstrate synergy between genital herpes and human immunodeficiency virus type 1 (HIV-1), which represents a major concern for global public health. **Objective**: The objective of this study was to evaluate the activity of crude extracts and isolated substances from *C. fluminensis* in the *in vitro* replication of the HSV-1 virus and HIV-1-RT activity. **Methods**: This study evaluated the activity of extracts and isolated compounds from *Clusia fluminensis* Planch. & Triana against HSV-1 using Vero cells in culture and against HIV-1 using a recombinant reverse transcriptase enzyme (HIV -1 RT). The percentage of inhibition against HSV-1 was determined from viral lysis plaque reduction assay, and the anti-HIV-1-RT test was performed by a fluorimetric assay. It was also evaluated the cytotoxic activity of the samples using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. **Results**: The crude extracts showed high percentage of inhibition against HSV-1, reaching 81.4 to 100.0% inhibition in non-cytotoxic concentration (50 µg/mL). The isolated compounds, lanosterol and clusianone, demonstrated 100% inhibition in non-cytotoxic concentration (50 µg/mL). We also examined the effects of the extracts and isolates on the activity of the HIV-1-RT. Among the crude extracts, only the methanolic extract of leaves and methanolic extract of stems showed inhibitory activity against HIV-1-RT. Regarding the isolated compounds, lanosterol showed a moderate activity. **Conclusion:** Our data demonstrate that extracts and isolates compounds *Clusia fluminensis* Planch. & Triana have promising antiviral activity inhibiting HSV-1 replication and HIV-1 by inhibiting the anti-RT activity.

Keywords: *Clusia fluminensis*; Clusiaceae; cytotoxic activity; antiviral activity; HSV-1; HIV-1 RT; lanosterol.

RESUMO

Introdução: A distribuição mundial do vírus herpes simplex tipo 1 (HSV-1) aliada ao surgimento de cepas resistentes torna necessária e urgente a busca e o desenvolvimento de novas substâncias capazes de prevenir e tratar infecções HSV-1. Estudos demonstram sinergia entre herpes genital e vírus da imunodeficiência humana tipo 1 (HIV-1), o que representa uma grande preocupação para a saúde pública global. **Objetivo:** O objetivo deste estudo foi avaliar a atividade de extratos brutos e substâncias isoladas de *Clusia fluminensis Planch. & Triana* na replicação *in vitro* do vírus HSV-1 e na atividade anti HIV-1-RT. **Métodos**: Este estudo avaliou a atividade de extratos e substâncias isoladas de *Clusia fluminensis Planch. & Triana* contra o HSV-1 utilizando células Vero em cultura e contra o HIV-1 utilizando a enzima transcriptase reversa recombinante (HIV-1 RT). A porcentagem de inibição contra o HSV-1 foi determinada a partir do ensaio de redução de placas de lise viral, e o ensaio anti-HIV-1 RT foi realizado por um ensaio fluorimétrico. Também foi avaliada a atividade citotóxica das amostras utilizando MTT [brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difeniltetrazólio]. **Resultados**: Os extratos demonstraram elevada percentagem de inibição contra o HSV-1, atingindo 81,4 a 100,0% de inibição em concentração não citotóxica (50 µg/mL). Os compostos isolados, lanosterol e clusianona, demonstraram 100% de inibição em concentração não citotóxica (50 µg/mL). Examinamos também os efeitos dos extratos e isolados sobre a atividade anti-HIV-1 RT. Entre os extratos brutos, apenas o extrato metanólico das folhas e caules apresentaram atividade anti-HIV-1 RT. Em relação aos compostos isolados, lanosterol mostrou uma atividade moderada. **Conclusão:** Nossos dados demonstram que os extratos e compostos isolados de *Clusia fluminensis Planch. & Triana* possuem atividade antiviral promissora inibindo a replicação do HSV-1 e HIV-1 através da inibição da atividade anti-RT.

Palavras-chave: *Clusia fluminensis*; Clusiaceae; atividade citotóxica; atividade antiviral; HSV-1; HIV-1 RT; lanosterol.

INTRODUCTION

Herpes simplex virus 1 (HSV-1) and herpes simplex virus 2 (HSV-2), two serious human pathogens, are members of the *Herpesviridae* family, a large family of DNA viruses that cause diseases in animals. Both are alpha-herpes viruses that are neurotropic, have a rapid replication cycle and are able to infect a wide variety of cells and hosts. These viruses establish latent infections in sensory neurons and can be reactivated by factors such as stress, fatigue, overexertion, fever, sun exposure, trauma, prolonged use of antibiotics and

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menstruation. Latency allows the maintenance of the viral genome in a non-pathogenic and nonreplicate form and serves as a reservoir for later viral attack of the host, which may increases the pathogenicity of HSV, and can become particularly severe in immunocompromised patients, to whom recurrent HSV-infection may be more extensive and/or aggressive, slow healing and extremely painful[1,2].

HSV-1 is commonly associated with orofacial infections and encephalitis. The public health impact of orolabial herpes caused by HSV-2 is probably small, being this virus primarily responsible for genital infections; however, recent studies have shown that HSV-1 infections also account for a substantial proportion of genital herpes infections[3].

Prevalence of HSV-1 infection is higher in most geographic areas worldwide and more prevalent than HSV-2 infection in non-high-risk populations. Exceptions are groups of immunocompromised patients, such as HIV-positive individuals. Most studies show that HSV-1 infection is acquired during childhood and adolescence, and HSV-1 prevalence increases consistently with age across the age spectrum or stabilizes after age 30[4].

Several clinical and epidemiological studies demonstrate synergy between genital herpes and human immunodeficiency virus type 1 (HIV-1). HIV-1 is the etiologic agent of acquired immunodeficiency syndrome (AIDS) and is still a major concern for global public health. HSV infections are regularly associated with transient depression of cell-mediated immunity, since there is a close relationship between viruses infections and cellular immunological competence of the host. Currently, there is no treatment capable of curing genital herpes and AIDS, and there is no effective vaccine for HSV and HIV-1 yet. Furthermore, only few antiviral drugs are able of shortening the disease and prevent breakouts. These antiviral drugs decrease the rate of virus replication, giving more opportunity for the immune system interference[5].

Although antiviral drugs such as acyclovir, famciclovir and valacyclovir show to be efficient and safe, the emergency of resistant HSV strains has been documented, and an aggravating situation is the fact that HSV is usually resistant to the three of them simultaneously, which decreases treatment options. In fact, the only tools to reduce morbidity and mortality associated with AIDS and its complications are still the prevention and the use of antiretroviral drugs. The antiretroviral drugs act by interacting with major viral proteins of the replication cycle of the virus. The resistance to antiretroviral drugs is largely unavoidable due to the error-prone nature of HIV reverse transcriptase (RT) and its lack of a proofreading function[6].

The need for search and development of new substances capable of preventing and treating infections with HSV-1, HSV-2 and HIV-1 makes natural products interesting targets for assays involving evaluation of antiviral activity.

Among the botanical families, Clusiaceae was chosen because of its great representativeness in Brazil and the description of the use of its species in folk medicine. It comprises 14 botanical genera and is chemically characterized mainly by the presence of xanthones, polyisoprenylated benzophenones, flavonoids and terpenes[7-11].

Clusia fluminensis Planch. & Triana is a native species from the Brazilian coast, with few studies exploring its chemical and biological aspects as a part of the investigation of the biological activities of this species, and in the search of substances with potential to become new drugs for the treatment of herpes and AIDS.

OBJECTIVE

The objective of this study was to evaluate the activity of crude extracts and isolated substances from *C. fluminensis* in the *in vitro* replication of the HSV-1 virus. Singh[12] demonstrated the anti-HIV-1 activity of polyisoprenylated benzophenones isolated from fruits of *Clusia torresi*, with EC_{50} values in the range between 0.02 and 0.8 μM. These results prompted us to also analyze the activity of the extracts and the isolated compounds on the enzyme reverse transcriptase of HIV-1.

METHODS

Plant material

Leaves, stems and flowers from a male individual and fruits from a female individual of *Clusia fluminensis* Planch. & Triana were collected at Forte Barão do Imbuhy, Niterói, in Rio de Janeiro State, Brazil. Flowers were collected in the summer, leaves, stems and partially ripe fruits were collected in the autumn, and completely ripe fruits were collected in the winter. The plant material was identified by Dr. Marcelo Guerra Santos, and a voucher specimen was deposited at the herbarium of the Faculdade de Formação de Professores, Universidade do Estado do Rio de Janeiro (RFFP), Brazil, registered under the number 9213.

Preparation of plant extracts

Leaves, fruits and stems of *C. fluminensis* were dried in an oven at 40°C and subsequently fragmented. The flowers were not subjected to the drying process. The crude extracts were obtained by static maceration of plant organs with the respective solvents at room temperature for 30 days for methanolic and hexanic extracts, and 15 days for acetonic extracts. The solvent was renewed at each seven days and followed by evaporation under reduced pressure.

Isolated substances

Clusianone (**Figure 1**) was previously isolated from the hexanic extract of the flowers of *C. fluminensis* by countercurrent chromatography using the solvent system *n*-hexane-acetonitrile-methanol (2:1.25:0.5, v/v/v)[13]. A new fractionation of this extract using the same procedure described by Silva *et al.*[13] now allowed the isolation of lanosterol (**Figure 1**), for the first time from the flowers of *C. fluminensis*. The chemical structure of lanosterol was determined by Gas Chromatography-Mass Spectrometry (GC-MS) and The Nuclear Magnetic Resonance (NMR) and spectra were compared with literature data[14]. GC-MS was performed on an Agilent model 6890N gas chromatograph equipped with a mass selective detector, model 5973N with data base. Conditions: carrier gas, helium at 2.0 mL.min^{-1}, split ratio 5:1, injector temperature 300ºC, ion source temperature 230 ºC. Oven temperature was programmed from 150 – 300 ºC at 10 ºC.min^{-1} and held at 300 ºC for 15 min. Sample injection volume was 1 μL. The NMR spectra were acquired using a BRUKER DRX 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Chemical shifts were measured relatively to an internal tetramethylsilane (TMS) reference.

Lanosterol (C30H50O) characterization is made by 1H-NMR (400MHz, CDCl3) and 13C-NMR (100 MHz, CDCl3). 1H-NMR (400MHz, CDCl3): δH: 0.76 (s, 3H, 18-Me), 0.81 (s, 3H, 29-Me), 0.88 (s, 3H, 30-Me), 0.86 (d, 3H, 21-Me), 0.96 (s, 3H, 19-Me), 1.01 (s, 3H, 28-Me), 1.13 (m, 1H, 5-H), 1.31 (m, 1H, 20-H), 1.52 (m, 1H, 17-H), 1.61 (s, 3H , 27-Me), 1.69 (s, 3H, 26-Me), 3.25 (dd, J = 4.4, 11.6 Hz, 1H, 3-H), 5.10 (s, 1H, 24-H); 13C-NMR (CDCl3): δC: 15.7 (C-18), 15.8 (C-29), 17.9 (C-27), 19.1 (C-6), 19.2 (C-21), 20.4 (C-19), 21.7 (C-11), 24.7 (C-30), 25.0 (C-23), 25.9 (C-26), 27.9 (C-7), 28.2 (C-2), 28.3 (C-28), 28.4 (C-16), 30.0 (C-15), 31.1 (C-12), 35.5 (C-20), 35.6 (C-22), 36.1 (C-1), 37.5 (C-10), 39.2 (C-4), 44.3 (C-13), 49.9 (C-14), 50.3 (C-5), 51.2 (C-17), 79.2 (C-3), 125.4 (C-24), 131.1 (C-25), 133.8 (C-9), 134.3 (C-8).

Cell culture and virus

Vero cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal bovine serum (FBS; HyClone, Logan, Utah), 100 U/mL penicillin, 100 µg/mL streptomycin, and amphotericin B (25 mg/mL; Cultilab, São Paulo, Brazil), at 37 ºC in a humidified 5% CO_2 atmosphere. Herpes simplex virus type 1 (HSV-1, strain KOS) obtained from Departamento de Virologia Universidade Federal de Santa Catarina, Brazil, was routinely grown. Vero cells and virus stock cultures were prepared from supernatants of infected cells and stored at -80 ºC until use.

Cell viability test

Monolayer of Vero cells in 96-multiwell plates were treated with 25, 50, 100 and 200 µg/mL of the plant extracts, and 50, 250, 500 and 1000 µM of the pure compounds, for 72 h/37 °C. After that, 1 mg/mL solution of 3-(4-5 dimethylthiazol-2yl)-2-5- diphenyl tetrazolium bromide (MTT; Sigma) was added (50 µL/well) to evaluate cell viability according to procedures described elsewhere[15]. The 50% cytotoxic concentration (CC_{50}) was calculated by linear regression analysis of the dose-response curves.

Viral inhibition percentage

The antiviral effect was evaluated by plaque reduction assay[6]. Substances and extracts at 50 µg/mL were added 1 h after addition of viral dilution and left for 20 h. After 20 h, cells were lysed, and the title of supernatant virus was determined by adding different dilutions on plate to verify the viral plaque reduction. The result was obtained by counting PFU, comparing cells treated with the substances and cells of viral control[16,17].

Bacteria and Plasmids

Escherichia coli strain BL21 (DE3) was used as a recipiente for DNA transformations. *E. coli* cells transformed with the plasmid containing RTp66 and RTp51 HIV-1 gene were cultured in Luria-Bertani (LB) containing ampicillin (100 µg/mL) under shaking, at 220 rpm at 37 °C; overnight. This culture was used as inoculum for 1L of LB medium containing 100 µg/mL of ampicillin. Cells were grown for 6 h at 37 °C with vigorous shaking, then induced with isopropyl-b-D-thiogalactopyranoside (IPTG) (1 mM) for 2 h. Cells were harvested by centrifugation (5,000 g, 15 min), and bacterial lysates were prepared using a lysis buffer (50 mM Tris-HCl (pH 7,9 at 4 °C), 60 mM NaCl, 1 mM EDTA and using lysozyme/DNAse I treatment. Clarified lysates were used for the isolation of the p51/p66 heterodimeric RT. The active RT heterodimer was purified by using MagneHis™ Protein Purification System according to the manufacturer's instructions.

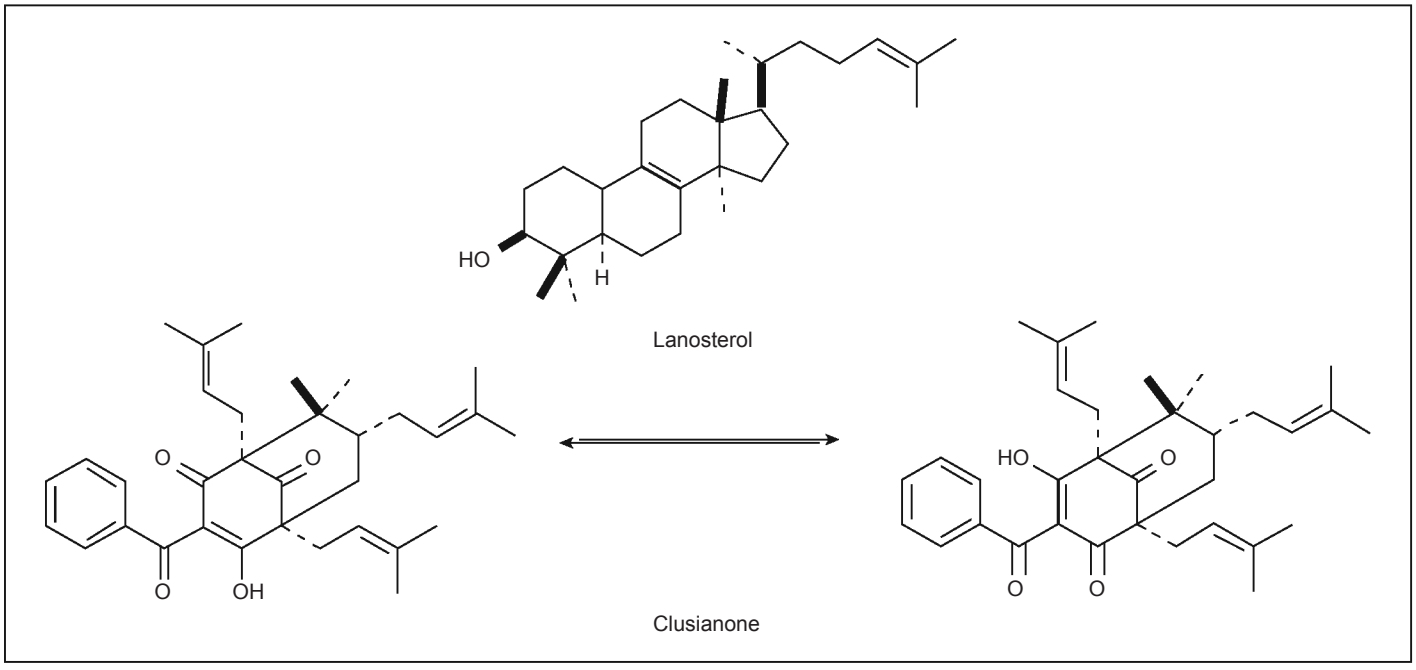

Figure 1 – Chemical structures of lanosterol and clusianone (as a tautomeric pair).

Fluorometic assay of HIV-1-RT activity

An EnzChek RT Assay Kit (Molecular Probes) was used for detecting the RT activity. The mixture of 350 base-poly (A) ribonucleotide template and oligo d(T)16 primer was incubated at room temperature for 1 h. Then, the mixture was added into the tube containing polymerizing buffer (63 mM Tris-HCl, pH 8.1, 8 mM MgCl2, 132 mM NaCl, and 13 mM DTT). The reaction was started by adding the purified enzyme (1 µg/mL) into reaction mixture. The samples were incubated at 37 °C for 30 minutes and stopped by adding 200 mM EDTA. Then, the polymerizing activity was measured using a fluorometric assay by adding PicoGreen in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to the EDTA-terminated reaction mixture and incubated for 10 minutes on ice and in the dark. The background fluorescence was normalized by subtracting a control reaction value, and the fluorescence intensity was measured in microplate reader Thermomax (Molecular Devices) by using excitation and emission at 502 and 523 nm, respectively.

Statistical analysis

Results of anti-HIV-1 RT activity were expressed as means ± SD of three determinations. The IC_{50} values were calculated using the Microsoft Excel program. Results were considered significant if the $p < 0.05$.

RESULTS

Isolation of lanosterol

A portion (1.0956 g) of the crude hexanic extract from flowers of *C. fluminensis* was fractionated through high speed counter-current chromatography as described by Silva *et al*.[13]. A fraction constituted of white crystals in needle shape was analyzed though GC-MS and revealed the presence of a major substance with the retention time of 18.85 min. and 71.55% purity. The mass spectrum showed the molecular ion of 426 Da and triterpene characteristic mass fragmentation. The ion fragments with m/z = 411 and 393 correspond, respectively, to [M+ - CH_3] e [M+ - (CH_3+H_2O)]. The fragment with m/z = 69 corresponds to the isopentenyl group. The major signal was accompanied by three minor signals, with the same fragmentation pattern of the major component, suggesting that the sample is a mixture of isomers. A portion of the sample was analyzed through ^1H and ^{13}C NMR, and the spectral data were compared to those described in literature for lanosterol[14], confirming its structure.

Cell viability, viral inhibition and anti-HIV-1-RT activity

The crude extracts showed high percentage of inhibition against the HSV-1 virus, reaching 81.4 to 100.0% inhibition in non-cytotoxic concentration (50 µg/mL) (**Table 1**). The isolated substances, lanosterol and clusianone, also showed some cytotoxicity compared to acyclovir and both demonstrated 100% inhibition in non-cytotoxic concentration (50 µg/mL) (**Table 2**). The crude extracts showed some cytotoxicity compared to acyclovir, except the hexanic extract of fruits (CFFRH) and methanolic extracts of leaves (CFLM) and fruits (CFFRM) (**Table 1**).

The anti-HIV-1 activities of extracts and isolated products from *C. fluminensis* were investigated using RT fluorimetric assay, where the enzyme activity is determined after treatments in the presence or absence (untreated control) of drugs. Inhibition of HIV-1 RT enzyme was evaluated based on their percentage of inhibition, compared to a negative control and with a standard drug. Only the methanolic extracts of leaves (CFLM) and stems (CFSM) showed inhibitory activity against HIV-1-RT. Among the isolated compounds, lanosterol showed a moderate inhibitory effect (**Tables 1** and **2**).

Previous studies showed that the hexanic extracts of leaves and stems of *C. fluminensis* (CFLH and CFSH, respectively) are composed primarily of terpenes, especially friedelin and epifriedelinol[18]. CFLH and CFSH showed 100% inhibiton of viral replication at 50 µg/mL, making these extracts interesting targets for the search of antiviral substances. However, in a study with friedelin and epifriedelinol, the terpenes did not inhibit herpes simplex virus 1 and 2[19], which means that the antiviral activity of CFLH and CFSH may be due to other substances present in the extracts, and these samples need to go through further chemical investigations.

The hexanic extract of the flowers of *C. fluminensis* showed a CC_{50} of 78 µg/mL and 100% inhibition of the virus in the concentration evaluated. Chemical investigations of the floral resins of *Clusia* species revealed that they are mainly constituted by polyisoprenylated benzophenones[13,20] which are also present in their fruits[21-23]. These substances have a broad spectrum of biological activities, which includes antiviral activity against HIV[20,10].

Table 1 – Anti-HSV-1, anti-HIV-1-RT and cytotoxic activities of extracts from *Clusia fluminensis.*

Samples	Code	CC_{50} (µg/mL)	HSV-1 Inhibition (%)[a]	HIV-1-RT Inhibition (%)[a]
Hexane extract of leaves	CFLH	100	100	Inactive
Hexane extract of stems	CFSH	131	100	Inactive
Hexane extract of fruits	CFFRH	303	95,5	Inactive
Hexane extract of flowers	CFFLH	78	100	ND
Acetone extract of stems	CFSAC	138	100	Inactive
Acetone extract of fruits	CFFRAC	154	100	ND
Methanol extract of fruits	CFFRM	304	81,4	Inactive
Methanol extract of leaves	CFLM	325	100	41,75 ± 11,19
Methanol extract of stems	CFSM	149	100	20,24 ± 6,24
Acyclovir	ACV	216	100	–
Efavirenz	EFV	–	–	92,16 ± 2,34

[a]Determined at 50 µM; ND: not determined.

Table 2 – Anti-HSV-1, anti-HIV-1-RT and cytotoxic activities of isolated substances from *Clusia fluminensis.*

Samples	Code	CC_{50} (µM)	HSV-1 Inhibition[a]	HIV-1 RT Inhibition[a]
Lanosterol	CFFLH16	74	100	77,31 ± 10,74
Clusianone	CFFLH35	121	100	37,6 ± 1,73
Acyclovir	ACV	960	100	–
Efavirenz	EFV	–	–	92,16 ± 2,34

[a]Determined at 50 µM.

Clusianone is the major component of the flowers of *C. fluminensis*, representing about 37% of the composition of the resin from male flowers of this species[24]. It has been isolated from the hexanic extract of the flowers of *C. fluminensis* by Silva *et al*.[13]. Lanosterol has already been isolated from the hexanic extract of fruits of *C. fluminensis*[25]; here we describe the isolation of this triterpene from the hexanic extract of flowers of this species. Both substances showed 100% inhibition of viral replication at 50 µg/mL. Lanosterol was more cytotoxic than clusianone, and this one less cytotoxic than the original crude extract.

In the study conducted with clusianone isolated from the fruits of *Clusia torresii*, this substance was evaluated for its activity against HIV-1 in C8166 cells (human T-lymphoblastoid cells), as well as other benzophenones. The substance was active in a very low concentration, 0,02 µM, however showed high citotoxicity. The benzophenone was more effective when added before or during the viral infection period and also neutralized more than 99% of viral infection when incubated with the virus in the concentration 0,05 µM for 60 min. at 37 °C. The study of the mechanism of action showed that clusianone inhibited the interaction gp120-sCD4, suggesting its interference with virus binding to CD4 cell receptor, preventing infection[23]. In the present study, clusianone showed a weak inhibition on HIV-1-RT. This result complements the information on the mechanism of action of clusianone on HIV, showing that the inhibition of the enzyme reverse transcriptase is not the main mechanism of action of this substance.

The acetonic extracts of fruits and stems are expected to be composed by substances with medium to high polarity, such as benzophenones, while the methanolic extracts of leaves, fruits and stems are expected to have a high content of substances with higher polarity, such as flavonoids and flavonoids glucosydes. Silva and Paiva[26] determined the flavonoid content (as flavones and flavonols) of these extracts, together with their antioxidant activity. The extracts evaluated showed a relative high percentage of these substances, which may also be responsible for their antiviral activities[27,28].

CONCLUSION

The results corroborate that plant extracts are a valuable source of substances with antiviral activities. This study showed that *C. fluminensis* is a promising target for studies in the search for new substances with anti-HSV and anti-HIV-1 activity, including *in vitro* studies of their action mechanisms. Also, the relative low cytotoxicity of the samples makes them potential candidates for *in vivo* studies.

Conflict of interests

The authors have declared no conflict of interest related to the manuscript.

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