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In vitro anti-HSV-1 activity of a chemically sulfated galactomannan from *Leucaena leucocephala* seeds

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ABSTRACT

A galactomannan extracted from the seeds of *Leucaena leucocephala* was sulfated chemically, yielding a polymer (LLS-2) with 15.2% sulfate by weight (degree of sulfation 0.60), and its effect on the replication of Herpes simplex virus 1 (HSV-1) in Vero cells was investigated. When added during infection and early replication, LLS-2 showed 93.7% inhibition of HSV-1 replication at a concentration of 2.5 µg/mL, according to the reduction in the number of viral plaques, and a selectivity index higher than 1,000, suggesting that it inhibits HSV-1 binding to the host cell.
Keywords: Leucaena leucocephala. Galactomannan. Sulfation. HSV-1; Antiviral activity.

INTRODUCTION

Leucaena leucocephala is a leguminous tree native to Central America, known in Brazil as leucena (white lead tree in English), that has diverse uses such as production of nutritious forage (as it fixes N efficiently), green manure, charcoal and firewood, reforestation (due to its ability to grow rapidly even in degraded soils) and, in some countries, its leaves and seeds ("jumbie beans") are an important food (Sethi & Kulkarni, 1995). However, the leaves contain a cytotoxic non-protein amino acid, mimosine, which is usually toxic to all mammals except some ruminants, which present bacteria that can break it down to a harmless substance.

The galactomannans from the endosperm of *Leucaena* spp. are storage polysaccharides whose extraction yield is nearly 15%. The chemical structure consists of a main chain of (1→4)-linked β-D-mannopyranosyl units with dispersed side-branches of single α-D-galactopyranosyl units joined by 1→6 linkages to mannose units (mannose: galactose ratio of 1.3:1) (Buckeridge et al., 2000) (Fig. 1).

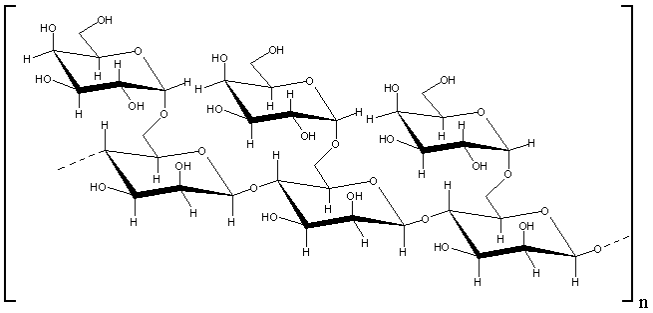

Fig.1 - Partial chemical structure of a highly substituted galactomannan with (1→4)-linked β-D-mannan backbone substituted at *O*-6 by single α-D-galactosyl units.

Herpes simplex virus (HSV) is included in the family *Herpesviridae* and subfamily *Alphaherpesvirinae*, consisting of a linear double-stranded DNA encoding at least 84 different polypeptides encased within an icosahedral protein capsid enveloped in a membrane, the whole virion measuring about 200 nm (Whitley & Roizman, 2001).

An important biological property of HSV is that, after an initial replication phase in epithelial cells, it can remain latent in sensory neurons innervating the site of primary exposure, persisting there during the lifetime of the host and causing recurrent disease upon reactivation, following various stimuli such as stress, fever, exposure to ultraviolet light, tissue damage and immune suppression. Another property that also influences the human disease is neurovirulence: the replication of HSV in nervous system tissue can result in severe neurological disease (Whitley & Roizman, 2001; Fatahzadeh & Schwartz, 2007).

Therefore, HSV infections, which are distributed worldwide, have clinical presentations ranging from asymptomatic to mucocutaneous manifestations such as primary oropharyngeal and recurrent orolabial herpes, primary and recurrent genital herpes, ocular

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herpes (common cause of corneal blindness) and severe disseminated disease with multiorgan infection in neonatal and immunocompromised hosts, either transplant, cancer or AIDS patients (Parker & Montrowl, 1994; Samraj & Patel, 2006).

The current prophylaxis and treatment of HSV infections include medication with acyclovir, gancyclovir and pencyclovir (acyclic nucleoside analogues), valacyclovir (prodrug of acyclovir), famcyclovir (prodrug of pencyclovir), cidofovir (acyclic nucleotide analogue), docosanol (22-carbon alcohol) and foscarnet (analogue of pyrophosphate). Acyclovir (ACV) is the compound of choice for anti-HSV therapy, which, after its sequential phosphorylation, acts by competitive inhibition of viral DNA polymerase, thus terminating the synthesis of the DNA chain. It targets infected cells, as it requires HSV-encoded thymidine kinase (TK) for the initial phosphorylation. None of these drugs has virucidal activity that eradicates HSV, but they can attenuate the clinical course, viral shedding and the severity of the disease (Brady & Bernstein, 2004).

Drug resistance to these antiviral agents after prolonged has been reported, being particularly important in severely immunocompromised patients, among whom the prevalence of resistant HSV is about 4%. This results mainly from mutations within the *TK* gene, causing a drop in TK activity in HSV-infected cells (95% of ACV-resistant isolates) and an alteration of TK substrate specificity (Morfin & Thouvenot, 2003).

Owing to the increased isolation of HSV resistant to current antiviral treatments, considerable attention has been focused on the study of sulfated polysaccharides that have been reported to show potent inhibition of HSV replication at noncytotoxic concentrations *in vitro*, such as the recently described agaran-carrageenan hybrids (Matsuhiro et al., 2005), spirulan-like polysaccharides (Rechter et al., 2006), galactans (Chattopadhyay et al., 2007) and xylomannans (Mandal et al., 2008).

The synthetically sulfated galactomannan obtained from *Leucaena leucocephala* seeds, studied here, was reported to have i*n vitro* and *in vivo* antiviral activities against the yellow fever virus (YFV) in the C6/36 cell line and in mice, respectively (Ono et al., 2003). The *in vitro* activity of this macromolecule seems to be related to the presence of negatively charged sulfate groups, which could inhibit virus adsorption to the host cells. The *in vivo* activity may also be related to an immunostimulatory action. Other chemical modifications of this galactomannan, such as a C-glycosidic 2-propanol derivative, and even the sulfated derivative itself, can enhance macrophage functions such as proliferation and phagocytosis (Gamal-Eldeen et al., 2007).

The aim of the present study was to assay the *in vitro* antiviral properties of a chemically sulfated galactomannan extracted from the seeds of *L. leucocephala* and to perform the basic chemical analysis of this polysaccharide.

MATERIALS AND METHODS

Sulfation and purification of the derivative

The galactomannan derivatized in this study was taken from a sample extracted previously (Ono et al., 2003) from the endosperm of *Leucaena leucocephala* seeds collected in the Federal University of Paraná, Curitiba, State of Paraná, Brazil.

Following a modified protocol of O'Neill (1955), 3.0g of the leucena galactomannan (LL) was allowed to swell in pyridine (600 mL), stirred at 25°C, until a finely-dispersed suspension was obtained. This was cooled to 4°C and 40 mL of chlorosulfonic acid was slowly added, followed by 100 mL of formamide, after which the mixture was stirred for 24h at 4°C. The resulting solution was neutralized with saturated aq. $NaHCO_3$ and dialyzed against water for 120h, centrifuged (8000x*g*, 25min) and filtered through acetate membranes with a 0.22µm pore diameter. Sodium chloride was added to the solution, to a final concentration of 0.1M, and the sulfated polysaccharide was precipitated with 2 volumes of ethanol. Two consecutive sulfations were carried out and the end-product was denominated LLS-2.

General analyses

Carbohydrate contents were determined as described by Dubois et al. (1956). Total protein was measured by the Hartree method (1972). The degree of sulfation was determined by a turbidimetric method using the barium chloride-gelatin reagent (Dodgson & Price, 1962).

The monosaccharide composition of the sulfated polysaccharide was determined by complete hydrolysis with 2M *trifluoroacetic acid* (TF*A)* for 6h at 100°C, the resulting mannose and galactose being converted into their alditol acetates (Wolfrom & Thompson, 1963). The mixture was examined by gas–liquid chromatography, with the HP 5890 chromatograph, incorporating a DB-225 capillary column with N_2 as carrier gas. Oven: 220°C, injector: 250°C, detector: FID, 250°C.

Infrared spectra (FTIR) of sulfated saccharides were recorded from KBr pellets, with a BOMEM MB-100 spectrometer.

Virus and cells

Herpes simplex virus 1 (HSV-1) was provided by the Marcos Enrietti Diagnostic Center – SEAB, Curitiba, State of Paraná, Brazil.

Vero cells, from *Cercopithecus aethiops* kidney, were cultured in Eagle´s Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 IU/mL), enrofloxacin (10 µg/mL) and amphotericin B (1.25 µg/mL). The cells were incubated in an atmosphere of 5% CO_2 at 37°C.

Virus stock was prepared in Vero cell culture and stored at -70°C until use. The HSV-1 infectious titer was determined by plaque count assay after 72h of infection and it was expressed as the number of plaque-forming units per unit volume (PFU/mL).

Cytotoxicity assay

Vero cells were cultured in 96-well microplates and the monolayers incubated with 50 µL MEM containing 2-fold serial dilutions of the polysaccharides at

concentrations ranging from 9.8 to 2,500 µg/mL for 1.5h, after which the test compounds were withdrawn, 200 µL MEM was added and the plates incubated for 72h.

The *in vitro* toxicity of the native (LL) and sulfated (LLS-2) polysaccharides was assayed as described by Denizot & Lang (1986), who counted the viable cells revealed by adding 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), which is reduced to a purple formazan by dehydrogenases in actively respiring mitochondria.

Fifty percent cytotoxic concentration (CC_{50}) was defined as the polysaccharide concentration that reduced by 50% the number of viable cells, relative to a control without polysaccharide, and was calculated by regression analysis of the dose-response curves.

Evaluation of the antiviral activity

The sulfated polysaccharide (LLS-2) was tested for antiviral activity against HSV-1 by the plaque reduction assay, at concentrations ranging from 2.5 to 20 µg/mL, and the native galactomannan (LL) was tested at 20 µg/mL, as a non-sulfated polysaccharide control. Vero cell monolayers grown in 24-well microplates for 24h at 37°C in a 5% CO_2 atmosphere were infected with HSV-1 at ~100 PFU/well (~100 PFU/0.2 µL) under two different treatment conditions, to evaluate the effect of the time of addition of the polysaccharides: LLS-2 at various concentrations (200 mL) was (a) present only during the virus early replication steps (added simultaneously with the virus inoculum and incubated for 1.5h at 37°C, 5% CO_2); after virus adsorption, the polysaccharide was replaced by maintenance MEM, containing 0.9% methylcellulose and 5% FBS; (b) added (in maintenance MEM containing 0.9% methylcellulose and 5% FBS) only after infection, remaining until the end of the 72h incubation.

After incubation at 37°C, in 5% CO_2 for 72h, in both treatments, the number of plaques was counted on cells fixed with 10% formalin and stained with 1% crystal violet. The 50% effective concentration (EC_{50}) was defined as the concentration of LLS-2 required to inhibit the growth of 50% of the viruses, relative to a polysaccharide-free infected control.

Statistical analysis

All the results of biological experiments were expressed as mean ± standard deviation, and analyzed by Student´s *t*-test, with P=0.05. Variables exceeding the upper quantitation limit were considered statistically significant.

RESULTS

Sulfation of the native galactomannan (LL) gave a product (LLS-2) with a yield of 35%, containing 15.2 wt% of sulfate, corresponding to a degree of sulfation (DS) of 0.60 sulfate groups per residue. The presence of sulfate in LLS-2 was confirmed in the FTIR assay by the presence of a stretching vibration band for S=O at 1250 cm^{-1} (Turvey, 1965), which was absent when the native polysaccharide was examined. The increase of the mannose:galactose ratio from 1.4:1 in LL to 1.8:1 in LLS-2, shown in Table 1, indicates partial loss of the galactopyranosyl side chains and degradation of the starting material by the sulfation process, while the decrease in the carbohydrate content also corroborates the occurrence of some *O*-substitution by sulfate groups.

Table 1 - Monosaccharide ratio, total carbohydrate, protein and sulfate contents of the native (LL) and sulfated (LLS-2) galactomannans of *L. leucocephala*

Polysaccharide	Man:Gal ratio[a]	Carbohydrate[b] (% anhydrous sugar)	Protein[c] (%)	Sulfate content[d] (%)	Degree of sulfation
LL	1.4	77.5	3.7	ND	ND
LLS-2	1.8	42.8	4.1	15.2	0.60

[a]Dubois et al. (1956), [b]Wolfrom & Thompson (1963), [c]Hartree (1972), [d]Dodgson and Price (1962). ND: not determined.

The MTT assay was carried out to assess the cytotoxicity of LL and LLS-2 in Vero cells, which was defined as the percentage of surviving cells, in comparison with an untreated control, after 72h of incubation. At a concentration of 2,500 µg/mL, LL decreased Vero cell viability by 19.5% and no significant toxicity was observed up to 1,250 µg/mL (Fig. 2A). LLS-2 started to show a significant toxic effect on Vero cells above 312.5 µg/mL, lowering the viability of the cells by 16.9%, as shown in Fig. 2B. Viability fell to 58.2% at 2,500 µg/mL, so that CC_{50} exceeded this concentration. When LLS-2 was added after the HSV-1 adsorption step (data not shown), remaining in contact with Vero cells for 72h, it lowered their viability by 21.7% at 19.5 µg/mL and also exhibited a CC_{50}>2,500 µg/mL.

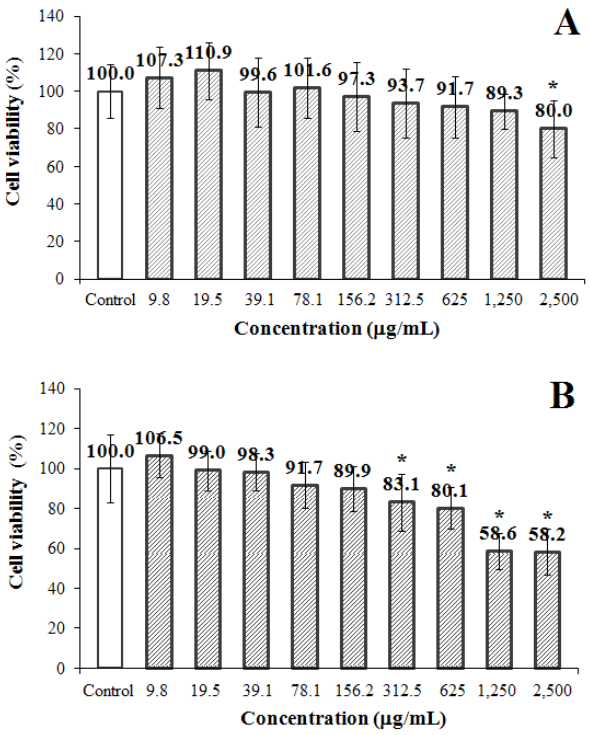

Fig. 2 - Evaluation of cytotoxicity of the native (LL) (A) and sulfated (LLS-2) (B) galactomannans from *Leucaena leucocephala* seeds in Vero cells by the MTT assay. Polysaccharides were initially incubated for 1.5h, removed and the cell cultures incubated for 72h at 37°C and 5% CO_2. Control: without polysaccharide treatment. *Bars* represent means, with *vertical lines* indicating standard deviations, n = 8, *P = 0.05.

The inhibitory effect of LL and LLS-2 on HSV-1 replication was assessed by performing a virus plaque reduction assay under two different treatment conditions, the polysaccharides being added either simultaneously with the virus suspension, during the virus early replication steps, or only after these steps. When LLS-2 was added to the Vero cells in the HSV-1 adsorption phase, the number of viral plaques was significantly reduced, in a dose-dependent way, by 98.3% to 93.7%, at the tested concentrations of 20 µg/mL to 2.5 µg/mL, respectively (Fig. 3A). Thus, the EC_{50} value obtained was lower than 2.5 µg/mL. Addition of LLS-2 to cells already exposed for 1.5 h to HSV-1 did not efficiently block the infection (Fig. 3B) as it did during the early replication steps. At the highest tested concentration of LLS-2 (20 µg/mL), the number of HSV-1 plaques was reduced by only 16%. Incubation with LL did not reduce the number of viral plaques. The selectivity index (SI) of LLS-2, defined as the ratio CC_{50}/EC_{50} against HSV-1, was estimated to be higher than 1,000.

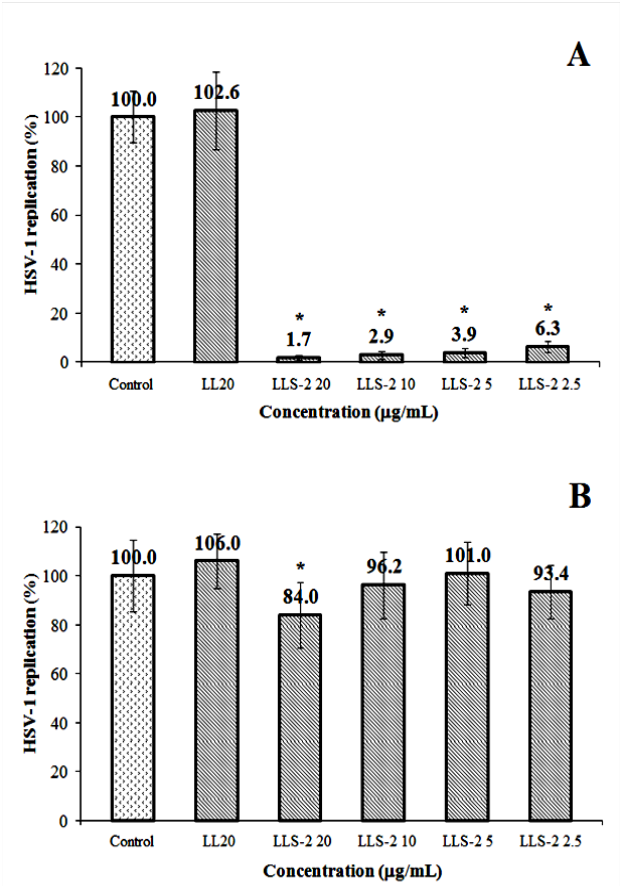

Fig.3 - Evaluation of antiviral activity of the sulfated galactomannan (LLS-2) from *Leucaena leucephala* seeds against HSV-1 in Vero cells by the plaque reduction method, (A) during and (B) after early stages of viral replication, after 72h of incubation at 37°C and 5% CO_2. The native galactomannan (LL) was used as non-sulfated control. Infected cells (100 PFU) without polysaccharide treatment were used as control. *Bars* represent means, with *vertical lines* indicating standard deviations, $n = 12$, $^*P = 0.05$.

DISCUSSION

The sulfated derivative of the galactomannan from *Leucaena leucocephala* seeds exhibited a DS of 0.6, lower than that obtained by Mestechkina et al. (2006), who synthesized sulfated galactomannans of various Man:Gal ratios, from 1.07 to 5.30, with DS values ranging from 1.42 to 1.85. Those authors used dimethylformamide as the reaction solvent and SO_3–pyridine complex as the sulfating agent, and employed various reaction temperatures between 0 and 80°C.

Sulfated polysaccharides have shown direct toxicity to cultured cells from tumoral cell lines. Thus, when hepatocellular carcinoma and human gastric carcinoma cells were exposed, respectively, to polyanions extracted from the Chinese herb *Gekko swinhonis* and the fungus *Grifola frondosa* (chemically sulfated fungal glucan), they promoted cell death by arresting the cell cycle at the G2/M phase (Wu et al., 2006) or by inducing apoptosis (Shi et al., 2007).

A sulfated polysaccharide obtained previously from *Leucaena leucocephala*, named LLS (sulfate content: 14.3%, DS: 0.50) (Ono et al., 2003), exhibited a lower value of CC_{50} (2,000 mg/mL) in the C6/36 clone of *Aedes albopictus* cells than that found in this study in Vero cells, showing that the degree of cytototoxicity depends on the cell line tested. Also, an *in vivo* toxicity study performed by intraperitoneal injection of LLS into young adult mice demonstrated that, below a dose of 244 mg/kg (totaling 7 doses), it was not lethal, with LD_{50}>5 mg/mL.

LLS-2 strongly reduced HSV-1 infectivity at nontoxic concentrations, in the early stages of viral infection (attachment and penetration), but showed a less potent antiviral effect thereafter. However, this inhibitory effect after the adsorption step is probably not related to the presence of sulfated polysaccharide, since during the 72h continuous exposure to LLS-2 (data not shown), in contrast to the 1.5h exposure, it reduced the number of viable cells, lowering the number of host cells available for HSV-1 replication. The non-sulfated polysaccharide LL did not show any antiviral effect, proving that the antiherpetic activity of LLS-2 depends on the presence of the negatively-charged groups.

The finding that this antiherpetic efffect is predominantly due to the inhibition of viral attachment to the host cell is partly supported by studies on sulfated galactans from seaweeds that suggest a possible interaction between viruses and sulfated polysaccharides that could result in the inhibition of HSV-1 adsorption to the cells (Matsuhiro et al., 2005; Chattopadhyay et al., 2007; Mandal et al., 2008;). Sulfated polysaccharides probably mimic heparan sulfate, a glycosaminoglycan chain found on cell-surface proteoglycans, needed for the initial binding of clustered basic aminoacids of the HSV envelope glycoproteins gC or gB to the host cell (Spillmann, 2001). The results obtained for antiviral activity after the attachment step also accord partially with González & Carrasco (1987), who, using radiolabeled HSV-1 virions, proposed that Iota-carrageenans inhibit viral replication upon entry of the polysaccharide–virus complex into the cells, which suggested that the inhibition step occurs after viral internalization, but before the onset of late viral protein synthesis.

The selectivity index (SI) of LLS-2 was similar to that of acyclovir (whose SI is 1,522), the antiviral drug used most frequently in the treatment of HSV-1 infections (Oh et

al., 2000). As the sulfated derivative of the galactomannan from *Leucaena leucocephala* seeds has also been reported to have immunomodulatory activity (Gamal-Eldeen et al., 2007; Ono et al., 2003), LLS-2 appears to be a good candidate for further investigation of its antiherpetic activity in animal models.

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RESUMO

Atividade anti-HSV-1 in vitro de galactomanana quimicamente sulfatada de sementes de Leucaena leucocephala

Obteve-se uma galactomanana quimicamente sulfatada (LLS-2) a partir de polissacarídeo extraído de sementes de *Leucaena leucocephala*, a qual apresentou 15.2% de sulfato e grau de derivatização de 0,60, e, seu efeito antiviral sobre a replicação do vírus Herpes simplex tipo 1 (HSV-1) em células Vero foi avaliado pela metodologia de redução do número de unidades formadoras de placas. LLS-2 apresentou 93.7% de inibição da replicação viral à concentração de 2,5 µg/ml, quando adicionado durante as etapas iniciais de replicação, com um índice de seletividade maior que 1.000, sugerindo que LLS-2 inibe a ligação de HSV-1 às células hospedeiras.
Palavras-chave: Leucaena leucocephala. Galactomanana. Sulfatação. HSV-1. Atividade antiviral.

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