Francielle Tramontini Gomes de Sousa Cardozo

CARACTERIZAÇÃO QUÍMICA E AVALIAÇÃO DAS ATIVIDADES ANTI-HERPÉTICA E CITOTÓXICA DE POLISSACARÍDEOS DO FUNGO *Agaricus brasiliensis* Wasser

Tese submetida ao Programa de Pós-Graduação em Biotecnologia e Biociências da Universidade Federal de Santa Catarina como requisito parcial para obtenção do título de Doutor e Biotecnologia e Biociências.

Orientadora: Profa. Dra. Cláudia Maria Oliveira Simões

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POR

FRANCIELLE TRAMONTINI GOMES DE SOUSA CARDOZO

Tese julgada e aprovada em sua forma final, pelo Orientador e membros da Comissão Examinadora.

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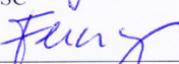
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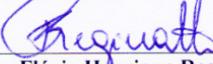
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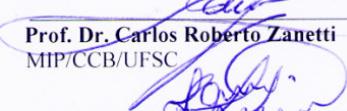
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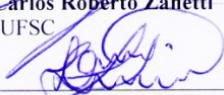
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Florianópolis, Agosto de 2012.

AGRADECIMENTOS

I would especially like to thank Dr Curtis Brandt for being supportive and encouraging and for his knowledgeable lessons. I am also thankful to all my colleagues from the Brandt' Lab, especially to Inna Larsen for her valuable help and patience.

A todos que contribuíram para o meu crescimento pessoal e profissional, cujos nomes não foram mencionados, meu sincero agradecimento.

Francielle Tramontini Gomes de Sousa Cardozo

Tentei achar epígrafe mais adequada, mas esta continua sendo a minha predileta:

"Ainda que eu falasse as línguas dos homens e dos anjos, e não tivesse amor, seria como o metal que soa ou como o sino que tine. Mesmo que tivesse o dom de profecia, e conhecesse todos os mistérios e toda a ciência, e ainda que tivesse toda a fé, de maneira tal que transportasse os montes, e não tivesse amor, nada seria."

I Coríntios, 13.

RESUMO

Agaricus brasiliensis (syn *A. subrufescens*) é um fungo basidiomiceto nativo no Brasil, que apresenta paredes celulares ricas em polissacarídeos, tais como β-(1→6)-(1→3)-glicanas nos corpos de frutificação e β-(1→2)-glico-β-(1→3)-mananas no micélio. Neste trabalho, estes polissacarídeos foram isolados e modificados quimicamente gerando seus respectivos derivados sulfatados. Os polissacarídeos nativos (FR e MI) e sulfatados (FR-S e-MI-S) foram caracterizados quimicamente e avaliados quanto as suas atividades citotóxica e anti-herpética *in vitro*. Uma vez que FR-S e MI-S apresentaram uma promissora ação anti-HSV-1 (cepas KOS e 29-R) e anti-HSV-2 (cepa 333), seu mecanismo de ação foi determinado. A atividade anti-herpética *in vivo* do MI-S também foi testada utilizando-se modelos murinos de infecção ocular, cutânea e genital. FR-S e MI-S apresentaram duas novas bandas de absorção no espectro de infravermelho em 1253 e 810 cm^{-1}, relacionados aos grupamentos C-S-O e S=O, respectivamente. A massa molecular do FR e MI foram estimados em 609 e 310 kDa, respectivamente. FR-S (127 kDa) e MI-S (86 kDa) apresentaram massas reduzidas provavelmente devido a hidrólise ocorrida durante a reação de sulfatação. FR-S e MI-S apresentaram ~14% de enxofre na análise elementar. Análise de RMN de ^{1}H e ^{13}C confirmaram as estruturas mencionadas anteriormente para FR e MI, sendo o FR-S totalmente sulfatado em C-4 e C-6, enquanto o MI-S parcialmente sulfatado em C-2, C-3, C-4 e C-6. Embora FR e MI não terem apresentado atividade, FR-S e MI-S exibiram promissora ação anti-HSV, com índices de seletividade (SI=CC$_{50}$/EC$_{50}$) superiores a 208. FR-S e MI-S não tiveram efeito virucida, mas significativamente inibiram a adsorção e penetração, provavelmente por interagir com glicoproteínas virais. Estudos de combinação revelaram que FR-S e MI-S atuam de forma sinérgica com o aciclovir. Além disso, MI-S inibiu a expressão das proteínas virais ICP27, UL42, gB e gD. Não foi observada toxicidade nos grupos de animais não infectados e tratados com MI-S. O tratamento tópico e oral com MI-S não foi efetivo na redução da doença ocular. A aplicação tópica de MI-S nas lesões cutâneas também não foi efetiva, mas os animais infectados cutaneamente e tratados oralmente com MI-S mostraram redução significativa dos escores da doença e cura acelerada das lesões pelo HSV-1 após o nono dia. A administração vaginal de MI-S (500 µg), 20 min antes do desafio viral, significativamente reduziu os escores da doença (dias 5 a 9), títulos virais (dias 1 e 3) e mortalidade em

comparação aos grupos controles. A sulfatação aumentou em 5X o efeito citotóxico do FR contra células tumorais A549, enquanto foi essencial para a atividade do MI. A combinação de fatores como o alto grau de substituição e a massa molecular relativamente menor teve impacto positivo sobre a atividade citotóxica do FR-S e MI-S. Os resultados mostram que o MI-S tem potencial a ser utilizado na redução da severidade das lesões cutâneas pelo HSV-1 e principalmente como um microbicida evitando a transmissão sexual das infecções genitais pelo HSV-2. Ainda, os resultados promissores obtidos na triagem preliminar estimulam novos estudos para avaliação do mecanismo da atividade citotóxica do MI-S e FR-S.

Palavras-chave: *Agaricus brasiliensis*; polissacarídeos sulfatados; análise química; atividade anti-HSV *in vitro*; Herpes simplex virus; mecanismo de ação; atividade anti-HSV *in vivo*; atividade citotóxica; células tumorais A549.

ABSTRACT

Agaricus brasiliensis (syn *A. subrufescens*) is a basidiomycete fungus native to Brazil, which presents cell-wall rich polysaccharides, such as β-(1→6)-(1→3)-glucans in the fruiting bodies and β-(1→2)-gluco-β-(1→3)-mannan in the mycelia. Herein, these polysaccharides were isolated and chemically modified to produce their sulfated derivatives. The native (FR and MI) and sulfated polysaccharides (FR-S and MI-S) were chemically characterized and had their *in vitro* antiherpetic and cytotoxic activities evaluated. Since FR-S and MI-S presented promising anti-HSV-1 (KOS and 29-R strain) and anti-HSV-2 (strain 333) activities, their mechanism of action was determined. MI-S was also tested for its *in vivo* antiherpetic activity in the ocular, cutaneous, and genital murine models. FR-S and MI-S presented two new absorption bands at 1 253 and 810 cm^{-1}, related to S=O and C-S-O sulfate groups, respectively, in the infrared spectra. The molecular weight (Mw) of FR and MI was estimated to be 609 and 310 kDa, respectively. FR-S (127 kDa) and MI-S (86 kDa) presented lower Mw, probably due to hydrolysis occurred during the sulfation reaction. FR-S and MI-S presented ~14% of sulfur content in elemental analysis. ^{1}H and ^{13}C NMR analysis confirmed the aforementioned structures for FR and MI, being FR-S fully sulfated at C-4 and C-6 while MI-S partially sulfated at C-2, C-3, C-4, and C-6. Although FR and MI were not active, FR-S and MI-S presented promising anti-HSV activity with with selectivity indices (SI = CC_{50}/EC_{50}) higher than 208. FR-S and MI-S had no virucidal effect, but significantly inhibited viral attachment and penetration, probably by interacting with viral glycoproteins. Combination studies revealed that FR-S and MI-S act synergistically with acyclovir. MI-S was also shown to inhibit the expression of ICP27, UL42, gB, and gD viral proteins. No toxicity was observed in the uninfected groups of animals treated with MI-S. The MI-S topical and oral treatments were not effective in reducing ocular disease. Topical application of MI-S on skin lesions was also not effective, but cutaneously infected mice treated orally with MI-S showed significantly reduced disease scores and accelerated healing of HSV-1 lesions after day 9. Single vaginal administration of 500 µg of MI-S 20 min before viral challenge, significantly reduced the mean disease scores (days 5 to 9), viral titers (days 1 and 3), and mortality in comparison to the control groups. Sulfation enhanced FR cytotoxic effect against A549 tumour cell by five times while was essential for the MI activity. Combination of high degree of substitution and relatively low molecular weight had a

positive impact on the cytotoxic activity of FR-S and MI-S. These results show that MI-S may be potentially useful as an oral agent to reduce the severity of HSV-1 cutaneous lesions and more importantly as a microbicide to block sexual transmission of HSV-2 genital infections. Still, the promising results in the preliminary screening stimulate further studies regarding the mechanism of the cytotoxic activity of MI-S and FR-S.

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LISTA DE ABREVIATURAS E SIGLAS

A549: linhagem de células de tumor pulmonar humano

AIDS: síndrome da imunodeficiência adquirida

ATCC: *American Type Culture Collection*

CC_{50}: concentração citotóxica a 50%, concentração que reduz a viabilidade celular em 50%

CCD: cromatografia de camada delgada

CE_{50}, EC_{50}: concentração efetiva a 50%

CIF: Departamento de Ciências Farmacêuticas (UFSC)

CLAE-UV: cromatografia líquida de alta eficiência com detector por ultravioleta

D_2O: água deuterada

Da: Dalton(s)

DEX-S: sulfato de dextrana

DMSO: dimetilsulfóxido

DS: grau de substituição (do inglês, *degree of substitution*)

dsDNA: DNA dupla fita (do inglês, *double-stranded DNA*)

DST: doença sexualmente transmissível

DTGA: Análise Termogravimétrica Diferencial (do inglês, *Differential Thermogravimetric Analysis*)

FDA: *Food and Drug Administration* (Estados Unidos)

FR: polissacarídeo obtido da frutificação de *A. brasiliensis*

FR-S: derivado sulfatado do polissacarídeo obtido da frutificação do *A. brasiliensis*

GAG(s): glicosaminoglicana(s)

gB, gC, gD, gH, gI, gJ, gK, gL, gM, gN: glicoproteína B, C, D, H, I, J, K, L, M e N, respectivamente.

GS: grau de substituição

HEP: heparina

HIV: vírus da imunodeficiência humana

HSV: Herpes simplex virus

HSV-1: Herpes simplex virus tipo 1

HSV-2: Herpes simplex virus tipo 2

HVEM: *Herpesvirus Entry Mediator*, mediador da entrada dos herpesvírus

IC: índice combinatório

ICP: *Infected cell protein(s)*, proteínas expressas em células infectadas por HSV.

IS ou SI: índice de seletividade

kDa: kilo Dalton(s)

LISTA DE ABREVIATURAS E SIGLAS (cont.)

kpb: kilo pares de base

LCME: Laboratório Central de Microscopia Eletrônica (UFSC)

MEM: Meio Essencial Mínimo, *Minimal Essential Medium*

MEV: microscopia eletrônica de varredura, *scanning electron microscopy*

MI: polissacarídeo obtido do micélio do *Agaricus brasiliensis*

MI-S: derivado sulfatado do polissacarídeo miceliano do *Agaricus brasiliensis*

MIP: Departamento de Microbiologia Imunologia e Parasitologia

MM: massa molecular

MTT: bromento de [3 (4,5-dimetiltiazol-2-il)-2,5 difenil tetrazólio], sal de tetrazólio

Mw: *molecular weight,* massa molecular

nm: nanômetro(s)

NMR: *Nuclear Magnetic Resonance*

p.i.: pós-infecção, *post-infection*

PBS: *Phosphate-buffered saline,* solução de tampão fosfato

PSA: mistura de penicilina, estreptomicina e anfotericina B

RMN: Ressonância Magnética Nuclear

SEM: *scanning electron microscopy,* microscopia eletrônica de varredura

SDS: dodecil sulfato de sódio, do inglês *Sodium Dodecyl Sulfate*

SDS-PAGE: gel de eletroforese de poliacrilamida dodecil sulfato de sódio (do inglês, *Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis*

SFB, BFS: soro fetal bovino, *bovine fetal serum*

TG: Termogravimetria

TGA: Análise Termogravimétrica (do inglês, *Thermogravimetric Analysis*)

Tmax: temperatura(s) máxima(s) de degradação

UFP/ml: unidades formadoras de placas por mililitros

UFP, PFU: unidades formadoras de placas, *plaque forming units*

VERO: linhagem celular de fibroblastos de rins do macaco verde da África (*Cercopithecus aethiops*)

VP: *virion polypeptides*, polipeptídeos virais

X *g*: gravidade(s), medida da força de uma centrífuga

SUMÁRIO

1. INTRODUÇÃO

As fontes naturais continuam sendo a principal base para o desenvolvimento de fármacos para o tratamento de várias doenças humanas. Por exemplo, 74,9% dos fármacos utilizados no tratamento do câncer, disponibilizados no mercado entre os anos de 1981 a 2010, foram derivados ou inspirados em produtos naturais. De 270 fármacos anti-infecciosos aprovados pelo *Food and Drug Administration* (FDA-EUA), entre os anos de 1981 e 2010, 109 são antivirais, dos quais 53 foram desenvolvidos com base em protótipos naturais. Alguns deles (4) foram modificados sinteticamente para melhorar suas atividades farmacológicas e/ou sua biodisponibilidade (NEWMAN; CRAGG, 2012). Estes dados mostram que os estudos envolvendo tais fontes, aliados à tecnologia de modificação química de biomoléculas, devem ser incentivados.

Dentre mais de 1 milhão de espécies de fungos existentes na natureza, 14 mil estão entre os que produzem cogumelos durante o seu ciclo de vida. Muitas de suas atividades farmacológicas são atribuídas aos polissacarídeos, principais componentes da parede celular do micélio, do escleródio (ambos da fase vegetativa) e da frutificação (fase reprodutiva), exercendo funções estruturais e protetoras (LINDEQUIST et al., 2005). Estes carboidratos podem ser constituídos por um único tipo de açúcar, sendo denominados homopolímeros, como as β-glicanas, ou por diferentes monômeros, denominados heteropolímeros, como as glicomananas.

Tais compostos apresentam estruturas altamente diversas cuja variabilidade se dá pela constituição monossacarídica, estereoquímica e posição das ligações glicosídicas, ramificações, massa molecular (MM) e substituintes. Sabe-se que diferenças estruturais estão relacionadas com ações farmacológicas distintas (DA SILVA et al., 2006) e, sendo assim, a caracterização química destes compostos é importante para o entendimento das suas relações estrutura-atividade.

Os vírus herpéticos dos tipos 1 e 2 (HSV-1 e HSV-2) são neurotrópicos e causam infecções latentes crônicas que podem ter episódios de recorrência. Estes fatores, além da disseminação e gravidade da doença em pacientes imunocomprometidos e os efeitos adversos dos fármacos atualmente disponíveis, têm limitado a cura e a eficácia do tratamento do herpes. Consequentemente, a busca por novos agentes anti-herpéticos eficazes, de baixa toxicidade e com características químicas e mecanismo de ação diferenciado dos antivirais atuais, é necessária e relevante (BRADY; BERNSTEIN, 2004).

A atividade antiviral contra vírus humanos e animais tem sido bastante descrita para diversos polissacarídeos sulfatados de origem semissintética ou de ocorrência natural, obtidos de diferentes espécies de algas marinhas, bactérias, fungos e animais (GHOSH et al., 2009). Particularmente, glicanas obtidas de fungos foram sulfatadas quimicamente para aumentar a sua solubilidade e modificar suas propriedades farmacológicas, incluindo a ação antiviral (ZHANG et al., 2004).

Agaricus brasiliensis Wasser (syn *A. subrufescens* Kerrigan), popularmente conhecido como cogumelo-de-deus, cogumelo-do-sol e *himematsutake*, é nativo do Brasil e distribuído nas Américas. Muitas das atividades farmacológicas relatadas para esta espécie, tais como imunomodulatória (KIM et al., 2005), antitumoral (NIU et al., 2009), antigenotóxica (ANGELI et al., 2009) e antiviral (CARDOZO et al., 2011), têm sido relacionadas à presença de polissacarídeos.

Sendo os polissacarídeos metabólitos primários de fungos, a sua utilização depende da produção em larga escala de biomassa que pode ser obtida das frutificações ou do micélio. Neste contexto, a produção de micélio em substrato líquido em biorreator constitui uma alternativa ao cultivo dos corpos de frutificação, pois permite um maior controle do processo e o aumento da escala produtiva do fungo, além da redução do tempo para obtenção de biomassa (CRUEGER; CRUEGER, 1990).

A produção de biomassa miceliana e o isolamento de polissacarídeos, tanto dos cogumelos quanto do micélio de *A. brasiliensis*, têm sido realizados pelo grupo de pesquisa do Laboratório de Bioprocessos, MIP, CCB, UFSC[1]. Tendo em vista a necessidade de estudos complementares que verifiquem a potencial aplicabilidade farmacêutica destes compostos, o objetivo desta tese foi realizar a caracterização química e a avaliação das atividades citotóxica e anti-herpética *in vitro* e *in vivo* de polissacarídeos obtidos da frutificação e do micélio deste fungo, bem como de seus derivados sulfatados.

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[1] O material de estudo desta tese foi gentilmente cedido pela Dra Carla Maísa Camelini, sob a orientação da Profa. Dra. Margarida Matos de Mendonça e do Prof. Dr. Márcio José Rossi (Programa de Pós-Graduação em Biotecnologia e Biociências da UFSC), a quem agradecemos esta frutífera colaboração.

2. REVISÃO BIBLIOGRÁFICA

2.1 Herpes vírus

Os Herpes simplex virus (HSV) pertencentes à ordem *Herpesvirales*, família *Herpesviridae*, subfamília *Alphaherpesvirinae* incluem dois sorotipos: o *Herpesvirus humano 1*[2] (HHV-1) ou Herpes simplex tipo 1 (HSV-1) e o *Herpesvirus humano 2*[2] (HHV-2) ou Herpes simplex tipo 2 (HSV-2) (DAVISON, 2010). Os vírions ou partículas infecciosas dos HSV possuem 120-200 nm de diâmetro e são compostos por núcleo, capsídeo, tegumento e envelope (Figura 1). O núcleo consiste de genoma viral empacotado como uma molécula única de DNA dupla fita (dsDNA) linear, embalado em uma matriz de líquido cristalino que preenche todo o volume interno do nucleocapsídeo ou core. O capsídeo, arranjado em simetria icosaédrica com número de triangulação (T=16) e diâmetro externo de aproximadamente 125 nm, é formado por 162 capsômeros (150 hexâmeros e 12 pentâmeros). O tegumento, um conjunto amorfo de diferentes proteínas que podem variar em abundância, apresenta simetria apenas na região imediatamente adjacente ao capsídeo. O envelope ou membrana viral é formado por uma bicamada lipídica, na qual glicoproteínas de superfície estão ancoradas desempenhando funções importantes nas diversas etapas da infecção viral. Os genomas do HSV-1 (152 kpb) e do HSV-2 (155 Kpb) codificam no mínimo 84 polipeptídios diferentes e compartilham, aproximadamente, 84% de homologia da sequência genômica. Estes dois tipos virais podem ser distinguidos pela composição do DNA; no entanto, diversidades antigênicas e propriedades biológicas também servem como métodos de diferenciação (FLINT et al., 2000; GUPTA et al., 2007; KING, 2009; LUPI et al., 2000; ROIZMAN et al., 2007; WHITE; FENNER, 1994; WHITLEY; ROIZMAN, 2001).

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[2] Esta é a nomenclatura taxonômica formal para a espécie do vírus, porém a designação Herpes simplex virus é a mais comumente usada.
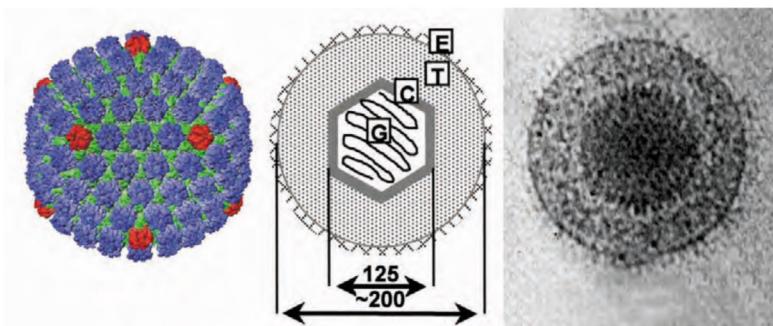
Figura 1: Morfologia do herpesvirus. (Esquerda) Reconstrução de um capsídeo do Herpes simplex virus (HSV-1) gerado a partir de imagens de microscópio crio-eletrônico, com hexons mostrados em azul e pentons em vermelho e o triplex em verde. (Centro) Representação esquemática de um vírion com diâmetros em nm. (G) genoma, (C) capsídeo, (T) tegumento e (E) envelope. (Direita) Imagem de microscópio crio-eletrônico de um virion do HSV-1. Fonte: (KING, 2009)

2.2 **Infecção herpética**

Os HSV causam desde infecções assintomáticas ou com lesões brandas de pele e mucosas (herpes labial, gengivomastite e herpes genital) até doenças graves, tais como a queratite herpética e aquelas com comprometimento do sistema nervoso central (SNC), como a encefalite aguda e a meningite necrosante, além de produzir infecções fatais em pacientes com deficiências imunes (LUPI et al., 2000; WHITE; FENNER, 1994). Apesar de serem transmitidos por diferentes rotas e usualmente envolverem áreas diferentes do corpo, há uma sobreposição na epidemiologia e manifestações clínicas destes vírus. Excluindo-se os casos resultantes da auto inoculação, transmissão vertical ou contato oro-genital, a transmissão dos HSV depende do contato pessoal direto entre um indivíduo soronegativo susceptível e um indivíduo soropositivo, que excreta o vírus sintomaticamente ou assintomaticamente na saliva ou em outras secreções muco cutâneas (genital, anal ou ocular). Um indivíduo naturalmente infectado pode ainda ser reinfectado, superinfectado ou auto inoculado através de uma infecção assintomática ou ativa (FATAHZADEH; SCHWARTZ, 2007; HYLAND; LAMEY, 1999).

O HSV-1 afeta principalmente a boca, lábios, olhos e locais acima da cintura em 75% dos casos. Em geral, a infecção oral causada

pelo HSV-1 é mais frequente do que pelo HSV-2; já a infecção genital pelo HSV-2 ocorre com maior constância do que pelo HSV-1 (HYLAND; LAMEY, 1999; LUPI et al., 2000). Porém, este padrão está mudando nos últimos anos, conforme alguns estudos que apontam o HSV-1 como maior causador em infecções herpéticas genitais do que o HSV-2 (BUXBAUM et al., 2003; PEÑA et al., 2010; PEREIRA et al., 2012; ROBERTS et al., 2003).

Para a maioria dos pacientes imunocompetentes infectados (aproximadamente 90%), as infecções pelos HSV são assintomáticas ou os sintomas são brandos. Contudo, as lesões genitais e a infiltração local de linfócitos, provocada pela infecção herpética, aumentam em três vezes o risco de adquirir o vírus da imunodeficiência humana (HIV) numa relação sexual. O herpes está entre as mais frequentes infecções virais em pacientes com AIDS. Além disso, indivíduos imunocomprometidos e neonatos são os que apresentam maior incidência de quadros clínicos severos, com morbidade e mortalidade significativas (FATAHZADEH; SCHWARTZ, 2007; GUPTA et al., 2007; VAN DE PERRE et al., 2008).

2.3 **Dados epidemiológicos**

O herpes genital é umas das DSTs mais frequentes em todo o mundo, sendo a causa mais comum de ulcerações da genitália de origem infecciosa, com estimativa de 640 mil novos casos diagnosticados anualmente. Estudos epidemiológicos indicam a idade, sexo, etnia, status socioeconômico, nível educacional, uso de preservativo, número de parceiros sexuais e histórico de DSTs prévias como os principais parâmetros que podem estar relacionados ao risco de aquisição da doença (LUPI, 2011; PEREIRA et al., 2012).

Um estudo transversal realizado no Brasil entre 1996 e 1997 com 1.090 indivíduos da população geral com idade entre um e 40 anos mostrou soroprevalência de 67,2% e 11,3% para HSV-1 e HSV-2, respectivamente. Este estudo também revelou que não houve diferença quanto ao sexo e as soroprevalências aumentaram com a idade, sendo que para o HSV-2 o maior aumento ocorreu na adolescência e entre adultos jovens. Indivíduos soropositivos para HSV-1 apresentaram maior risco de serem positivos para HSV-2 (15,7%), quando comparados com os negativos para HSV-1 (4,7%). Além disso, o histórico de doenças sexualmente transmissíveis (DSTs) aumentou significativamente a probabilidade de soropositividade para HSV-2 (CLEMENS; FARHAT, 2010).

A exposição ao HSV-1 ocorre de maneira precoce, conforme o estudo realizado por Cowan e colaboradores (2003), no qual a idade média em que a soroprevalência atingiu 50% da população infantil brasileira avaliada foi de sete anos. O mesmo estudo mostrou que o Brasil apresentou 2,4% de soroprevalência para o HSV-2 em crianças, semelhante à da Índia com 2,2%.

2.4 **Herpes e HIV**

O herpes genital é frequentemente associado com a infecção pelo HIV. Mecanismos moleculares, através da indução da replicação do HIV, celulares, pelo recrutamento de linfócitos T ativados para o local na resposta imunológica à infecção herpética, e teciduais, pelo rompimento do epitélio devido às ulcerações genitais, são descritos como facilitadores da transmissão do HIV. Adicionalmente, observa-se que os pacientes HSV-2/HIV coinfectados apresentam sinais clínicos mais severos com aumento da morbidade e mortalidade (REYNOLDS; QUINN, 2005).

A avaliação de anticorpos anti-HSV-2 em populações de todo o mundo mostram uma alta soroprevalência em pacientes com HIV, com valores de 63–77% nos Estados Unidos, 81% no Reino Unido e 88% na China (LUPI, 2011).

Uma análise soroepidemiológica, realizada no Brasil, em cem pacientes HIV positivos, demonstrou a soroprevalência de 73% para o HSV-2. Este estudo também evidenciou que uma população de comportamento sexual promíscuo, soronegativa para o HIV, teve 41,9% de prevalência para este vírus. Outro estudo realizado entre 2001-2002, em Niterói-RJ, de pacientes HIV-positivos revelou uma soroprevalência para o HSV-2 de 53%, dos quais 21,8% apresentaram histórico da doença (herpes genital) (SANTOS et al., 2006). Já um estudo realizado no Rio de Janeiro, durante o ano de 1994, demonstrou que uma população de doadores de sangue voluntários, de comportamento sexual não promíscuo, apresentou soroprevalência para o HSV-2 de 29,1% (LUPI et al., 2000; SANTOS et al., 1996). Desta maneira, a multiplicidade de parceiros sexuais e a co-infecção com o HIV estão correlacionadas com o aumento na soroprevalência do HSV-2 (LUPI et al., 2000).

Vale ainda ressaltar que o herpes é considerado uma epidemia silenciosa, pois, apesar de apresentar alta soroprevalência, as manifestações clínicas das doenças relacionadas tem baixa incidência e são pouco diagnosticadas. Considerando o total de pacientes

sintomáticos e assintomáticos HSV-2 positivos, estima-se que as chances de adquirir HIV aumentam em cinco vezes, sendo a infecção subclínica pelo HSV-2 também um importante fator para o aumento da suscetibilidade à infecção pelo HIV (REYNOLDS; QUINN, 2005).

2.5 **Ciclo de multiplicação dos HSV**

Estudos realizados com virions purificados de HSV-1 e HSV-2 sugeriram que eles contêm mais de 30 proteínas distintas, as quais são designadas de polipeptídeos virais (VP, do inglês *virion polypeptides*). Destas, estão descritas 12 glicoproteínas de superfície (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM e gN), que estão ancoradas na membrana dos vírus ou presentes no citoplasma das células infectadas, e desempenham funções importantes nas diversas etapas da infecção viral. Por exemplo, as gC, gB, gD, gH, e gL são responsáveis pela adsorção do vírus às membranas celulares, e as quatro últimas são exigidas na fusão. Além disso, as glicoproteínas são capazes de induzir a resposta imune no hospedeiro infectado, sendo duas delas (gB ou gD, ou ambas) usadas na pesquisa de vacinas (HELDWEIN et al., 2006; ROIZMAN et al., 2007).

O ciclo de multiplicação viral acontece em várias etapas. Primeiramente, as partículas virais interagem com as membranas celulares, adsorvendo e posteriormente penetrando nas células. Uma vez no citoplasma, seguem-se as etapas de transcrição, tradução e replicação do genoma viral, que culminam com a formação de novas partículas que serão liberadas ao espaço intercelular e infectarão as células adjacentes (FLINT et al., 2000).

Entrada ou penetração: é um processo complexo, envolvendo cinco das 12 glicoproteínas de superfície. Pode ser dividido em três fases: adsorção, ligação e fusão.

O contato inicial do HSV com uma célula se inicia pela interação do virion com as cadeias de glicosaminoglicanas (GAG) das proteoglicanas de superfície celular. Na **adsorção**, o sulfato de heparana é preferencialmente a molécula que interage com as glicoproteínas virais gC e/ou gB. Apesar desta interação aumentar significativamente a eficiência da infecção por HSV, ela não é absolutamente essencial e irreversível, ao menos pelo que foi observado *in vitro*, pois mutantes que não expressam a gC não perderam a capacidade de infectar células em cultura (BENDER et al., 2005).

O sulfato de heparana é um tipo de GAG expressa na maioria das células fixadas em tecidos, diferentemente do que ocorre nas células circulantes, como as do sistema imune. É uma molécula composta por dissacarídeos de unidades repetidas de N-acetil-glicosamina ligada ao

ácido glicurônico e modificada, em algumas regiões específicas da cadeia, por uma sequência de reações enzimáticas, incluindo acetilação de glicosaminas, sulfatação de grupamentos amino, epimerização do ácido glicurônico a ácido idurônico, e O-sulfatação da hidroxila na posição 2 do ácido idurônico e das hidroxilas nas posições 6 e 3 do amino açúcar. Estas reações geram sítios de ligação a proteínas (aproximadamente 6-12 resíduos) com maior especificidade. O sítio de ligação para a gD, por exemplo, é gerado pela ação de 3-O-sulfotransferases. Os sítios de ligação à gB ou gC ainda não foram determinados; no entanto, sabe-se que gB e gC se ligam em diferentes estruturas e que a ligação de gC é divergente entre os tipos 1 e 2 do HSV, sendo que a gC é a principal mediadora da adsorção do HSV-1, enquanto a gB é preponderante na adsorção do HSV-2 (AGUILAR et al., 2006; SPEAR, 2004).

Após a adsorção dos HSV nas células, ocorre a etapa de **ligação,** envolvendo a interação da gD com um dos seus receptores, que incluem nectina-1 e nectina-2, o mediador específico da entrada dos herpesvírus [*Herpesvirus Entry Mediator* (HVEM)], ou o sulfato de heparana, especificamente modificado (BENDER et al., 2005; HELDWEIN et al., 2006).

O HVEM é considerado um membro da superfamília de receptores do fator de necrose tumoral. Também conhecido como HveA, ATAR, TR2 ou TNFRSF-14, é expresso em uma variedade de tecidos, incluindo linfócitos T e B, outros leucócitos, células epiteliais, endoteliais e fibroblastos e, provavelmente, não está presente em neurônios (SPEAR, 2004). Entre as cinco glicoproteínas que atuam na penetração, somente a gD é capaz de ligar-se a esse receptor, permitindo a entrada do vírus em células nas quais ele é expresso (WHITBECK et al., 1997).

As nectinas-1 e 2 são membros da superfamília de receptores de imunoglobulinas, sendo expressas em vários tecidos e tipos celulares, incluindo células epiteliais, fibroblastos e neurônios. São moléculas de adesão colocalizadas com caderinas, junções aderentes e outras estruturas de adesão, que dimerizadas na membrana de uma célula, irão interagir com outro dímero de nectinas da célula vizinha ou com a gD do HSV (SPEAR, 2004).

O HSV-1 e o HSV-2 divergem na preferência pelos receptores, apesar do HVEM e da nectina-1 serem importantes receptores de entrada para ambos os sorotipos. A nectina-2 é inativa para HSV-1 e tem uma fraca atividade na entrada do HSV-2; a recíproca acontece para o sulfato de heparana modificado (SPEAR, 2004).

A ligação da gD a um dos seus receptores é mais estável e provoca uma mudança conformacional na sua cadeia polipeptídica, liberando o segmento carboxi-terminal. Este, por sua vez, pode interagir com a gB, ou com o complexo gH/gL, desencadeando rearranjos moleculares e, finalmente, a **fusão**. Os mecanismos de atuação da gB e da gH/gL são desconhecidos. Suas estruturas são conservadas entre todos os membros da família dos herpesvírus, ao contrário da gC e da gD, conservadas apenas entre os α- herpesvírus. Tanto a gB quanto o heterodímero gH/gL são necessários para a entrada do vírus na célula, e qualquer um ou ambos, presumivelmente, recebem o sinal da gD e sofrem alterações conformacionais (HELDWEIN et al., 2006; SPEAR, 2004).

Um estudo realizado por Bender et al. (2005) mostrou que mutantes que não expressam a gC exibem redução na adsorção, porém a infecciosidade viral é mantida. Já os vírus privados da gB não são capazes de penetrar nas células-alvo. Por isso, a gC é considerada uma glicoproteína não essencial de entrada, ao contrário da gB, que é essencial.

Após a **fusão** do envelope viral com a membrana celular ocorre a liberação do nucleocapsídeo e proteínas do tegumento no citoplasma da célula hospedeira (ROIZMAN et al., 2007). Após entrar na célula, o nucleocapsídeo é transportado via microtúbulos até as proximidades do núcleo, onde o capsídeo vazio é deixado no citoplasma. Em seguida, o DNA penetra no núcleo, através dos poros nucleares, onde irá ocorrer a transcrição, replicação e montagem de novos capsídeos (MIRANDA, 2002).

Dependendo do tipo de célula, a rota de entrada dos HSV pode variar. Uma segunda forma de entrada, menos comum, chamada de forma secundária, envolvendo a endocitose do capsídeo envelopado, foi relatada para os HSV (NICOLA; STRAUS, 2004).

Transcrição, tradução e replicação: o processo de transcrição, no qual a RNA polimerase celular sintetiza RNAm a partir do DNA viral, e a síntese proteica acontecem de forma coordenada, em três fases: imediata, precoce, e tardia. Após a ativação da maquinaria transcricional pelas proteínas da fase imediata inicia-se a fase precoce. Nesta fase, são sintetizadas as enzimas necessárias para a replicação do genoma viral, sendo a DNA polimerase a principal delas. Com o acúmulo de DNA viral, ocorre a sinalização para que os genes tardios sejam transcritos, gerando proteínas estruturais do capsídeo e do tegumento, e as glicoproteínas do envelope viral (BOEHMER; LEHMAN, 1997; ROIZMAN et al., 2007).

Sabe-se que durante a infecção produtiva ocasionada pelos vírus herpéticos, mais de 84 diferentes proteínas são expressas de uma forma coordenada e regulada em três fases sequenciais (COLBERE, 1975; ROIZMAN et al., 2007), a saber:

- **Fase imediata (α):** indutores transcricionais cruciais na indução e regulação do ciclo de replicação viral, sendo sintetizadas em torno de 2 - 3 h pós-infecção (p.i.). Exemplos das proteínas α: *Infected cell proteins* (ICPs) 0, 4, 22, 27, 47 e US1.5;
- **Fase precoce (β):** proteínas que promovem a replicação do DNA viral e a ativação da fase γ, com pico de expressão entre 5 - 7 h p.i. As proteínas de fase β incluem as enzimas que são requeridas para a síntese do genoma viral: DNA polimerase viral, proteína de ligação de DNA fita simples (ICP8), DNA helicase-primase, proteína UL9 e as enzimas necessárias para o metabolismo do ácido nucléico viral (ribonucleotídeo redutase, timidina quinase, dUTPase, uracil DNA glicosilase, etc);
- **Fase tardia (γ):** proteínas estruturais do nucleocapsídeo e todas as outras que formarão os vírions, como as glicoproteínas do envelope, com pico de expressão em torno de 12 h p.i. Exemplo das proteínas γ: gB, gC, gD e gE.

Montagem e liberação dos vírions: as proteínas do capsídeo são transportadas para o núcleo, onde são reunidas em pró-capsídeos vazios ou preenchidos com DNA viral. Os nucleocapsídeos ligam-se em porções modificadas da membrana nuclear interna, que contém glicoproteínas virais, e são temporariamente envelopados e liberados no espaço nuclear intermembranário. Posteriormente, eles são conduzidos ao citoplasma, onde ocorre a adição das proteínas do tegumento e sua introdução em vesículas derivadas do complexo de Golgi, nas quais também há glicoproteínas virais inseridas. Após o envelopamento, os vírions infecciosos migram para a superfície celular, fundem-se com a membrana citoplasmática e, finalmente, são liberados no espaço extracelular (JOHNSON; HUBERT, 2002; METTENLEITER, 2002; ROIZMAN et al., 2007).

2.6 **Desenvolvimento de fármacos antivirais**

O desenvolvimento de fármacos antivirais teve início na década de 50. Entre as principais razões das dificuldades encontradas nesta área

específica está a natureza intrínseca dos vírus, os quais são totalmente dependentes da maquinaria das células infectadas para sua multiplicação e sobrevivência. Dessa maneira, fármacos que inibem a replicação viral ou provocam a inativação das partículas infecciosas virais, causam também toxicidade às células hospedeiras. O compromisso com a especificidade pelas células infectadas, a eficácia e um baixo nível de toxicidade são, portanto, critérios indispensáveis para a seleção de novos agentes antivirais (JONES, 1998; WHITE; FENNER, 1994).

Posteriormente, o entendimento da replicação viral em nível molecular conduziu ao desenvolvimento de novos fármacos, que interferem em etapas específicas do ciclo de multiplicação dos vírus. Um ótimo exemplo desse tipo de atuação foi a introdução na terapêutica, no final da década de 70, do aciclovir, que atua de forma seletiva sobre os HSV, sendo ativado por uma enzima, a timidina quinase, que é essencial para a sua replicação (ELION et al., 1977).

O atual arsenal quimioterápico para infecções virais consiste em aproximadamente 50 fármacos licenciados pelas autoridades governamentais. A maioria foi aprovada nos últimos anos, sendo que metade deles é utilizada para o tratamento da infecção pelo HIV. Os demais antivirais são utilizados contra os vírus das hepatites B e C, vírus influenza, e vírus herpéticos, incluindo nesse último grupo o vírus da varicela zoster, o citomegalovírus e os vírus herpes simplex (DE CLERCQ, 2010; ROTTINGHAUS; WHITLEY, 2007).

Os fármacos com atividades clinicamente relevantes e aprovados pelo FDA para o tratamento de infecções pelos HSV são: aciclovir, valaciclovir, penciclovir, fanciclovir, idoxuridina, trifluridina, brivudina, vidarabina, cidofovir e foscarnet, que inibem a replicação viral; e docosanol, que é um inibidor de entrada dos víroins nas células, porém. Contudo, eles apresentam efeitos adversos e toxicidade, além de já existirem cepas virais resistentes (MAMIDYALA; FIRESTINE, 2006; ROTTINGHAUS; WHITLEY, 2007).

Portanto, a pesquisa de novos agentes anti-herpéticos eficazes, com mecanismos de ação diferenciados e com efeitos deletérios mínimos, é muito importante e deve ser incentivada.

2.6.1.1 Avaliação da atividade antiviral *in vitro*

A citotoxicidade foi definida por Nardone (1977) como sendo o conjunto de alterações da homeostase celular, levando a uma série de modificações que interferem na capacidade adaptativa das células, bem como na sua sobrevivência, reprodução e realização de suas funções metabólicas. Os programas de pesquisa e desenvolvimento de fármacos

envolvem, além da pesquisa das atividades farmacológicas, o estudo da citotoxicidade dos mesmos, pois ambos são importantes para caracterizar sua eficácia terapêutica e segurança (WILSON, 2000).

É importante salientar que qualquer efeito biológico de um composto ou extrato frente a um microrganismo deve ser diferenciado da sua citotoxicidade, que necessita ser avaliada previamente ou em paralelo. Assim, o componente intrínseco da avaliação da atividade antiviral de uma amostra é a determinação do seu índice de seletividade (IS), que é a razão entre a concentração que causa 50 ou 90% de citotoxicidade (CC_{50} ou CC_{90}) e a concentração que inibe a multiplicação viral na mesma proporção (CI_{50} ou CI_{90}) (COS et al., 2006).

Teoricamente, uma amostra com propriedades antivirais pode proteger as células de várias maneiras: inativando diretamente os vírions ou interferindo em etapas do ciclo de multiplicação viral. A inativação direta ou atividade virucida pode ser causada por desintegração total dos vírions, solubilização do envelope viral ou por modificação química, degradação ou interação com proteínas essenciais do envelope (ZHU et al., 2004).

Além da polimerase viral, proteínas que funcionam durante as múltiplas etapas da multiplicação viral podem fornecer alvos úteis para novos agentes terapêuticos. Moléculas que atuam na adsorção e penetração dos vírus têm as vantagens de prevenir a infecção, impedindo a produção de proteínas virais citotóxicas, além de não exigirem a sua entrada na célula para produzir o efeito. Estudos estão sendo realizados nesse sentido e já se conhecem algumas moléculas, a exemplo da heparina, que se ligam à glicoproteínas do envelope viral (COEN; SCHAFFER, 2003). O docosanol, entre outros mecanismos, é um inibidor de entrada e já está à disposição em alguns países para o tratamento do herpes labial (BARBARASH, 2001). Este panorama, aliado à disponibilidade de técnicas para o estudo das interações específicas de moléculas com componentes da partícula viral, incentiva a busca por novos agentes mais eficazes e com mecanismos de ação diferenciados.

2.6.1.2 Sinergismo

O uso de fármacos combinados pode ser efetivo contra múltiplos alvos, subpopulações ou doenças, simultaneamente. O uso de uma combinação de fármacos com diferentes mecanismos de ação pode ter efeito sinérgico no tratamento de uma doença, por aumentar a eficácia do efeito terapêutico, permitir a diminuição da dose diminuindo a

toxicidade, diminuir o desenvolvimento de resistência, e melhorar a farmacodinâmica e a farmacocinética dos mesmos. A terapia combinada, conhecida popularmente como coquetel, é amplamente usada e compõe o tratamento de escolha para doenças graves como câncer e AIDS (CHOU, 2006).

Chou (2006) definiu o sinergismo como um efeito maior do que simplesmente o efeito aditivo, ou seja, o somatório dos dois efeitos isoladamente, e o antagonismo com um efeito menor do que o aditivo. Chou e Talalay (1981, 1983, 1984) deduziram um modelo matemático para calcular o denominado índice combinatório (IC, em inglês CI, *combination index*), que quantifica o sinergismo ou antagonismo entre duas substâncias. Neste teorema, valores de IC < 1, =1 e >1 indicam sinergismo, efeito aditivo e antagonismo, respectivamente.

De acordo com os valores de IC, diferentes graus de sinergismo ou antagonismo podem ser determinados, conforme mostra o Quadro 1 a seguir**.**

Quadro 1: Descrição de sinergismo e antagonismo em estudos de combinação de substâncias, através do método de determinação do Índice Combinatório (IC), de acordo com Chou (2006)**.**

Faixa do IC	Interpretação	Símbolo
< 0,10	Sinergismo muito forte	+++++
0,10-0,30	Sinergismo forte	++++
0,30-0,70	Sinergismo	+++
0,70-0,85	Sinergismo moderado	++
0,85-0,90	Sinergismo fraco	+
0,90-1,10	Aditivo	+/-
1,10-1,20	Antagonismo fraco	-
1,20-1,45	Antagonismo moderado	- -
1,45-3,30	Antagonismo	- - -
3,30-10,00	Antagonismo forte	- - - -
>10,00	Antagonismo muito forte	- - - - -

2.7 **Fungos que produzem cogumelos**

O termo cogumelo é sinônimo de corpo de frutificação e corresponde a estruturas aéreas em forma de guarda-chuva, suficientemente grandes para serem coletadas e vistas a olho nu. Estas

estruturas fazem parte de ciclo reprodutivo de macrofungos distribuídos principalmente na classe dos Basidiomicetos, mas também em algumas espécies na classe dos Ascomicetos (LINDEQUIST et al., 2005).

Os cogumelos medicinais têm uma história estabelecida de uso em terapias orientais tradicionais. A título ilustrativo pode-se citar *Ganoderma lucidum* (reishi), *Lentinus edodes* (shiitake), *Inonotus obliquus* (chaga), *Grifola frondosa* (maitake), *Flammulina velutipes* (winter) e *Pleurotus ostreatus* (hiratake), que são usados há centenas de anos na Coréia, China, Japão e Rússia (CHANG; BUSWELL, 1996; COHEN et al., 2002; LULL et al., 2005; WASSER, 2002b).

Estes fungos representam uma grande e pouco estudada fonte de potenciais agentes terapêuticos. O número de diferentes espécies no planeta é estimado em 140.000, sendo apenas 10% conhecidas. Dessas 14.000 espécies, aproximadamente 50% são consideradas potencialmente comestíveis, mais de 2.000 são seguras para o consumo humano, e em torno de 700 apresentam alguma propriedade farmacológica (LINDEQUIST et al., 2005; MATTILA et al., 2000).

O interesse no uso de cogumelos com propriedades terapêuticas tem aumentado nos últimos anos. Além do amplo número de espécies existentes, os fungos apresentam metabólitos primários e secundários diferenciados dos das plantas, com efeitos farmacológicos em diferentes alvos celulares e moleculares, com comprovada eficiência no tratamento de inúmeras doenças. Entre as atividades farmacológicas estudadas estão: antitumoral, antialérgica, anti-inflamatória, antioxidante, antidiabética, anti-hipertensiva, anti-hiperlipêmica, antitrombótica, imunomoduladora, hepatoprotetora, antibacteriana, antifúngica, antiparasitária e antiviral (FAN et al., 2006; LINDEQUIST et al., 2005; POUCHERET et al., 2006; WASSER, 2002a; ZAIDMAN et al., 2005).

Na revisão feita por Stamets (2002), foram descritos entre outras atividades a ação antiviral de várias espécies de cogumelos, como por exemplo, *Agaricus brasiliensis, Fomes fomentarius, Grifola frondosa, Ganoderma lucidum, Inonotus obliquus, Lentinula edodes, Coriolus versicolor* e *Trametes versicolor*. Os efeitos antivirais dos cogumelos são descritos não só para os extratos totais como também para compostos deles isolados. Tais efeitos podem ser causados pelo efeito virucida direto, por inibição das enzimas virais, da síntese dos ácidos nucléicos virais, da adsorção e/ou da penetração viral nas células do hospedeiro. Efeitos indiretos resultantes da atividade imunoestimulante também são descritos para polissacarídeos isolados ou complexados com outras moléculas (LINDEQUIST et al., 2005).

Há inúmeros exemplos de compostos isolados de fungos produtores de cogumelos que apresentaram atividade antiviral. Entre elas, a ação anti-HIV foi verificada para os triterpenos (ganoderiol, ganodermanontriol e ácido ganodérico) da espécie *Ganoderma lucidum*, para as ligninas do *Inonotus obliquus,* e para as ligninas e lentinanas sulfatadas do *Lentinula edodes*. A ação anti-influenza foi relatada para os terpenos (ganodermadiol e lucidadiol) isolados do *Ganoderma pfeifferi* e para polissacarídeos do *Lentinula edodes* e para a hispidina do *Inonotus hispidus*. A ação anticitomegalovírus foi descrita para os polissacarídeos do *Trametes versicolor*. A ação anti-HSV foi relatada para as proteínas do *Rozites caperata e Grifola frondosa* e para a iludina S, um sesquiterpeno extraído do *Omphalotus illudens* (ALI et al., 2003; BRANDT; PIRAINO, 2000; FAN et al., 2006; GU et al., 2007; PIRAINO, 1999).

Aliado a isso, muitas pesquisas relatam que extratos e metabólitos isolados de cogumelos estimulam ou suprimem componentes específicos do sistema imune, apresentando efeito imunomodulador. Esses extratos ou compostos podem ser eficazes na prevenção e tratamento de doenças infecciosas, atuando como agentes terapêuticos principais ou em combinação com outros fármacos, ou ainda, como adjuvantes de vacinas (LULL et al., 2005).

2.8 *Agaricus brasiliensis*

O objeto de estudo desta tese é conhecido popularmente no Brasil como cogumelo-do-sol, e no Japão como *himematsutake*, sendo amplamente utilizado como alimento e na medicina tradicional (LAKHANPAL; RANA, 2005). No entanto, sua classificação taxonômica é bastante controversa e merece especial atenção.

Taxonomicamente, este fungo pertence à classe dos Basidiomicetos, ordem Agaricales, família Agaricomycetideae, tribo Agariceae, seção Arvenses e gênero *Agaricus*. A denominação da espécie de estirpes brasileiras é denominada, mais comumente, de *Agaricus brasiliensis* Wasser.
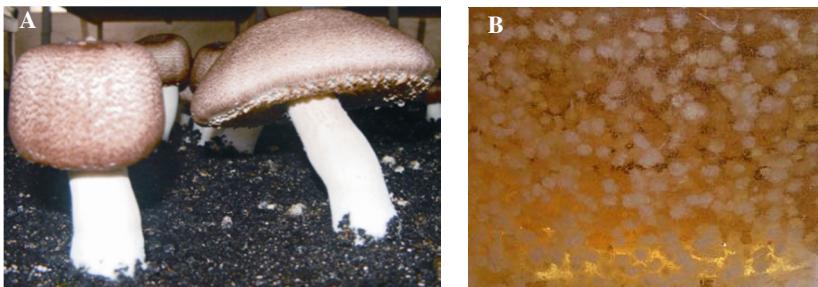
Figura 2: A) Corpos de frutificação e B) micélio em meio líquido do fungo de *Agaricus brasiliensis* Autora: Carla Maísa Camelini.

Este cogumelo é nativo no sudeste do Brasil, tendo sido descoberto na cidade de Piedade, São Paulo, em 1960, pelo produtor e pesquisador japonês, Takatoshi Furumoto, que o enviou ao Japão para ser investigado. Sua primeira identificação como *A. blazei* Murrill sucedeu-se sete anos depois pelo botânico belga Heinemann. Com a morte de Furumoto, o cultivo deste cogumelo foi abandonado, e o interesse pela espécie só retornou em 1990. Em seguimento, ocorreu um intenso cultivo e ampla utilização desta espécie na culinária e na medicina tradicional, atuando contra o estresse físico e mental, estimulando o sistema imune, aumentando a qualidade de vida de diabéticos, reduzindo o colesterol e auxiliando no tratamento do câncer. Tais usos têm sido fortemente explorados do ponto de vista publicitário, somente com fins comerciais, sem que haja estudos toxicológicos e farmacológicos pré-clínicos e clínicos suficientes que comprovem sua segurança e eficácia (DIAS et al., 2004; FIRENZUOLI et al., 2008).

Em 2002, (Wasser) e colaboradores publicaram uma análise histórico-botânica e concluíram que *A. blazei* ss. Murrill difere em alguns aspectos do *A. blazei* ss. Heinemann, devendo ser consideradas duas espécies distintas. Eles propuseram para as linhagens identificadas por Heinemann, que incluem as brasileiras, uma nova denominação, *Agaricus brasiliensis*. No entanto, Colauto e colaboradores (2002) verificaram pouca variabilidade genética entre espécimes de *Agaricus* comercializadas no Brasil. Além disso, Fukuda et al. (2003) compararam sete estirpes cultivadas no Japão e uma brasileira, e verificaram que a espécime do Brasil não foi compatível com as japonesas. Já Kerrigan (2005) afirmou que os cogumelos originados do Brasil e do Japão são biologicamente e filogeneticamente idênticos à espécie norte-americana (*Agaricus subrufescens)*. Como a discussão ainda permanece (DIAS et al., 2008; KERRIGAN, 2005, 2007;

WASSER, 2007, 2010; WASSER et al., 2005), a determinação desta espécie é considerada controversa até que um consenso internacional oficial seja obtido.

No presente trabalho, a denominação *A. brasiliensis* será utilizada, visto que é a mais frequentemente empregada para as estirpes brasileiras.

No dia 09 de agosto de 2012, foi realizada uma busca na base de dados *Scopus* (http://www.scopus.com) com o termo *Agaricus blazei*, e obtiveram-se 384 artigos, entre os quais estão relatadas as atividades: antitumoral (60) imunomoduladora (33), antimutagênica (15), antioxidante (14), citotóxica (6), antidiabética (4), anti-inflamatória (3), antibacteriana (2), antiviral (2), anti-hipertensiva (2), antilipêmica (4), hepatoprotetora (3), antialérgica (3), antiparasitária (2) e antitrombótica (1).

Utilizando-se o termo *Agaricus brasiliensis* obtiveram-se 21 artigos, e dentre as ações farmacológicas estudadas estão as atividades: antiviral (3), antitumoral (2), imunoestimulante (2), antimutagênica (1), antidiabética (1), e antioxidante (1).

Para o termo *Agaricus subrufescens*, foram detectados quatro artigos: um relata a atividade antioxidante e os outros três discutem a denominação da espécie e seus sinônimos.

Este levantamento mostrou que, embora alguns usos populares deste fungo tenham sido confirmados, a sua atividade antiviral foi pouco pesquisada. Entre os trabalhos anteriores, foi demonstrada a inibição *in vitro* da multiplicação do vírus da encefalite equina (SORIMACHI et al., 2001), do HSV-1 (BRUGGEMANN et al., 2006), do herpes bovino tipo 1 (BRUGGEMANN et al., 2006; MINARI et al., 2011) e do poliovírus tipo 1 (FACCIN et al., 2007). Além disso, um estudo realizado com camundongos, que receberam uma vacina de DNA recombinante contra o vírus da hepatite B, e também extratos deste cogumelo, detectou um aumento de 3-4 vezes na produção de anticorpos específicos contra o vírus (anti-HBc), em relação aos controles (CHEN et al., 2004).

2.9 **Polissacarídeos**

Polissacarídeos são macromoléculas naturais encontradas em todos os organismos vivos, constituindo um grupo abundante e importante da biosfera, tais como a celulose e o amido das plantas, o glicogênio dos animais e as glicanas dos fungos. Esses polímeros são constituídos de unidades monossacarídicas, unidas por ligações glicosídicas, diferindo entre si na unidade e no grau de ramificação destas, no tipo de ligações que as unem e no comprimento de suas

cadeias, apresentando diferentes composições e funções (DA SILVA et al., 2006).

Com relação à sua utilização terapêutica, os polissacarídeos são compostos de baixa toxicidade para mamíferos e apresentam inúmeras propriedades farmacológicas, entre as quais, a atividade antiviral que foi mostrada em vários estudos utilizando diversas fontes, como plantas (JASSIM; NAJI, 2003), líquens (OLAFSDOTTIR; INGÓLFSDOTTIR, 2001), bactérias (MATSUDA et al., 1999), algas marinhas (SMIT, 2004) e fungos (EO et al., 2000).

Nos fungos, estas moléculas representam um importante constituinte da biomassa. A parede da célula fúngica, por exemplo, pode conter mais de 75% de polissacarídeos, predominantemente glicanas e mananas e, em menor quantidade, quitina. Além de atuarem como elemento de suporte para as hifas, alguns polissacarídeos formam uma capa extracelular ao redor do micélio, proporcionando um suporte para adesão das enzimas excretadas e participando na degradação da lignina, como uma fonte indireta de peróxido de hidrogênio. Sua contribuição é importante também para proteger as células da desidratação e regular a concentração de glicose extracelular (DA SILVA et al., 2006; GOMPERTZ et al., 2002).

Os polissacarídeos representam uma classe de macromoléculas com grande variabilidade estrutural. A polimerização dos monossacarídeos pode ocorrer em diferentes posições da molécula de açúcar, gerando uma ampla variedade de estruturas lineares ou ramificadas. A estrutura secundária da molécula também pode variar, dependendo da conformação dos seus componentes, da sua massa molecular e das interações inter e intracadeias (PAULSEN, 2002). Contribuindo também para esta diversidade, existem processos de modificação de polissacarídeos, como a sulfatação, que é verificada em organismos marinhos e na matriz extracelular de vertebrados (KIRKWOOD, 1974).

Diversos estudos já relataram a atividade antiviral de polissacarídeos contra alguns vírus, tais como HSV-1 e HSV-2 (EO et al., 2000; LIU et al., 2004), HIV (JASSIM; NAJI, 2003), vírus da hepatite B (LEE et al., 2002), citomegalovírus e vírus influenza (KANEKIYO et al., 2005) e coxsackie virus B3 (LEE et al., 2010). Para alguns polissacarídeos naturalmente sulfatados ou produzidos por semi-síntese foram relatadas atividades frente aos vírus: HSV-1 e HSV-2 (LIU et al., 2004; TALARICO et al., 2004; ZHU et al., 2004), Vírus Herpes Humano tipo 6 (NAESENS et al., 2006), vírus da dengue (QIU et al., 2007), HIV (TALYSHINSKY et al., 2002), citomegalovírus, vírus

respiratório sincicial, vírus influenza, adenovírus, entre outros (WITVROUW; DE CLERCQ, 1997). Além disso, em uma revisão realizado por Ghosh et al., (2009) foi verificado que muitos dos polissacarídeos avaliados nos últimos 20 anos apresentam atividade contra mais de uma espécie de vírus, demostrando que estes compostos geralmente apresentam amplo espectro de atividade antiviral.

O principal modo de ação antiviral dos polissacarídeos sulfatados relaciona-se às suas características aniônicas, o que lhes permite interagir com as cargas positivas presentes no envelope viral ou na superfície celular, inibindo a adsorção, fusão e penetração dos vírus nas células do hospedeiro (EO et al., 2000). Todavia, o efeito antiviral indireto *in vivo*, através do estímulo da resposta imune inata e adaptativa também já foi verificado (HAYASHI et al., 2008).

2.9.1.1 Absorção intestinal de macromoléculas

A superfície interna do intestino delgado é revestida por uma camada única formada por dois tipos de células especializadas: os enterócitos, correspondendo ao tipo celular majoritário, e as células M, que estão presentes nas Placas de Peyer e compõem menos de 0,1% do total de células. Ambos os tipos celulares entram em contato com as células adjacentes através de interdigitações que são junções protéicas que interligam as membranas celulares. No entanto, células M e enterócitos diferem entre si em muitos aspectos como mostra a Figura 3. Os microvilos das células M são mais curtos e estão em menor número do que nos enterócitos. A membrana basolateral das células M é geralmente bastante invaginada, formando um bolso intra-epitelial, ocupado por macrófagos e linfócitos B e T. Além disso, as células M também são ausentes de glicocálice, muco e secreção de anticorpos IgA e têm níveis muito baixos de lisossomos e secreção de enzimas na borda em escova. As macromoléculas com MM > 1.000 Da penetram na barreira da mucosa intestinal por endocitose, através das células M, podendo ser absorvidas sem uma degradação significativa. Estas células M são responsáveis pela captação e checagem de polissacarídeos, proteínas e microorganismos no tecido linfóide associado à mucosa intestinal, regulando a resposta imune no local. No entanto, devido ao fato destas células serem pouco abundantes e não possuirem marcadores específicos, pouco se sabe sobre a cinética e o mecanismo de absorção de macromoléculas nestas células (COOPER et al., 2002; LIANG et al., 2001).
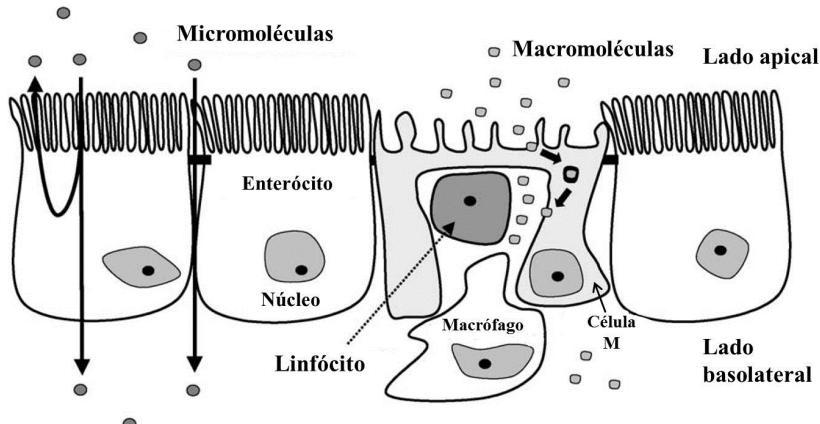
Figura 3: Diagrama mostrando as diferenças morfológicas entre os enterócitos e células M. O transporte transepitelial de compostos de baixa massa molecular através dos enterócito e de alta massa molecular através das células M também está representado. (Fonte: adapatado de Liang et al., (2001).

2.10 **Glicanas**

As glicanas são um extenso grupo de polímeros de D-glicose unidas por ligações glicosídicas. Entre elas, as mais comumente encontradas na parede celular de fungos são as de configuração β. Como a glicose é um açúcar de seis carbonos, a ligação pode ocorrer entre qualquer combinação das seis posições. Assim, quando um polissacarídeo é referido como (1→6)-β-D-glicana, a ligação β-glicosídica está entre a posição 1 (carbono 1) em uma molécula de glicose e a hidroxila localizada na posição 6 (carbono 6) da próxima molécula. Ramificações laterais também podem ocorrer em alguns pontos particulares da molécula, tal como as ramificações (1→3)-β- na cadeia principal (1→6)-β-D-glicana, sendo esta a configuração predominante das glicanas presentes nas frutificações de *A. brasiliensis* (MCGINNIS, 1996; CAMELINI, 2005). Os polissacarídeos de cadeia longa, tais como as β-glicanas, não são produzidos pelo corpo humano e podem ser obtidos a partir de fungos e também de plantas. Sabe-se que certos cogumelos são as fontes mais ricas de β-glicanas já conhecidas, que se apresentam como componentes menores do citosol e da parede celular, e como polissacarídeos excretados no meio (DA SILVA et al., 2006).

2.11 **Câncer**

O termo câncer refere-se à neoplasia, especificamente aos tumores malignos. O câncer é uma doença complexa, caracterizada pela proliferação descontrolada de células transformadas devido à ativação de oncogenes e/ou desativação de supressores de tumor. Existem aproximadamente 200 tipos de cânceres diferentes, correspondentes às diferentes linhagens de células do corpo, os quais se distinguem pela capacidade de invadir tecidos e órgãos, vizinhos ou distantes (ALY, 2012; DE ALMEIDA et al., 2005).

Segundo a Organização Mundial da Saúde (OMS), 7.6 milhões de pessoas morreram de câncer no mundo em 2008 e há projeção de aumento para 13 milhões em 2030 (WHO, 2008). No Brasil, estima-se a ocorrência de mais de 518 mil novos casos de câncer para o biênio de 2012-2013, sendo os tipos mais incidentes os cânceres de pele não melanoma, próstata, pulmão, cólon e reto e estômago no sexo masculino; e os cânceres de pele não melanoma, mama, colo do útero, cólon e reto e glândula tireoide para o sexo feminino (INCA/MS, 2011).

2.12 **Cancer de pulmão**

Os cânceres de traquéia, brônquios e pulmão são a causa mais importante de morte por câncer no mundo, sendo responsáveis por 1,39 milhões de mortes em 2008, com uma razão mortalidade/incidência de aproximadamente 86%. A mais recente estimativa mundial apontou uma incidência de 1,61 milhão de casos novos de câncer do pulmão para o ano de 2008, representando 12,7% de todos os novos casos de câncer. Na maioria das populações, os casos de câncer do pulmão tabaco-relacionados representam 80% ou mais dos casos, pois o tabagismo é responsável pelo aumento de cerca de 20 a 30 vezes do risco de desenvolver câncer pulmonar. Outros importantes fatores de risco conhecidos para o câncer do pulmão incluem exposição à carcinógenos ocupacionais e ambientais, tais como amianto, arsênio, radônio, hidrocarbonetos aromáticos policíclicos e queima de carvão. Além desses, repetidas infecções pulmonares, tuberculose, deficiência ou excesso de vitamina A e história familiar também são considerados fatores de risco para o desenvolvimento desse tipo de neoplasia (INCA/MS, 2011).

No Brasil, foram estimados para o ano de 2012, 17.210 casos novos de câncer de pulmão em homens e 10.110 em mulheres. Esses valores correspondem a um risco estimado de 18 novos casos a cada 100 mil homens e 10 a cada 100 mil mulheres. Sem considerar os tumores da

pele não melanoma, o câncer do pulmão em homens é o segundo mais frequente nas regiões Sul (37/100 mil) e Centro-Oeste (17/100 mil), ocupando a terceira posição nas regiões Sudeste (20/100 mil), Nordeste (8/100 mil) e Norte (8/100 mil). Para as mulheres, é o terceiro mais frequente na região Sul (19/100 mil), o quarto na região Centro-Oeste (9/100 mil) e o quinto nas regiões Sudeste (11/100 mil), Nordeste (6/100 mil) e Norte (5/100 mil) (INCA/MS, 2011).

Devido às diferenças de tratamento e prognóstico, o câncer pulmonar é classificado em duas formas: carcinoma de pequenas células e carcinoma de não pequenas células. O carcinoma de pequenas células caracteriza-se pelo crescimento rápido, alta probabilidade de metástase e uma boa responsividade ao tratamento com quimioterapia e radioterapia. O carcinoma de não pequenas células é responsável por mais de 75% dos cânceres e inclui três tipos de tumores: adenocarcinoma, carcinoma de células escamosas também denominado carcinoma epidermóide, e carcinoma de células grandes (CERSOSIMO, 2002).

2.13   **Avaliação do potencial anticâncer**

As alternativas disponíveis para o tratamento do câncer incluem a cirurgia, para remoção do tumor, a radioterapia, que consiste na administração de raios-X, e a quimioterapia, com a utilização de agentes antineoplásicos. Todas elas são associadas a efeitos colaterais com eficácia e especificidade ainda insuficientes (ALY, 2012). Sendo assim, a pequisa de novos agentes anticâncer á bastante importante e se inicia com a avaliação *in vitro* das amostras disponíveis ou selecionadas através de ensaios de citotoxicidade em linhagens celulares tumorais.

Segundo o *National Cancer Institute* (NCI-EUA), os termos citotóxico, antiproliferativo, antitumoral e anticâncer são diferentemente definidos. O termo citotóxico é utilizado para agentes tóxicos para células tumorais *in vitro*, podendo causar morte celular por diferentes mecanismos. A atividade antitumoral é determinada por ensaios *in vivo* em modelos animais. Já o termo anticâncer é reservado a agentes que apresentam atividade em humanos (**Developmental Therapeutics Program NCI/NIH**, 2012).

Este Instituto realiza, desde 1955, o mais importante programa de triagem de compostos com atividade citotóxica. Atualmente, no âmbito desse programa, os compostos são testados quanto às atividades citotóxica e antiproliferativa através de um painel de 60 diferentes linhagens celulares, originadas de sete diferentes tipos de tumores (cerebral, cólon, leucemia, pulmão, melanoma, ovário e renal) e

incluindo também linhagens resistentes a fármacos (SUGGITT; BIBBY, 2005).

Os procedimentos realizados pelo programa incluem uma triagem inicial com apenas uma concentração da amostra e, posteriormente, outra triagem com cinco concentrações das amostras mais promissoras. A metodologia inclui a utilização do ensaio colorimétrico da sulforrodamina B, com tratamento das células por 48 h, e a fixação das cavidades controle no tempo zero (antes do tratamento). Utilizando controles do tempo zero podem-se corrigir os valores de absorbância obtidos no tempo final (48 h) e diferenciar a atividade citotóxica da citostática. Três parâmetros de concentração-resposta são calculados a partir dos dados obtidos:

- ✓ GI_{50}= (*Growth Inhibition of 50%*), que corresponde à concentração que causa 50% de inibição do crescimento celular em relação ao controle não tratado.
- ✓ TGI= (*Total Growth Inhibition*), que corresponde à concentração que inibe totalmente o crescimento celular, ou seja, a concentração na qual o número de células nas cavidades que receberam o tratamento é igual ao número de células nas cavidades controle (tempo zero).
- ✓ LC50= (*Lethal Concentration at 50%*), que corresponde à concentração que resulta em 50% de redução do número de células em comparação ao tempo inicial.

Desta forma, uma amostra só é considerada citotóxica se o número total de células diminuir em relação ao tempo zero, ou seja, tiver menos células que inicialmente. Se a amostra somente reduzir o número de células em relação ao controle celular é considerada citostática, pois reduz a proliferação celular (**Developmental Therapeutics Program NCI/NIH**, 2012)

2.14   **Polissacarídeos com atividade citotóxica**

O primeiro importante estudo de avaliação da atividade citotóxica de substâncias obtidas de fungos foi realizado por Gregory, em 1966, que isolou substâncias ativas de mais de 200 espécies de Basidiomicetos e mostrou que polissacarídeos de 22 espécies de cogumelos apresentaram efeito inibitório contra células tumorais (ZHANG et al., 2007).

Diferentes mecanismos de ação foram descritos para polissacarídeos sulfatados com atividade citotóxica. Entre eles, a inibição da metástase e proliferação de células tumorais por ligação a moléculas de adesão celular e fatores de crescimento, por exemplo,

inibindo a heparinase, enzima responsável pela clivagem das cadeias de sulfato de heparana das proteoglicanas e a liberação de fatores de crescimento tumoral, ou por ligação direta a fatores de crescimento. Alguns polissacarídeos sulfatados também foram relatados como indutores da apoptose e diferenciação de células tumorais (NIE et al., 2006). Esta classe de compostos pode ainda atuar melhorando a resposta imune inata e adaptativa às células tumorais. Mais especificamente, polissacarídeos sulfatados estimulam a resposta inata pelo aumento da atividade tumoricida de macrófagos e células *natural killer*. A resposta adaptativa é aumentada pelo aumento da produção de citocinas, como interleucina-1 beta e TNF-alfa, que por sua vez estimulam as células T citotóxicas, as quais atuam diretamente sobre as células tumorais. Estes compostos também podem reconhecer estruturas de adesão celular como as CD2, CD3 e CD4 e aumentar a proliferação de linfócitos T (WU; CHEN, 2006).

Apesar do *Agaricus blazei*, espécie relacionada ao *Agaricus brasiliensis*, estar entre as 28 espécies de fungos mais avaliadas quanto à atividade antitumoral/citotóxica de seus polissacarídeos, a atividade de seus derivados sulfatados não foi ainda estudada. Sendo assim, foi incluída nos objetivos deste trabalho a avaliação da atividade citotóxica dos polissacarídeos nativos e derivados sulfatados, obtidos tanto do cogumelo quanto do micélio de *A. brasiliensis*.

3. OBJETIVOS

3.1 **Objetivo geral**

Realizar a caracterização química e a avaliação das atividades citotóxica e anti-herpética de polissacarídeos obtidos do fungo *Agaricus brasiliensis* e de seus derivados sulfatados.

3.2 **Objetivos específicos**

✓ Realizar a caracterização química, físico-química e morfológica dos polissacarídeos obtidos dos corpos de frutificação (FR) e do micélio (MI) de *A. brasiliensis*, bem como de seus respectivos derivados sulfatados, FR-S e MI-S;

✓ Avaliar *in vitro* o efeito citotóxico das amostras frente à linhagem de células tumorais A549;

✓ Avaliar *in vitro* a citotoxicidade frente às células Vero e a atividade anti-herpética das amostras frente à diferentes cepas dos Herpes simplex virus;

✓ Avaliar *in vitro* o mecanismo da atividade anti-herpética das amostras sulfatadas, em diferentes etapas do ciclo de replicação viral;

✓ Avaliar *in vitro* o sinergismo da atividade anti-herpética das amostras sulfatadas com o fármaco aciclovir;

✓ Avaliar *in vivo* a atividade anti-herpética do MI-S utilizando diferentes modelos murinos de infecção.

CAPÍTULO I:
**CARACTERIZAÇÃO QUÍMICA E AVALIAÇÃO DA
ATIVIDADE CITOTÓXICA DE POLISSACARÍDEOS DO
FUNGO *Agaricus brasiliensis***

1. APRESENTAÇÃO

A caracterização das quatro amostras obtidas (FR, MI, FR-S e MI-S) foi uma das estratégias deste trabalho, permitindo o conhecimento das estruturas nativas e das modificações ocorridas no processo de sulfatação, contribuindo para a qualidade dos resultados e para um melhor entendimento das relações estrutura-atividade dos compostos testados.

Sendo assim, através de diferentes metodologias foram realizadas análises químicas, físico-químicas e morfológicas das amostras, a fim de caracterizá-las e compará-las entre si.

Os dados obtidos foram compilados em um artigo, que constitui o primeiro capítulo desta tese. Nele também foram incluídos os resultados da avaliação da atividade citotóxica.

2. ARTIGO SUBMETIDO PARA AVALIAÇÃO AO INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES

Characterization and cytotoxic activity of sulfated derivatives of polysaccharides from *Agaricus brasiliensis*

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Abstract

Agaricus brasiliensis cell-wall polysaccharides isolated from fruiting body (FR) and mycelium (MI) and their respective sulfated derivatives (FR-S and MI-S) were chemically characterized and had their cytotoxic activity evaluated against A549 tumor cells. FR-S was characterized as a $(1\rightarrow6)$-$(1\rightarrow3)$-β-glucan fully sulfated at C-6 and partially substituted at C-4. MI-S was shown to be a $(1\rightarrow3)$-β-D-gluco-$(1\rightarrow2)$-β-D-mannan, partially sulfated at C-2, C-3, C-4, and C-6. The combination of high degree of sulfation and low molecular weight was correlated with the increased cytotoxic activity of both FR-S (EC_{50}=605.6 µg/mL) and MI-S (EC_{50}=342.1 µg/mL) compared to the non-sulfated polysaccharides FR and MI (EC_{50}>1500 µg/mL).

Keywords: *Agaricus brasiliensis*; sulfated polysaccharide; chemical characterization; cytotoxic activity.

1. Introduction

Agaricus brasiliensis is an edible Basidiomycete fungus belonging to the Brazilian biota and has traditionally been used to treat cancer and other diseases. In the last few decades, numerous studies have reported the cytotoxic and antitumor properties of *A. brasiliensis* polysaccharides, which mainly act through immunomodulatory mechanisms, but also by direct cytotoxic effects on tumor cells [1].

The species found in Brazil was originally named *Agaricus blazei* Murrill *sensu* Heinemann. Since 2005, this binominal nomenclature has been considered incorrect and replaced by two botanical names: *Agaricus subrufescens* Peck or *Agaricus brasiliensis* Wasser, with the latter being adopted for the fungus cultivated in Brazil [2-5].

Polysaccharides are a structurally diverse class of macromolecules for which physicochemical and biological properties are dependent on a combination of factors such as sugar composition, molecular weight, and chain conformation. For sulfated polysaccharides, the degree of substitution and position of sulfated groups are also important [6]. There are many reports demonstrating that sulfation improves the biological activity of polysaccharides, including anti-coagulant [7], antiviral [8], immunostimulant [9], hypoglycemic [10], anti-oxidant [11], cytotoxic [12, 13], and antitumor [14] properties.

In our previous work [15], we evaluated the anti-herpetic activity of an *A. brasiliensis* mycelial polysaccharide (MI) and its sulfated derivative (MI-S) and found that sulfation of MI significantly improved its antiviral activity. In this paper, MI and MI-S as well as *A. brasiliensis* fruiting body polysaccharide (FR) and its sulfated derivative (FR-S) were chemically characterized and had their cytotoxic activity evaluated. To the best of our knowledge, this is the first report on the cytotoxic activity of sulfated derivatives of polysaccharides from this species.

2. Materials and methods

2.1 Fungal materials

The fruiting bodies of *Agaricus brasiliensis* Wasser (syn *A. subrufescens* Peck) were collected in Biguaçu, Santa Catarina state, Brazil, and designed as strain UFSC 51. A voucher specimen is deposited in the FLOR Herbarium at UFSC (FLOR 11797) and at Coleção Brasileira de Micro-organismos de Ambiente e Indústria - CBMAI/UNICAMP (code number: 1449, available at http://webdrm.cpqba.unicamp.br/catalogo/pycat/index.py).

The *A. brasiliensis* mycelium was isolated and cultivated as previously described [15].

2.2 Isolation of mycelial and fruiting body polysaccharides

A. brasiliensis polysaccharides were isolated as previously described [15, 16], with minor modifications. Briefly, 50 g of dried fruiting bodies or mycelial biomass from the submerge-cultivated state were blended twice with 0.5 L of distilled water, refluxed at 100°C for 3 h and filtered through a Whatman filter paper no. 42. The extracts were precipitated with three volumes of 95% ethanol and recovered by centrifugation (2000 x g, 15 min) to obtain the crude mycelial (cMI) and fruiting body (cFR) polysaccharide fractions. Finally, the higher molecular weight polysaccharides from the mycelium and fruiting body were obtained through dialysis (5 kDa cutoff membrane - Spectrum Laboratories, New Brunswick, USA) and, after lyophilization, were designated as MI and FR, respectively. Both polysaccharides were chemically sulfated using the chlorosulfonic acid/pyridine method as described by Zhang et al. [17], generating their respective sulfated derivatives MI-S and FR-S.

2.3 Sample characterization

2.3.1 Scanning electron microscopy (SEM)

The surface morphology of gold coated samples was analyzed by the scanning electron microscope (JSM-6390 LV, Jeol, Japan).

2.3.2 Thermogravimetry combined with differential thermogravimetric analysis (TGA-DTGA)

Thermograms of samples (1 mg) were obtained in a thermogravimetric analyzer (TGA-50, Shimadzu) from room temperature up to 900°C, at a scan rate of 10°C per min.

2.3.3 High-performance gel permeation chromatography (HPGPC)

The molecular weight (Mw) determination was carried out by HPGPC using a Perkin-Elmer series 200 instrument (USA) equipped with a refractive index detector and a gel filtration column (TSK-Gel 5000 PW 7.8 × 300 mm connected to a TSK PWH 5 × 75 mm guard column; Tosoh, Japan). Samples were eluted with 0.2 M NaCl mobile phase at a flow rate of 1 mL/min. The Mw was estimated by reference to

the calibration curves of standard dextrans (5, 12, 50, 150, 410, and 670 kDa; Sigma, USA).

2.3.4 Analytical methods

Total sugar content was determined using the phenol-sulfuric acid method [18] adapted to a 96 well microassay plate [19] using glucose as standard. The Bradford method [20] was used to determine the protein content using calibration curves built with bovine serum albumin. The sulfate content was determined by the $BaCl_2$ method [21]. All reagents were purchased from Sigma. Results, determined from calibration curves obtained in three different days, are expressed as mean ± standard deviation (% w/w ± s.d.).

2.3.5 Elemental analysis

Elemental analysis (carbon, hydrogen, nitrogen, and sulfur) was performed with a Perkin Elmer 2400 series II elemental analyzer. The percentage of sulfur (% S) and carbon (% C) were used to calculate the degree of substitution (DS) according to the formula:
DS= 2.25 × % S / % C [22].

2.3.6 Monosaccharide composition analysis

The qualitative monosaccharide composition of MI and FR was evaluated by thin layer chromatography (TLC) using xylose, arabinose, mannose, glucose, and galactose as reference compounds. Briefly, the samples were hydrolyzed with 3M trifluoroacetic acid (TFA) for 4 h and analyzed on PEI-cellulose sheets (Merck, Germany), developed with *n*-butanol/ethyl acetate/pyridine/water (6:1:5:4). The chromatograms were visualized after spraying aniline-*o*-phthalic acid reagent followed by heating [23].

Quantification of monosaccharides was performed using High Performance Liquid Chromatography (HPLC) in a Perkin-Elmer series 200 instrument equipped with an UV detector, at 250 nm, according to Lv et al. [24]. Briefly, hydrolyzed samples (4 M TFA, 2 h) were derivatized with 1-phenil-3-methyl-5-pyrazolone (PMP). Calibration curves, constructed with PMP-labeled standard monomers (mannose, glucose, galactose, and glucuronic acid), were used for determining the sugar concentrations of the samples. Arabinose was employed as internal standard. Analyses were undertaken at room temperature (25 ºC) on a C18 column (4.6 mm × 250 mm, 5 µm, Perkin-Elmer,

USA) using a gradient elution of 0.045% KH_2PO_4 - 0.05% triethylamine buffer (A) / acetonitrile (B) as follows: 10% to 14% B over 40 min, at a flow rate of 1.0 mL/min.

2.3.7 Fourier transformation infrared (FT-IR) spectroscopy

The FT-IR spectrum was recorded on a Perkin-Elmer Spectrum One spectrometer in the region between 650 and 4.000 cm^{-1}.

2.3.8 Nuclear Magnetic Resonance (NMR) analysis

The spectra were recorded at room temperature for samples (60 mg/mL) dissolved in D_2O in a Bruker Avance III 500 NMR instrument, operating at 500 MHz for ^1H and 125 MHz for ^{13}C. Chemical shifts were expressed in ppm relative to internal acetone (= 32 ppm).

2.4 Cytotoxic activity evaluation

2.4.1. Cell lines

Human lung adenocarcinoma (A549, ATCC, CCL-185) and Vero cells (ATCC, CCL-81) were grown in Minimal Essential Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, 100 µg/mL streptomycin and 25 µg/mL amphotericin B in a humidified 5% CO_2 atmosphere at 37°C.

2.4.2 MTT assay

The effect on cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular viability assay [25]. In brief, 1×10^4 cells/well were grown in 96-well microplates for 24 h. Cells were then treated with different concentrations of the samples for 48 or 72 h. Negative controls were treated with medium only. After the exposure period, the culture medium was replaced by MTT solution (1 mg/mL) and plates were further incubated for 4 h. Formazan crystals were dissolved by addition of DMSO (Merk, Germany) and the optical densities were read at 540 nm (Infinite 1200 TECAN, Austria). The 50% effective concentration (EC_{50}) was defined as the concentration that reduced cell proliferation by 50% when compared to untreated controls. Paclitaxel (Glenmark, Brazil) was used as positive control.

2.4.3 Sulforhodamine assay

The sulforhodamine assay [26] was also used to evaluate the cytotoxicity of selected samples. Briefly, A549 or VERO (ATCC: CCL 81) cells, cultured in 96-well plates, at a density of 1×10^4 cells/well, were exposed to eight concentrations of samples for 48 h. After subtracting the absorbance values of the initial cell cultures (time zero cell control), the GI_{50} (50% growth inhibitory activity), TGI (total growth inhibition, cytostatic activity), and LC_{50} (50% lethal concentration, cytotoxic activity) values were calculated.

2.5 Statistical analysis

GraphPad Prism 5 Software was used to calculate EC_{50} values and their 95% confidence intervals through a nonlinear fit- curve (log of compound concentration *versus* normalized response - variable slope). GI_{50}, TGI, and LC_{50} values were calculated using linear fit- curves in the same program.

3 Results and Discussion

Since the introduction of sulfate groups with an appropriate degree of substitution can improve the bioactivity of polysaccharides, the goal of this study was to modify polysaccharides isolated from *A. brasiliensis* fruiting body and mycelium and evaluate their physicochemical properties and cytotoxic activity.

Analysis of surface morphology by scanning electron microscopy (SEM) is a qualitative tool to characterize fungal polysaccharides, comparing with standards and assessing morphological differences of modified derivatives. Microstructural irregularities observed in SEMs presented in Fig. 1 show that *A. brasiliensis* polysaccharides are an amorphous solid. The FR polysaccharide (Fig. 1 A,) showed a rough appearance with no porosity that may be due to high molecular packing as a result of inter and intramolecular hydrogen bonds. Following sulfation, FR-S (Fig. 1 C, D) displayed a smoother surface with an internal porous structure, suggesting that the sulfate groups expanded intermolecular spaces. The MI polysaccharide (Fig. 1 E, F) consisted of flakes that were smaller in size than those of FR and FR-S. MI-S (Fig. 1 G, H) presented particles of variable size with smoother surfaces and rounded shapes. Porous structures were also observed in the MI-S preparation. These particle structures are consistent with previous results. For example, an *A. brasiliensis* mycelial exopolysaccharide was

previously reported to be an amorphous solid by Lima et al. [27]. In contrast, Hong and Choi [28] described a spherical shape for an *A. blazei* protein-polysaccharide complex, prepared by spray-drying process. This variation on morphology may be related to the different methods used for fungus cultivation, sample extraction and drying.
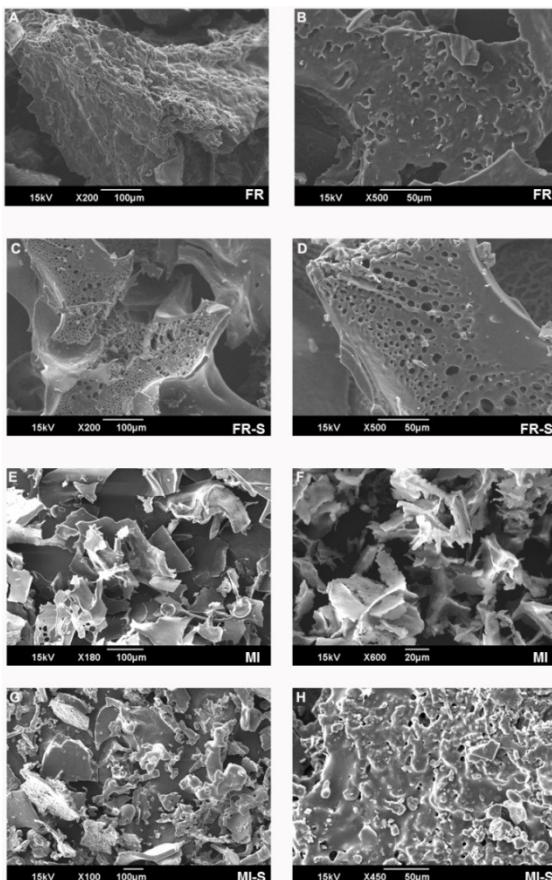
Fig. 1: *Agaricus brasiliensis* polysaccharides observed under scan electron microscopy. Lyophilized samples were gold coated and analyzed by a JSM-6390 LV microscope. A and B: FR, *A. brasiliensis* fruiting body polysaccharide; C and D: FR-S, sulfated derivative of *A. brasiliensis* fruiting body polysaccharide; E and F: MI, *A. brasiliensis* mycelial polysaccharide; G and H: MI-S, sulfated derivative of *A. brasiliensis* mycelial polysaccharide.

Data from thermal analysis performed by TGA-DTGA are compiled in Table 1 and thermograms are available in Supplementary Fig. 1. According to the thermograms, polysaccharide decomposition occurred in three steps. The small initial drop in mass represents the loss of water. The second stage of decomposition began above 266°C for the non-modified compounds (FR and MI) and above 218°C for the sulfated derivatives (FR-S and MI-S). The temperature peak (Tmax) recorded in the DTGA curve is characteristic of an endothermic reaction and can be attributed to thermal decomposition of the polysaccharides [29]. The Tmax obtained for FR and MI was around 300°C. Hong and Choi [28] found a similar profile for a polysaccharide-protein complex isolated from *A. blazei* fruiting body. A similar behavior has been described for other polysaccharides such as chitosan [30] and galactomannan [31]. The sulfation reduced the temperature of degradation range to 225-250°C, demonstrating the molecular change. Jayakumar et al. [32] also reported a slight decrease in the thermostability of chitin after sulfation.

Table 1: Thermal analysis of *Agaricus brasiliensis* polysaccharides

Sample	TGA[a]			DTGA[b]
	Range of temperature (°C)	Tf-Ti	Mass loss (%)	Tmax (°C)
FR	272.68 – 343.72	71.04	51.59	313.92
FR-S	224.96 – 277.36	52.40	39.09	248.89
MI	266.12 – 343.98	77.86	44.83	301.33
MI-S	217.21 – 260.30	43.09	27.23	236.32

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative; MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.
[a]Thermogravimetric analysis; (Tf - Ti)= range of reaction (final temperature - initial temperature);
[b]Differential thermogravimetric analysis; Tmax= maximal temperature of degradation.
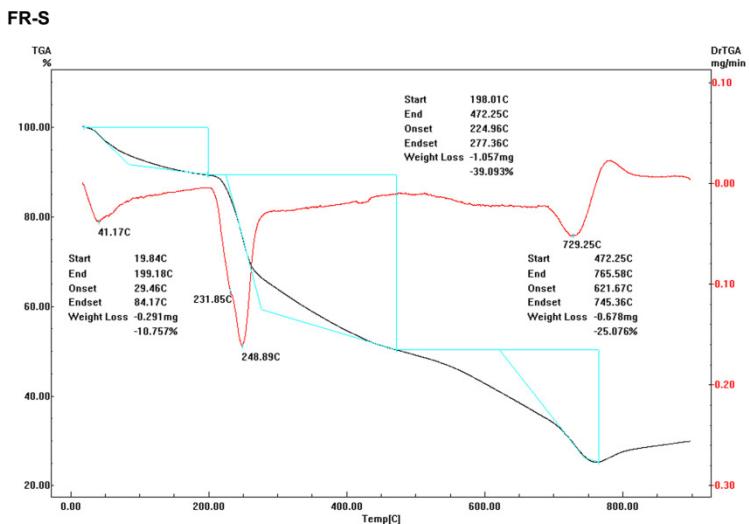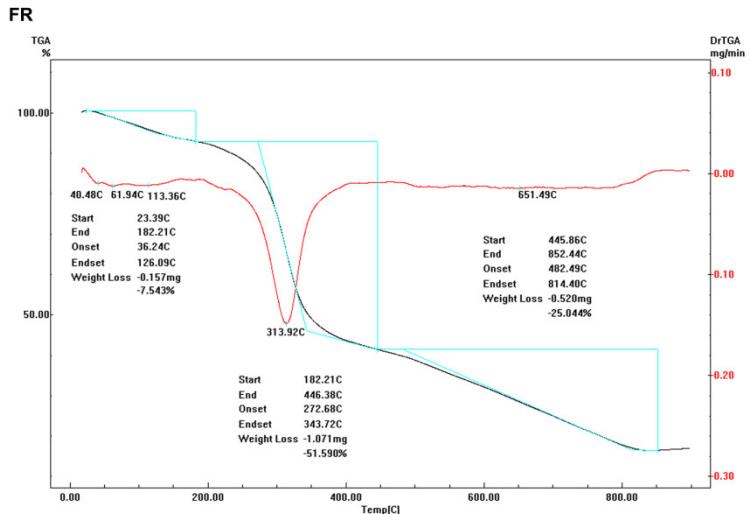
Supplementary Fig. 1: Thermograms of *Agaricus brasiliensis* polysaccharides obtained by TGA-DTGA thermal analysis. FR and FR-S, fruiting body polysaccharide and its sulfated derivative; MI and MI-S, mycelial polysaccharide and its sulfated derivative.
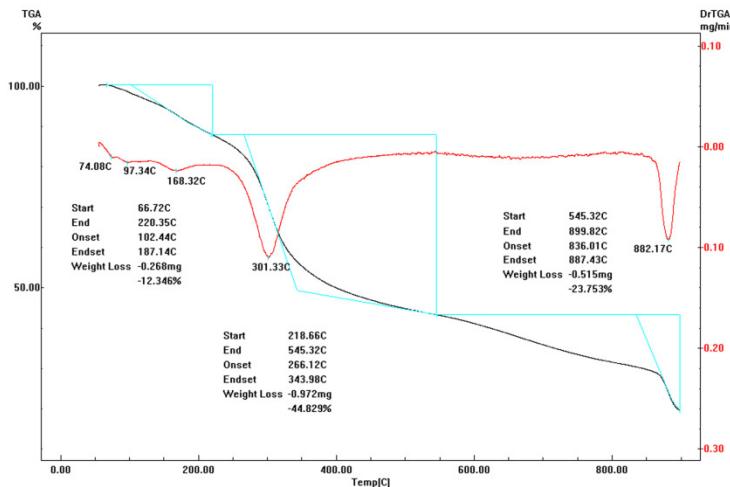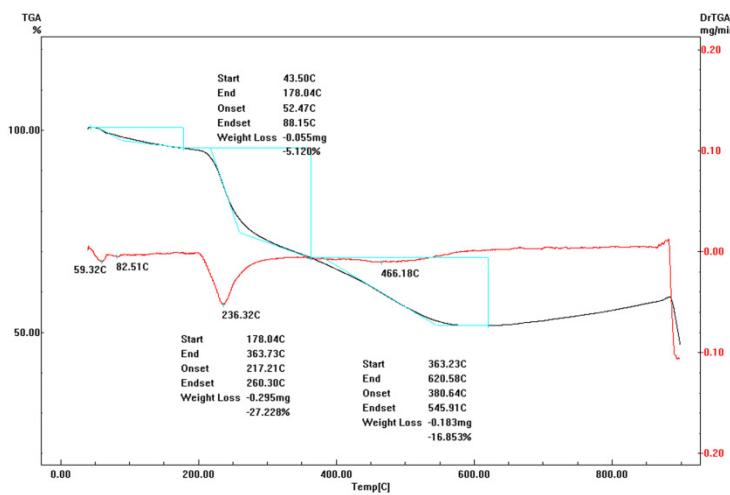
Supplementary Fig. 1 (cont.): Thermograms of *Agaricus brasiliensis* polysaccharides obtained by TGA-DTGA thermal analysis. FR and FR-S, fruiting body polysaccharide and its sulfated derivative; MI and MI-S, mycelial polysaccharide and its sulfated derivative.

The data on the apparent molecular weights of *A. brasiliensis* polysaccharides are presented in Table 2. The polysaccharide isolated from the *A. brasiliensis* fruiting body (FR) was found to have a Mw of 609 kDa, which falls within the wide range (390 kDa and 2000 KDa) previously described for similar preparations [33-36]. The molecular weight of 310 kDa determined for the mycelial polysaccharide (MI) showed good correspondence with the values reported by Fujimiya et al. (380 kDa) [37] and Lin and Yang (274 kDa) [38]. The sulfation process carried out in acid medium at high temperature probably induced hydrolysis of the polymers, thus accounting for the lower Mw of the sulfated derivatives (127 and 86 kDa for FR-S and MI-S, respectively). This effect has been previously described by Lanteri [39].

Table 2: Molecular weights and chemical composition of *Agaricus brasiliensis* polysaccharides

Sample	Molecular Weight (kDa)	Monosaccharides (%)			Content mean ± SD [% (w/w)]		
		Glucose	Mannose	Galactose	Total sugar	Protein	Sulfate
cFR	617.93 ± 12.01 and 17.88 ± 0.98	60.19 ± 2.12	8.86 ± 3.46	0.94 ± 0.03	n.t.	n.t.	n.t.
FR	608.73 ± 4.11	63.67 ± 4.08	1.76 ± 0.13	4.55 ± 1.44	78.97 ± 7.52	1.76 ± 0.22	n.d.
FR-S	127.20 ± 6.95	68.98 ± 1.26	1.16 ± 0.34	2.86 ± 1.31	65.91 ± 9.39	n.d. (< 0.16)	40.25 ± 1.78
cMI	309.89 ± 2.22 and 30.20 ± 9.22	65.05 ± 5.12	9.95 ± 0.21	n.d.	n.t.	n.t.	n.t.
MI	310.11 ± 1.08	19.35 ± 3.12	58.65 ± 2.74	n.d.	77.33 ± 10.41	1.06 ± 0.06	n.d.
MI-S	85.52 ± 5.33	24.72 ± 4.12	55.28 ± 3.12	n.d.	76.60 ± 12.66	0.97 ± 0.09	36.07 ± 2.74
DEX	410	n.t.	n.t.	n.t.	75.11 ± 12.07	n.d. (< 0.16)	n.d.
DEX-S	>500	n.t.	n.t.	n.t.	70.70 ± 10.90	n.d. (< 0.16)	48.67 ± 1.40

cFR and cMI, *A. brasiliensis* fruiting body and mycelial crude polysaccharide fractions, respectively.
FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative.
MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.
DEX and DEX-S, dextran and dextran sulfate, purchased from Sigma (USA).
n.d., not detected or the concentration is below the detection limit between parentheses.
n.t. not tested.

The monosaccharide composition of cFR and cMI was initially accessed by TLC analysis (Supplementary Fig. 2). cFR was found to contain primarily glucose while cMI had predominantly glucose, with minor amounts of other sugars.
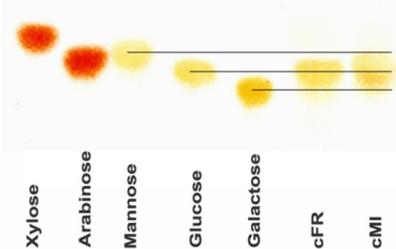
Supplementary Fig 2: TLC chromatogram obtained for the crude polysaccharide fractions of *Agaricus brasiliensis*. cFR: crude fruiting body polysaccharide fraction; cMI: crude mycelial polysaccharide fraction.

The quantitative carbohydrate composition was assayed by HPLC-UV analysis of PMP derivatized samples. As shown in Table 2, the major sugar found in FR was glucose (63.67%), while MI contained predominantly mannose (58.65%), but also had significant amounts of glucose (19.35%), which is characteristic of a heteropolymer glucomannan. Uronic acids were not detected in the analyzed samples. The total carbohydrate content varied from 66% to 79%, similar to the concentrations (80-95%) previously reported for *A. blazei* polysaccharides purified by anion exchange chromatography [33, 35]. Similarly, the low percentage of protein (1-1.8%) was comparable to values obtained previously [16, 27]. The low percentage of nitrogen (Table 3) was in agreement with the results of protein determination by the Bradford assay (Table 2). According to Fernandes et al. [40] the temperature, solvent, and pH used for the extraction and drying procedures can break peptide bonds thereby reducing the protein content. Therefore, different extraction and drying procedures may explain the higher protein levels reported by some groups for polysaccharides obtained from *A. brasiliensis* and *A. blazei* fruiting body [28, 35, 41, 42] and mycelium [43]. Sulfate analysis (Table 2) confirmed that the parental polysaccharides have no detectable sulfate content, whereas the derivatized samples contain over 35% sulfate. These results were also confirmed by data from elemental analysis (Table 3).

Table 3: Elemental analysis of polysaccharides from *Agaricus brasiliensis*

Sample	Elements (% w/w)				
	Carbon	Hydrogen	Nitrogen	Sulfur	DS[a]
FR	34.42	5.56	2.50	0	0
FR-S	17.15	3.52	1.62	14.37	1.88
MI	32.07	4.36	1.63	0	0
MI-S	21.10	3.60	1.80	14.77	1.58
DEX-S	14.90	3.10	0	10.72	1.62

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative;
MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.
DEX-S, dextran sulfate, purchased from Sigma.
[a]Degree of substitution DS= 2.25 x (S% /C %)

The IR spectra recorded for the samples (Supplementary Fig. 3) confirmed their polysaccharide constitution, disclosed by the intense stretching bands between 3200-3470 cm^{-1} (ν O-H) and 1075 cm^{-1} (ν C-O) [44]. A reduction in the intensity of the bands, observed for the derivatives, was related to the sulfation of the hydroxyl groups [45]. The absence of uronic acids was confirmed by the lack of carbonyl bands around 1700 cm^{-1} [16]. Polysaccharides can easily be hydrated due to their affinity for water and this is consistent with the presence of the water absorption bands at 1623-1636 cm^{-1}. The small peaks at 1370-1540 cm^{-1} confirmed that the preparations contained low amounts of protein. The weak absorption bands present in all the *A. brasiliensis* polysaccharides spectra at 876-898 cm^{-1} are indicative of β stereochemistry [6]. The sulfation was confirmed by the appearance of two new absorption bands around 1200 and 800 cm^{-1} in FR-S and MI-S spectra, characteristic of asymmetric (S=O) and symmetric (C-O-S) vibrations, respectively [17].
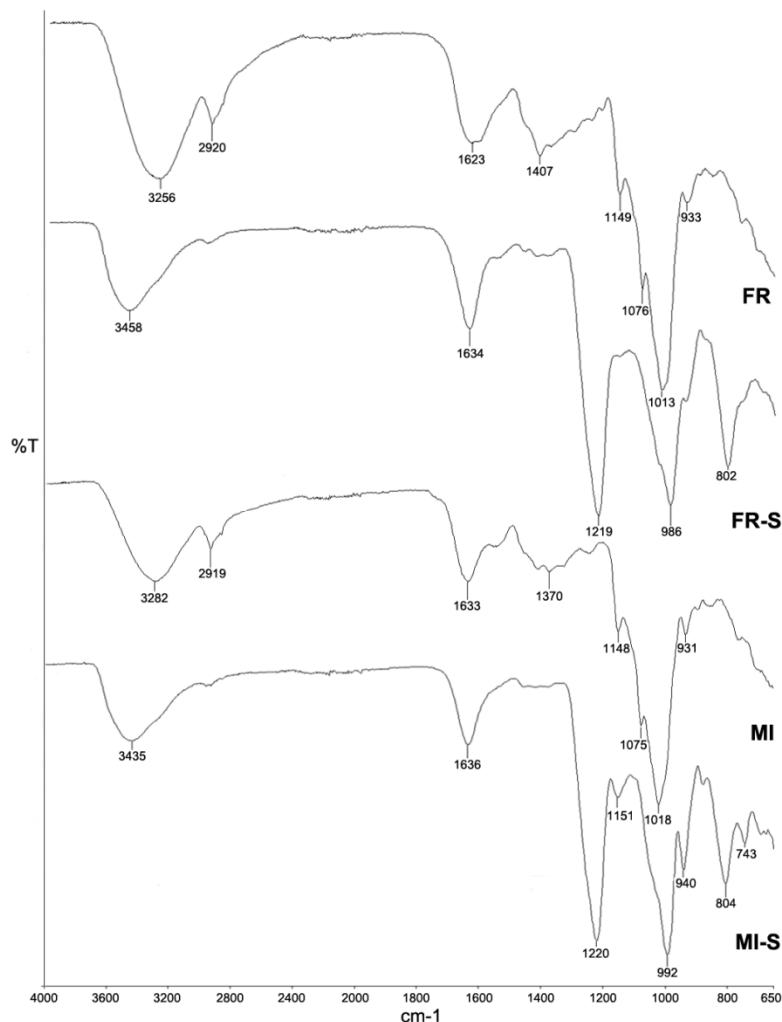
Supplementary Fig. 3: FT-IR spectra of *Agaricus brasiliensis* polysaccharides. FR and FR-S, fruiting body polysaccharide and its sulfated derivative; MI and MI-S, mycelial polysaccharide and its sulfated derivative.

The attribution of the chemical shifts from the ^{13}C and ^{1}H NMR spectra (Supplementary Figs. 4 and 5, respectively) obtained for the native polysaccharides and the sulfated derivatives are listed in Table 4.
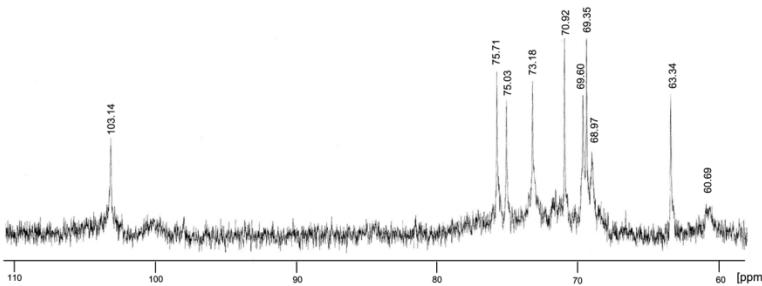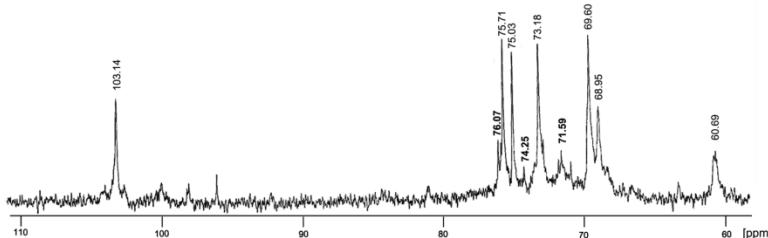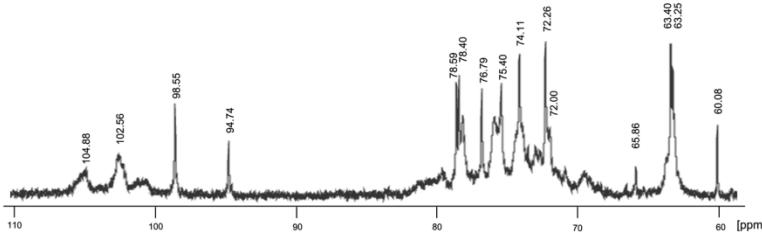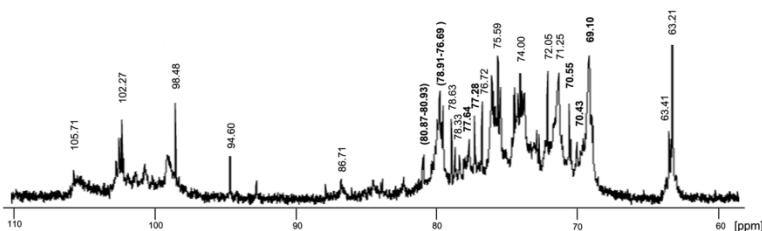
Supplementary Fig 4: ^{13}C NMR spectra of *Agaricus brasiliensis* polysaccharides. FR and FR-S, fruiting body polysaccharide and its sulfated derivative; MI and MI-S, mycelial polysaccharide and its sulfated derivative.
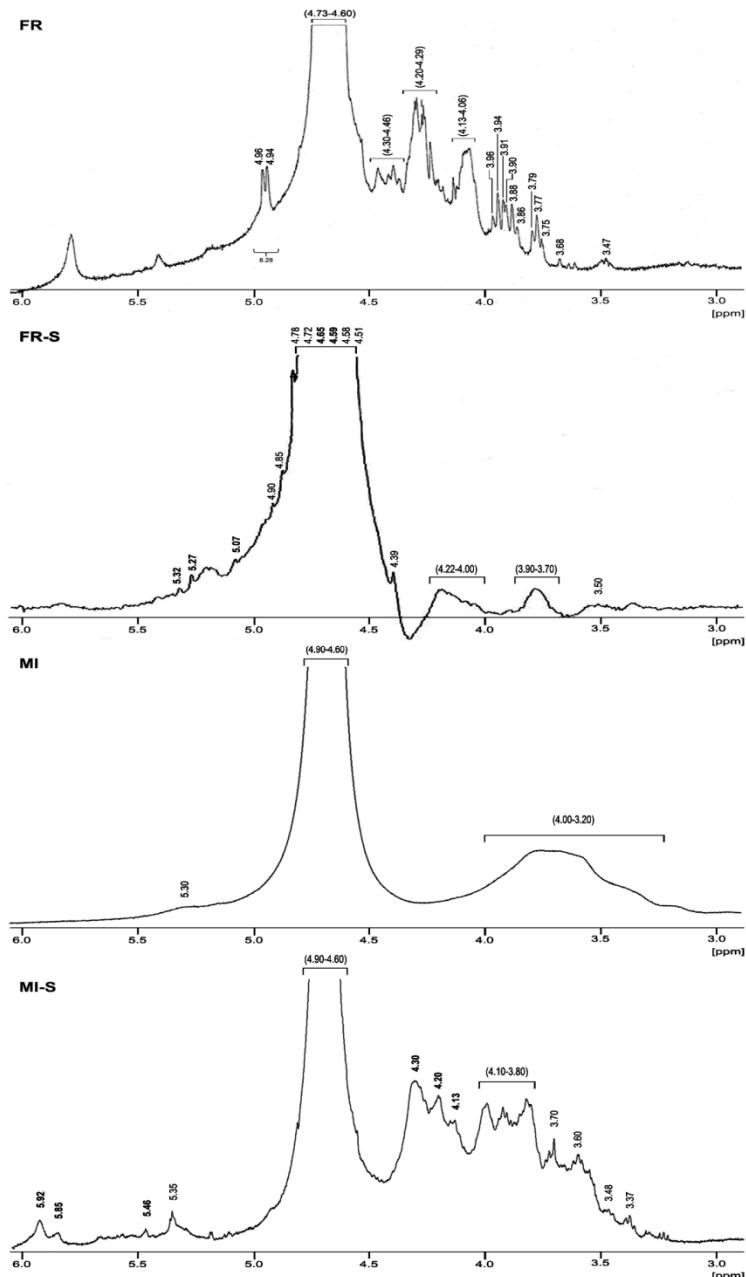
Supplementary Fig 5: ^{1}H NMR spectra of *Agaricus brasilie*nsis polysaccharides. FR and FR-S, fruiting body polysaccharide and its sulfated derivative; MI and MI-S, mycelial polysaccharide and its sulfated derivative.

Table 4: ^{13}C and ^1H NMR spectroscopic data of *Agaricus brasiliensis* polysaccharides and its sulfated derivatives

| Sample	Residue	Chemical shift (ppm)						
		C/H-1	C/H-2	C/H-3	C/H-4	C/H-5	C/H-6	C/H-6 term[c]
FR	β-(1→6)-D-glucose	103.14 (4.60-4.73)	73.18 3.47	75.71 3.75	(70.92, 68.97)[b] 3.68	75.03 3.88	69.60 (4.06-4.13)	63.34 (4.30-4.46)
FR-S	β-(1→6)-D-glucose	103.14 (4.72-4.78)	73.18 3.50	75.71 (3.70-3.88)	n.d. (**76.07**)[a], 68.95[b] n.d. (**4.65**)[a]	75.03 (4.00-4.22)	69.60 4.39	n.d. (**71.59**)[a] n.d. (**5.32**)[a]
FR	β-(1→3)-D-glucose	103.14 (4.96, 4.94)	73.18 3.86	n.d. (3.94, 3.96)	69.35 3.79	75.71 3.77	60.69 (3.90, 3.91)	63.34 (4.20-4.29)
FR-S	β-(1→3)-D-glucose	103.14 (4.85, 4.90)	73.18 (3.70-3.88)	n.d. 4.58	n.d. (**74.25**)[a] n.d. (**4.59**)[a]	75.71 (3.70-3.88)	60.69 n.d. (**5.07**)[a]	n.d. (**71.59**)[a] n.d. (**5.27**)[a]
MI	β-(1→2)-D-mannose	104.88, 98.55 5.30	78.59 (3.20-4.00)	74.11 (3.20-4.00)	72.26 (3.20-4.00)	78.40 (3.20-4.00)	63.25, 3.40 (3.20-4.00)	60.08 (3.20-4.00)
MI-S	β-(1→2)-D-mannose	105.71, 98.48 5.35	78.63 (3.80-4.10)	74.00 (**78.91-79.69**)[a] 3.70 (**4.30**)[a]	72.05 (**77.64**)[a] 3.60 (**4.20**)[a]	78.33 3.37	63.21 (**70.43**)[a] (3.80-4.10) (**5.46**)[a]	n.d. (**69.10**)[a] (3.80-4.10) (**5.92**)[a]
MI	β-(1→3)-D-glucose	102.56, 94.74 (4.60-4.90)	75.40 (3.20-4.00)	n.d. (3.20-4.00)	72.00 (3.20-4.00)	76.79 (3.20-4.00)	63.40 (3.20-4.00)	60.08 (3.20-4.00)
MI-S	β-(1→3)-D-glucose	102.27, 94.60 (4.60-4.90)	75.59 (**80.87-80.93**)[a] 3.60 (**4.20**)[a]	86.71 (3.80-4.10)	71.25 (**77.28**)[a] 3.48 (**4.13**)[a]	76.72 3.37	63.41 (**70.55**)[a] (3.80-4.10) (**5.85**)[a]	n.d. (**69.10**)[a] (3.80-4.10) (**5.92**)[a]

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative.

MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative.

[a]Assignments corresponding to the respective sulfation sites are shown in bold font between parentheses.

[b]Assignment for the C4 of β-(1→6)-D-glucose linked to the side chain in C3.

[c]term.: corresponding to the terminal sugar residues.

n.d.: not detected.

Chemical shifts were assigned using the CASPER program [60] and according to previously published data [16, 35, 42, 50]..

FR was characterized as a pure glucan, showing a major component with β-(1→6) linkages, and a minor amount of (1→3)-β-linkages. The β-(1→3)-linked H-1 of the side chain appeared as one resolved doublet centered at 4.95 ppm (*J*= 8.28 Hz), whose coupling constant value allowed to define its β-configuration. Although the signal around 86 ppm ascribed to C-3 of (1→3)-β-linkage was not detected in the FR spectrum, as reported by Ohno et al. [46], the remaining signals indicate the presence of (1→3)-β-glucan component. These results show that FR consists of a backbone (1→6)-β-glucan with (1→3)-β-glucan side chains attached to C-3, as previously described for the polysaccharides isolated from *A. brasiliensis* [16, 42] and *A. blazei* [35] fruiting body.

The sulfation of hydroxyl groups results in downfield shift of the carbons bearing sulfates and the protons linked to them by about 7-10 ppm and 0.5-2 ppm, respectively [47]. Hence, the ^{13}C and ^{1}H NMR spectra of FR-S showed downfield shifts of signals at 70.92/3.68 ppm and 63.34/4.30-4.46 ppm respectively to 76.08/4.65 and 71.59/5.32 ppm, indicating that the (1→6)-β-glucan portion was fully sulfated at C-4 and C-6 of the terminal residues. Similarly, the hydroxyl groups of (1→3)-β-glucan moiety in FR-S appear to have been fully sulfated at positions 4 and 6 of the terminal residues. The signal at 60.69 assigned to C-6 in the 1,3-β-chain increased in FR-S ^{13}C NMR spectrum in comparison to the one found for FR. However, the corresponding H-6 signal was not detected in ^{1}H NMR spectrum of FR-S, which most likely indicates the partial sulfation of C-6 hydroxyl groups. The low reactivity of other carbon positions could be attributed to steric hindrance [48].

As expected, the signals of the hydrogens linked to carbons bearing sulfate groups experienced downfield shifts from 0.60 to 2.03 ppm with respect to the unsubstituted polysaccharides in the ^{1}H NMR spectra of sulfated derivatives. Minor changes in chemical shifts were also observed on protons located in the vicinity of sulfation sites [49].

According to ^{13}C and ^{1}H NMR data, MI was characterized as a (1→3)-β-D-gluco-(1→2)-β-D-mannan, as previously described [15, 50]. The signal at 65.86 ppm probably results from the branching in C6 of the main chain. With regard to MI-S, sulfation at C-6 position of the terminal residue was nearly complete, both in the main and side chains, disclosed by the downfield shift of C-6 to a broad peak at 69.10 ppm. Partial sulfation was observed at C-3, C-4, and C-6 positions of the (1→2)-β-D-mannan moiety, as well as at C-2, C-4, and C-6 sites of the (1→3)-β-D-glucan side chain. A splitting pattern of C-3 signal (74.00

ppm) from $(1\rightarrow2)$-β-D-mannan and C-2 (75.59 ppm) from $(1\rightarrow3)$-β-D-glucan moiety can be attributed to the sulfation of adjacent carbons.

It is well known that sulfation of polysaccharides is responsible for changes in the original chain conformation usually resulting in alterations in their biological actions, including antiviral, cytotoxic, and antitumor activities [13, 51-57]. The inhibitory effects on A549 cell proliferation were concentration- and time-dependent and the results, expressed as EC_{50} values for the 48 and 72 h treatment period, are presented in Table 5. FR and MI had no cytotoxic effect in the 48 h treatment at 1,500 µg/ml. Sulfation of both preparations increased the cytotoxic activity with EC_{50} values of 605.6 and 342.1 µg/ml, respectively for FR-S and MI-S. Similarly DEX-S was more active than DEX.

Table 5: Inhibitory effect of *Agaricus brasiliensis* polysaccharides in A549 cell proliferation (MTT assay)

Sample	48h		72h		Increase in Cytotoxicity
	EC_{50}[a]	95% Confidence Interval	EC_{50}[a]	95% Confidence Interval	EC_{50} 72h/ EC_{50} 48h
FR	>1500	-	1147	1006 to 1307	>1.3
FR-S	605.6	440.6 to 832.5	222.5	153 to 323.6	2.7
MI	>1500	-	>1500	-	-
MI-S	342.1	275.4 to 425.1	60.66	50.41 to 73	5.6
DEX	>1500	-	>1500	-	-
DEX-S	991.6	600.5 to 1638	783.1	560.6 to 1094	1.3
Paclitaxel	0.40	0.27 to 0.58	0.056	0.03 to 0.11	7

FR and FR-S, *A. brasiliensis* fruiting body polysaccharide and its sulfated derivative;
MI and MI-S, *A. brasiliensis* mycelial polysaccharide and its sulfated derivative;
DEX and DEX-S, dextran (410 kDa) and dextran sulfate (> 500 kDa), Sigma.
[a]50% effective concentration (µg/mL)

In order to differentiate the cytotoxic (LC_{50}) and cytostatic (TGI) effects in the detected activity, FR-S and MI-S were evaluated by the sulforhodamine assay using A549 and Vero cells. Table 6 shows that FR-S and MI-S were cytotoxic in the higher concentrations tested, with MI-S appearing to be slightly more active. Moreover, Vero cells were found to be resistant to the cytotoxic effects of FR-S and MI-S. These results raise the possibility that these sulfated polysaccharides might

have selectivity for human cancer cells, but further studies are needed to confirm this hypothesis. As observed by other authors, the antiproliferative effect of sulfated polysaccharides depends on cell type [51, 58].

Table 6: Inhibitory effect of sulfated *Agaricus brasiliensis* polysaccharides in A549 and Vero cells growth by the sulforhodamine method

Parameter	FR-S	MI-S	Paclitaxel
		A549	
GI_{50}[a]	155.4	160.9	0.23
TGI[b]	598.8	488.3	0.19
LC_{50}[c]	1042	815.7	0.62
		Vero	
GI_{50}[a]	>1500	>1500	0.12
TGI[b]	>1500	>1500	0.46
LC_{50}[c]	>1500	>1500	1.05
SI[d]	>1.4	>1.8	1.7

FR-S, sulfated derivative of *A. brasiliensis* fruiting body polysaccharide; MI-S, sulfated derivative of *A. brasiliensis* mycelial polysaccharide.
[a]Median growth inhibition; [b]Total growth inhibition; [c]Median lethal inhibition. Values are expressed in µg/mL.
[d]Selectivity index: calculated as LC_{50} Vero/LC_{50} A549.

Previous studies have shown that there is an optimum degree of sulfation (DS) to reach the maximal biological response, which varies according to the polysaccharide type. For instance, Liu et al. [52], comparing polysaccharides with similar Mw (~20 kDa), observed a stronger inhibition of Hep 2 cells grown by polysaccharides with a DS of 1.8 in comparison to those with lower DS values (1.52), while the activity was reduced when the DS was increased to 2.02. Similarly, Bao et al. [55] demonstrated that sulfated polysaccharides with low DS (0.11-0.14) were less cytotoxic than those with higher values (0.28-066), whereas further increases in DS (1.06) reduced the activity. No direct relationship between the cytotoxic activity and DS values were observed in the present work and the differences on effectiveness might be related to other polymer characteristics. MI-S had a slightly higher cytotoxic activity than FR-S and both were more effective than the commercial DEX-S. Since DEX-S has a much higher Mw, we believe that this is an important feature for its reduced efficacy. Yang et al., [59] found a significantly higher cytotoxic activity for partially hydrolyzed fucoidans (Mw= 490 kDa) compared to the native polymers (Mw= 5,100 kDa). However, it is important to note that the sulfated polysaccharides

evaluated herein have different sugar compositions, chain conformations, and sulfation positions, and these features probably also contribute to the differences in their cytotoxicity.

4. Conclusion

The current study showed that the sulfation increased the cytotoxic activity of *A. brasiliensis* fruiting body polysaccharide, and was essential for the activity of the mycelial polysaccharide. Despite using identical conditions for chemical derivatization, distinct patterns of sulfation were obtained for MI-S and FR-S, most likely due to the differences in their native carbohydrate composition and structure. After chemical modification, structural analysis revealed that the chain structure of the compounds was preserved. MI-S, FR-S, and the control DEX-S presented cytotoxic effects inversely proportional to their Mw. The present findings increase the understanding of FR-S and MI-S structure-activity relationships and raise the possibility that these sulfated polysaccharides might display selective toxicity against tumor cells. Additional studies of these polysaccharides are clearly warranted.

5. Acknowledgments

Conflict of interest

Authors disclose any actual or potential conflict of interest in this work.

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CAPÍTULO II:
AVALIAÇÃO DO MECANISMO DA ATIVIDADE ANTI-HERPÉTICA DO DERIVADO SULFATADO DO POLISSACARÍDEO OBTIDO DOS CORPOS DE FRUTIFICAÇÃO DO FUNGO *Agaricus brasiliensis*

1. APRESENTAÇÃO

Uma vez que os polissacarídeos isolados das frutificações (FR) e do micélio (MI) do *A. brasiliensis* não apresentaram atividade anti-herpética *in vitro* e com base no conhecimento de que polissacarídeos sulfatados apresentam potencial ação antiviral, foi realizada a sulfatação química destes polímeros gerando os derivados FR-S e MI-S. Ambas as amostras apresentaram promissora atividade anti-herpética *in vitro* e tiveram seu mecanismo de ação determinado. Assim, foram elaborados dois artigos. O primeiro que trata do FR-S, incuído no capítulo II desta tese e será submetido à revista *Letters of Applied Microbiology*. O segundo artigo trata do MI-S, incluído no Capítulo III, tendo sido publicado, em 2011, no periódico *Antiviral Research*.

2. ARTIGO A SER SUBMETIDO PARA AVALIAÇÃO AO PERIODICO LETTERS OF APPLIED MICROBIOLOGY

TITLE: Antiherpetic mechanism of a sulfated derivative of *Agaricus brasiliensis* fruiting bodies polysaccharide

RUNNING HEADLINE: Anti-HSV mechanism of a sulfated polysaccharide

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Abstract

Aims: This study aimed to evaluate the cytotoxicity and mechanism of antiherpetic activity of the sulfated derivative of a polysaccharide extracted from *Agaricus brasiliensis* fruiting bodies (FR-S).

Methods and Results: The mechanism of anti-HSV activity was evaluated by viral plaque assay applying different methodological strategies. FR-S suppressed HSV-1 and HSV-2 attachment ($EC_{50}=0.32$ and $0.10\mu g$ ml^{-1}) and penetration ($EC_{50}=8.39$ and $2.86\mu g$ ml^{-1}), respectively. FR-S efficiently reduced cell-to-cell spread of HSV-1 (57.14%) and HSV-2 (54.76%), at $6.74\mu g$ ml^{-1} and $4.72\mu g$ ml^{-1}, respectively. The synergistic antiviral effect between FR-S and acyclovir was also verified using Calcusyn software.

Conclusions: FR-S was categorized as a potent viral entry inhibitor since it significantly reduced HSV attachment and penetration, probably by interacting with viral and cellular components. FR-S also displayed an efficient reduction of HSV-1 and HSV-2 cell-to-cell spread as well as synergistic antiherpetic effect with acyclovir.

Significance and Impact of Study: In this study we demonstrated that FR-S displays an interesting mechanism of antiviral action, and might represent a promising drug candidate for the treatment of herpetic infections, useful as a single therapeutic agent or in combination with acyclovir.

Keywords: *Agaricus brasiliensis*; sulfated polysaccharide; antiherpes activity; Herpes Simplex Virus type 1 (HSV-1); Herpes simplex virus type 2 (HSV-2); mechanism of action; synergistic effect.

1. Introduction

Herpes simplex virus (HSV) types 1 and 2 belong to the family Herpesviridae, subfamily Alphaherpesvirinae, and are responsible for a variety of mucocutaneous infections highly prevalent worldwide. Factors such as the high morbidity in immunocompromised patients, the emergence of drug-resistant virus strains, and the undesirable side effects of the current antiviral chemotherapy has encouraged the search for new antiherpetic agents (Roizman et al. 2007).

The HSV attachment and entry into target cells, as well as virus cell-to-cell spread, entail the interaction of viral envelope glycoproteins with host cell surface receptors. These complex processes include a series of sequential steps and are essential for a productive infection in humans and, therefore, represent suitable targets for drug development. Three viral entry inhibitors have already been approved by the FDA: *n*-docosanol for oro-labial herpes infections, and enfuvirtide and maraviroc for HIV-1 infections (FDA 2012).

Agaricus brasiliensis (syn *A. subrufescens*) is closely related to *A. blazei*. This Brazilian mushroom has been traditionally used worldwide for prevention and treatment of various illnesses, including cancer, physical and mental stress, osteoporosis, diabetes, hyperlipidemia, arteriosclerosis, and hepatitis (Firenzuoli et al. 2008). *In vitro* and *in vivo* studies have shown mainly immunomodulatory (Kozarski et al. 2011), antitumoral (Gonzaga et al. 2009), and antigenotoxic activities for this species (Angeli et al. 2009). Some authors have previously demonstrated the antiviral activity of *A. brasiliensis* extracts against HSV, Poliovirus, and Western equine encephalitis virus (Sorimachi et al. 2001), HSV-1 (Bruggemann et al. 2006) and bovine herpesvirus 1 (Bruggemann et al. 2006; Minari et al. 2011), and poliovirus 1 (Faccin et al. 2007). Recently, our group has reported the anti-HSV-1 and 2 activity of a sulfated derivative of a polysaccharide obtained from *A. brasiliensis* mycelia (Cardozo et al. 2011). Herein we endeavored to underline the antiherpetic mechanism of the sulfated derivative of a polysaccharide obtained from fruiting bodies of the same species by evaluating its action on virus viability, attachment, penetration, and cell-to-cell spread.

2. Materials and methods

2.1 Fungal Material

Agaricus brasiliensis fruiting bodies were collected in Biguaçu (Santa Catarina State, Brazil), and cultivated in the Bioprocess Laboratory - UFSC. Species identification was performed by Dr Maria Alice Neves and a voucher specimen was deposited in the FLOR Herbarium (FLOR 11 797, UFSC). Afterward, the dried fruiting bodies were ground, lyophilized, and stored at -80ºC until use.

2.2 Isolation of fruiting bodies polysaccharide

The polysaccharide was obtained as reported by Cardozo et al., (2011). Briefly, 50g of lyophilized fruiting bodies were extracted with water at 100ºC (3h), precipitated with ethanol, and centrifuged (1100g, 10min). The obtained polysaccharide (FR) was freeze-dried and chemically sulfated according to the methodology described previously (Zhang et al. 2003). The highest molecular mass polysaccharides were separated by dialysis (Cut-off 3.5kDa) and freeze-dried generating the sulfated derivative (FR-S).

2.3 Cells and viruses

Vero (ATCC:CCL 81, Rockville, MD, USA) and GMK AH1 (Department of Clinical Virology, Göteborg University, Göteborg, Sweden) cells were grown in minimum essential medium (MEM, Cultilab, Brazil), supplemented with 10% fetal bovine serum (Cultilab), penicillin (100U ml^{-1}), streptomycin (100µg ml^{-1}), and amphotericin B (25µg ml^{-1}) (Cultilab), and maintained at 37ºC in a humidified 5% CO_2 atmosphere. The virus strains used were: HSV-1 KOS and HSV-1 29R (Faculty of Pharmacy, University of Rennes, France); HSV-1 gC-null variant gC⁻39 (Holland et al. 1983); HSV-2 strain 333 (Duff and Rapp 1971) and its gC-negative designated HSV-2 gCneg1 (Trybala et al. 2000). HSV-1 and HSV-2 were propagated in Vero and GMK AH1 cells, respectively. Virus titers were determined by plaque assay and expressed as plaque forming units (PFU ml^{-1}), as reported by Burleson et al. (1992).

2.4 Cytotoxicity evaluation

The cytotoxicity of samples on Vero and GMK-AH1 cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann 1983), with minor modifications. Briefly, 2×10^4 cells per well were cultivated in 96-well plates for 24h. Then, the growth medium was replaced by 1:2 serial dilutions of each sample diluted in MEM. After 72h of incubation, the medium was substituted by MTT solution (1mg ml^{-1}) and plates were further incubated for 4h. MTT solution was removed and DMSO was added to dissolve formazan crystals. The optical densities were read at 540nm and concentrations of the samples sufficient to reduce cell viability by 50% (CC_{50}) were estimated by linear regression of concentration-response curves generated from the data. Acyclovir (ACV, A4669) and heparin (HEP, H9399) were purchased from Sigma (USA) and were employed as controls in all experiments. Stock solutions of samples and controls were prepared in MEM and maintained at -20°C until use.

2.5 Evaluation of antiherpes activity

For the plaque number reduction assay, confluent cells were infected with HSV-1 KOS or HSV-2 333 (100 PFU per well) at 37°C for 1h. Different concentrations of samples were added either simultaneously with virus (simultaneous treatment) or after virus infection (post-infection treatment). After virus infection, plates were further incubated at 37°C for 48 and 72h for HSV-1 and HSV-2, respectively. Viral plaques were visualized by staining with naphtol blue-black (Sigma), and enumerated using a stereomicroscope. The concentrations of samples required to reduce plaque number by 50% (EC_{50}) and the respective selectivity indices ($SI=CC_{50}/EC_{50}$) were calculated by standard methods (Burleson et al. 1992).

2.6 Evaluation of mechanism of action

2.6.1 Virucidal assay

The direct effect of FR-S on HSV infectivity was assessed according to procedures described previously (Cardozo et al. 2011). Equal volumes of samples (20μg ml^{-1}) and 4×10^4 PFU of HSV (KOS or HSV-2) in serum-free MEM were co-incubated for 20min at 4, 22, or 37°C. Ethanol (70% v/v) was used as a positive control. The samples were then diluted to non-inhibitory concentrations (1:1000) to determine

residual infectivity by plaque number reduction assay, as described above (item 2.5).

2.6.2 Effect on virus adsorption

Four distinct experimental protocols (treatments A to D) were employed to investigate the inhibition of HSV-1 (strains KOS and gC-39) and HSV-2 (strains 333 and gCneg1) adsorption by the samples.

Treatment A (attachment assay): cells monolayers grown in 24-well culture plates were pre-chilled at 4°C for 1h and exposed to viruses (100 PFU per well) in the absence or presence of serial dilutions of the sample (FR-S) and controls (HEP and ACV). After incubation for 2h at 4°C (adsorption period), cells were washed with ice-cold PBS to remove samples and unbound viruses. As a control, wells in which viruses had been preattached to cells were treated with citrate buffer (pH 3.0) to verify that incubation at 4°C allowed only viral attachment avoiding viral entry. Subsequently, cells were overlaid with CMC medium (MEM containing 1.5% carboxymethylcellulose) and the inhibitory activity of the samples was determined by the plaque number reduction assay, as described above (item 2.5).

Treatment B (pretreatment of virions): virus suspensions were incubated with the samples for 2h at 4°C. After that, the mixtures were added to pre-chilled cell monolayers to allow viral attachment. After the adsorption period (2h, 4°C), the same procedures described for the treatment A were performed.

Treatment C (pretreatment of cells): confluent cells grown in 24-well culture plates were pretreated for 1h at 37°C with different concentrations of the samples and then washed with PBS. Afterward, cells were chilled at 4°C for 1h, and exposed to viruses for another 2h. The inhibitory effect was then determined as described in the treatment A.

Treatment D (post-attachment assay): pre-chilled cells were firstly incubated with virus suspensions at 4°C for 2h to allow a stable attachment of HSV to cells. Unbound viruses were removed by aspiration and cells were washed with ice-cold PBS. Samples were then added and plates were further incubated at 4°C for another 2h. The remaining steps were conducted as described above.

2.6.3 Penetration assay

In order to examine the effect of samples on penetration of HSV-1 (KOS or gC-39) and HSV-2 (333 or gCneg1), cells were incubated

with viruses for 2h at 4°C. After the removal of unbound viruses, the temperature was shifted to 37°C to allow penetration and cells were treated with different concentrations of pre-warmed samples. After the penetration period (1h, 37°C), non-penetrated viruses were inactivated with citrate buffer (pH 3.0). Cells were washed twice with PBS, and CMC overlay medium was added to allow plaque formation as described previously. The percentage of inhibition was calculated based on the reduction of plaque number.

2.6.4 Plaque size reduction assay

The effect of tested samples on HSV cell-to-cell spread was investigated as previously described (Cardozo et al. 2011). In brief, infected cells (100 PFU per well) were treated with FR-S, HEP, or ACV at concentrations equivalent to their corresponding EC_{50} values and incubated throughout the entire period of viral plaques development. Results were obtained by analyses of the images of 20 plaques per each sample concentration, and compared with the untreated controls. The area of each plaque was determined using the Image J software (http://rsb.info.nih.gov/ij/).

2.7 Synergistic effect in combination with acyclovir

The effects of FR-S in combination with ACV was evaluated by plaque reduction assay as described above and according to the experimental design proposed by Chou (2006). Briefly, each drug alone or in combination was tested at a fixed ratio of its corresponding EC_{50} value (i.e. at EC_{50} x 0.25, x 0.5, x 1, x 2, and x 4). The interaction degree between FR-S and ACV was calculated through combination index (CI) equation, using Calcusyn software (version 2.1, Biosoft®). According to the CI theorem, CI values < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively.

2.8 Anticoagulant assay

The anticoagulant activity of FR-S was evaluated using the activated partial thromboplastine time (APTT) assay (Andersson et al. 1979). Heparin was used as reference substance at the same concentration (10 µg ml^{-1}). PBS was used as negative control. Briefly, 50 µL of each sample was added to 100 µL of pooled human plasma and 100 µL of APTT reagent (Biotécnica, Brazil). The mixtures were

incubated for 3 min at 37ºC. After that, preheated calcium chloride (100 µL, 20 mmol l^{-1}) was added and clot time was recorded.

2.9 Statistical analysis

Data are presented as mean ± standard deviation of three independent experiments. The differences between tested samples and controls were analyzed by one-way ANOVA followed by Dunnett test. A *p* value less than 0.05 was considered to be statistically significant.

3. Results

3.1 Antiviral activity

Different *A. brasiliensis* polysaccharides were initially evaluated in terms of cytotoxicity by assessing their effects on Vero and GMK AH1 cells viability. The antiviral activity was evaluated by plaque number reduction assay using simultaneous and post-infection treatments. These results reveal that FR has no antiviral activity, while FR-S inhibited both HSV-1 and HSV-2 replication (Table 1).

Table 1: Cytotoxicity and antiherpetic activity of *Agaricus brasiliensis* polysaccharides

Sample	Cytotoxicity		Simultaneous treatment				Post-infection treatment			
	VERO	GMK AH1	HSV-1 (KOS)		HSV-2 (333)		HSV-1 (KOS)		HSV-2 (333)	
	CC_{50}*	CC_{50}*	EC_{50}[†]	SI[‡]	EC_{50}[†]	SI[‡]	EC_{50}[†]	SI[‡]	EC_{50}[†]	SI[‡]
FR	2370 ± 60	>2500	NI	-	NI	-	NI	-	NI	-
FR-S	2650 ± 190	2520 ± 460	1.43 ± 0.06	1853	0.62 ± 0.08	4064	6.74 ± 0.01	393	4.62 ± 0.38	545
HEP	>2500	>2500	1.15 ± 0.30	>2174	0.58 ± 0.01	>4310	12.68 ± 1.13	>197	8.06 ± 1.46	>310
ACV	>2500	>2500	NI	-	NI	-	0.51 ± 0.05	> 22727	3.91 ± 0.41	>639

*50% cytotoxic concentration; [†]EC_{50}: 50% effective concentration. [‡]Selectivity index (=CC_{50}/EC_{50}); NI: No inhibitory activity; FR: *A. brasiliensis* fruiting bodies polysaccharide; FR-S: Sulfated *A. brasiliensis* fruiting bodies polysaccharide; HEP: Heparin; ACV: Acyclovir. Values represent the mean ± standard deviation of three independent experiments in μg ml^{-1}.

3.2 Virucidal activity

Preincubation of virus suspensions with FR-S, HEP, and ACV at 4, 22, and 37ºC had no significant inactivating effects on HSV-1 KOS and HSV-2 333 at the tested concentrations (data not shown). These results indicate that virucidal effects do not seem to be involved in the detected antiviral activity of FR-S. Ethanol (70% v/v) presented 100% inhibition of residual virus infectivity, confirming the assay validity.

3.3 Inhibition of viral adsorption and penetration

The EC_{50} values obtained on different adsorption and penetration assays are summarized in Tables 2 and 3, respectively. Results showed that FR-S strongly inhibited viral adsorption in all treatments, while HEP was only effective in treatments A and B. The results presented in Table 3 reveal that FR-S inhibited the HSV penetration more significantly than HEP for all virus strains tested. As expected, acyclovir did not impair viral adsorption and penetration.

Table 2: Effect of *Agaricus brasiliensis* sulfated polysaccharide (FR-S) on HSV adsorption

Treatment protocol	Virus(strain)	EC_{50}[†]		
		FR-S	HEP	ACV
(A)Attachment assay	HSV-1(KOS)	0.32 ± 0.05	0.33 ± 0.04	NI
	HSV-2(333)	0.10 ± 0.04	0.07 ± 0.01	NI
	HSV-1(gC⁻39)	2.07 ± 0.06	2.16 ± 0.41	NI
	HSV-2(gCneg1)	0.51 ± 0.13	0.54 ± 0.03	NI
(B)Pretreatment of virions	HSV-1(KOS)	0.20 ± 0.01	0.23 ± 0.03	NI
	HSV-2(333)	0.08 ± 0.04	0.03 ± 0.01	NI
	HSV-1(gC⁻39)	1.00 ± 0.05	0.91 ± 0.04	NI
	HSV-2(gCneg1)	0.11 ± 0.01	0.02 ± 0.01	NI
(C)Pretreatment of cells	HSV-1(KOS)	68.26 ± 2.21	> 100.00	NI
	HSV-2(333)	6.09 ± 1.81	> 10.00	NI
	HSV-1(gC⁻39)	3.49 ± 1.56	> 10.00	NI
	HSV-2(gCneg1)	2.43 ± 1.07	> 10.00	NI
(D)Post-attachment assay	HSV-1(KOS)	3.00 ± 0.01	> 10.00	NI
	HSV-2(333)	7.95 ± 0.93	> 10.00	NI
	HSV-1(gC⁻39)	8.76 ± 0.83	> 10.00	NI
	HSV-2(gCneg1)	6.70 ± 1.11	> 10.00	NI

[†]EC_{50}: 50% effective concentration ($\mu g\ ml^{-1}$). NI = no inhibitory activity; FR-S: Sulfated *A. brasiliensis* fruiting bodies polysaccharide; HEP: Heparin; ACV: Acyclovir. Values represent the mean ± standard deviation of three independent experiments.

Table 3: Effect of *Agaricus brasiliensis* sulfated polysaccharide (FR-S) on HSV penetration

Virus (strain)	EC_{50}[†]		
	FR-S	HEP	ACV
HSV-1(KOS)	8.39 ± 0.36^{b}	13.34 ± 3.64^{c}	NI
HSV-2(333)	2.86 ± 0.83^{a}	6.47 ± 0.21^{b}	NI
HSV-1(gC⁻39)	9.53 ± 0.90^{b}	17.99 ± 4.06^{c}	NI
HSV-2(gCneg1)	1.97 ± 0.21^{a}	5.21 ± 0.69^{b}	NI

[†]EC_{50}: 50% effective concentration ($\mu g\ ml^{-1}$). NI = no inhibitory activity; FR-S: Sulfated *A. brasiliensis* fruiting bodies polysaccharide; HEP: Heparin; ACV: Acyclovir. Values represent the mean ± standard deviation of three independent experiments. Different letters indicate statistically significant differences (one way ANOVA/Tukey's test).

3.4 Inhibition of viral cell-to-cell spread

The reduction of HSV-1 and HSV-2 lateral spread was evaluated by measuring 20 viral plaque areas in infected cells treated with FR-S, HEP, and ACV at concentrations equivalent to their EC_{50} values (Fig. 1).
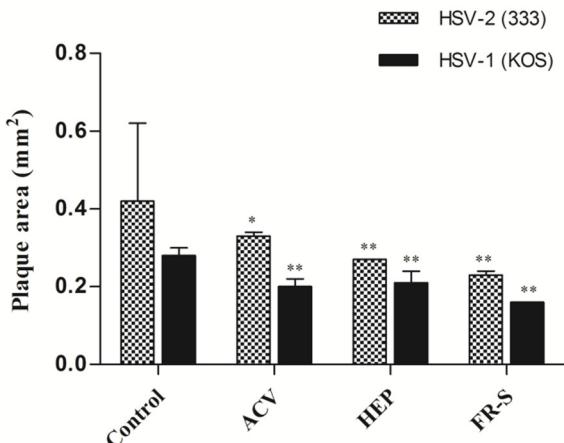
Fig 1: Inhibition of HSV cell-to-cell spread by FR-S (sulfated *A. brasiliensis* polysaccharide), HEP (heparin), and ACV (acyclovir) at their corresponding EC_{50} concentrations. FR-S, HEP, and ACV were evaluated at 6.74, 12.68, and $0.51 \mu g\ ml^{-1}$ for HSV-1(KOS); and at 4.62, 8.06, and $3.91 \mu g\ ml^{-1}$ for HSV-2(333), respectively. Results are expressed as mean area $\pm$ SD of viral plaques developed in cells sample-treated and untreated (controls). ANOVA/Dunnett's test showed significant differences in relation to controls with p values $<0.05(*)$ and $<0.01(**)$.

3.5 Synergism with acyclovir

Results of combinations between FR-S and acyclovir are compiled in Table 4. Different concentrations of both compounds, based in their respective EC50 values, were tested. The percentagens of inhibition increased in comparison to each compound alone and since all

combinations tested presented CI values less than 1, FR-S was shown to have a synergistic anti-HSV effect with ACV for both tested viruses.

Table 4: Synergistic anti-HSV effects of sulfated *Agaricus brasiliensis* polysaccharide (FR-S) in combination with acyclovir (ACV)

Compounds combination ratio	Sample concentration (μg ml^{-1})		Mean percentage of inhibition (%) FR-S + ACV	Experimental CI values	Description (Graded symbols)
	FR-S	ACV			
HSV-1 (KOS)					
4 x EC$_{50}$	26.96	2.04	100.00	0.662	Synergism (+ + +)
2 x EC$_{50}$	13.48	1.02	100.00	0.572	Synergism (+ + +)
1 x EC$_{50}$	6.74	0.51	99.22	0.286	Strong synergism (+ + + +)
0.5 x EC$_{50}$	3.37	0.26	69.15	0.239	Strong synergism (+ + + +)
0.25 x EC$_{50}$	1.68	0.13	55.19	0.443	Synergism (+ + +)
HSV-2 (333)					
4 x EC$_{50}$	18.88	15.64	100.00	0.550	Synergism (+ + +)
2 x EC$_{50}$	9.44	7.82	100.00	0.312	Strong synergism (+ + + +)
1 x EC$_{50}$	4.72	3.91	94.51	0.171	Strong synergism (+ + + +)
0.5 x EC$_{50}$	2.36	1.96	86.20	0.192	Very strong synergism (+ + + + +)
0.25 x EC$_{50}$	1.18	0.98	52.63	0.367	Strong synergism (+ + + +)

CI: Combination Index is a quantitative index calculated by Calcusyn software, which quantifies the interaction between the tested compounds, as described by Chou (2006). In detail, the ranges of CI <0.1, 0.10-0.30, 0.31-0.70, 0.71-0.85, and 0.86-0.90 mean very strong synergism, strong synergism, synergism, moderate synergism, and slight synergism, respectively. Obtained values represent the mean of three independent experiments.

3.6 Anticoagulant activity

The APTT obtained values for FR-S (15 µg ml-1) and PBS were 82.01 ± 7 and 56.67 ± 1.53 s, respectively. Differently, no coagulation occurred for HEP at 1 µg ml-1 under experimental conditions. These results indicated that, differently from HEP, FR-S has a slight anticoagulant activity at its 100% anti-HSV concentration.

4. Discussion

The chemical modification of carbohydrate polymers, especially sulfation, has been often applied to improve their intrinsic biological properties or provide new functional effects, including antiviral activities (Ghosh et al. 2009; Karmakar et al. 2010; Liu et al. 2011). In this work, attempts were made to chemically sulfate a polysaccharide isolated from *A. brasiliensis* fruiting bodies (FR). Since its sulfated derivative (FR-S) presented a promising antiherpetic activity, further investigations focused on determining its mechanism of action and comparing its activity with that of the sulfated derivative from the *A. brasiliensis* mycelial polysaccharide (MI-S) previously published (Cardozo et al. 2011).

Firstly, the viral plaque number reduction assay was performed under two distinct conditions: simultaneous treatment (samples were added to the cells at the same time of virus infection) and post-infection treatment (samples were added 1h after virus infection). FR-S was considerably more effective on simultaneous treatment than on post-infection treatment (Table 1), probably due to its inhibitory capacity on virus adsorption and penetration as discussed further, agreeing to previous reports on sulfated polysaccharides (Carlucci et al. 1999; Zhang et al. 2004; Karmakar et al. 2010). In the simultaneous treatment, EC_{50} values obtained for FR-S and HEP were similar, while in the post-infection treatment the inhibitory effect of HEP was approximately 50% lower than FR-S effect, for both tested viruses (Table 1). The structural diversity between HEP and FR-S, even though both compounds are sulfated polysaccharides, seemed to interfere particularly with their inhibitory capacity when samples were added after viral entry. The HEP used in this study is a linear glycosaminoglycan with molecular mass lower (~18kDa) than FR-S (127kDa), a branched $(1{\rightarrow}6)$-$(1{\rightarrow}3)$-β-glucan fully sulfated at C-6 and partially sufated at C-4 (Cardozo et al.). When comparing the inhibition of HSV-1 and HSV-2 replication, HSV-2 was observed to be more sensitive to FR-S and HEP, while HSV-1 was more sensitive to acyclovir. This relationship has already been reported for these two control drugs (Herold et al. 1996; Li et al. 2003).

Heparin, whose *in vitro* anti-HSV activity was firstly published by Nahmias and Kibrick (1964), is a sulfated polysaccharide analogous to heparan sulfate and widely used as anticoagulant (Rabenstein 2002). Since sulfated polysaccharides may interfere with the blood coagulation process, the FR-S anticoagulant activity was also evaluated. Differently from HEP, FR-S had only a slight anticoagulant activity at 100% viral

inhibitory concentration, which represents an advantage for a drug candidate. However, *in vivo* studies are needed to further assess potential side-effects likeness and clinical applicability of FR-S for human use.

Previous reports demonstrated the virucidal activity against HSV for sulfated polysaccharides types isolated from marine algae, such as lambda carrageenan (Carlucci et al. 1999), kappa-2 carrageenan, fucan, galactan, and galactofucan (Harden et al. 2009). In order to investigate if FR-S exerts its antiviral activity by direct virus particle inactivation, the virucidal assay was carried out. Since HSV infectivity was not impaired by FR-S, we were led to believe that the antiherpetic activity detected by plaque number reduction assay was due to the interference on the HSV multiplication cycle. Bruggemann et al. (2006) reported the HSV-1 virucidal activity for an aqueous extract of *A. brasiliensis*, but these researchers have employed different experimental design and the sample extraction was performed in water at room temperature without sulfation step.

Attachment and entry are among the multiple steps of HSV life cycle which have attracted researcher's attention as potential anti-infective targets. Adsorption and penetration are complex biochemical processes involving five (gB, gC, gD, gH-gL, gK) of the twelve known HSV surface glycoproteins. gC-1 has been reported to play a more important role in the attachment of wild-type HSV-1 than gB-1, while gB mediates the binding of gC-null mutants. Regarding gB, its contribution has been suggested to be higher for HSV-2 than for HSV-1 binding (Gerber et al. 1995; Trybala et al. 2000). According to the most accepted opinion, antiviral sulfated polysaccharides act by interfering with electrostatic interaction between viral glycoproteins and cell surface glycosaminoglycans (Bergefall et al. 2005). Herein, mutant virus devoid of gC were used in an effort to investigated the involvement of this glycoprotein in the antiherpetic activity of FR-S. Four different experimental strategies (A to D) were applied in the adsorption assays. Data shown in Table 2 demonstrated that HSV-1 gC-negative mutant virus was considerably less sensitive to FR-S than the parental strain (HSV-1 KOS) in the protocols A and B. Similarly, but to a less extent, EC_{50} values for HSV-2 333 in treatment A were lower than for HSV-2 g-C negative variant. These results indicate a possible interaction between gC and FR-S, as occurs for HEP (Rux et al. 2002). The findings also suggest differences in FR-S binding proprieties between HSV-1 and HSV-2 or simply might be associated to the fact that gC plays a more important role in the attachment of HSV-1 than that of HSV-2.

Furthermore, FR-S could bind to other membrane targets in addition to gC, similarly to HEP, which binds mostly to gC, but also gB (Gerber et al. 1995; Cheshenko and Herold 2002). Whether FR-S interacts with these HSV glycoproteins remains to be confirmed.

When virions where pretreated with samples (treatment B), FR-S and HEP were more active than in the treatment A. These results support the possible interaction of FR-S with viral particles, avoiding virus adsorption into host cells, which has already been described for heparin (Herold et al. 1996).

Interestingly, when cells were treated with samples prior to viral attachment (treatment C), FR-S has also reduced the viral infectivity, even though this effect occurred at higher concentrations, suggesting that FR-S may further interact with cell surface components in addition to virus, blocking virus-cell interactions in entry events. Differently, HEP had no inhibition at tested concentrations.

When added to cell cultures after the initial binding period (treatment D), FR-S was also able to inhibit virus infectivity, whereas HEP was not active at tested concentrations. Taken together, these considerations support that, differently from HEP, FR-S may act by interacting with both viral and cellular structures, being able to inhibit viral adsorption and post-binding events.

After the initial viral attachment to cellular glycosaminoglycans, further interaction of viral glycoproteins, specially gB, gD, and gH, with cell receptors is required for entry process (Roizman et al. 2007). As shown in Table 3, FR-S (branched polymer) inhibited the penetration of all HSV tested strains more efficiently than HEP (linear polymer). These results bring the hypothesis that branching affects positively the ability of polysaccharides to inhibit viral adsorption and penetration. There were no statistical differences between the EC_{50} values of wild type and gC-null strains for FR-S and HEP during the penetration assay. This behavior may be due to the minor role played by gC in HSV penetration into cells.

Viral glycoproteins interfering with HSV entry (gB, gD and gH/gL) are also involved in HSV cell-to-cell spread, as well as the heterodimer gE/gI. Since the rapid spread between adjacent cells allows virus escape from host's immune response, the inhibition of intercellular diffusion is a potential target for therapeutic intervention (Roizman et al. 2007). Figure 1 shows the mean plaque areas of infected cells (HSV-1 KOS or HSV-2 333) treated with samples at their corresponding EC_{50} post-infection values, i.e. at concentrations in which the reduction of plaque number was almost identical (50%). Despite all tested

compounds significantly reduced plaque areas in comparison to untreated controls, infected cells treated with FR-S displayed the smallest plaque areas for both viruses. In our previous study we have also found a virus cell-to-cell spread inhibition for the sulfated derivative from *A. brasiliensis* mycelia (MI-S) (Cardozo et al. 2011). The HSV-1 cell-to-cell spread inhibition by sumarin, a polysulfonated napthylamine, was reported by Aguilar et al., (Aguilar et al. 1999). Other sulfated compounds that inhibited HSV-1 and HSV-2 intercellular diffusion were a capsular sulfated polysaccharide from *E. coli* (Pinna et al. 2008), disulfated cyclitols (Ekblad et al. 2006), a sulfonated oligosaccharide (PI-88) (Nyberg et al. 2004) and its glycoside derivative (Ekblad et al. 2010). A study performed by Cheshenko et al. (2004) showed that some sulfated/sulfonated compounds (PRO 2000, polystyrene sulfonate, cellulose sulfate, and polymethylenehydroquinone sulfonate) inhibited HSV-2 attachment, penetration, and cell-to-cell spread. However, Bergefall et al. (2005) did not find any inhibition of HSV-1 and HSV-2 cell-to-cell spread by the chondroitin sulfate polysaccharide.

Our previous paper demonstrated that a sulfated derivative from a polysaccharide obtained from *A. brasiliensis* mycelia has a similar antiherpetic mechanism and effectiveness (Cardozo et al. 2011), showing the potential for drug development of polysaccharides from this species. In this paper, FR-S was also found to have a synergistic anti-HSV effect with acyclovir, and therefore their combination might be useful on antiherpetic therapy.

In summary, FR-S presents a promising antiherpetic activity proved through the efficient inhibition of viral adsorption, penetration, and cell-to-cell spread, as well as acting synergistically with acyclovir.

Acknowledgements

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**CAPÍTULO III:
AVALIAÇÃO DO MECANISMO DA ATIVIDADE
ANTI-HERPÉTICA DO DERIVADO SULFATADO DO
POLISSACARÍDEO MICELIANO DO FUNGO
*Agaricus brasiliensis***

1. APRESENTAÇÃO

Conforme mencionado na apresentação do capítulo anterior, este capítulo trata da avaliação do mecanismo da atividade anti-herpética do MI-S, dados que foram compilados em um artigo, que já foi publicado no periódico *Antiviral Research*.

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Antiherpetic activity of a sulfated polysaccharide from *Agaricus brasiliensis* mycelia

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ABSTRACT

Sulfated polysaccharides are good candidates for drug discovery in the treatment of herpetic infections. *Agaricus brasiliensis* (syn *A. subrufescens*, *A. blazei*) is a Basidiomycete fungus native to the Atlantic forest region of Southeastern Brazil. Herein we report the chemical modification of a polysaccharide extracted from *A. brasiliensis* mycelia to obtain its sulfated derivative (MI-S), which presented a promising inhibitory activity against HSV-1 [KOS and 29R (acyclovir-resistant) strains] and HSV-2 strain 333, with selectivity indices (SI = CC_{50}/IC_{50}) higher than 439, 208, and 562, respectively. The mechanisms underlying this inhibitory activity were scrutinized by plaque assay with different methodological strategies. MI-S had no virucidal effects, but inhibited HSV-1 and HSV-2 attachment, penetration, and cell-to-cell spread, as well as reducing the expression of HSV-1 ICP27, UL42, gB, and gD proteins. MI-S also presented synergistic antiviral effect with acyclovir. These results suggest that MI-S presents multiple modes of anti-HSV action.

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1. Introduction

Herpes Simplex Virus types 1 and 2 (HSV-1 and HSV-2) are human neurotropic viruses usually associated with infections of the skin and mucosae of different locations, most commonly the oral and genital regions. Although infections are often subclinical, HSV can cause mild to severe diseases, especially in neonates and immunocompromised individuals. Currently, there is no cure for the persistent infection, and prolonged therapy with the available antiherpes drugs has induced the emergence of drug-resistant virus strains. Moreover, HSV has been described as a risk factor for HIV infection (Roizman et al., 2007). This scenario has triggered the search for new antiherpetic agents, especially those with mechanisms of action different from that of nucleoside analogs, the major class of antiviral agents used for the management of HSV infections. Besides, a treatment based on the combination of different antiviral agents can be considered a promising approach to increase antiviral selectivity while simultaneously enabling the reduction of the active concentrations of the drugs (Chou, 2006).

Many synthetic or naturally occurring sulfated polysaccharides from different species of marine algae, bacteria, fungi, and animals have been previously shown to have antiviral activity against human and animal viruses (Ghosh et al., 2009). In the case of fungi, cell wall polysaccharides have been chemically modified to increase their solubility and enhance their biological activities (Liu et al., 2010), including their antiviral action (Zhang et al., 2004).

The pharmacological effects of *Agaricus brasiliensis*, a Basidiomycete fungus native to the Brazilian Atlantic forest region, have been mainly related to the presence of polysaccharides and protein–polysaccharide complexes (Firenzuoli et al., 2008). Concerning its previous antiviral evaluation, Sorimachi et al. (2001) showed that the ethanolic fractions of *A. brasiliensis* mycelium and fruiting bodies inhibited HSV, poliovirus, and Western equine encephalitis virus replication. The inhibition of HSV-1 and herpes bovine virus by an aqueous extract of *A. brasiliensis* fruiting bodies was also demonstrated by Bruggemann et al. (2006). Additionally, both aqueous and ethanolic fruiting bodies extracts and an isolated polysaccharide from this species displayed antiviral activity against poliovirus 1, as reported by Faccin et al. (2007).

Considering that fungal biomass is an important source of cell wall polysaccharides and that sulfated compounds are promising antiviral agents, this study attempted to produce a sulfated derivative of a polysaccharide obtained from *A. brasiliensis* mycelia. Since this compound presented a potential anti-HSV activity, its mechanism of action was also evaluated.

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2. Materials and methods

2.1. Fungal material

The fruiting bodies of *Agaricus brasiliensis* Wasser strain UFSC 51 (syn *A. subrufescens*, *A. blazei*) were collected in Biguaçu, Santa Catarina State, Southern Brazil. The characterization of the species was performed by Dr. Maria Alice Neves, and a voucher specimen (FLOR 11 797) was deposited in the FLOR Herbarium (Universidade Federal de Santa Catarina). The mycelium of *A. brasiliensis* was isolated and cultivated on potato dextrose agar (PDA) (Oxoid, UK) at 25 °C during 7 days. The liquid inoculum was produced by transference of mycelial disks to flasks containing Melin-Norkrans Modified medium (MNM) (Marx, 1969) and cultivated at 25 °C during 10 days. Mycelia were filtered and fragmented in 300 mL of NaCl 0.8%. The inoculum was then added to MNM in an airlift bioreactor (5 L) and cultivated during 7 days at 26 °C. The liquid culture was centrifuged and the mycelial biomass was dehydrated at 55 °C until constant weight.

Agaricus brasiliensis polysaccharide was isolated as previously described (Camelini et al., 2005), with minor modifications. Fifty grams of dried mycelial biomass were blended twice with five volumes of distilled water and refluxed at 100 °C for 3 h. The material was filtered under vacuum through a Whatman n°42 filter paper. Three volumes of ethanol were added to the filtrate. The mixture was maintained at 4 °C for 24 h and centrifuged (1100 g, 10 min). The mycelial polysaccharide was freeze-dried and designated as MI.

To produce the sulfated derivative, MI was sulfated using the pyridine-chlorosulfonic acid reagent as described by Zhang et al. (2003). After sulfation, resulting polysaccharides were dialyzed through a 5 kDa molecular weight cut-off membrane (Spectrum Laboratories, Rancho Dominguez, CA) against distilled water and freeze-dried yielding the sulfated derivative (MI-S).

MI and MI-S were characterized by spectroscopic methods [Fourier transform infrared (FTIR) and ^{13}C Nuclear magnetic resonance (^{13}C NMR)] and elemental analyses (C, H, O, S). Determination of homogeneity and molecular weight (M_w) was carried out by high-performance gel filtration chromatography (HPGFC) using a Perkin Elmer series 200 equipment coupled with a RI detector, using a gel filtration column (TSK-Gel 5000 PW 7.8 × 300 mm connected to a TSK PWH 5 × 7 mm guard column; Tosoh, Japan). Samples were eluted with 0.2 M NaCl mobile phase at a flow rate of 1 mL/min. Mean M_w was estimated by comparison with retention times of standard dextrans.

2.2. Cells and viruses

Vero cells (ATCC:CCL 81) were grown in minimum essential medium (MEM), supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (25 μg/mL). Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere chamber. The virus strains used were: HSV-1 KOS and 29 R (Faculty of Pharmacy, University of Rennes, France), and HSV-2 333 (Department of Clinical Virology, Göteborg University, Sweden). Virus titers were determined by plaque assay and expressed as plaque forming units (PFU/mL) (Burleson et al., 1992).

2.3. Antiherpes evaluation

The cytotoxicity of samples was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, confluent Vero cells were exposed to different sample concentrations for 72 h. The medium was then substituted by the MTT solution and incubated for 4 h. After dissolution of formazan crystals, optical densities were read (540 nm) and the concentration of each sample that reduced cell viability by 50% (CC_{50}) was calculated based on untreated controls.

Subsequently, the potential antiherpetic activity was evaluated by the plaque reduction assay as previously described (Silva et al., 2010). Monolayers of Vero cells grown in 24-well plates were infected with 100 PFU per well of each virus for 1 h at 37 °C. Treatments were performed by adding samples either simultaneously with the virus (simultaneous treatment) or after the virus infection (post-infection treatment). Cells were subsequently covered with CMC medium (MEM containing 1.5% carboxymethylcellulose) and incubated for 72 h. Cells were then fixed and stained with naphthol blue black and viral plaques was counted. The concentration of each sample required to reduce the plaque number by 50% (IC_{50}) was calculated by standard method (Burleson et al., 1992). Acyclovir (ACV), dextran sulfate (DEX-S), and heparin (HEP) were purchased from Sigma (St. Louis, MO) and used as positive controls. IC_{50} and CC_{50} values were estimated by linear regression of concentration–response curves generated from the data. The selectivity index (SI = CC_{50}/IC_{50}) was calculated for each sample.

2.4. Evaluation of the mechanism of anti-HSV action

The virucidal assay was conducted as described by Ekblad et al. (2006), with minor modifications. Mixtures of equal sample volumes (20 μg/mL) and $4 × 10^5$ PFU of HSV-1 (KOS and 29-R) or HSV-2 333 in serum-free MEM were co-incubated for 20 min at 4 or 37 °C. Samples were then diluted to non-inhibitory concentrations (1:1000) to determine the residual infectivity by plaque reduction assay as described above. Ethanol 70% (v/v) served as a positive control.

The attachment and penetration assays followed the procedures described by Silva et al. (2010). In the attachment assay, pre-chilled Vero cell monolayers were exposed to viruses (100 PFU per well), in the presence or absence of the samples. After incubation for 2 h at 4 °C, samples and unabsorbed viruses were removed by washing with cold phosphate-buffered saline (PBS) and cells were overlaid with CMC medium. Further procedures were the same as described above for the plaque reduction assay.

In the penetration assay, viruses (100 PFU per well) were adsorbed for 2 h at 4 °C on pre-chilled cells. After the removal of unbound viruses, the temperature was shifted to 37 °C to allow penetration. Then, the cells were treated with different concentrations of pre-warmed samples, and incubated for 1 h at 37 °C. Unpenetrated viruses were inactivated with citrate-buffer (pH 3.0). Cells were washed with PBS and covered with CMC medium. The percentage of inhibition was calculated based on the reduction of plaque number as mentioned previously.

Time-of-addition study was performed by virus yield reduction assay as reported by Carlucci et al. (1999), with some modifications. Briefly, monolayers of Vero cells were inoculated with HSV-1 at a MOI (multiplicity of infection) of 0.04, incubated for 60 min at 4 °C and 30 min at 37 °C to ensure synchronous viral replication. After removing virus inoculum, cells were maintained at 37 °C and treated with MI-S (20 μg/mL), DEX-S (20 μg/mL), or ACV (2 μg/mL) at 2, 4, 8, 12, 16, and 20 h post-infection (p.i.). After a 24 h period, cells were lysed by freeze-thawing three times and cellular debris were removed by centrifugation. Subsequently, virus titration was carried out by plaque assay. The percentage of viral inhibition of each sample treatment was calculated by comparing it with virus titers of untreated controls.

The effect of tested samples on HSV cell-to-cell spread was investigated as described by Ekblad et al. (2010). In brief, different concentrations of MI-S, ACV, or DEX-S were added to Vero cells 1 h after their infection with 100 PFU per well of HSV, and the plates were incubated throughout the entire period of plaque

development. Results were obtained by analyses of the images of 20 viral plaques formed in the absence (viral control) or the presence of different concentrations of each sample concentration. Images were captured using a cooled digital camera coupled to an Olympus BX41 microscope and the area of each plaque was determined using the Image J software (http://rsb.info.nih.gov/ij/).

2.5. Western blotting analysis

Western blotting analysis was performed as previously described (Kuo et al., 2001). Briefly, monolayers of Vero cells were inoculated or not with HSV-1 KOS at a MOI of 0.1. Plates were incubated for 60 min at 4 °C and 30 min at 37 °C to ensure synchronous viral replication. Then, infected cells were treated with MI-S (20 µg/mL), DEX-S (20 µg/mL) or ACV (2 µg/mL) at 1, 4, or 8 h p.i., and the plates were incubated for 18 h. Next, cells were lysed and protein quantification was carried out (Bradford, 1976). Each sample (5 µg of protein) was separated electrophoretically on a 12% SDS–PAGE gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes. After blocking, membranes were incubated overnight with either anti-ICP27 (1:700, Millipore, Billerica, MA), or anti-UL42 (1:1000, Millipore), or anti-gB (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-gD (1:5000, Santa Cruz Biotechnology). After incubation with the corresponding secondary antibodies conjugated to horseradish peroxidase, protein bands were revealed by chemiluminescence on X-ray films (IBF, Brazil) using Pierce ECL substrate (Thermo Scientific, Waltham, MA) according to the Manufacturer's protocol. The relative amounts of protein in the detected bands were quantified by Image J software. The anti-β-actin antibody was used as a control for total protein loading.

2.6. Synergistic effect in combination with acyclovir

Potential synergistic effects of MI-S in combination with ACV was evaluated by plaque reduction assay, according to experimental design proposed by Chou (2006). Therefore, each drug alone or in combination was tested at an equipotency ratio, based on its corresponding IC$_{50}$ value. The degree of interaction between MI-S and ACV was calculated through combination index (CI) equation, based on the median-effect principle of the mass-action law, using Calcusyn software (version 2.1, Biosoft®). According to the CI theorem, CI values <1, =1, and >1 indicate synergism, additive effect, and antagonism, respectively.

3. Results

3.1. Characterization of A. brasiliensis polysaccharides

Assignment of ^{13}C NMR spectrum (Fig. 1) was based on the previously published spectrum by Mizuno and colleagues (1999). Anomeric signals (C1) at δ 105.1 and 101.9 ppm were assigned to β-glucopyranosyl and β-mannopyranosyl residues, respectively. The signals at δ 98.1 and 94.3 ppm were assigned to the corresponding reducing end-groups. The characteristic resonances of C2, C3, C4, C5, and C6 of β-(1 → 2)-linked components were observed at δ 78.2, 73.7, 71.8, 77.9, and 62.9 ppm, respectively. The signals of β-(1 → 3)-linked components were assigned as C2 (75.0), C3 (86.6), C4 (71.9), C5 (76.3), and C6 (62.9). This result suggested that MI is a glucomannan with a main chain of β-1,2-linked ᴅ-mannopyranosyl residues and β-ᴅ-glucopyranosyl-3-O-β-ᴅ-glucopyranosyl residues as side chains. A symmetric single peak was obtained by gel permeation chromatography of MI-S, suggesting that the polymer is homogeneous. Based on calibration curves with standard dextrans, the apparent *M*w of MI-S was 86 kDa. In the MI-S spectrum, obtained by FTIR analyses, two new absorption bands
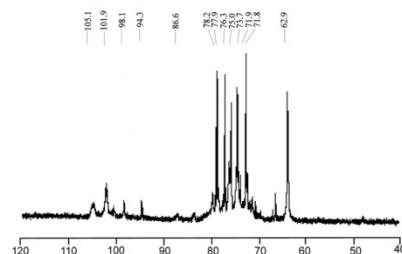
Fig. 1. ^{13}C-NMR spectrum of MI (*Agaricus brasiliensis* mycelial polysaccharide). ^{13}C NMR analysis of MI (60 mg, dissolved in D₂O) was performed at 25 °C with VARIAN Unity Plus spectrometer operating at 100,582 MHz (9.4 Tesla), using TSPA-d4 as external reference.

appeared at 1253 and 810 cm^{-1} (data not shown). These bands are related to S=O and C–S–O sulfate groups respectively, confirming that sulfation had actually occurred (Silverstein et al., 2005). In addition, the content of sulfur determined by elemental analyses was 14.77% and 10.72% for MI-S and DEX-S, respectively.

3.2. Antiherpetic activity

The cytotoxicity and antiviral activity results were used to calculate the selectivity index of each sample (SI = CC$_{50}$/IC$_{50}$) (Table 1). The data show that MI presented no antiviral activity, whereas MI-S inhibited both HSV-1 and HSV-2 replication, indicating that chemical sulfation was required for the antiviral activity. Since the simultaneous treatment was more efficient than the p.i. treatment, a direct inactivation of viral particles or inhibition of virus replication at the initial phases of the viral replication cycle could be involved.

Preincubation of virus suspensions with MI-S or controls (DEX-S and ACV), at 4 and 37 °C, had no significant inactivating effects (data not shown) on HSV-1 KOS and HSV-2 333 at the tested concentrations (20 µg/mL), which suppressed 100% of viral replication in the plaque reduction assay. These results indicate that the virucidal effect does not seem to be involved in the MI-S antiviral activity detected.

Along with the adsorption, the effect of MI-S on HSV penetration was also investigated (Table 2). The results demonstrated that MI-S, as well as DEX-S and HEP, strongly inhibited attachment of all viruses tested. Similarly to DEX-S, MI-S was also able to prevent penetration of all HSV strains into the cells, whereas HEP was much less effective for the HSV-2 strain.

To further clarify which steps of HSV infection are targeted by the samples, a time-of-addition study was performed (Fig. 2). The observed inhibition of HSV-1 KOS yield was higher than 50%, even when MI-S was added 16 h p.i. This might indicate that MI-S exerts some effect on virus cycle step(s), other than adsorption and penetration, as verified by the following results.

After penetration, HSV-1 expresses immediate early genes about 2–3 h p.i., early genes about 7 h p.i., and late genes after the viral DNA synthesis has begun. Western blotting analyses were carried out to evaluate if the MI-S antiviral mechanism was related to the inhibition of HSV-1 protein expression. To reduce the interference with any prior step of each protein expression stage in the viral replication cycle, samples were added at 1, 4, and 8 h p.i. for analysis of α, β, and γ proteins, respectively (Fig. 3). The results shown in Fig. 3B represent the quantification of each band in relation to the β-actin expression. As shown in Fig. 3, MI-S significantly

Table 1
Cytotoxicity and antiherpetic activity of *Agaricus brasiliensis* polysaccharides.

| Sample	CC₅₀	IC₅₀ (SI)					
		Simultaneous treatment			Post-infection treatment		
		HSV-1 (KOS)	HSV-1 (29R)	HSV-2 (333)	HSV-1 (KOS)	HSV-1(29R)	HSV-2 (333)
MI	3352.11 ± 206.64	NI	NI	NI	NI	NI	NI
MI-S	2415.29 ± 389.21	1.24 ± 0.05 (**1948**)	1.59 ± 0.25 (**1519**)	0.39 ± 0.17 (**6193**)	5.50 ± 0.58 (**439**)	11.62 ± 1.01 (**208**)	4.30 ± 0.36 (**562**)
HEP	>2,500	1.15 ± 0.30 (**>2174**)	2.01 ± 0.38 (**>1244**)	0.58 ± 0.01 (**>4310**)	12.68 ± 1.13 (**>197**)	NT	8.06 ± 1.46 (**>310**)
DEX-S	2465.53 ± 200.13	0.59 ± 0.01 (**4179**)	0.92 ± 0.08 (**2680**)	0.47 ± 0.16 (**5246**)	6.23 ± 0.96 (**396**)	11.92 ± 1.31 (**207**)	15.36 ± 2.79 (**160**)
ACV	>2500	NI	NI	NI	0.51 ± 0.05 (**>4902**)	760.00 ± 80 (**>3**)	3.91 ± 0.41 (**>639**)

CC₅₀, 50% cytotoxic concentration for Vero cells (µg/mL); IC₅₀, 50% inhibitory concentration (µg/mL). Selectivity index values (SI = CC₅₀/IC₅₀) are presented between parenthesis; NI, no inhibitory activity; NT, not tested; MI, *A. brasiliensis* mycelial polysaccharide; MI-S, sulfated *A. brasiliensis* mycelial polysaccharide; HEP, heparin; DEX-S, dextran sulfate; ACV, acyclovir. These values represent the mean ± SD of three independent experiments.

Table 2
Effect of *Agaricus brasiliensis* polysaccharides on HSV adsorption and penetration.

Sample	Inhibitory effect (IC₅₀)					
	Attachment			Penetration		
	HSV-1 (KOS)	HSV-1 (29 R)	HSV-2 (333)	HSV-1 (KOS)	HSV-1 (29 R)	HSV-2 (333)
MI	NI	NI	NI	NI	NI	NI
MI-S	0.10 ± 0.01	0.26 ± 0.03	0.05 ± 0.01	1.04 ± 0.06	0.49 ± 0.10	1.21 ± 0.28
HEP	0.33 ± 0.04	0.62 ± 0.03	0.07 ± 0.01	13.34 ± 3.64	25.74 ± 2.64	17.99 ± 4.06
DEX-S	0.12 ± 0.04	0.11 ± 0.00	0.02 ± 0.00	0.85 ± 0.13	0.82 ± 0.11	0.69 ± 0.09
ACV	NI	NI	NI	NI	NI	NI

IC₅₀, 50% inhibitory concentration (µg/mL); NI, no inhibitory activity; MI, *A. brasiliensis* mycelial polysaccharide; MI-S, Sulfated *A. brasiliensis* mycelial polysaccharide; HEP, heparin; DEX-S, dextran sulfate; ACV, acyclovir; These values represent the mean ± SD of three independent experiments.
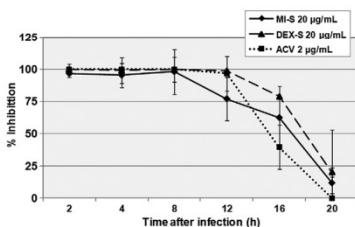
Fig. 2. Time-of-addition study. Percentage of inhibition of MI-S (sulfated *Agaricus brasiliensis* mycelial polysaccharide), ACV (acyclovir), and DEX-S (dextran sulfate) when added at different times after HSV-1 (KOS strain) infection. Each point represents the mean value ± SD of two independent experiments.

reduced the expression of ICP27, UL42, and gB. Moreover, the combination of MI-S and acyclovir (lane 4) reduced all the proteins expression more strongly than these compounds tested separately.

The reduction of HSV-1 and HSV-2 cell-to-cell spread was evaluated by comparing viral plaque areas between treated cells and untreated controls. Considering that significant differences in plaques sizes were only observed at concentrations higher than the IC₅₀ values of all tested samples (data not shown), as well as the small number of plaques at this condition, an additional experiment was performed with samples at concentrations equivalent to their IC₅₀ values. Mean plaque areas for each treatment and untreated controls are shown in Fig. 4. Regarding to HSV-1 (KOS strain), MI-S reduced the viral plaque size more extensively than did DEX-S and ACV. Although HSV-2 lateral diffusion was significantly reduced by all tested samples, MI-S resulted in the smallest mean plaque areas for both viruses. Even though the tested concentrations in this experiment were different, the reduction of viral plaque numbers was similar (~50%).

Given the fact that MI-S seemed to act in a different way than acyclovir, the potential synergistic effects between them were tested at different concentrations (Table 3). Since all combinations tested presented CI values less than 1, synergistic anti-HSV-1 and HSV-2 effects of MI-S with ACV were demonstrated.

4. Discussion

In order to evaluate the influence of the treatment period on the anti-HSV activity of MI-S, the plaque number reduction assay was performed under two different conditions. As shown in Table 1, MI-S was considerably more effective by simultaneous rather than post-infection treatment. The same result was observed for the other sulfated polysaccharides tested, HEP and DEX-S, as expected due to their similar nature. These results are in agreement with those of other authors who tested different sulfated polysaccharides, such as carrageenans (Carlucci et al., 1999), fucoidans (Karmakar et al., 2010), and sulfated β-glucans (Zhang et al., 2004), and found a stronger inhibition of HSV replication in the simultaneous treatment with these compounds than in post-infection treatments.

Although similar IC₅₀ values were obtained for MI-S and HEP in the simultaneous treatment, we have not found an anticoagulant activity for MI-S at a 100% inhibitory concentration (data not shown), which represents an advantage for an antiherpes agent with these chemical features. Moreover, in the post-infection treatment, the inhibitory effect of MI-S was stronger than those of HEP for HSV-1 (KOS strain) and HSV-2, and of DEX-S for HSV-2. Differences among these results may be related to their structural diversity since, differently from MI-S and DEX-S, HEP is a linear polymer (Rabenstein, 2002), with a lower molecular mass (~18 kDa) than either MI-S (86 kDa) or DEX-S (500 kDa). Furthermore, the higher content of sulfur present in MI-S (14.77%) can be correlated to its stronger effect at inhibiting HSV-2 than DEX-S (10.79%). Indeed, the antiherpetic properties of sulfated polysaccharides are determined by a combination of structural features such as molecular
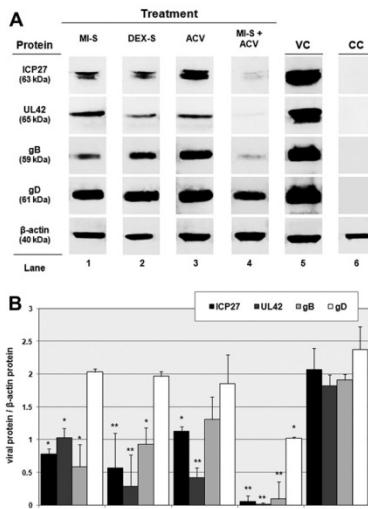
**Fig. 3.** Effect of MI-S (sulfated *Agaricus brasiliensis* mycelial polysaccharide) on HSV-1 (KOS strain) protein expression. Treated and untreated cells were analyzed by SDS–PAGE/Western blotting using specific antibodies for viral (ICP27, UL42, gB, or gD) and cellular (β-actin) proteins. (A) Lanes 1 to 4: HSV-1 infected cells treated with MI-S (20 μg/mL), dextran sulfate (DEX-S, 20 μg/mL), acyclovir (ACV, 2 μg/mL), and MI-S (20 μg/mL) plus ACV (2 μg/mL), respectively. Samples were added at 1, 4, and 8 h p.i. for analyses of α, β, and γ HSV proteins, respectively. Lane 5: untreated viral control; Lane 6: untreated cellular control. (B) The graph indicates the ratio of each viral protein to β-actin protein. Statistical analysis (one-way ANOVA followed by Newman-Keuls test) demonstrated significant differences in relation to viral controls with p values <0.05 (∗) and <0.01 (∗∗). The results are representative of two independent experiments.

Fig. 4. Inhibition of HSV-1 and HSV-2 cell-to-cell spread by MI-S (sulfated *Agaricus brasiliensis* mycelial polysaccharide), DEX-S (dextran sulfate), and ACV (acyclovir) at their corresponding IC_{50} concentrations. The interference on cell-to-cell spread of MI-S, DEX-S, or ACV was evaluated at 5.50, 6.23, and 0.51 μg/mL for HSV-1 KOS, and at 4.30, 15.36, and 3.91 μg/mL for HSV-2 333, respectively. Results were expressed as average area of viral plaques developed in sample-treated cells and compared to untreated controls. Statistical analysis (one-way ANOVA followed by Dunnett's test) demonstrated significant differences in relation to the controls with p values <0.05 (∗), <0.01 (∗∗), and <0.005 (∗∗∗). Values shown represent means of three independent experiments.

mass, branching degree, charge density, and molecular composition of uncharged portions (Ghosh et al., 2009).

Sulfated polysaccharides may present an antiherpetic activity through different mechanisms, including virucidal effects. In this study, however, MI-S showed no virucidal effects, indicating that the antiherpes activity detected by the plaque reduction assay was due to the interference with some step(s) of the HSV replication cycle. By contrast, Bruggemann et al. (2006) have shown an HSV-1 virucidal activity for an aqueous extract of *A. brasiliensis*, but they used different methodologies for virucidal evaluation and extract preparation, which did not include the sulfation reaction. Still, other studies on the antiviral activity of sulfated polysaccharides have similarly reported no virucidal effects (Adhikari et al., 2006; Chattopadhyay et al., 2007, 2008; Karmakar et al., 2010; Matsuhiro et al., 2005; Zhu et al., 2006), or the virucidal action was detected only at concentrations higher than their IC_{50} values (Carlucci et al., 1999; Mazumder et al., 2002; Zhu et al., 2004).

Among the steps of HSV infection and replication, attachment and entry have been considered as potential targets. The findings presented in Table 2 are in agreement with those published by other authors, who stated that the mechanism underlying the antiherpes activity of polysaccharides, especially sulfated ones, may be related to the inhibition of HSV adsorption (Carlucci

et al., 1999; Eo et al., 2000; Zhang et al., 2007). Since there was no detectable loss of HSV residual infectivity at 4 °C in the presence of MI-S, the virucidal mechanism in the adsorption assays was dismissed. Table 2 shows that MI-S and DEX-S displayed IC_{50} values lower than 1.21 μg/mL, whereas HEP showed values higher than 13.34 μg/mL. Since HEP was the only tested sulfated polysaccharide with a linear chain, it can be suggested that the presence of lateral branches could be important for the inhibition of the herpes virus penetration. The lack of inhibition of viral adsorption and penetration by the non-sulfated polysaccharide (MI) confirmed that the presence of sulfate groups is required for such activities.

In addition to the inhibition of HSV replication at 1 h p.i. treatment, MI-S presented inhibitory activity even when added at longer times after infection (Fig. 2), suggesting an action in post-entry events. This hypothesis was investigated by Western blotting analyses, in which a considerable reduction of α (ICP27), β (UL42), and γ (gB) HSV-1 proteins expression was found when MI-S was added at 1, 4, and 8 h p.i., respectively. Differently, infected cells treated with MI-S resulted in a slight reduction of gD expression. As for now, considering the performed experiments, the authors are unable to point out the reason for differences observed in reduction of expression of the late proteins gB and gD. Furthermore, the detected general reduction of protein production by MI-S could be associated with a secondary effect on another step of the viral cycle, as observed for ACV, for which inhibition of protein expression was due to an indirect effect on suppression of viral DNA replication. Although we are not aware of previous reports indicating the inhibition of HSV protein expression by sulfated polysaccharides, one study described the reduction of HIV protein expression by a sulfated oligosaccharide as well as by dextran sulfate (Artan et al., 2010).

Since an efficient dissemination of virus has an important role in its infectivity, the inhibition of viral intercellular diffusion is an attractive target for new antiviral drugs. In the plaque size reduction assay, MI-S significantly reduced plaque areas. Recently, Ekblad and colleagues (2010) have shown the inhibition of HSV cell-to-cell spread by a sulfated tetrasaccharide.

Here, a synergistic effect of MI-S with ACV was also found, supporting the results of their combination by Western blotting assay. These findings suggest that MI-S can be used either alone or in combination with ACV for the treatment of herpes infections, espe-

Table 3

Synergistic effects of combination of sulfated *Agaricus brasiliensis* polysaccharide (MI-S) with acyclovir (ACV) on anti-HSV activity.

Compounds combination ratio	Sample concentration (µg/mL)		Mean percentage of inhibition (%) MI-S + ACV	Experimental CI values	Description (graded symbols)
	MI-S	ACV			
HSV-1 (KOS)					
$4 \times IC_{50}$	22.00	2.04	100.00	0.398	Synergism (+ + +)
$2 \times IC_{50}$	11.00	1.02	100.00	0.199	Strong synergism (+ + + +)
$1 \times IC_{50}$	5.50	0.51	100.00	0.099	Strong synergism (+ + + +)
$0.5 \times IC_{50}$	2.75	0.26	64.00	0.431	Synergism (+ + +)
$0.25 \times IC_{50}$	1.38	0.13	39.00	0.383	Synergism (+ + +)
HSV-2 (333)					
$4 \times IC_{50}$	17.20	15.64	100.00	0.372	Synergism (+ + +)
$2 \times IC_{50}$	8.60	7.82	100.00	0.186	Strong synergism (+ + + +)
$1 \times IC_{50}$	4.30	3.91	86.86	0.472	Synergism (+ + +)
$0.5 \times IC_{50}$	2.15	1.96	59.79	0.646	Synergism (+ + +)
$0.25 \times IC_{50}$	1.08	0.98	19.59	0.816	Slight synergism (+)

CI, combination index, a quantitative measure calculated by Calcusyn Software. This index quantifies the interaction between the tested compounds as described by Chou (2006). In detail, CI from 0.10 to 0.30 means strong synergism, 0.30–0.70 means synergism, 0.70–0.85 means moderate synergism, and 0.85–0.90 means slight synergism. Obtained values represent the mean of two independent experiments.

cially those in which patients do not respond to ACV. Some other HSV entry inhibitors have already been reported to present synergistic effects with ACV. For example, a complex polysaccharide–protein from *Ganoderma lucidum* (Eo et al., 2000), docosanol (Marcelletti, 2002), and oxyresveratrol (Chuanasa et al., 2008).

In summary, our findings indicate that MI-S interferes with various steps of the HSV replication cycle, mainly adsorption and penetration, but also viral protein expression, as well as with HSV cell-to-cell spread. Taking into account the prospect of an economically feasible biotechnological production of this polysaccharide and its promising antiherpetic activity herein reported, further investigation is needed to clarify the potential of such compound for clinical application.

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CAPÍTULO IV:
AVALIAÇÃO *IN VIVO* DA ATIVIDADE ANTI-HERPÉTICA DO
DERIVADO SULFATADO DO POLISSACARÍDEO MICELIANO
DO FUNGO *Agaricus brasiliensis*

1. APRESENTAÇÃO

Depois de realizada a caracterização química e a determinação do mecanismo da atividade anti-herpética dos polissacarídeos sulfatados de *A. brasiliensis*, o próximo objetivo deste trabalho foi avaliar a atividade *in vivo* destas amostras. Com a produção de biomassa miceliana do fungo em biorreator de meio líquido no Laboratório de Bioprocessos, do Departamento de Microbiologia, Imunologia e Parasitologia (MIP), da UFSC, houve a disponibilidade de mais material para testes. Devido aos resultados promissores, o presente estudo tratou da avaliação *in vivo* da atividade anti-herpética do MI-S em camundongos. Os resultados obtidos nos três modelos avaliados (ocular, cutâneo e genital) foram organizados e serão submetidos para avaliação ao periódico *Antiviral Research*. Esta parte da tese foi realizada durante o estágio sanduíche de seis meses no *Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison*, sob a orientação do Professor Dr. Curtis R. Brandt.

2. ARTIGO A SER SUBMETIDO PARA AVALIAÇÃO AO PERIÓDICO *ANTIVIRAL RESEARCH*

In vivo anti-HSV activity of a sulfated derivative of *Agaricus brasiliensis* mycelial polysaccharide

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Abstract

Agaricus brasiliensis (syn *A. subrufescens*) is a Basidiomycete fungus native to the Atlantic forest in Brazil that contains cell walls rich in glucomannans polysaccharides. The $(1{\rightarrow}3,\ 1{\rightarrow}2)$-$\beta$-D-glucomannan was isolated from *A. brasiliensis* mycelia and chemically modified in order to produce its sulfated derivative (MI-S), which presented promising *in vitro* anti-HSV activity, with multiple mechanisms of action including inhibition of viral entry, protein expression and cell-to-cell spread. To further clarify the potential of MI-S for clinical applications we assessed its in vivo antiherpetic activity in murine models of Herpes Simplex ocular, cutaneous, and genital infection. Groups of 10 mice were infected with HSV-1 (KOS strain) or HSV-2 (strain 333) under various pre- and post-treatment schemas. The severity of disease was scored and virus titers of ocular and vaginal samples were determined. No toxicity was observed in the MI-S uninfected treated groups. The topical and oral treatments with MI-S were not effective in reducing ocular disease. Topical application of MI-S on skin lesions was not effective but cutaneously infected mice treated orally with MI-S showed significantly reduced disease scores and accelerated healing of HSV-1 lesions after day 9. Vaginal administration of MI-S 20 min before viral challenge significantly reduced the mean disease scores (days 5 to 9), viral titers (days 1 and 3), and mortality in comparison to the control groups (untreated and vehicle treated). These results show that MI-S may be potentially useful as an oral agent to reduce the severity of HSV cutaneous and mucosal lesions and more importantly as a microbicide to block sexual transmission of HSV-2 genital infections.

1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) are responsible for a wide range of diseases, affecting the skin or mucus membranes (cold sores, genital herpes and gingivostomatitus), the eye (herpetic keratitis), or the central nervous system (necrotizing encephalitis, and meningitis). Ocular HSV infections are the leading cause of infectious blindness in developed countries and neonatal HSV-2 infection has a high mortality rate (Liesegang, 2001; Roizman et al., 2007).

In the United States, 57.7% of the population was seropositive for HSV-1 between 1999 and 2004. For HSV-2 infection, the incidence in the US is approximately 20% for those older than 12 years of age (Xu et al., 2006).

A study performed in Brazil between 1996 and 1997, with 1,090 people from the general population aged from 1 to 40 years, showed a seroprevalence of 67.2% and 11.3% for HSV-1 and HSV-2, respectively (Clemens and Farhat, 2010). Another study performed in 2000 showed a HSV-2 seroprevalence of 42.9% in females and 25.9% in males of a blood donor population (Cowan et al., 2003).

Genital herpes is a common sexually transmitted infection (STI) and HSV is among the most frequent viral infections in AIDS patients, intensifying their morbidity and mortality. Moreover, HSV genital infection increases the risk of acquiring human immunodeficiency virus (HIV) in a sexual unprotected relationship by 3-fold (Fatahzadeh and Schwartz, 2007; Gupta et al., 2007; Van de Perre et al., 2008) so it is clearly a co-factor for the spread of HIV-1. Agents that would reduce the rate of acquisition of HSV genital infection alone could have a significant effect on the HIV-1 epidemic.

Several antivirals effective against HSV are approved for use including acyclovir, valacyclovir, and fancyclovir. Although they are effective, they cannot eliminate latent virus, breakthrough reactivations occur in the presence of the drugs, resistant strains of virus can emerge, particularly in immunosuppressed patients, and toxic side effects can occur in some people. Given that once an infection is established it cannot be cleared, one attractive antiviral strategy is to block infection of new hosts. One potential approach to reduce or eliminate transmission is the use of microbicidal preparations prior to the initiation of genital contact.

Microbicides are prophylactic agents that can be applied topically in the vagina or rectum as a single agent or with other

components which have ability to prevent the transmission of STIs. A promising candidate in addition to have efficacy, must be easy to use, non-irritating, non-toxic, and preferably have a broad spectrum of activity against common pathogens in the genital tract. Considerable effort has been applied to the development of microbicides, especially to reduce the risk of sexual acquisition of HIV. Sulfated polysaccharides such as dextrin-2-sulfate, carrageenan, and cellulose sulfate have recently been evaluated in clinical trials. This class of compounds are thought to act primarily through inhibition of fusion between the membranes of the pathogen and mucosal cells and/or by binding to the pathogen and preventing attachment to the host (Abdool Karim et al., 2012). However, the clinical trials for carrageenan and cellulose sulfate were halted early due to lack of significant effect on viral transmission (carrageenan) (Skoler-Karpoff et al., 2008) or insufficient power (cellulose sulfate) (Van Damme et al., 2008).

Since there is no vaccine or microbicide available to avoid HSV infections, and current antiherpes drugs cannot eliminate latent infections, may have side effects, or induce the emergence of drug-resistant virus strains, the search for new agents capable of preventing and/or treating HSV infections is still needed.

In previous papers, we characterized the structure and toxicity of the sulfated derivative of a cell-wall glucomannan obtained from *Agaricus brasiliensis* mycelia (MI-S) (Cardozo et al.) and we evaluated *in vitro* anti-HSV-1 and 2 activities (Cardozo et al., 2011). Considering the economically feasible biotechnological production of *A. brasiliensis* mycelia biomass and the promising *in vitro* antiherpetic activity of MI-S, the goal of this study was to characterize the anti-HSV spectrum of activity of MI-S, using different virus strains, as well as assess its in vivo anti-HSV-1 and anti-HSV-2 activity in murine models of herpes ocular, cutaneous, and genital infection.

2. Materials and methods

2.1 Compound

The sulfated derivative of *Agaricus brasiliensis* Wasser (syn *A. subrufescens* Peck) mycelial polysaccharide was prepared and characterized as previously described (Cardozo et al., ; Cardozo et al., 2011).

2.2 In vitro antiviral activity

The antiviral spectrum of activity of MI-S against HSV-1 strains CJ311, CJ360, CJ394, OD4 (Berdugo et al., 2012; Grau et al., 1989), and KOS; as well as HSV-2 strain 333 was evaluated by yield reduction assay. Briefly, Vero cells (2.5×10^5 cells/mL) were seeded into 24-well plates and incubated for 24 h at 37°C in a CO_2 incubator. Cells were infected with a MOI of 1 and treated following two different treatments: simultaneous, when the sample was added at the same time of viral infection; and post-infection (p.i.) treatment, when the sample was added after viral infection period (1h). Treated cells were then incubated at 37°C in a CO_2 incubator for 24 h. Viral loads of freeze-thaw collected samples for each well were determined by plaque assay (Brandt et al., 2007). The 50% effective concentrations (EC_{50}) were determined by comparison to the untreated controls using the Graphpad Prism software.

2.2 In vivo antiviral activity

2.2.1 Animals

Groups of ten female BALB/c mice, four to six-week-old, which were approved by the UW-Madison Institutional Animal Care and Use Committee and conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, were used in all the experiments.

2.2.2 HSV-1 corneal infection

The right corneas of mice were scratched three times vertically and three times horizontally with a sterile needle under isoflurane anesthesia. Mice were infected by application of 5 µL of DMEM (2% serum) containing 1.0×10^5 plaque-forming units (PFU) of HSV-1 KOS strain to the scarified cornea, and the different groups were treated as follows: group 1 (topical treatment): eye drops (3 µL) of MI-S solution (1mg/mL), prepared in DMEM, were administered starting 4 hours after infection and continued five times per day, for 10 days. Group 2 (topical treatment-toxicity): an uninfected group was treated in the same conditions as group 1 in order to evaluate the cutaneous toxicity of MI-S. Group 3 (oral treatment): Infected mice received orally MI-S prepared in PBS buffer in a dose of 1 mg twice a day from 4 hours until 7 days after infection. Groups 4 and 5 were tested in parallel and

correspond to PBS treated and untreated infected control groups, respectively (Brandt et al., 2007).

2.2.2.1 Ocular disease scoring

The mice were examined microscopically to determine ocular disease severity by using a score system as described previously (Brandt et al., 2007). Briefly, blepharitis was scored: 1+, puffy eyelids; 2+, puffy eyelids with some crusting; 3+, eye swollen shut with severe crusting; and 4+, eye completely swollen shut and crusted over. Vascularization was scored: 1+, < 25% of the cornea involved; 2 +, 25% to 50% corneal involvement; and 3 +, >50% corneal involvement. Stromal disease was scored: 1 +, cloudiness, some iris detail visible; 2 +, iris detail obscured; 3 +, cornea totally opaque; and 4 +, corneal perforation.

2.2.2.2 Measurement of ocular viral titers

On days 1, 3, 5, 7, 9, 11, and 13 after infection, samples were harvested from the mice as follows. The mice were anesthetized with isoflurane, and the infected corneas were flushed with 10 µL of serum-free DMEM. The rinse was added to 190 µL of serum-free DMEM and stored at -80°C until use. Serial 10-fold dilutions were quantified by using a standard plaque assay on Vero cells (Brandt et al., 2007).

2.2.3   HSV-1 cutaneous infection

The right midflank of each mouse were clipped and depilated with a chemical depilatory hair remover. Three days later, the naked skin was scratched in a grid-like pattern using 27-guage needles and 15 µL of HSV-1 KOS strain suspension (1×10^5 PFU) were applied to the scarified area (Chuanasa et al., 2008). A base cream, formulated with 70% (wt/wt) of hydrophilic petrolatum (Eucerin), 15% (wt/wt) of white mineral oil (USP, Sigma, USA), and 15% (wt/wt) of water, was used to prepare the MI-S cream at 2% (wt/wt). Different groups were treated as follows: 1) topical treatment: 2% MI-S cream was applied topically on the scratched area 4 hours after infection and continued five times per day, for 7 days; 2) vehicle treated Group; 3) oral treatment: mice receive orally MI-S solution prepared in PBS at a dose of 1 mg twice a day; 4) prophylactic + post-infection treatment: infected mice were orally treated with 1 mg of MI-S twice a day, from three days before virus inoculation to one week post-inoculation; 5) group treated only with PBS vehicle.

2.2.3.1 HSV-1 cutaneous disease scoring

The herpes cutaneous was scored according to Park et al., (2005) with minor modifications, as follows: 0, no visible infection; 1, yellowish colored swelling visible (pre-lesions) or healing ulcers; 2, ulcers at inoculation site only with swelling, crust, and or erythema; or healing ulcers with crusting; 3, spreading ulceration, crusting, some clear ulcers; or healing ulcers beyond shaved region; 4, zosteriform rash; 5, rash confluent but not yet necrotic or ulcerated; 6, Complete rash with necrosis or ulceration; hind limb paralysis, bloating, or death.

2.2.4   HSV-2 genital infection

This experiment was performed based as previously reported by Bernstein et al., (2008) with minor modifications. Five and one day prior to intravaginal challenge, mice were subcutaneously injected with 2 mg of medroxy-progesterone acetate (Greenstone®, Peapack, New Jersey, USA) in the upper back. Afterward, mice were inoculated intravaginally with 10 µL of virus suspension (HSV-2 strain 333, 1 x 10^5 PFU) and the different groups were treated as follows: 1) infected untreated; 2) MI-S p.i. treatment, 25 µl of MI-S solution at 10 mg/ml was administered intravaginally to mice three times a day, from 4 h to 7 days post-inoculation; 3) vehicle p.i. treatment: 25 µl of the 2% methylcellulose vehicle was administered intravaginally to mice three times a day, from 4 h to 7 days after virus inoculation; 4) MI-S pre-treatment/ microbicidal capacity, 25 µl of MI-S solution at 20 mg/ml was administered intravaginally to mice 20 min before instillation of virus suspension; 5) vehicle pre-treatment: 25 µl of the 2% methylcellulose vehicle was administered intravaginally to mice 20 min before virus inoculation; 6) sample local treatment/ toxicity: non infected animals received intravaginally 25 µl of MI-S at 10 mg/ml in the 2% methylcellulose vehicle, three times a day, starting and finishing at same time of group 2.

2.2.4.1 Disease scoring

Clinical signs of infection were scored at days 1, 3, 5, 6, 7, 8, and 9 post-infection, according to a composite scale from 0 to 6, as described by Gill et al., (2005), with minor modifications. The disease was graded as: 0, no sign of infection; 1, slight redness of external vagina; 2, swelling and redness of external vagina, and/or pus/mucous; 3, severe swelling of external vagina with pus/mucous, and sometimes hair loss in the surrounding area; 4, ulceration of vaginal tissue, redness,

and swelling; 5, continued ulceration, redness, swelling, hind limb paralysis, death. Mice that became moribund with hind limb paralysis were killed according to guidelines of University of Wisconsin-Madison. Mice were observed daily for toxicity signs and the body weights were determined on before infection (baseline) and at days 3 and 7. The mortality rates of mice intravaginally infected with HSV-2 were estimated for 9 days after virus inoculation.

2.2.4.2 Measurement of vaginal viral titers

Viral shedding was carried out before the administration of MI-S and at days 1, 3, 5, 7, and 9 after infection by vaginal swabbing with a DMEM-moistened calgiswab (Pur-Wraps®). Each swab was added to 200 µL ml of DMEM supplemented with 2% serum and 2% of PSA and stored at -80°C until use. Viral titers of samples serial dilutions were quantified by plaque assay on Vero cells (Brandt et al., 2007).

2.2.4.3 Histopathological analysis

After death, the mouse genital tracts were excised, fixed in 4% buffered paraformaldehyde, embedded in paraffin, sectioned, stained with hematoxylin-eosin. Slides were examined under 200× and 400× magnification using the Qimage analysis system. The transversal sections of vaginal tissue were examined for epithelial exfoliation, vascular congestion, leukocyte infiltration, and lamina propria thickness (edema).

2.2.5 Statistical analysis

Statistical analyses were performed in the GraphPad Prism version 5.01. Differences in disease score data (Mean ± SEM) among groups were compared for each day through analysis of variance (ANOVA) followed by the Bonferroni or Student-Newman-Keuls Multiple Comparison test. Viral titers were compared by ANOVA and the Dunn's Multiple Comparison test. Survival curves comparison between treated and control groups were performed by the Lo-rank (Mantel-Cox) test. A p value lower than 0.05 was considered statistically significant.

3. Results

3.1 In vitro antiviral evaluation

Previously we characterized the antiviral activity of MI-S against a single HSV-1 and HSV-2 strain. To determine if MI-S had broad spectrum activity against Herpes simplex virus, we assessed the activity against four ocular HSV-1 isolates, CJ 360, CJ311, CJ394, and OD4 (Berdugo et al., 2012; Grau et al., 1989), as well as KOS and HSV-2 333 in a yield reduction assay adding the MI-S either simultaneously with the virus or one hour after the adsorption period. The resulting EC_{50} values in µg/ml are shown in Table 1. The EC_{50} values for the HSV-1 strains when MI-S was added simultaneously ranged from 2.35 to 17.27 µg/ml. When MI-S was added after the adsorption period, the EC_{50} values increased for all of the HSV-1 strains tested from 1.1 to 4.56-fold. For HSV-2 333, the EC_{50} value for the post-infection treatment was 2.8-fold higher than when MI-S was added simultaneously. These results are consistent with our previous findings (Cardozo et al., 2011) and show that MI-S is a broad spectrum inhibitor of Herpes simplex virus.

Table 1: Spectrum of anti-HSV activity of *Agaricus brasiliensis* sulfated polysaccharide (MI-S)

Virus strain	EC_{50} (µg/mL)	
	Simultaneous treatment	Post-infection treatment
HSV-1 CJ 360	15.76 ± 3.84	17.79 ± 1.18
HSV-1 CJ 311	2.35 ± 0.83	29.98 ± 2.54
HSV-1 CJ 394	5.67 ± 0.77	25.97 ± 0.93
HSV-1 KOS	17.27 ± 3.95	31.75 ± 3.35
HSV-1 OD4	10.41 ± 1.96	37.53 ± 5.34
HSV-2 333	4.73 ± 0.04	13.32 ± 2.68

EC_{50}: 50% inhibitory concentration.
Values represent the mean ± SD of three independent experiments.

3.2 In vivo toxicity assessment

The *in vivo* toxicity of MI-S was assessed by including MI-S treated but non-infected groups for each of the models. No local signals of toxicity, such as edema, erythema, or swelling were seen at the administration sites in all of the topical application models. We also did not observe evidence of systemic toxicity such as ruffled fur, behavioral changes or weight loss in any of the animals given MI-S topically. In a pilot study where mice were given MI-S 2 mg of MI-S by gavage twice

per day, we found that the mice developed signs of toxicity that included ruffled fur and lethargy. When the dose of MI-S was lowered to 1 mg twice per day, there were no signs of toxicity. Thus, topical MI-S is tolerated well but when given orally at high doses it displays toxic effects.

3.3 Activity against HSV-1 keratitis

To determine if MI-S was active when topically applied to the cornea, we infected mice with KOS and then applied MI-S in PBS topically 5 times per day for 7 days. We found there was no significant reduction in the severity of blepharitis, stromal keratitis, or corneal neovascularization (data not shown) when comparing the MI-S treated group with either infected-untreated mice or mice treated with vehicle alone (PBS). When viral titers were measured, we also found no significant differences in the amount of virus shed from the eyes of infected mice (data not shown). Thus, MI-S was not effective against HSV-1 keratitis, at least at the concentration tested formulated in PBS. We also did not find significant effects on keratitis or viral shedding in mice given MI-S 1 mg MI-S orally (data not shown).

3.4 Antiviral activity against cutaneous HSV-1 infection

The activity of MI-S against cutaneous HSV infection was tested by scarifying the skin on the right flank of the mouse, infecting with HSV-1 KOS, and applying 2% MI-S in cream 5 times per day to the site of infection. The site of infection was swabbed every other day to measure viral titers. In this model, we found no significant differences in the disease scores between the infected untreated group, the vehicle (cream) only group, and the group treated with MI-S cream. None of the swabs from any of the groups yielded infectious virus so we were not able to determine if there was any effect on viral replication (data not shown).

The results from treating cutaneously infected mice orally with MI-S are shown in Figure 1. Signs of infection were first seen on day 3 or 5 in both groups and continued to increase until day 8 when the scores peaked at approximately 3.0. The skin lesions then began healing and had almost completely healed by day 15 post-infection. When comparing the vehicle (PBS) only group and the MI-S group, there were no significant differences in the disease scores through day 8. However, beginning at day 9, the disease scores in the MI-S treated mice were significantly lower than the vehicle only group through day 13,

indicating that oral administration of MI-S enhances healing of cutaneous HSV-1 lesions. The scores were not different on day 15 because the lesions were healed or almost healed.
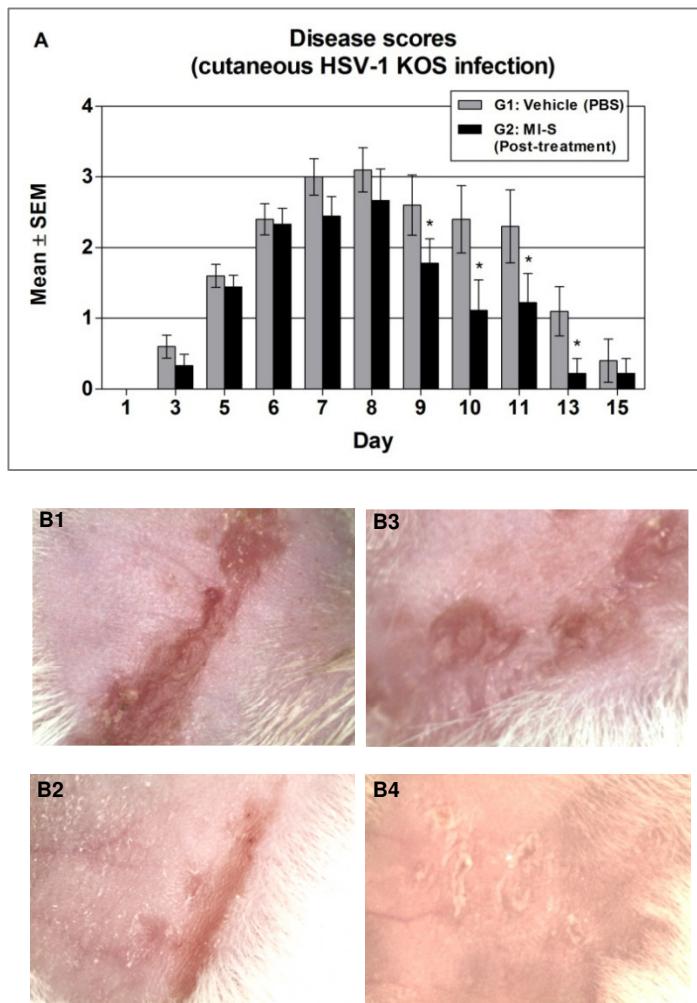
Fig. 1: Effect of MI-S post-infection oral treatment on HSV-1 cutaneous disease. A) Groups of 10 mice were treated after infection by oral gavage with 100 µL of MI-S solution (10 mg/ml) twice a day. The severity of disease was scored from day 1 to 15 and compared to control group by two-way ANOVA followed by Bonferroni post test. (*) $p < 0.001$. B 1) Zosteriform lesion, B2) Zosteriform healing lesion; B3) Spreading ulceration, B4) Healing ulcers.

3.5 Activity against HSV-2 genital infection

To determine if MI-S was effective against genital herpes, mice were either treated intravaginally one single time 20 minutes prior to infection with HSV-2 333 (microbicide model) or 5 times daily for 7 days beginning within 4 h post-infection. The results are shown in Figure 2. Signs of infection began to appear on day 5 and then continued to increase in severity through day 9 when the study was halted (see below). Post-infection treatment with MI-S did not significantly reduce the severity of genital herpes on any of the days. Differently, there was a significant reduction in disease severity in the group given a single intravaginal dose of MI-S (all days).
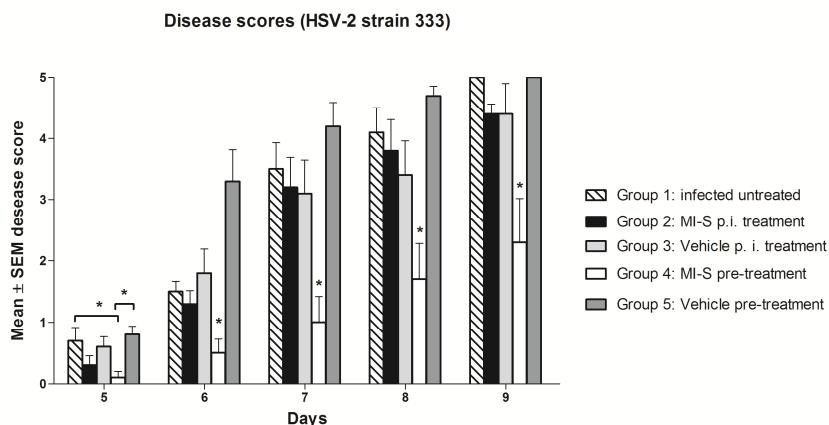
Fig. 2. Effect of MI-S on HSV-2 mean genital pathology scores. Mice (n=10) were intravaginally treated as follows: Group 1) infected untreated; 2) MI-S treatment at 20 mg/ml, 3 times/daily after infection, for 9 days; 3) Vehicle treatment, 3 times/daily after infection, for 9 days; 4) MI-S treatment at 40 mg/ml, once, 20 min before infection 5) Vehicle treatment, once, 20 min before infection. The lesion scores were determined as described in Materials and Methods. Statistical analysis (one-way ANOVA followed by the Student-Newman-Keuls test) demonstrated significant differences among groups with $p < 0.05$ (*).

When viral titers were measured, we found that there was a significant reduction of approximately 150-fold in the amount of virus shed in the MI-S pre-treated group compared to the untreated and vehicle only treated groups on day 1 post-infection (Figure 3A). We also

observed significant reductions in viral shedding on day 3 and day 9 in the MI-S pretreated mice. In the mice treated after infection (Figure 3B), significant differences in viral shedding were seen on day 1 and day 5 p.i. but post-infection treatment overall did not appear to be as effective as the single pre-infection dose in reducing viral shedding.

The mortality data for the HSV-2 333 infected mice are shown in Figure 4. By day 9, when the study was terminated due to high mortality in the untreated groups, only 40% of the mice given MI-S gel 20 minutes prior to infection had to be sacrificed due to extreme morbidity. In contrast, the infection was lethal to 100% of the mice in the infected-untreated and vehicle pre-treated only mice. The differences in mortality were significant comparing the MI-S group to the control groups (Figure 4A) while the two control groups were not significantly different. Thus, not only does a single pre-infection dose of MI-S reduce the severity of disease and viral shedding, it protects the mice from lethal infection.

A
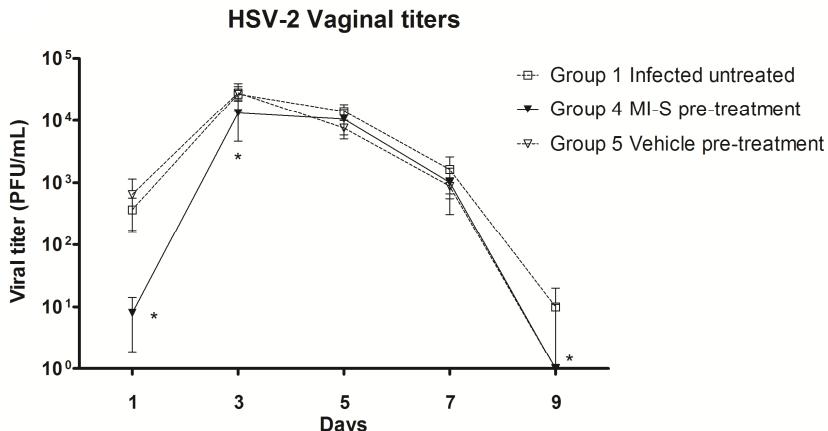
B
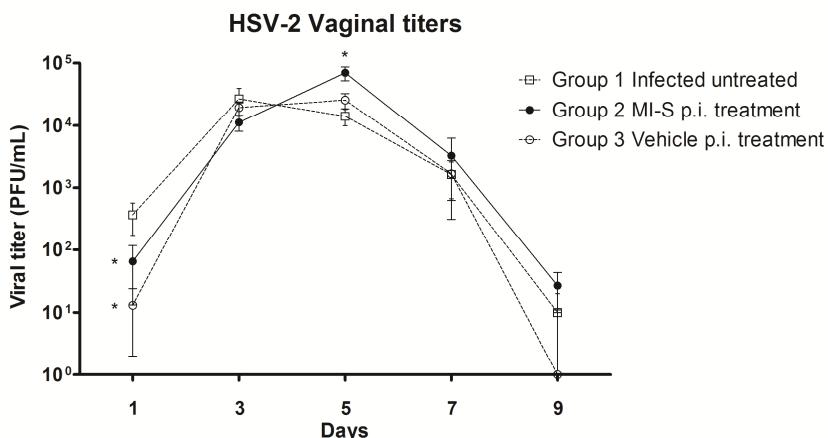
Fig. 3. Viral titers of vaginal swab samples. Mice (n=10) were intravaginally treated as follows: Group 1) infected untreated; 2) MI-S treatment at 40 mg/ml, once, 20 min before infection; 3) Vehicle treatment, once, 20 min before infection; 4) MI-S treatment at 20 mg/ml, 3 times/daily after infection, for 9 days; 5) Vehicle treatment, 3 times/daily after infection, for 9 days. The viral titers were determined by plaque assay as described in Materials and Methods. A) Pre-treatment. B) Post-infection treatment. *Statistically differences ($p<0.05$) by one-way ANOVA followed by the Student-Newman-Keuls test.

A
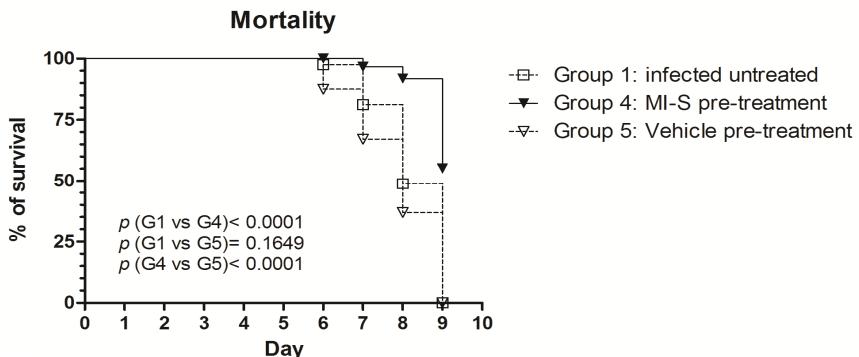
B
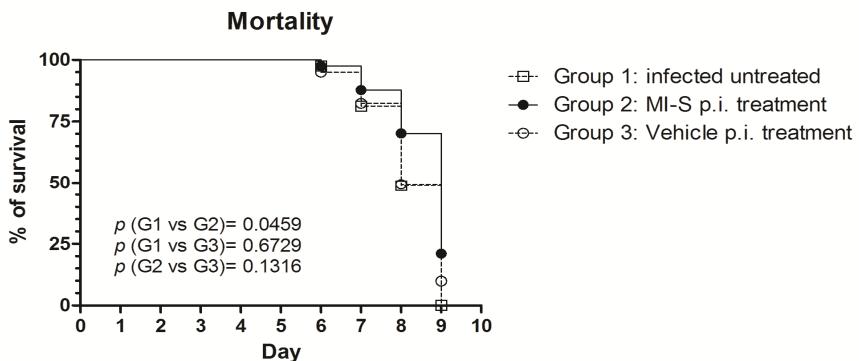
Fig. 4. The effect of MI-S treatment on mortality of infected mice after HSV-2 vaginal challenge. Mice (n=10) were intravaginally treated as follows: Group 1) infected, untreated; 2) MI-S treatment at 20 mg/ml, 3 times/daily after infection, for 9 days; 3) Vehicle treatment, 3 times/daily after infection, for 9 days; 4) MI-S treatment at 40 mg/ml, once, 20 min before infection; 5) Vehicle only treatment, once, 20 min before infection. Survival was monitored for up to 9 days after virus infection. A) Pre-treatment. B) Post-infection treatment. The data for the different groups were compared by Log-rank (Mantel-Cox) Test; p values < 0.05 were considered statistically significant.

3.6 Histopathology of Genital Tissues

Vaginal tissues were collected from mice sacrificed on day 9, fixed, sectioned, and stained with hematoxylin and eosin for analysis. Representative results are shown in Figure 5. The induction of diestrus by medroxy-progesterone acetate was confirmed by the presence of a mucified vaginal epithelium, desquamated epithelial cells, and mucus (Figure 5 C, D, and H). Figure 5A, which is from a group 1 infected but untreated animal, shows a partially denuded mucosal epithelium with dying cells and inflammatory infiltrates consisting of neutrophils and mononuclear cells in the lumen. Multinucleated cells with intranuclear inclusion bodies, characteristic of HSV-2 infection, were also seen (Figure 5B). Figures 5 C and D show a representative section from an animal in group 4 (MI-S pretreatment) showing normal epithelia and a lack of inflammation. Sloughed epithelial cells were also seen in the lumen of the MI-S pretreated mice (Figure 5D). Figures 5 G and H show sections from uninfected animals treated with MI-S. We found a normal modified columnar epithelium with mucus and epithelial cells indicating that MI-S alone was not toxic and did not induce abnormalities in the tissue. In the vehicle only animals (Figure 5 E and F) we found severe epithelial exfoliation and inflammatory infiltrate indicating that the vehicle did not inhibit the infection.
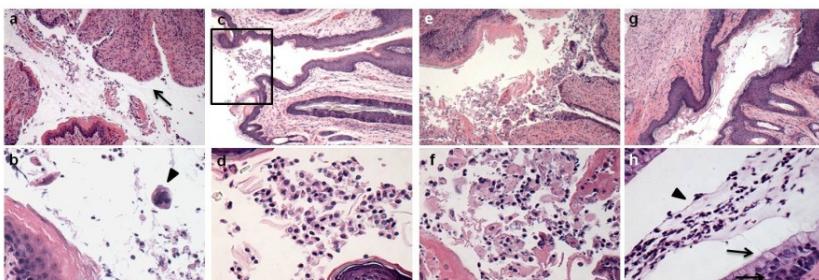
Fig. 5: Representative photomicrography of vaginal tissue sections. a, b) Group 1 (infected untreated), denuded epithelia (arrow) and multinucleated cell (arrowhead); c, d) Group 4 (infected MI-S pre-treated), vaginal orifice (square); e, f) Group 5 (infected vehicle pre-treated); g, h) Group 6 (uninfected MI-S treated), mucified columnar epithelium (arrow), mucus with epithelial cells in the lumen (arrowhead). Slides were stained with H&E, magnification: first and second row, X 100 and X 400, respectively.

Discussion

Sulfated polysaccharides have long been known to have antiviral effects (Green and Woolley, 1947). These long-chain anionic polymers predominantly inhibit viral entry, but also may interfere with later stages of viral cycle and are potential candidates to treat or prevent herpes infections (Baba et al., 1988; Carlucci et al., 2004; Cooper et al., 2002; Hayashi et al., 2008; Huheihel et al., 2002; Ohta et al., 2009; Piret et al., 2000) and may have potential utility as microbicides to block transmission of sexually transmitted viral infections (Buckheit Jr et al., 2010; Obiero et al., 2012). Recently, we described the anti-HSV-1 and HSV-2 activity of a sulfated derivative of *Agaricus brasiliensis* mycelial polysaccharide, MI-S (Cardozo et. al, 2011). We have also recently characterized the structure of MI-S (Cardozo et al.). The present paper presents a comprehensive evaluation of the in vivo activity of MI-S using ocular, cutaneous, and genital HSV infections as well as of the spectrum of activity of MI-S against several strains of HSV.

The results presented in Table 1 clearly show that MI-S has broad spectrum activity against HSV. MI-S was active against a panel of ocular HSV-1 isolates, as well as HSV-1 KOS and HSV-2 333, but the EC50 values were higher than the prior obtained results (Cardozo et al., 2011). This can be explained by the fact that, in the previous study, we used a MOI of 0.002 in a plaque reduction assay compared to the current study, in which an MOI of 1.0 was used in a yield reduction assay. The better activity found in the simultaneous treatment in comparison to the p.i. treatment is related to the fact that MI-S mainly acts in the early stages of virus cycle, inhibiting the adsorption and penetration of HSV (Cardozo et al., 2011).

For the ocular model, topical application (0.015 mg/day) of MI-S did not significantly reduce the ocular disease scores nor the viral titers in comparison to the vehicle treated groups. Differently, a sulfated polysaccharide from the red microalga Porphyridium sp. was shown to be effective against HSV-1 ocular infection (Huheihel et al., 2002). Differences in the chemical features of the polysaccharides, as well as the doses and treatment schedule applied, might be related to the discrepancy. Huheihel et. al., (2002) used rabbits and higher doses of polysaccharide (0.12 mg/day) and they started treatments immediately after infection.

In another study, oral administration of fucoidan (9 kDa) in mice, at a dose of 5 mg/day, from 1 week before virus inoculation to 1 week p.i., or for 1 week p.i. effectively suppressed the HSV-ocular infection

(Hayashi et al., 2008), whereas oral administration of MI-S (2mg/day) had no effect on ocular herpes in our study.

Similar to the topical treatment for ocular HSV, we found that topical application in the cutaneous model had no effect on the disease scores. Skin swabs of the infected area did not yield infectious virus so we were unable to determine if there was an effect on virus titers. Although MI-S was not effective in the topical cutaneous treatment model, mice treated orally with 2 mg of MI-S per day had lower disease scores (all days) and an accelerated healing of lesions (after day 9). Similarly to our results, Cooper et al., (2002) reported that the ingestion of a preparation of Undaria pinnatifida alga, enriched in sulfated polyanions such as galactofucan, increased the healing rates of active herpes infections in humans and reduced reactivation in patients with latent infections. We also tested an oral 3-day pre-treatment with 7 days p.i. treatment, however no differences, in comparison to the 7-day p.i. oral treatment, on cutaneous HSV infection were found (data not shown).

The oral bioavailability of MI-S has not been determined, but since it is a polymer with an intermediate molecular weight of 86 kDa (Cardozo et al.) it might display some degree of oral bioavailability. Previous studies have shown that dextran sulfates (10-500 kDa) can cross the intestinal epithelial layer in a cell culture system (Liang et al., 2001), and dermatan sulfate (475 kDa) is bioavailable following oral administration to humans (Dawes et al., 1989). The human oral bioavailability of some other sulfated polysaccharides such as heparin (7 kDa) and chondroitin sulfate (16 kDa) has also been demonstrated (Volpi et al., 1995).

A potential explanation for the effect of orally administered MI-S on cutaneous HSV infection is that the polysaccharide stimulates one or more components of the innate immune system and/or enhances adaptive immune responses. A number of different fungal extract components, including polysaccharides, have been shown to stimulate cytokine production, enhance activation of NK cells and T-Cells, and increase nitric oxide production by macrophages (Brandt and Piraino, 2000; Hayashi et al., 2008; Yang et al., 2012). Further studies are needed to determine if MI-S has immunostimulatory properties when administered orally.

Genital herpes infections cause significant morbidity and are a co-factor for transmission of HIV, thus agents that could prevent transmission are needed. When we tested the effect of post-infection topical treatment with MI-S in the genital model, we found that disease

scores and viral titers were not significantly different from the vehicle only control group. This finding is consistent with the lack of effect of topical treatments in the ocular and cutaneous models and observations that topical acyclovir treatment is less effective that oral acyclovir for treating genital herpes. When we tested MI-S in a microbicidal model where it was prepared in a gel and administered into the vagina 20 minutes prior to infection, we found a significant reduction in the vaginal mean disease scores (days 5 to 9) and viral titers (days 1 and 3) in comparison to the controls groups (untreated and vehicle treated). Since MI-S was shown to be a potent in vitro HSV entry inhibitor (Cardozo et al., 2011), we hypothesize that this was the mechanism by which MI-S prevented the in vivo genital infection. At a dose of 500 µg MI-S had an approximately 50% in vivo protective effect which was at least 2 x 103 times higher than the in vitro EC_{50} value (Table 1, EC_{50} (HSV-2 333)= 4.73 µg/mL which correspond to a 0.24 µg dose). According to Zeitlin et al., (1997), the in vivo 50% effective concentration of the sulfated polysaccharides carrageenan, dextran sulfate, fucoidan, heparin, and heparin sulfate against HSV-2 genital infection in mice ranged from 101 to 105 times higher than the in vitro data in cell culture.

Other sulfated polysaccharides have previously been shown to have anti-HSV-2 activity in the murine genital model with increased effectiveness occurring when mice received intravaginal treatments both pre and post-infection, but we did not tested this treatment schema (Carlucci et al., 2004; Ohta et al., 2009).

There is a significant need for antiviral preparations that women can use without the necessary involvement of their partner and a great deal of effort and funding has been invested in developing microbicide products. Several clinical trials have recently been conducted using a variety of products with mixed effects. Some of these included sulfated polysaccharides (eg. carrageenan; Carraguard TM) or other polyanions. Carraguard was not effective and this trial was terminated early. Other trials were halted early because of a lack of sufficient power in the design. The fact that these early trials have yielded mixed results could lead one to conclude that sulfated polysaccharides or other polyanions should not be developed further, however, it is clear that all sulfated polysaccharides are not equivalent in their antiviral activity. For example, MI-S was protective in the genital mouse model we used, whereas dextran sulfate was not (Piret et. al., 2000). The biological activity of sulfated polysaccharides depends on different factors such as sugar composition, molecular weight, chain conformation, degree of

substitution and position of sulfated groups. Therefore, before concluding sulfated polysaccharides should not be pursued for development, several different preparations, including perhaps MI-S, need to be tested.

In summary, we have shown the in vivo potential of MI-S for the oral treatment of cutaneous herpes. We have also shown that MI-S was active against genital HSV-2 infection when administered before vaginal challenge with HSV-2 and therefore has a potential to be developed as a microbicide. Further studies to determine the mechanism of action in vivo, including potential immunomodulatory activities, the pharmacokinetics and in vivo toxicity, and activity against other sexually transmitted pathogens, including HIV-1 are warranted in order to fully explore the potential use of MI-S as an antiviral.

Acknowledgements

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4. DISCUSSÃO GERAL

Baseado no fato de que a introdução de grupos sulfato pode melhorar a bioatividade de polissacarídeos, este trabalho teve como objetivo modificar quimicamente polissacarídeos que foram isolados do micélio (MI) e dos corpos de frutificação (FR) do fungo *Agaricus brasiliensis* gerando os respectivos derivados sulfatados (MI-S e FR-S). Este foi o primeiro relato encontrado na literatura da realização da sulfatação de polissacarídeos isolados desta espécie de fungo, o que demonstra o carácter inovador desta pesquisa.

Os polissacarídeos sulfatados apresentam um amplo espectro de atividades, principalmente associadas à sua alta capacidade de interagir com biomoléculas, tais como proteínas plasmáticas, moléculas de adesão, fatores de crescimento e glicoproteínas virais. Além disso, as características do polímero, incluindo massa molecular, grau e posição da sulfatação, composição monossacarídica e configuração de cadeia, são conhecidas por afetarem as atividades farmacológicas dos polissacarídeos sulfatados. Desta maneira, é importante associar o estudo de uma determinada atividade farmacológica com a caracterização química das amostras avaliadas a fim de se obter dados de relação estrutura-atividade, além de permitir a comparação com outros compostos e garantir a reprodutibilidade dos resultados (HAROUN-BOUHEDJA et al., 2000).

Inicialmente, realizou-se a caracerização química, físico-química e morfológica das amostras, através de diferentes experimentos, sendo alguns deles em parceria com outros laboratórios dentro e fora da UFSC.

A análise da morfologia superficial de polissacarídeos por microscopia eletrônica é uma ferramenta qualitativa que auxiliam na identificação de polissacarídeos obtidos de fungos comparando-os com padrões conhecidos e na verificação das diferenças morfológicas de derivados modificados. Segundo as fotomicrografias obtidas, os polissacarídeos de *A. brasiliensis* foram classificados como sólidos amorfos, de acordo com a descrição de Lima et al. (2008). A modificação estrutural (sulfatação) resultou no aparecimento de poros em uma superfície mais lisa.

Os termogramas obtidos na análise termogravimétrica permitiram a análise dos perfis de degradação das amostras, que foram característicos de polissacarídeos conforme já descrito na literatura (CASTRO et al., 2005; CERQUEIRA et al., 2011; HATAKEYAMA; QUINN, 1999; HONG; CHOI, 2007), inclusive da espécie relacionada *A. blazei* (HONG; CHOI, 2007). A sulfatação foi responsável por uma

leve redução da termoestabilidade das amostras, conforme demonstrado previamente por Jayakumar e colaboradores (2008).

A massa molecular (MM) aparente das amostras foi determinada através de cromatografia liquida de alta eficiência em coluna de permeação em gel utilizando padrões de dextrana de diferentes massas moleculares. A amostra FR apresentou uma MM de 609 kDa, que está dentro da faixa (390-2000 kDa) de valores encontrados por outros autores (DONG et al., 2002; LIU, J. et al., 2011; MIZUNO et al., 1990; WU et al., 2012). Vale ressaltar que a forma de preparação dos polissacarídeos e o método utilizado podem influenciar na determinação da massa molecular das amostras. A MM aparente do MI (310 kDa) foi menor que a do FR e similar aos valores encontrados por Fujimiya et al. (380 kDa) (1999) e Lin e Yang (274 kDa) (2006). As MM dos derivados sulfatados (FR-S=127 kDa e MI-S=86 kDa) foram menores em relação aos respectivos polissacarídeos nativos. Esta redução na MM provavelmente está relacionada à hidrólise dos polímeros devido às condições empregadas (alta temperatura e baixo pH) na reação de sulfatação, conforme descrito por Lanteri (1978).

Os teores de açúcares totais das quatro diferentes amostras ficaram entre 66 e 78%, que são valores condizentes com o método de isolamento utilizado (ultrafiltração/diálise), escolhido por ser mais rápido e permitir o isolamento de grande volume de biomassa, porém com obtenção de polissacarídeos com grau de pureza um pouco inferiores aos obtidos através de cromatografia de troca iônica (80-95%) (DONG et al., 2002; LIU, J. et al., 2011).

O baixo teor de proteínas (1-1.8%), determinado pelo método de Bradford, foi comparável aos valores obtidos por outros autores (CAMELINI et al., 2005; LIMA et al., 2008) e de acordo com os dados de análise elementar do nitrogênio. De acordo com Fernandes et al. (2010) a temperatura, o solvente e o pH utilizado nos processos de extração e secagem podem interferir com estruturas secundárias e terciárias das proteínas, quebrando as ligações peptídicas e reduzindo o conteúdo protéico das amostras. Portanto, diferentes procedimentos de extração e secagem podem explicar os maiores níveis de proteína relatados por outros autores para polissacarídeos obtidos da frutificação do *A. brasiliensis* obtidos do cogumelo (DONG et al., 2002; FUJIMIYA et al., 1998; GONZAGA et al., 2005; HONG; CHOI, 2007) e do micélio (KER et al., 2005).

A sulfatação do FR-S e do MI-S foi confirmada através da determinação do conteúdo de sulfato (>36%), enxofre (~14%) e espectroscopia no infravermelho, na qual as amostras FR-S e MI-S

mostraram duas novas bandas de absorção em 1253 e 810 cm^{-1}, relacionados aos grupamentos C-S-O e S=O, respectivamente (SILVERSTEIN et al., 2005). De acordo com a determinação da composição monossacarídica por CCD e CLAE-UV e pela análise dos espectros de RMN de ^{1}H e ^{13}C, o **FR** foi caracterizado como sendo uma **β-(1→6)-(1→3)-glicana**, e o **MI** como uma **β-(1→2)-glico-β-(1→3)-manana**. Ambas as estruturas confirmaram a configuração descrita anteriormente para polissacarídeos de *A. brasiliensis* (CARDOZO et al., 2011; MIZUNO et al., 1999). Uma vez que a sulfação resulta na alteração dos valores de deslocamento químico dos carbonos (7-10 ppm) e dos hidrogênios (0.5-2 ppm), a análise dos espectros de RMN permite determinar o sítio de ligação dos grupamentos sulfato na cadeia polissacarídica (DUUS et al., 2000; HRICOVÍNI et al., 2003). A partir dos dados obtidos pode-se concluir que o FR-S foi totalmente sulfatado nos carbonos 4 e 6 das porções β-1,6- e β-1,3-glicana. As estruturas sugeridas para o FR e FR-S estão mostradas na Figura 4. Diferentemente, o MI-S foi totalmente sulfatado no C-6 terminal da cadeia principal e parcialmente substituído nos C-3, C-4, e C-6 da porção (1→2)-β-D-manana e nos C-2, C-4, and C-6 da (1→3)-β-D-glicana das cadeias laterais. As estruturas sugeridas para o MI e MI-S estão mostradas na Figura 5. Apesar das reações de sulfatação seguirem o mesmo protocolo, os diferentes padrões de sulfatação observados para MI-S e FR-S, provavelmente foram devido à variações na composição/configuração dos carboidratos nativos (MI e FR).
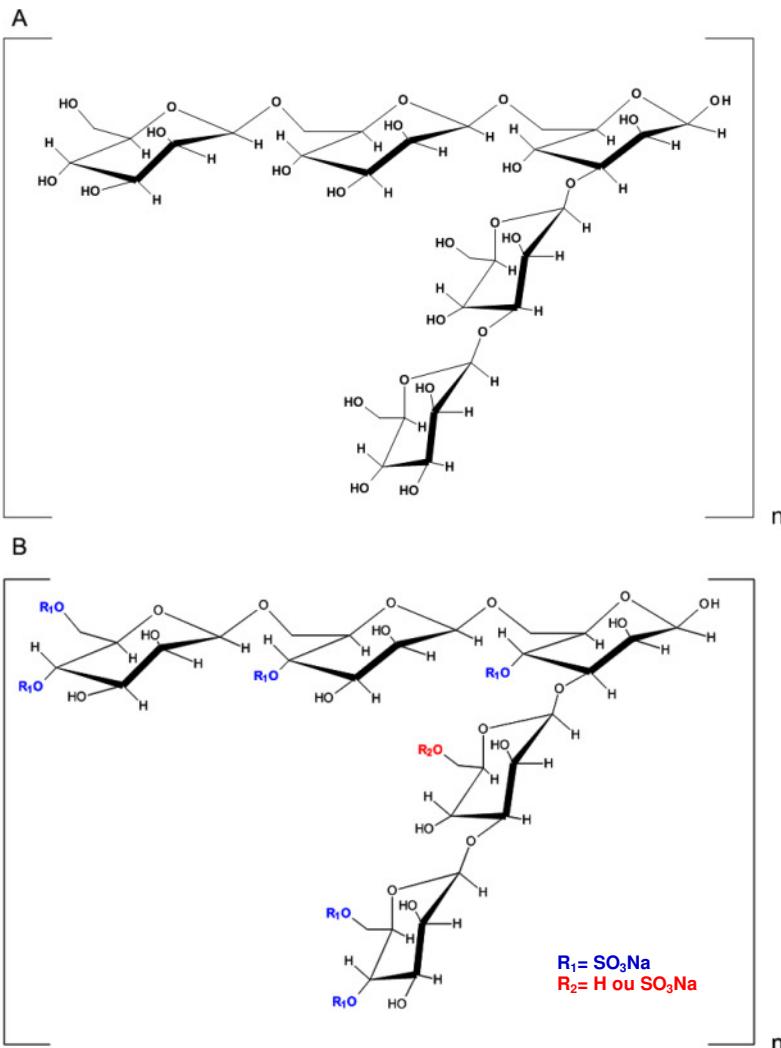
Figura 4: Estrutura do FR (A), uma β-(1→6)-(1→3)-glicana, e seu derivado sulfatado FR-S (B). Em azul e vermelho os sítios de substituição total e parcial, respectivamente.
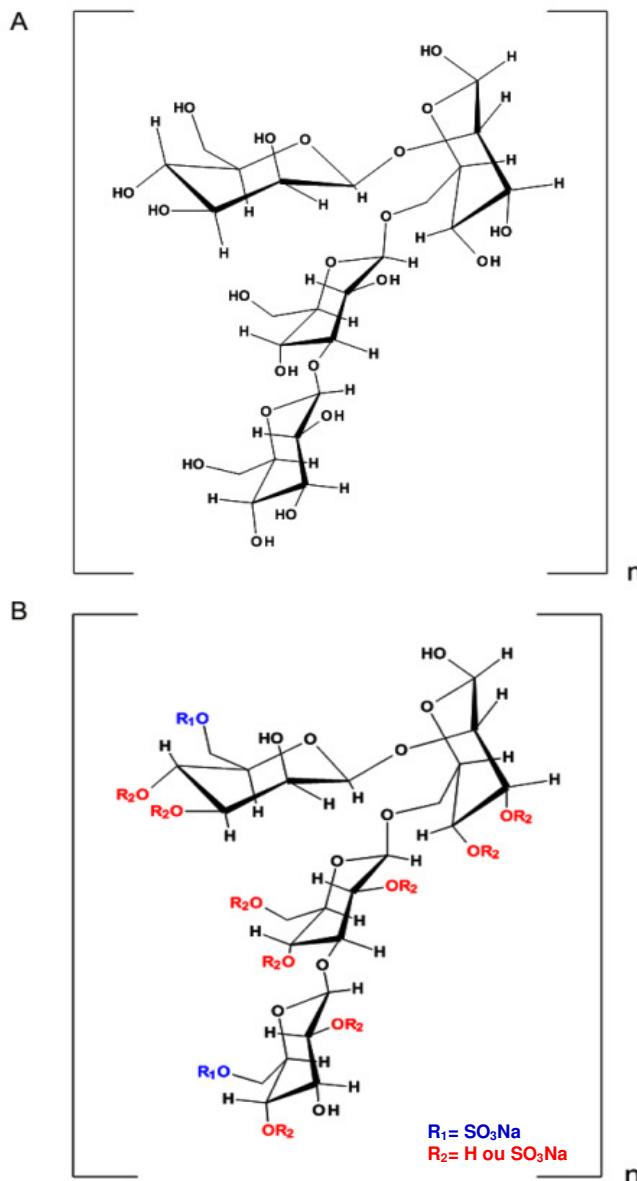
Figura 5: Estrutura do MI (A), uma β-(1→2)-glico-β-(1→3)-manana, e seu derivado sulfatado MI-S (B). Em azul e vermelho os sítios de substituição total e parcial, respectivamente.

A derivatização de polissacarídeos através da sulfatação já é reconhecidamente responsável por alterações na conformação de cadeia original, geralmente resultando no aumento das suas ações farmacológicas, incluindo as atividades citotóxica e/ou antitumoral (BAO et al., 2010; CAO; IKEDA, 2009; LIN et al., 2004; LIU et al., 2009; NIE et al., 2006; TAO et al., 2009; WANG; ZHANG, 2009; ZHANG et al., 2012). De fato, neste trabalho a sulfatação aumentou a atividade citotóxica contra células tumorais A549 do polissacarídeo obtido dos corpos de frutificação de *A. brasiliensis*, e foi essencial para a atividade do polissacarídeo isolado do micélio. Os resultados dos ensaios de citotoxicidade pelo MTT indicaram que as atividades são concentração- e tempo-dependentes, sendo que as amostras foram mais eficazes quando permaneceram em contato com as células, em maiores concentrações, por mais longo período (72 h).

Estudos comparando derivados sulfatados produzidos com diferentes graus de substituição (GS) têm demonstrado que existe um valor ideal para este parâmetro, que varia de acordo com o tipo de polissacarídeo, para alcançar uma melhor eficácia, em vez de uma relação direta com o efeito citotóxico. Por exemplo, Liu et al. (2009), ao compararem polissacarídeos com MMs semelhantes (~ 20 kDa), observaram uma maior atividade citotóxica frente às células Hep 2 para aqueles com GS de 1,8 em relação aos com GS inferiores (1,52), enquanto que a atividade foi reduzida quando o GS foi aumentado para 2,02. Da mesma forma, Bao et al. (2010) demonstraram que polissacarídeos sulfatados com baixo GS (0,11-0,14) foram menos ativos do que os com GS entre 0,28-066, enquanto o aumento do valor de GS para 1,06 reduziu a atividade citotóxica. Com os dados aqui obtidos, não foi possível fazer uma correlação direta entre a atividade citotóxica e o GS, uma vez que a amostra mais ativa (MI-S) teve o menor valor de GS (1.58). Sendo assim, é necessária a produção de derivados sulfatados do MI e FR com diferentes graus de sulfatação para determinar o DS ideal para cada polissacarídeo e melhor entender as suas relações estrutura-atividade.

O MI-S apresentou uma atividade citotóxica um pouco maior em comparação com o FR-S, e ambos foram mais ativos em comparação ao DEX-S, um controle adquirido comercialmente. Uma vez que o DEX-S apresenta um MM (>500 kDa) muito maior que o do FR-S (141 kDa) e do MI-S (86 kDa), acredita-se que este seja um fator importante na redução da sua eficácia, como observado por Yang e colaboradores (2008), que encontraram uma citotoxicidade significativamente maior para uma fucoidana parcialmente hidrolisada (490 kDa), em comparação

ao polímero nativo (5.100 kDa). É importante mencionar que os polissacarídeos sulfatados aqui avaliados têm diferentes composição de açúcares, conformação de cadeia e posição da sulfatação, sendo que essas características provavelmente contribuem para a observada variação na sua eficácia.

Após a triagem da atividade citotóxica pelo ensaio do MTT, as amostras com atividade mais promissora foram avaliadas pelo ensaio da sulforrodamina B, que permite diferenciar a ação citotóxica do efeito citostático. Os dados obtidos mostraram que MI-S e FR-S possuem efeito citotóxico em células A549, sem nenhum efeito citotóxico ou citostático observado nas células VERO, não tumorais, na maior concentração testada (1500 µg/ml). Isso mostra que ambos, MI-S e FR-S, parecem ter efeito citotóxico seletivo sobre células tumorais. Porém, a citotoxicidade frente a outras linhagens de células saudáveis e tumorais deve ser avaliada, para melhor estabelecer o perfil de seletividade das amostras.

A sulfatação de polímeros de carboidratos também tem demonstrado aumentar a atividade antiviral (GHOSH et al., 2009; KARMAKAR et al., 2010; LIU, X. et al., 2011). Neste trabalho, os dois derivados sulfatados obtidos tiveram sua atividade anti-herpética e mecanismo de ação determinados.

A fim de avaliar a influência do período de tratamento na atividade anti-HSV do FR-S e do MI-S, o ensaio de redução do número de placas de lise foi realizado sob duas condições diferentes: 1) tratamento simultâneo (amostras foram adicionadas às células ao mesmo tempo da infecção viral); 2) tratamento pós-infecção (amostras foram adicionadas 1 h após a infecção viral). Como resultado, todos os polissacarídeos sulfatados testados [amostras: FR-S e MI-S e controles: heparina (HEP) e DEX-S] foram consideravelmente mais efetivos no tratamento 1 que no tratamento 2. Este mesmo comportamento foi verificado para carragenanas (CARLUCCI et al., 1999), fucoidanas (KARMAKAR et al., 2010) e glicanas sulfatadas (ZHANG et al., 2004) e já era esperado, uma vez que essa classe de compostos é descrita como forte inibidora de entrada dos vírus nas células.

Comparando-se os valores de EC_{50} verificou-se que a HEP teve valores maiores que os demais polímeros testados. Devido ao fato de que a HEP é o único polissacarídeo de cadeia linear, há um forte indício de que a presença de ramificações seja importante para a atividade anti-HSV de polissacarídeos sulfatados.

Ao contrário da HEP, as amostras FR-S e MI-S não apresentaram ação anticoagulante, em concentrações correspondentes àquelas que

causam 100% de inibição da multiplicação viral, evidenciando a especificidade da ação anti-herpética. O tratamento oral de camundongos com 2 mg/dia do MI-S também não mostrou sinais de toxicidade ou sangramento, porém a redução do tempo de coagulação *in vivo* não foi avaliada.

Os compostos FR-S e MI-S não apresentaram efeito virucida direto sobre as partículas virais. Outros estudos também detectaram a ausência de efeito virucida de polissacarídeos sulfatados (ADHIKARI et al., 2006; CHATTOPADHYAY et al., 2008; CHATTOPADHYAY et al., 2007; KARMAKAR et al., 2010; MATSUHIRO et al., 2005; ZHU et al., 2006), ou o efeito foi detectado apenas em concentrações superiores ao valores de EC_{50} (CARLUCCI et al., 1999; MAZUMDER et al., 2002; ZHU et al., 2004). Bruggemann et al. (2006) detectaram atividade virucida contra o HSV-1 para um extrato aquoso de *A. brasiliensis*, porém além deles terem utilizado outro ensaio virucida, a amostra foi extraída em água fria, diferentemente da extração em água fervente do FR e MI.

Entre as diferentes etapas do ciclo dos HSV, a adsorção e a penetração têm atraído a atenção de pesquisadores como alvos terapêuticos. A adsorção e penetração dos HSV são processos bioquímicos complexos, envolvendo cinco (gB, gC, gD, gH-gL, GR) das doze glicoproteínas de superfície do HSV. A gC-1 é descrita por desempenhar um papel mais importante em comparação à gB-1 na adsorção de vírus HSV selvagens, enquanto que a gB medeia a ligação de mutantes gC-negativos. Diferentemente, a contribuição da gB parece ser mais importante na adsorção do HSV-2 (GERBER et al., 1995; TRYBALA et al., 2000).

Segundo a opinião mais aceita, polissacarídeos sulfatados apresentam atividade antiviral por interferirem na interação eletrostática entre as glicoproteínas virais e as glicosaminoglicanas de superfície celular (BERGEFALL et al., 2005). Neste trabalho, quatro diferentes estratégias experimentais do ensaio de adsorção foram testadas utilizando vírus recombinantes desprovidos de gC, a fim de investigar o envolvimento dessa glicoproteína na atividade antiherpética do FR-S. Os dados obtidos demonstraram que os vírus HSV-1 e HSV-2 gC-negativos foram menos sensíveis ao FR-S do que as cepas parentais, o que indica uma possível interação do FR-S com a gC, como ocorre para a HEP (CHESHENKO; HEROLD, 2002; GERBER et al., 1995). Porém, a interação do FR-S com glicoproteínas do HSV ainda não foi determinada. É possível que ocorra, ainda, a interação do FR-S com

componentes de membrana celular, uma vez que, diferentemente da HEP, o pré-tratamento das células com FR-S inibiu a adsorção viral.

Após a adsorção viral às glicosaminoglicanas celulares, a interação irreversível das glicoproteínas virais, gB, gD e gH, com receptores celulares desencadeiam o processo de fusão de membranas e entrada viral. O FR-S inibiu a penetração de todas as cepas testadas do HSV mais eficientemente do que a HEP. Esses resultados permitiram postular a hipótese de que a presença de ramificações na cadeia polimérica poderia afetar positivamente a capacidade de inibir a penetração e a adsorção viral. O fato da gC desempenhar um papel menos importante na penetração dos HSV pode explicar por que não houve diferença entre os valores de EC_{50} para o FR-S e a HEP no ensaio de penetração, quando compara-se os vírus do tipo selvagem com os mutantes gC-nulos.

A amostra MI-S também foi avaliada em diferentes tempos após a infecção viral, apresentando atividade inibitória superior a 50%, mesmo quando adicionada 16 h após a infecção. Este resultado também nos permitiu postular a hipótese de que ela estaria agindo em etapas pós-entrada viral. Foi realizado, então, o ensaio de *Western blotting*, no qual a amostra MI-S mostrou uma considerável inibição da expressão de proteínas nos diferentes estágios do ciclo viral: α (ICP27), β (UL42), e γ (gB). Apesar de poucos trabalhos relatarem a inibição de etapas pós-entrada viral por polissacarídeos sulfatados, Artan et al., (2010) descreveram a inibição da expressão de proteínas do HIV por um oligossacarídeo sulfatado bem como para o DEX-S.

As glicoproteínas virais que interferem com a entrada do HSV (gB, gD e gH/gL), bem como o heterodímero gE/gI, estão envolvidos na propagação viral de célula para célula. A propagação intercelular dos vírus entre células adjacentes permite o seu escape da resposta imune do hospedeiro, sendo alvo potencial para uma intervenção terapêutica (NYBERG et al., 2004; ROIZMAN et al., 2007).

A inibição da transmissão intercelular dos HSV por compostos sulfatados foi também demonstrada para a sumarina (AGUILAR et al., 1999), ciclitóis dissulfatados (EKBLAD et al., 2006), o oligossacarídeo PI-88 (NYBERG et al., 2004) e seu derivado glicosídico (EKBLAD et al., 2010), e um polissacarídeo capsular de *E. coli* (PINNA et al., 2008). No entanto, Bergefall et al. (2005) não encontraram qualquer inibição da difusão célula a célula do HSV-1 e HSV-2 para o polissacarídeo sulfato de condroitina. As amostras FR-S e MI-S foram responsáveis por uma redução significativa das áreas das placas de lise, em relação aos controles não tratados, o que poderia contribuir para a sua ação antiviral.

O detectado efeito sinérgico do MI-S e do FR-S com o ACV sugeriu que esses compostos apresentam potencial para serem usados sozinhos ou em combinação com o ACV no tratamento de infecções herpéticas. Alguns outros inibidores de entrada de vírus nas células também apresentaram efeito sinérgico com o ACV, como por exemplo, um complexo polissacarídeo-proteína obtido do fungo *Ganoderma lucidum* (EO et al., 2000), o docosanol (MARCELLETTI, 2002) e o oxiresveratrol (CHUANASA et al., 2008).

Conforme anteriormente mencionado para a atividade citotóxica, as propriedades anti-HSV de polissacarídeos sulfatados também são determinadas por uma combinação de características estruturais (GHOSH et al., 2009). Apesar de apresentarem diferentes características químico-estruturais, as amostras FR-S, MI-S e DEX-S apresentaram atividade anti-HSV com mecanismos de ação bastante semelhantes, com exceção da HEP, que foi significativamente menos efetiva na inibição da penetração viral, provavelmente relacionada à ausência de ramificações na sua cadeia (RABENSTEIN, 2002).

A ação anti-HSV equivalente do FR-S e do MI-S permite a utilização do micélio como alternativa à produção de biomassa a partir dos cogumelos, que é mais cara e demorada. Ao verificarmos a promissora atividade anti-herpética *in vitro* e a viabilidade de produção de biomassa em quantidades adequadas, decidiu-se continuar o estudo avaliando a atividade anti-herpética *in vivo* do MI-S, através de modelos murinos de infecção ocular, cutânea e genital. Além disso, foi avaliada a atividade anti-HSV *in vitro* do MI-S contra um painel de diferentes cepas virais, incluindo as testadas anteriormente, HSV-1 cepa KOS e HSV-2 cepa 333, usando o ensaio de redução do número de partículas infecciosas (*yield reduction assay*). Apesar dos valores obtidos de EC_{50} terem sido maiores que os obtidos anteriormente, quando a atividade antiviral foi testada em um MOI inferior (0,002) pelo ensaio de redução do número de placas de lise (CARDOZO et al., 2011) o MI-S apresentou, ainda, uma ação anti-HSV promissora quando foi usado o MOI de 1.

A toxicidade *in vivo* do MI-S foi avaliada em animais não infectados, que receberam os tratamentos local (ocular - solução em DMEM; cutânea - creme; e genital - solução de metilcelulose) e oral (solução em PBS). Não foram observados sinais locais ou generalizados de toxicidade, tais como eritema ou edema no local da administração, alteração nos pêlos, letargia e dificuldade respiratória. Além disso, não houve diferenças estatísticas significativas nos pesos dos animais antes e

após os tratamentos, indicando que o MI-S não apresentou toxicidade, nas vias e nas concentrações testadas.

Com relação ao modelo murino de infecção ocular, os tratamentos tópico (0,015 mg/dia) e oral (2 mg/dia) com o MI-S não reduziram significativamente os escores da doença, nem os títulos virais, em comparação com os grupos controle. Não foram encontrados muitos relatos recentes de atividade anti-herpética *in vivo* de polissacarídeos sulfatados. Um polissacarídeo sulfatado da microalga vermelha *Porphyridium* sp. mostrou-se eficaz n o tratamento da infecção ocular pelo HSV-1, em um estudo realizado por Huheihel et al. (2002). O modelo animal utilizado, o desenho experimental do tratamento e a escolha das doses devem ser levados em consideração, uma vez que, diferentemente do nosso estudo no qual os camundongos foram tratados com uma dose de 0,015 mg/dia a partir de 4h p.i,. por uma semana, estes autores usaram doses mais elevadas (0,12 mg/dia) para tratar coelhos, iniciando o tratamento imediatamente após a infecção (15-20 min), durante duas semanas. Outro estudo mostrou que a administração oral de fucoidana (9 kDa) em camundongos, na dose de 5 mg/dia, a partir de uma semana antes da inoculação viral e até uma semana p.i., ou por somente um semana p.i., suprimiu efetivamente a infecção ocular pelo HSV-1, sendo que o pré-tratamento aliado ao pós-tratamento foi mais eficaz que o tratamento p.i. sozinho (HAYASHI et al., 2008). Apesar do MI-S não ter sido eficaz no tratamento tópico, os camundongos infectados cutaneamente pelo HSV-1 e tratados oralmente com 2 mg/dia de MI-S apresentaram menores escores da doença (todos os dias) e uma cura acelerada das lesões (a partir do nono dia). Similarmente, Cooper et al. (2002) mostraram que a ingestão de uma preparação da alga *Undaria pinnatifida*, enriquecida em polissacarídeos sulfatados, como a galactofucana, acelerou a cura de infecções herpéticas ativas em humanos e evitou a sua reativação em pacientes com infecções latentes.

Apesar da biodisponibilidade oral do MI-S (um polímero com MM intermediário de 86 kDa) não ter sido avaliada, acreditamos que ele possa exibir algum grau de biodisponibilidade oral e/ou atuação através da ativação indireta do sistema imunológico associado ao intestino, conforme descrito para outros polissacarídeos sulfatados com atividade antiviral (HAYASHI et al., 2008; YANG et al., 2012). De fato, estudos anteriores demonstraram a biodisponibilidade oral *in vitro* para o sulfato de dextrana (10-500 kDa) (LIANG et al., 2001), e em seres humanos para o sulfato de dermatana (475 kDa) (DAWES et al., 1989).

Com relação aos experimentos no modelo genital, os resultados obtidos mostraram que o pré-tratamento com o MI-S reduziu

significativamente os escores da doença (todos os dias) e os títulos virais (dias 1 e 3), enquanto o tratamento pós-infecção não foi estatisticamente diferente dos controles.

Uma vez que foi comprovado que o MI-S é um potente inibidor da entrada do HSV *in vitro* (CARDOZO et al., 2011), é provável que esse seja o mecanismo pelo qual o MI-S impediu a infecção genital *in vivo*. Uma única dose de 500 µg do MI-S promoveu cerca de 50% de efeito protetor *in vivo*, que é pelo menos 2×10^3 vezes maior que o valor de sua EC_{50} *in vitro* [$EC_{50\ (HSV-2\ 333)} = 4,73$ µg/mL, que corresponde a uma dose de 0,24 µg]. Segundo Zeitlin et al. (1997), os valores de EC_{50} dos polissacarídeos sulfatados carragenana, sulfato de dextrana, fucoidana, sulfato de heparana e heparina obtidos *in vivo* contra a infecção genital por HSV-2 foram de 10^1 a 10^5 vezes maiores do que os valores obtidos em cultura de células.

Outros polissacarídeos sulfatados foram anteriormente descritos por possuirem atividade anti-HSV-2 no modelo murino genital, com eficácia aumentada quando os animais receberam pré-tratamento e tratamento p.i. (CARLUCCI et al., 2004; OHTA et al., 2009). Diferentemente, o pré-tratamento intravaginal com o sulfato de dextrana não demonstrou atividade anti-HSV-2, em um desafio vaginal realizado em camundongos (PIRET et al., 2000).

Os resultados obtidos neste trabalho mostraram o potencial *in vivo* do MI-S no tratamento oral do herpes cutâneo. O MI-S também preveniu significativamente a infecção pelo HSV-2, quando administrado antes do desafio vaginal e, portanto, tem um potencial a ser desenvolvido como microbicida. A imunoestimulação pode estar relacionada ao efeito positivo na cicatrização de lesões cutâneas dos animais tratados oralmente com o MI-S, como já foi descrito para esta classe de compostos. Uma vez que o MI-S demonstrou ser um potente inibidor *in vitro* da entrada dos HSV nas células, é provável que seja este o mecanismo pelo qual o MI-S preveniu a infecção genital. Porém, são necessários mais estudos para elucidar o mecanismo das atividades detectadas *in vivo*.

5. CONCLUSÃO

Através dos resultados aqui obtidos pode-se verificar que o processo de sulfatação possibilitou a geração de dois diferentes compostos sulfatados, uma β-(1→6)-(1→3)-glicana e uma β-(1→2)-glico-β-(1→3)-manana, isolados das duas formas de vida do fungo *Agaricus brasiliensis*, frutificação e micélio, respectivamente. Os dois polissacarídeos sulfatados produzidos, FR-S e MI-S, apresentaram promissoras atividades anti-herpética e citotóxica. Comparando-se quimicamente o FR-S e o MI-S verificaram-se algumas características bastante semelhantes, como a termoestabilidade, os teores de açúcares totais, de proteínas e de enxofre, mas com alguma diferença na massa molecular, sendo a estrutura da cadeia, a composição monossacarídica e os sítios de sulfatação as diferenças mais marcantes entre estes dois compostos. Apesar disso, FR-S e MI-S apresentaram atividade anti-herpética *in vitro* similar, o que direcionou os estudos *in vivo* somente com o MI-S, polissacarídeo sulfatado com produção mais rápida e barata.

A atividade anti-HSV *in vitro* do MI-S, com mecanismo de ação envolvendo principalmente a inibição da entrada, mas também com atividade em outros estágios do ciclo de replicação viral (expressão de proteínas e dispersão intercelular), foi confirmada *in vivo*. Através de modelos murinos, determinou-se a eficácia do MI-S no tratamento da infecção cutânea e prevenção da infecção genital causadas, respectivamente, pelo HSV-1 KOS e pelo HSV-2 333.

No que diz respeito à avaliação da atividade citotóxica, as diferenças estruturais resultaram em uma atividade mais promissora para o MI-S, em relação ao FR-S e ao FR, mostrando mais uma vez a vantagem da utilização do micélio como alternativa à produção de biomassa a partir dos cogumelos.

6. **CONSIDERAÇÕES FINAIS**

Este trabalho demonstrou a potencial aplicabilidade farmacêutica dos polissacarídeos do *Agaricus brasiliensis*, fornecendo dados promissores de eficácia e preliminares de segurança *in vitro* e *in vivo*, além de impulsionar outros projetos envolvendo a produção biotecnológica de biomassa miceliana. Para que os estudos continuem, o processo de sulfatação necessita ser padronizado a fim de garantir que os derivados sulfatados mantenham as mesmas características físico-químicas e permitam o aumento na sua escala de produção. No âmbito do processo de pesquisa e desenvolvimento do MI-S como um potencial candidato a fármaco, os próximos passos incluem o desenvolvimento de formas farmacêuticas, os estudos de estabilidade, toxicidade e farmacocinética, bem como a avaliação clínica de sua eficácia e segurança.

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APÊNDICE A - OUTROS ARTIGOS CORRESPONDENTES ÀS ATIVIDADES REALIZADAS NO PERÍODO

Artigos publicados

Freitas, A.M.; Almeida, M.T.R.; Andrighetti-Fröhner, C.R.; **Cardozo, F.T.G.S.**; Barardi, C.R.M.; Farias, M.R.; Simões, C.M.O. Antiviral activity-guided fractionation from *Araucaria angustifolia* leaves extract. Journal of Ethnopharmacology, v. 126, p. 512-517, 2009.

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Artigos submetidos para publicação

Cruz, A.C.C; Silva, M.L.; **Cardozo, F.T.G.S.**; Simões, C.M.O. Retinoic acid and bone morphogenetic protein-2 association improves osteogenic differentiation of human adipose-derived stem cells. Archives of Oral Biology, submetido em 17/05/2012, status: *under review.*

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Cardozo, F.T.G.S.; Galdino, S.L.; Simões, C.M.O. Cytotoxic activity of acridines and indole-imidazolidine derivatives.

Gomez, L.S.A; **Cardozo, F.T.G.S.**; Barardi, C.R.M.; Simões, C.M.O.; Galvis, L.B. Antiherpetic mechanism of labdadienedial.

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