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Antiherpetic activity of an *Agaricus brasiliensis* polysaccharide, its sulfated derivative and fractions

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ABSTRACT

Agaricus brasiliensis is an edible mushroom, traditionally used for the treatment of several diseases. In this paper, a polysaccharide (PLS) from *A. brasiliensis*, its carboxymethylated (CPLS) and sulfated (SPLS) derivatives, as well as, fractions (F1–F3) obtained from the PLS were investigated for their effect in the replication of herpes simplex virus and bovine herpes virus in HEp-2 cell cultures. The PLS, SPLS and F3 inhibited both virus strains similarly, in a dose-dependent curve. F1, F2 and CPLS did not show significant effect even at higher concentrations. All the compounds showed neither virucidal or viral adsorption inhibition activities nor effect when cells were treated prior to infection. Our study demonstrated that the extracts of *A. brasiliensis*, can be promising for future antiviral drug design and its biotechnological production is economically feasible.

1. Introduction

Herpes simplex virus (HSV) and bovine herpes virus (BoHV) are enveloped, double-stranded DNA virus, belonging to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, responsible for mild to severe diseases in human and bovine. HSV-1 also referred to as human herpes virus type 1 (HHV-1), a member of the *Simplexvirus* genus, is one of the most regular human pathogens, being a public health problem and the causal agent of several diseases estimated to occur in approximately 40–80% of world population [1]. The variety of infections is manifested with different degrees of severity, from superficial lesions to encephalitis, the latter, especially in immunocompromised patients. In Brazil, HSV-1 is one of the main causes of viral encephalitis [2].

BoHV-1, classified under the *Varicellovirus* genus, shares many biological properties with HSV-1 [1]. The virion is associated to several syndromes in cattle and buffaloes, including infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB). These diseases occur throughout the world and are responsible for significant losses in the cattle industry. It has been estimated that BoHV-1 infects 50–90% of the Brazilian cattle population [3].

The infections caused by both agents are characterized by a short replication cycle and the ability to induce latent infection, mainly in sensory neurons [4]. These viruses establish a persistent infection and can be life threatening and affect greatly the quality of the host life. The latency reactivation cycle of herpesvirus has a deep epidemiological impact, since it is responsible for the maintenance of HSV-1 and BoHV-1 in human and cattle population, respectively [4]. No proved effective vaccines are presently available for HSV infections and, although vaccines for BoHV-1 are available, none is able to prevent the establishment of latency [5]. Acyclovir continues to be the reference drug for HSV diseases treatment even after three decades of its development [6]. However, in long-term treatment and especially in immunocompromised individuals, acyclovir can lead to the selection of resistant mutants [7]. These features highlight the need to develop new antiherpetic drugs. Over the past few years great efforts have been made to identify new and potent antiviral drugs which are also active against latency establishing virus [8].

Agaricus brasiliensis (syn *Agaricus blazei*, *Agaricus subrufescens*), commonly known as the Cogumelo do Sol®, ABM, Himematsutake, and Cogumelo de Deus, is a native Brazilian basidiomycete [9]. It is often consumed as food and tea worldwide, because of its medicinal effects, such as, relief of physical and emotional stress, treatment for high cholesterol, diabetes, gastric disorders and osteoporosis. It is also used as antimutagenic, antioxidant, and as immune

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stimulator [10]. Some studies report the antiviral activity of extracts and compounds from mycelium or fruiting body of *A. brasiliensis* at replication of poliovirus (PV), Western equine encephalitis (WEE), Hepatitis B (HBV) and C (HCB) virus and HSV-1 and HSV-2 [11–16]. The aim of the present study was to investigate the antiviral activity of an *A. brasiliensis* polysaccharide, its fractions, as well as, its carboxymethylated and sulfated derivatives in the replication of HSV-1 and BoHV-1.

2. Materials and methods

2.1. Cells and virus

HEp-2 cells (human laryngeal epithelial carcinoma cell, ATCC CCL-23) were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (*Invitrogen – Gibco, USA), supplemented with 10% fetal bovine serum (*) and treated with 100 μg/ml streptomycin (*), 100 IU/ml penicillin (Novafarma Indústria Farmacêutica, Brazil) and 2.5 μg/ml amphotericin B (Meizler Biopharma S/A, Brazil). Herpes simplex virus type 1 (HSV-1) and bovine herpesvirus type 1 (BoHV-1) provided, respectively, by DV/IMPG/UFRJ, Brazil and DMVP/UEL, Brazil, were propagated in HEp-2 cells, stocked at −20 °C with 10% glycerol.

2.2. Compounds

The *A. brasiliensis* PLS, its fractions F1–F3, and its derivatives sulfated (SPLS) and carboxymethylated (CPLS) were prepared, as previously described [17–19].

2.3. Cytotoxicity assay

Cell viability was assayed by MTT method (Sigma Chem. Co., USA), according to manufacturer's instructions. Briefly, 70% confluent cell cultures in 96-well microplates (Nunc A/S, Denmark) were treated with 500 μg/ml, 1000 μg/ml, 1500 μg/ml, 2000 μg/ml and 2500 μg/ml of the compounds, in maintenance medium, with appropriate controls and maintained at 37 °C with 5% CO_2, during 72 h. The medium was replaced with 10 μl of the MTT reagent (dimethylthiazolyldiphenyltetrazolium bromide) (1.25 μg/ml) and incubated, under same conditions, for 3 h. The solubilizer agent was added and after 15 min the absorbance read at 570 and 690 nm The 50% cytotoxic concentration (CC_{50}) was calculated by the regression analysis of the obtained curves, and defined as the compound concentration capable of reducing the OD of the MTT product by 50% in relation to the control.

2.4. Plaque reduction assay (PRA)

Antiviral activity was evaluated by plaque reduction assay, as previously [20]. Briefly, HEp-2 cell monolayers grown in 24-well plates (TPP, Switzerland) were infected with 50–100 PFU and treated with 200 μg/ml, 400 μg/ml, 600 μg/ml and 800 μg/ml of each compounds. Infected and treated cells were washed and overlaid with nutrient agarose (2× DMEM/1.8% agarose) and 25 mM of $MgCl_2$ was added [21]. After 40 h cells were fixed with 10% formaldehyde in PBS, pH 7.3, and stained with 0.5% crystal violet in 20% ethanol. Plaques were counted and the percentage of viral inhibition (%VI) was calculated as follows: $\%VI = [1 - (V_d/V_c)] \times 100$, where V_d and V_c refer to the number of plaques in the presence and absence of the compounds, respectively [22]. The inhibitory concentration 50% (IC_{50}), the amount of the compounds capable to reduce in 50% the number of the plaques, was calculated. The selectivity index (SI) was calculated as the ratio of CC_{50} and IC_{50}. Acyclovir (Zynvir, Novafarma Indústria Farmacêutica, Brazil) and human interferon alfa-2B (Meizler Biopharma S/A, Brazil) were used as positive control against HSV-1 and BoHV-1 replication.

For the time-of-addition assay, the concentrations of the compounds, as before, were tested as previously described [23]. Briefly Protocol 1: cell cultures were placed in contact with the compounds either for 1 h or 2 h, followed by compounds removal prior to infection. Protocol 2: cell cultures treatment with the compounds simultaneously to infection (0 h) and left throughout and Protocol 3: cell cultures treatment with the compounds 1 h or 2 h post-infection and left throughout. For the inhibitory effect of the compounds on virus adsorption, cell cultures were maintained at 4 °C for 1 h followed by the infection in the presence of the compounds at the same concentrations as before, for 80 min at 4 °C. The cell cultures were washed with cold PBS to remove nonadsorbed virus followed by PRA [24]. For the virucidal assay, virus suspension was incubated with varying concentrations of the compounds (v/v), for 1 h at 37 °C in water bath. Treated virus suspension was diluted to the tenth and the residual infectivity determined by plaque assay [25].

2.5. Immunofluorescence assay (IFA)

IFA was performed with HEp-2 cells grown in 24-well plates with glass coverslips, infected with 100 μl of HSV-1 or BoHV-1 (moi approximately to 1) and treated with the compounds at 200 μg/ml, 400 μg/ml, 600 μg/ml and 800 μg/ml, at the time of infection (0 h). Briefly, 24 h post-infection, the cells were washed with 0.05% Tween-20 PBS, fixed with cold acetone (−20 °C) and blocked with powdered skimmed milk PBS. The cells were incubated either with mouse anti-HSV-1 (Santa Cruz Biotechnology, USA) or bovine anti-BoHV-1 antibodies (DMVP/UFSM, Brazil) and further washed with 0.05% Tween-20 PBS, then incubated with goat anti-mouse IgG (Sigma Chem. Co., USA) and rabbit anti-bovine IgG FITC conjugate (Sigma Chem. Co., USA), respectively. The cells were examined in a Zeiss fluorescence microscope (Imager A1 with Axio Cam MRc5) and 100 cells/coverslip were counted and the percentage of fluorescent cells calculated in comparison to control infected and nontreated cells [33].

2.6. Statistics

Anova's test followed by Turkey's test (BioEstat 5.0 for Windows XP, 2007) was used throughout. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Compounds and characterization

A polysaccharide-complex and its fractions (F1–F3) were extracted and characterized by ^{13}C and ^{1}H NMR [17], presenting $\beta(1 \rightarrow 6)$ and $\alpha(1 \rightarrow 4)$ glucan–protein polysaccharide constituted mainly by β-glucans. The $\alpha(1 \rightarrow 4)$ configuration showing a significant reduction on the fractionation proceeded (F1–F3) [17].

3.1.1. ^{13}C NMR spectroscopy from PLS, CPLS and SPLS

The ^{13}C NMR results of the isolated polysaccharides (Table 1) were in good agreement with those presented previously [17]. The structural characteristics of the polysaccharide and its fractions (F1–F3) presented $\beta(1 \rightarrow 6)$ and $\alpha(1 \rightarrow 4)$ glucan with the predominance of $\beta(1 \rightarrow 6)$ reported in literature as having the most effective pharmacological response relative to antitumoral and immunosuppressive activities. The $\alpha(1 \rightarrow 4)$ configuration showing a significant reduction on the fractionation proceeded (F1–F3). The ^{13}C NMR spectrum of CPLS showed a peak of (C=O) in 175 ppm, demonstrating the carboxymethylation (Table 1). The group sulfate generally

Table 1
^{13}C NMR assignments for PLS, CPLS and SPLS systems from *Agaricus brasiliensis*.

System	Chemical shift (ppm)						
	C1	C2	C3	C4	C5	C6	C=O
PLS/β (1 → 6)	103	73,1	75.6	69.5	74.9	68.3	–
PLS/α (1 → 4)	99.8	71.5	75.3	78.7	71.2	60.1	175.0
CPLS/β (1 → 6)	103.5	75.5	82.9	73.9	76.7	70.7	–
SPLS	103.5	73.8	76.4	70.7[a]	75.6	61.7[b]	

[a] β configuration.
[b] α configuration.

occurs at the carbon 4 (C4) of configuration β(1 → 6) and at the carbon 6 (C6) of configuration α(1 → 4). The introduction of sulfate groups in the SPLS was confirmed by displacement of C4 (glucan in β configuration), from 69.5 to 70.7 ppm and of C6 (glucan in α configuration) from 60.1 to 61.7 ppm (Table 1). The introduction of the sulfate group generally occurs at the carbon 4 (C4) of configuration β(1 → 6) and preferably at the carbon 6 (C6) of configuration α(1 → 4).

3.1.2. FT-IR spectroscopy

The FT-IR spectra of PLS, CPLS and SPLS (Table 2) showed characteristic bands of the presence of carbohydrates [26] relative to OH (3000–3500 cm^{-1}), CO (1034 cm^{-1}) groups, to the C—O—C (1150 cm^{-1}) and of proteins (1653, 1562 and 1242 cm^{-1}) [27,28]. The band around 1739 cm^{-1} of the CPLS spectrum is characteristic of stretch C=O due to carboxymethylation. The sulfatation was confirmed by the presence of the band in 1262 cm^{-1} that refers to the group (S=O) observed in the SPLS spectrum [29,30].

3.2. Cytotoxicity assay

The cytotoxic concentration of PLS, SPLS, CPLS and fractions F1–F3 for HEp-2 cells was above the highest concentration of the compounds used in this assay (2500 μg/ml) (Table 3).

3.3. Antiviral activity

3.3.1. Anti-HSV-1

Out of the tested compounds, only PLS, SPLS and F3 presented antiviral activity against HSV-1 (Fig. 1). The time-of-addition assay demonstrated that these extracts showed highest %VI when added at the time of infection. The PLS presented the most effective result, moreover, this activity was detected at the times 1 and 2 h after the infection. The PLS %VI for the times 0 h, 1 h and 2 h were 77.5%, 83.6% and 2%, respectively, at the concentration of 800 μg/ml. At the same conditions, F3 showed %VI of 35.9%, 32.3% and 15%, respectively, and the SPLS presented inhibition only at the time 0 h, about 82%. The PLS and F3 did not inhibit HSV-1 when added at 2 h and 1 h before infection and did not demonstrate any direct effect on virus particle or inhibited virus adsorption either, at the indicated concentrations. The CPLS derivative was tested at higher concentrations and demonstrated low activity (not shown). The compounds F1 and F2 demonstrated highest %VI when added 1 h after infection,

Table 2
Assignments of the most important IR bands for PLS, CPLS and SPLS systems from *Agaricus brasiliensis*.

System			Assignment
PLS	CPLS	SPLS	
3396	3436	3418	ν (O—H) and (N—H)
2930	2923	2927	ν (C—H)
–	1739	–	ν (C=O)
1653	1638	1647	ν_{as} (COO^-), amide I (C=O)/protein
1562	1547	1547	(δ NH, ν CN, ν HCS) amide II/protein
1411	1420	1422	ν_s (COO^-)
–	–	1262	ν (S=O) ester sulfate
1242	1250	1240	(δ NH, ν CN) amide III/protein
1150	1149	1154	ν (C—O—C) glycosidic ether linkage
1079	–[a]	1081	ν (C1—H) anomeric carbon
1034	1039	1039	ν (C—O) primary alcohol
858	–[a]	857	δ (C1—H) β-glycosidic
–	–	820	δ (C—O—S) group associated to the C—O—SO_3

[a] Not clearly visible.

Table 3
Antiviral activity of polysaccharide (PLS), sulfated derivative (SPLS) and fraction F3 of *Agaricus brasiliensis* against herpes simplex virus-1 (HSV-1) and bovine herpesvirus-1 (BoHV-1) by the plaque reduction assay, in HEp-2 cells. Cell cultures were treated with the compounds (200–800 μg/ml) at the time of the infection (time 0 h).

	CC_{50}[a]	HSV-1		BoHV-1	
		IC_{50}[b]	SI[c]	IC_{50}	SI
PLS	>2500	454	>5.5	634	>3.9
SPLS	>2500	346	>7.2	830	>3.0
F3	>2500	0	–	674	>3.7

[a] Fifty percent cytotoxic concentration (CC_{50}) for HEp-2 cells (μg/ml).
[b] Fifty percent inhibitory concentration (IC_{50}) (μg/ml).
[c] Selectivity index (CC_{50}/IC_{50}).

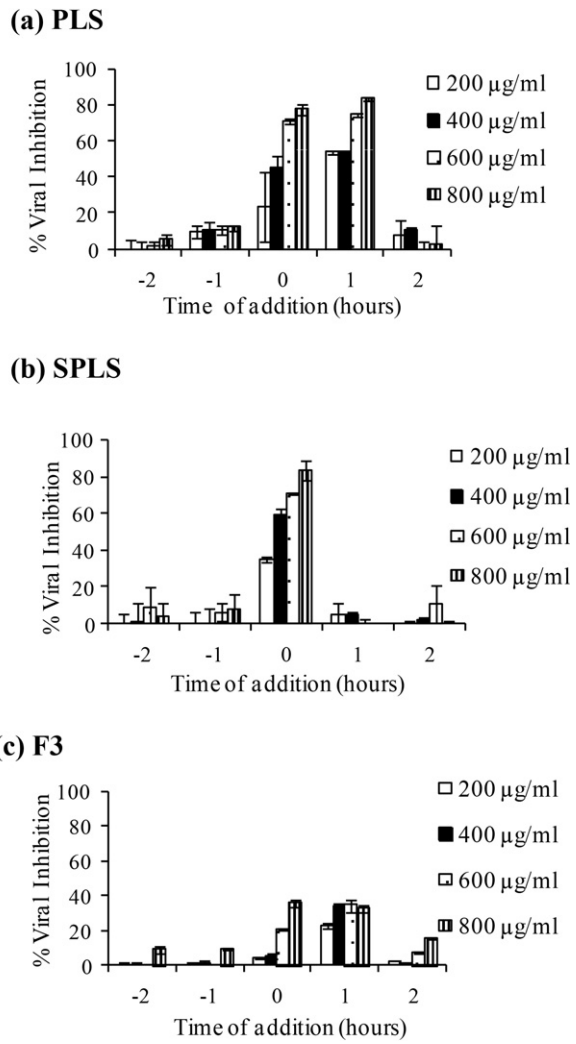

Fig. 1. The time-of-addition assay of polysaccharide (PLS) (a), the sulfated derivative (SPLS) (b) and fraction F3 (c) of *Agaricus brasiliensis* in the replication of herpes simplex virus (HSV-1) by plaque reduction assay, in HEp-2 cells. The substances were added at varying concentrations (200–800 μg/ml) before (−2 h and −1 h), during (0 h) and after (1 h and 2 h) infection. The data are expressed as mean ± S.D ($n = 3$).

Table 4
The effect of polysaccharide (PLS), sulfated derivative (SPLS) and fraction F3 of *Agaricus brasiliensis* against herpes simplex virus-1 (HSV-1) and bovine herpesvirus-1 (BoHV-1) replication by immunofluorescence assay, in HEp-2 cells. The substances were added at the time 0 h of infection.

	HSV-1			BoHV-1		
	PLS	SPLS	F3	PLS	SPLS	F3
200[a]	14.8[b]	7.4	1.6	30.3	5.1	9.9
400	18.5	34.4	10.6	37.1	17.4	17
600	33.3	60.0	36.9	48.1	24.6	33.7
800	53.7	85.2	44.5	59.1	44.9	50.5

[a] μg/ml.
[b] Percent of fluorescent cells reduction in comparison to infected and nontreated cells.

with 20% of viral inhibition, and, at the same conditions, F3 inhibited 32.3% of HSV-1 replication. The IC_{50} for PLS was 454 μg/ml with a SI > 5.5, while SPLS IC_{50} was 346 μg/ml with SI > 7.2 (Table 3).

The PLS, SPLS and F3 effect in the synthesis of HSV-1 protein demonstrated reduction of fluorescent cells number up to 53.7%, 85.2% and 44.5%, respectively, at the highest tested concentration, when added at the time 0 h of the infection (Table 4).

3.3.2. Anti-BoHV-1

The effect of PLS, SPLS and F3 for BoHV-1 was similar to that found for HSV-1. Significant inhibition was detected when PLS, SPLS and F3 were added at the time 0 h, 1 h and 2 h after the infection. Fig. 2 shows the results of time-of-addition assay of PLS, SPLS and F3 at the indicated concentrations. When the compounds were added at the highest concentration at 0 h, and 1 h and 2 h post-infection, the inhibition rates were 29.3%, 69.2% and 23.4%, respectively, for PLS. For SPLS, 53.6%, 28.6% and 41.5%, and for F3 28.0%, 77.4% and 37.3%. The CPLS, F1 and F2 showed no significant inhibition (not shown). None of the compounds showed direct effect on BoHV-1 or inhibited viral adsorption. For PLS the IC_{50} was 634 μg/ml and SI > 3.9. For SPLS the IC_{50} was 830 μg/ml and SI > 3, while, for F3 the IC_{50} was 674 μg/ml and SI > 3.7 (Table 3).

The results of IFA showed that, when the compounds were added at the moment of infection (time 0 h), there were reductions of fluorescent cells up to 59.1%, 44.9% and 50.5%, for PLS, SPLS and F3 respectively, at the concentration of 800 μg/ml (Table 4).

3.4. Positive control

Acyclovir at 2500 μg/ml inhibited the HSV-1 by 67%, while BoHV-1 was completely inhibited by interferon at 1000 U/ml.

4. Discussion

The antiviral activity of compounds (PLS, SPLS, CPLS and fractions) obtained from *A. brasiliensis* mycelium and fruiting body was studied in the replication of human and bovine herpesvirus. We demonstrated that the PLS, SPLS and F3 inhibited the replication of both virus with %VI varying from 30 to 80% at the highest used concentration (800 μg/ml). The inhibition occurred when cells were treated with the compounds simultaneously to the infection and when treatment was performed after infection. However, no effect was shown for virucidal, attachment inhibition or treatment prior to infection protocols, at the concentrations used. These results are in agreement with similar experiments carried out with extracts of *A. brasiliensis* in herpesvirus and poliovirus infection, in that inhibitory effect was observed when cells were treated with extracts at the time of infection or when treatment was followed the infection [13,14,16]. Interestingly, we demonstrated that original nonsulfated PLS showed better inhibition than the sulfated derivative and F3 fraction due to the extended effect observed at

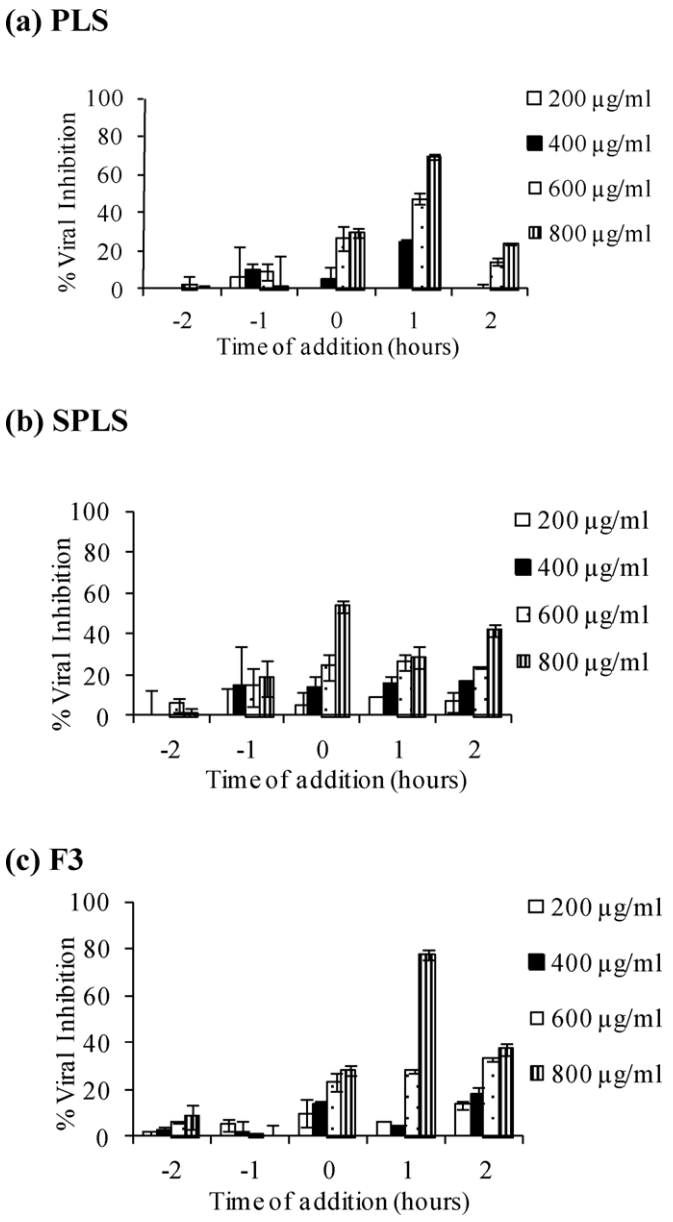

Fig. 2. The time-of-addition assay of polysaccharide (PLS) (a), the sulfated derivative (SPLS) (b) and fraction F3 (c) of *Agaricus brasiliensis* in the replication of bovine herpesvirus (BoHV-1) by plaque reduction assay, in HEp-2 cells. The substances were added at varying concentrations (200–800 μg/ml) before (−2 h and −1 h), during (0 h) and after (1 h and 2 h) infection. The data are expressed as mean ± S.D ($n = 3$).

the time 1 h after infection. The %VI was 83.6% to HSV-1 and 69.2% to BoHV-1 at the concentration of 800 μg/ml. It was demonstrated that aqueous extract of *A. brasiliensis* inhibited HSV-1 by 47.3% and BoHV-1 by 20.9% at the concentration of 100 μg/ml. Additionally, it was also found that the inhibition of poliovirus, a single-stranded RNA virus (PV-1) was inhibited by 67% with similar PLS at the concentration of 200 μg/ml [13,14]. However, it was also shown that *A. brasiliensis* mycelium original nonsulfated PLS did not inhibit HSV-1 at the concentration of 20 μg/ml, but, the sulfated derivative did [16]. These results demonstrated that the antiviral effect of natural compounds from *A. brasiliensis*, without chemical modification, requires concentrations of above 100 μg/ml. Of the PLS derivatives presently used, only the sulfated form demonstrated stronger inhibition for both HSV-1 and BoHV-1 replication, when the cells were treated simultaneously to infection. Similar findings were described, for carrageenans [31], fucoidans [32], PLS from

Azadirachta indica [33], sulfated β-glucans [34] and sulfated derivatives. Overall, we suggest that antiherpetic activity of sulfated polysaccharides could be through different mechanisms, including virucidal and adsorption inhibition effects. In our study, only SPLS presented a small %VI (34%) for the inhibition adsorption protocol and 6% for virucidal assay even at the highest concentration used (800 μg/ml). The insignificant inhibition of viral adsorption by the original nonsulfated polysaccharide (PLS) confirmed that the presence of sulfate groups is required for such activities [31,35,36]. Other studies similarly reported no virucidal effects of sulfated polysaccharides [37,38,32,39,40], however, when this effect was described it was detected only at concentrations much higher than their IC_{50} [31,41,24]. Nevertheless, SPLS activity was improved only when used at the moment of the infection, therefore, this chemical modification, as well as, carboxymethylation, could not be necessary.

The non-significant virucidal, adsorption inhibition and pretreatment activities found in our study are in agreement with previous studies [11,12,14–16]. In contrast, it was found that aqueous extract of *A. brasiliensis* presented virucidal activity to herpesvirus [13], however, this effect could be synergistically promoted by substances present in crude preparation, but, absent in purified PLS.

The present results suggest that the compounds (PLS, F3 and SPLS) act at early steps of the HSV-1 and BoHV-1 replication. The inhibition of viral protein synthesis could be one of the steps, as demonstrated by dose-dependent inhibition of HSV-1 by SPLS followed by other compounds for both viruses when treatment was simultaneously to infection by IFA.

The *A. brasiliensis* PLS is a glucan–protein complex, presenting α and β glycosidic linkage. β-Glucans being predominant to which the antiviral activity may be attributed. Some PLS rich in β-glucans, like PSK from kawaratake, lentinan from shiitake and shizophyllan from suehirotake are effective against several viral agents [42]. The β-glucans can decrease viral nucleic acid levels in cells infected and stimulate the immune system by binding to Toll-like receptors and dectins and causes the induction of various cytokines, among them the interferon [43]. The polysaccharides can induce interferon and, consequently, antiviral state by stimulation of intracellular signaling pathways [44]. This mechanism has already been proposed for *A. brasiliensis* for patients infected with hepatitis B [15] and for hepatitis C virus [12], in which it was detected the induction of the interferon receptor suggesting that the extracts could be useful for patients who do not respond to interferon treatment.

In addition, of the fractions (F1–F3) analyzed, only F3 showed small inhibition both HSV-1 and BoHV-1 replication. When the PLS is fractioned, the β-glucans content increased [17], therewith F3 has a higher % of β-glucans that may be related to their greater activity compared to F1 and F2.

In summary, our findings indicated that of the tested compounds, PLS and SPLS were the most efficient to inhibit herpesvirus replication, interfering at the initial steps, such as, penetration, uncoating and protein synthesis inhibition. Our results alongside to those previously obtained suggest that *A. brasiliensis* extracts can be promising for the future antiviral drug design.

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