KRISTIE AIMI YAMAMOTO

ATIVIDADE ANTIVIRAL DE POLISSACARÍDEO E FRAÇÕES OBTIDOS DE PRODUTOS NATURAIS NA REPLICAÇÃO DO POLIOVÍRUS E HERPESVÍRUS

Londrina
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Dissertação apresentada junto ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina, como requisito parcial para obtenção do título de Mestre em Microbiologia.

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Londrina, 28 de março de 2011.

DEDICO

Aos meus pais e irmãos, os que amo incondicionalmente.

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Não somos responsáveis apenas pelo que fazemos,
mas também pelo que deixamos de fazer.
(Molière, dramaturgo francês)

Yamamoto, Kristie Aimi. **Atividade antiviral de polissacarídeo e frações obtidos de produtos naturais na replicação do poliovírus e hrepesvírus.** 2011. 56 f. Dissertação (Mestrado em Microbiologia) - Universidade Estadual de Londrina - UEL, Londrina.

RESUMO

O estudo dos antivirais teve início na década de 1950, porém somente por volta de 1960, um grupo de cientistas desenvolveu compostos seguros e seletivos para os vírus através dos análogos de nucleotídeos. Atualmente, cerca de 40 compostos encontram-se aprovados para uso clínico e estão focados em um número pequeno de vírus, além disso, vem aumentado o aparecimento de cepas virais resistentes à quimioterapia atual. Novos vírus continuam emergindo e a procura de substâncias com maior ação seletiva tem incentivado a pesquisa de novos agentes antivirais, principalmente a partir de produtos naturais. Várias pesquisas com antivirais naturais vêm reportando a atividade de polissacarídeos isolados de plantas e fungos. Neste trabalho foi avaliada a atividade antiviral de frações e polissacarídeo isolado de *Agaricus brasiliensis*, um basidiomiceto que apresenta propriedades medicinais como anticarcinogênico, dentre outras. Foi estudado também o efeito antiviral de polissacarídeo isolado da planta *Azadirachta indica*, conhecida popularmente como neem, amplamente utilizada na medicina alternativa e conhecida por possuir uma vasta gama de compostos biologicamente ativos que são quimicamente diferentes e estruturalmente complexas. As substâncias testadas não apresentaram efeito citotóxico significativo e, em sua maioria, foram capazes de inibir a replicação dos herpevirus bovino e humano e também do poliovirus. Estas substâncias agiram principalmente nas etapas iniciais da replicação viral.

Palavras-chave: Agentes antivirais. Poliovírus. Vírus do herpes. Produtos naturais.

Yamamoto, Kristie Aimi. **Antiviral activity of fractions obtained polysaccharide and natural products replication of poliovirus and herpes.** 2011. 56 p. Dissertação (Mestrado em Microbiologia) - Universidade Estadual de Londrina - UEL, Londrina.

ABSTRACT

The study of antiviral began in the 1950s, but only around 1960, a group of scients has developed safe and selective compounds for viruses by nucleotide analogs. Currently, about 40 compounds for viruses by nucleotide analogs. Currently, about 40 compounds are approved for clinical use and are focused on a small number of viruses, moreover, has increased the emergence of resistant viral strains to current chemotherapy. New viruses continue to emerge and the demand for substances with more selective action has encouraged the search for new antiviral agents, especially from natural products. Several researches have reported with natural antiviral activity of polysaccharides fractions isolated from *Agaricus brasiliensis,* one basidiomycete that has medicinal properties such as anticarcinogenic, among others. The antiviral activity of polysaccharides isolated from Azadirachta was also studied indicates, popularly known as neem, widely used in alternative medicine and known to possess a wide range of biologically active compounds that are chemically and structurally different complex. The substances tested showed no significant cytotoxic effect, and most were able to inhibit the replication of bovine and human herpevirus and also the poliovirus. These substances act mainly in the initial stages of viral replication.

Key words: Antiviral agents. Natural products. Agaricus brasiliensis. Azadirachta indica.

SUMÁRIO

1 INTRODUÇÃO

Vírus são partículas nucleoprotéicas metabolicamente inertes, acelulares e ultramicroscópicas, contendo um "pool" de genes de RNA ou DNA, cercado ou não por um envelope lipídico. Diferente de bactérias de vida livre, os vírus são parasitas intracelulares obrigatórios que utilizam a maquinaria celular do hospedeiro para propagar novos vírus, podendo causar uma variedade de injúrias ao mesmo. Dessa forma, os vírus podem ser denominados como "parasitas acelulares de hospedeiros celulares" (CHATTOPADHYAY *et al.*, 1999).

Cada cepa de vírus é única na sua estrutura antigênica de superfície, nos seus receptores nas células hospedeiras e no seu ciclo de vida. Como conseqüência da variação genética, variedade no modo de transmissão, replicação eficiente e habilidade de persistir no hospedeiro, os vírus se adaptaram a todas as formas de vida e ocuparam numerosos nichos ecológicos, resultando em doenças frequentes em humanos, animais e plantas (CHATTOPADHYAY; NALK, 2007).

Evidências sobre a existência das infecções virais surgiram desde os primeiros registros de atividades humanas (FERREIRA *et al.,* 2008*),* acompanhando o homem, provavelmente, desde a formação das primeiras civilizações e causando algumas das doenças mais devastadoras à humanidade. Estima-se que a varíola seja o primeiro caso conhecido de infecção viral e tenha aparecido há mais de 5000 anos, matando milhões de pessoas, antes de ser erradicada oficialmente em 1979 (JEROME, 2005). Esse fato representou um grande marco no controle das doenças infecciosas e na medicina preventiva, pois foi a primeira doença infecciosa viral a ser erradicada pela vacinação.

De forma semelhante, no começo do século 20, o vírus da poliomielite foi responsável por causar paralisia em milhares de crianças todos os anos, se tornando um problema de saúde pública, até a introdução das vacinas a partir de 1950. Atualmente, o número de doentes caiu vertiginosamente, porém, casos registrados na África e Ásia continuam representando uma ameaça mundial (POLIO ERADICATION, 2010).

Ainda hoje é possível observar impactos profundos na qualidade de vida provocados por algumas viroses, tais como as pandemias causadas pelo vírus influenza e pelo vírus da imunodeficiência humana (HIV) (JONES, 1998). O aumento da população mundial, a concentração de pessoas em áreas densamente povoadas e a modernização dos meios de transportes facilitam a disseminação de novas infecções (RÁCZ, 2008). O número de pessoas infectadas com o vírus da hepatite B e C (HBV e HBC) e vírus da dengue, por exemplo,

cresce a cada ano no mundo todo. Em 2006, cerca de 4 milhões de pessoas foram infectadas pelo HIV, acarretando até 8.000 mortes diariamente (BURGO, 2008) e em 2007, o número de pessoas infectadas passou para quase 39 milhões, sendo que 65% destes encontravam-se na África do Sul (DE CLERCQ, 2007).

A medicina conta com poucas alternativas para combater esses agentes infecciosos. A vacinação é a alternativa mais efetiva contra doenças causadas por vírus (ARVIN; GREENBERG, 2006), pois proporciona uma resposta imune mais eficiente e duradoura. Entretanto, apesar dos benefícios das vacinas, nem todas as doenças virais podem ser controladas por elas. O HIV e o HCV são instáveis geneticamente, sofrendo rápidas variações antigênicas, enquanto outros vírus como por exemplo, os herpesvírus possuem potentes mecanismos de escape da resposta imune, incluindo o estabelecimento de longos períodos de latência. Por não ser capaz de vencer obstáculos como estes, juntamente com a emergência de outros vírus para os quais os mecanismos de infecção são pouco conhecidos, a vacinação, embora ideal, é uma ferramenta restrita e muitas vezes ineficaz (DE CLERCQ, 2002; JEROME, 2005).

Para algumas doenças virais foram desenvolvidos quimioterápicos que são clinicamente efetivos, propiciando alívio aos doentes e melhoria na qualidade de vida. Na atualidade, destaca-se a terapia antiretroviral para portadores do HIV, onde a expectativa de vida é aumentada devido ao tratamento.

Dessa forma, devido à ameaça que algumas viroses representam à saúde pública seja pela ampla distribuição, facilidade de transmissão ou dificuldade no controle das mesmas, a busca de medicamentos é essencial no combate a estas infecções.

ANTIVIRAIS

As doenças virais eram intratáveis há 40 anos. A pesquisa por compostos antivirais teve início na década de 1950 e, devido ao pouco conhecimento sobre a biologia molecular dos vírus, muitos achavam ser impossível desenvolver um medicamento seguro e efetivo contra doenças virais, sem interferir no metabolismo celular (JONES, 1998; WIGG, 2008). Antibióticos sulfonamidas foram testados nos primeiros ensaios antivirais *in vitro* utilizando ovos embrionados (BROWNLEE; HAMRE, 1951) e camundongos infectados com o vírus vaccínia (SNEADER, 1985 apud in JONES, 2003). Em 1952, na Europa, 142 extratos de plantas também foram testados em ovos embrionados infectados, sendo que 12 deles inibiram a multiplicação do vírus influenza A (CHANTRILL *et al.*,1952).

Uma década de trabalho culminou em 1960 no desenvolvimento da metisazona, introduzida para prevenção da varíola, apesar de seus efeitos colaterais (JONES, 1998; WIGG, 2008). Nessa mesma época, um grande número de análogos de nucleosídeos foi desenvolvido e teve sua atividade antiviral testada. Os análogos iododeoxiuridina (IDU) e trifluorotimidina (TFT) passaram a ser utilizados no tratamento tópico de ceratite herpética, devido à toxicidade em uso sistêmico (KAUFMAN**N**; HEIDELBERGER, 1964). A vidarabina (Ara-A) foi o primeiro análogo de nucleosídeo a ser administrado via sistêmica contra herpes simplex vírus (HSV) e vírus varicella-zoster (VZV), com toxicidade moderada para o hospedeiro (SCHABEL, 1968; BUCHANAN; HESS, 1985).

As primeiras drogas antivirais, eram capazes de atuar no DNA viral, mas também na síntese do DNA celular, com consequentes efeitos tóxicos aos pacientes (WIGG, 2008). As pesquisas por novos compostos continuaram e no final de 1970, o desenvolvimento do acyclovir (ACV), um análogo de guanosina com excelente índice de seletividade e eficácia, marcou o início da terapia antiviral (JONES, 1998). O ACV também deu origem a compostos relacionados, tais como valaciclovir, penciclovir e famciclovir, que apresentam espectros de atividade antiviral ligeiramente diferentes e/ou melhor farmacodinâmica e, ainda hoje, o ACV continua sendo um importante composto no tratamento de infecções herpéticas. (JEROME, 2005).

A pesquisa por análogos de nucleosídicos ainda perdurou por muitos anos na terapia antiviral. Porém, em meados de 1980, com a descoberta do HIV como agente etiológico da síndrome da imunodeficiência humana (AIDS), a pesquisa adquiriu um novo perfil (JONES, 1998). Devido ao elevado custo com o desenvolvimento de antivirais, os estudos foram concentrados nas viroses epidemiologicamente mais importantes (WIGG,

2008). Investimentos no campo acadêmico e industrial beneficiaram enormemente as pesquisas, levando ao avanço da biologia molecular e genética reversa e ao melhor entendimento da bioquímica da replicação viral, permitindo uma abordagem mais racional para a pesquisa de terapias antivirais, tendo como alvos principais a fusão ou ligação viral, intermediários de replicação, DNA/RNA polimerases, proteínas envolvidas na maturação, supressão da expressão do gene viral ou clivagem de RNAm (JONES, 1998; CHATTOPADHYAY *et al.*, 2009).

Vários compostos foram testados para diferentes vírus na ultima década, mas apenas cerca de 40 antivirais estão disponíveis para uso clínico (DE CLERCQ; FIELD 2006; CHATTOPADHYAY *et al.*, 2009). Desses, cerca de metade é utilizada para tratamento de infecções por HIV, enquanto os outros se concentram a um número pequeno de vírus como HBV, HCV, VZV, citomegalovírus (CMV) e vírus influenza (DE CLERCQ, 2004; DE CLERCQ, 2005)

Deve-se ressaltar que, até o momento, nenhum antiviral é capaz de curar o paciente da virose. O tratamento é apenas preventivo ou de controle e o uso contínuo e indiscriminado pode favorecer a seleção de mutantes resistentes, dificultando ainda mais o combate e o controle das infecções virais (WIGG, 2008). Dessa forma, a emergência e re-emergência de novos vírus e o desenvolvimento de resistência viral têm incentivado a pesquisa de novos agentes antivirais, principalmente a partir de produtos naturais. Diversas substâncias encontradas em fungos, bactérias, algas e, principalmente plantas superiores têm sido testadas em todo o mundo.

Por milênios, todas as culturas antigas do mundo como China, Índia, Pérsia, etc. utilizaram e continuam a utilizar plantas medicinais para o tratamento de diversos males. De acordo com Halberstein (2005), evidências arqueológicas dos tempos pré-históricos indicam que as plantas com fins medicinais representam a mais velha forma de medicação. Os cogumelos medicinais também estabeleceram história de uso nas terapias tradicionais antigas. Esses produtos naturais fornecem cerca de 30-40% das novas substâncias anticancerígenas e antimicrobianas à indústria farmacêutica (CHATTOPADHYAY *et al.*, 2009; WASSER, 2010).

Vários estudos têm relatado os efeitos inibitórios de extratos de plantas medicinais sobre a replicação de diversos vírus, dentre eles, HSV, HIV, HBV e alguns vírus emergentes, como o vírus da síndrome respiratória aguda grave (SARS). Além disso, estudos recentes mostraram a atividade de extratos vegetais contra cepas virais resistentes aos antivirais convencionais (MUKHTAR *et al*. 2008).

Ngai e Ng (2003) isolaram uma proteína do corpo de frutificação do cogumelo comestível *Lentinula* edodes chamada lentinan, que apresentou atividade inibitória sobre a transcriptase reversa do HIV-1. PELO KIT DA BOHERINGER MANNHEIM (GERMANY)

Wang e Ng (2004) isolaram a enzima lacase do corpo de frutificação de *Tricholoma giganteum* e mostraram que esta proteína de baixo peso molecular foi capaz de inibir a transcriptase reversa do HIV-1.

Niedermeyer *et al.,* (2005) isolaram quatro esteróis e dez triterpenos do corpo de frutificação de *Ganoderma pfeifferi* para avaliação da atividade antimicrobiana. Três compostos exibiram uma alta atividade inibitória contra o HSV. Talarico *et al.,* (2005) analisaram a atividade antiviral de dois polissacarídeos sulfatados, obtidos das algas vermelhas marinhas *Gymnogongrus griffithsiae* e *Cryptoneia crenulata*, contra quatro sorotipos de vírus da dengue (DENV), mostrando que suas atividades antivirais são dependentes do sorotipo do vírus e da célula hospedeira.

Tait *et al.,* (2006) determinaram a atividade antiviral *in vitro* de homoisoflavonóides, uma classe de flavonóides, contra vários enterovírus, em cultura de células BGM. Nenhuma das substâncias foi efetiva contra o poliovírus 1, mas a maioria apresentou atividade antiviral contra coxsachievírus B1, B3, B4, A9 e echovírus 30. Gebre-Mariam *et al*., (2006) testaram a atividade antiviral de plantas medicinais da Etiópia, usadas no tratamento de doenças de pele. Entre estas, havia espécies que apresentaram atividade contra HSV-1, coxsachievírus B3 e contra o vírus da influenza A, em cultura de células.

Zuo *et al.,* (2007) mostraram atividade antiviral de frações e compostos isolados de extratos etanólicos de rizomas da erva medicinal chinesa *Rhodiola kirilowii* (Regel) Maxim contra a NS3 serina protease do HCV. Gu *et al.,* (2007) purificaram uma proteína do corpo de frutificação de *Grifola frondosa,* que demonstrou inibir a replicação do HSV-1 *in vitro*.

Serkedjieva, Gegova e Mladenov (2008) mostraram que o extrato rico em polifenol, obtido de raízes aéreas da planta medicinal *Geraniium sanguineum* L., protegeu os ratos da morte por infecções com vírus influenza (H3N2). Cirne-Santos *et al*., (2008) investigaram propriedades antiretrovirais de um diterpeno isolado da alga marinha *Dictyota pfaffii* (Dolabelladienetriol), demonstrando que este é um inibidor de transcriptase reversa não-nucleosídico (NNRTI).

Harden *et al.,* (2009) verificaram uma alta atividade virucida contra o HSV-1 e 2 de polissacarídeos extraídos de quatro espécies de algas, *Undaria pinnatifica,*

Splachnidium rugosum, Gigartina atropurpurea e *Plocamium cartilagineum*. Ghaemi *et al.,* (2009) demonstraram que o polissacarídeo isolado de *Echinacea purpurea* estimulou a resposta imune de camundongos, reduzindo a taxa de latência do HSV-1 quando tratados antes da infecção.

ERMOLENKO, *et al.,* (2010) pesquisaram a inibição da replicação do HSV-1 por bactérias probioticas e observaram que sobrenadantes de culturas de *Lactobacillus sp.* e *Enterococcus sp.* inibiram o efeito citopático do HSV-1, em cultura de células de rim de macaco verde (Vero). García *et al.,* (2010) isolaram óleo essencial de sete plantas aromáticas do centro oeste da Argentina e testaram contra o HSV-1, DENV-2 e Junin vírus, mostrando ação virucida variável de acordo com o vírus.

Javed *et al.,* (2011) testaram dez plantas medicinais de diferentes áreas do Paquistão e mostraram que extratos metanólicos e clorofórmicos de sementes de *Solanum nigrum* são capazes de inibir o HCV *in vitro* em 37 e 50%, respectivamente.

AGARICUS BRASILIENSIS

Agaricus brasiliensis (*Agaricus Blazei* Murill ss. Heinemann), conhecido popularmente como Cogumelo do Sol, Cogumelo de Deus, "Himematsutake" é um basideomiceto natural da cidade de Piedade, interior de São Paulo, Brasil (WASSER, 2002; BRAGA; EIRA; CELSO,1998). É frequentemente consumido em diferentes partes do mundo na alimentação e na forma de chá, devido aos seus efeitos medicinais, em casos de estresse físico e emocional, colesterol alto, diabetes, distúrbios gástricos e osteoporose. Também é utilizado como agente antimutagênico, antioxidante e estimulador do sistema imune (MENOLI *et al.* 2001).

O corpo de frutificação do *A. brasiliensis* consiste em 85-87% de água e, quando desidratado, é rico em proteínas (40-45%), apresenta carboidratos (3-4%), fibras dietéticas (6-8%), lipídios (3-4%) e vitaminas (especialmente B1, B2 e niacina). Ergosterol (0,1-0,2%) e ácido linoléico (70-80% de lipídios totais) são os lipídios predominantes (BELLINI *et al.,* 2003). A partir de resíduos solúveis em água, foi isolado do corpo de frutificação do *A. brasiliensis* um complexo proteína-polissacarídeo, constituído de 50,2% de carboidrato e 43,3% de proteína (KAWAGISHI *et al*., 1989 e 1990). A análise do polissacarídeo indica a presença de 93,8% de glicose, 3,54% de manose e 2,25% de arabinose, sendo que esses polissacarídeos são glucanas, compostos principalmente por 1,6-β-glucana (SUI *et al.*, 2010).

Os efeitos antitumorais dos polissacarídeos se diferem na sua composição química, configuração e propriedades físicas como solubilidade em água, tamanho das moléculas, taxa de ramificação e forma do polissacarídeo (WASSER, 2002). Para aumentar a utilização dos polissacarídeos, varias modificações químicas, como sulfatação, metilação e carboximetilação têm sido amplamente realizadas e seus derivados, extensivamente estudados, tanto pelo uso industrial como pelo interesse científico (WANG; YU; MAO, 2009).

Os efeitos antivirais de *A. brasiliensis,* entretanto, são pouco relatados. Sorimachi *et al*., (2001) verificaram que frações obtidas pela precipitação etanólica a 44 e a 50% do extrato aquoso micelial deste cogumelo inibiram completamente o efeito citopático do vírus da encefalite eqüina do oeste (WEE) em células Vero. Faccin *et al*., (2007) mostraram que o polissacarídeo e as frações aquosa e etanólica obtidos do corpo de frutificação do *A. brasiliensis,* inibiram de forma significativa o número de plaques de poliovírus em células HEp-2 em relação ao controle. Além disso, Hsu *et al.,* (2008) reportaram que o extrato do *A. brasiliensis* pode normalizar a função hepática de pacientes com hepatite B.

AZADIRACHTA INDICA

Azadirachta indica A. Juss é uma árvore perene respeitada no sub-continente indiano por mais de 2000 anos e conhecida localmente como neem. Nos antigos escritos em sânscrito (antiga língua indiana), é referido como Arishtha, "apaziguador de doença" devido à sua ampla aplicação na medicina Ayurvedica e Unani. Neem também tem sido amplamente utilizada na medicina homeopática e tornou-se um centro de atração da medicina moderna, possuindo uma vasta gama de compostos biologicamente ativos que são quimicamente diferentes e estruturalmente complexas. Mais de 140 compostos isolados de diferentes partes do neem: folhas, flores, sementes, frutos, raízes e cascas têm sido utilizadas tradicionalmente para o tratamento de inflamações, infecções, dores, febre, doenças de pele e problemas dentários. As utilidades medicinais têm sido descritas, especialmente as folhas de neem, que tem se apresentado como imunomoduladores, antiinflamatórios, antidiabéticos, antiulcerosos, antimaláricos, antilepra, antifúngico, antibacteriano, propriedades antiviral (HIV/AIDS), antioxidante, antimutagênica e anticarcinogênica (SUBAPRYA; NAGINI, 2005; VEITCH; BOYER; LEY, 2008; ANAYAEHIE, 2009). Além de suas eficácias terapêuticas, neem também é fonte natural de inseticidas, pesticidas e agrotóxicos. (BRAHMACHARI, 2004).

A avaliação de propriedades antivirais é limitada a poucos vírus. Badam, Joshi e Bedekar (1999) mostraram que frações do extrato metanólico das folhas de neem inibiram a formação de plaque de 6 tipos antigênicos de coxsackievírus B. Segundo SaiRam *et al.,* (2000), fração do óleo de neem é capaz de suprimir a replicação do poliovírus. Parida *et al.,* (2002) mostraram o efeito inibitório do extrato aquoso das folhas de neem sobre o vírus da dengue tipo 2 em sistemas in vivo e in vitro, enquanto Tiwari *et al.,* (2010) identificaram uma potente atividade anti-HSV do extrato aquoso da casca de neem, sugerindo um efeito direto sobre a partícula viral, além de inibir a fusão célula-célula mediada pela glicoproteína viral e a formação de policariócitos em cultura de células. Além disso, Saha *et al.,* (2010) sugerem que a atividade anti-BoHV-1 do polissacarídeo obtido das folhas de neem interfere diretamente nas estruturas do envelope viral ou mascaram as estruturas virais que são necessárias para adsorção ou entrada do vírus na célula hospedeira.

HERPESVÍRUS

Os herpesvírus pertencem a família *Herpesviridae,* constituída por três sub-famílias, a saber *Alphaherpesvirinae*, responsáveis por lesões na pele e mucosas, e sub-famílias *Betaherpesvirinae* e *Gammaherpesvirinae*, que ocasionam manifestações sistêmicas (MEHNERT; CANDEIAS, 2005). São capazes de produzir infecções líticas e estabelecer infecções latentes e/ou persistentes em gânglios nervosos sensitivos ou em leucócitos (MIRANDA, 2002), podendo ser reativadas periodicamente ao longo da vida, (JENSSEN, *et al*., 2008). Durante a latência, nenhum dos genes virais expressos durante a fase lítica é detectado, apenas alguns RNAs, denominados transcritos associados a latência (*Latency-Associated Transcripts* – LATs) (MIRANDA, 2002).

O herpes simplex vírus tipo 1 (HSV-1) e o herpesvírus bovino tipo 1 (BoHV-1) são alphaherpesvírus, caracterizados por abranger uma gama de hospedeiros, possuir um ciclo de replicação curto e capacidade de induzir infecção latente principalmente, mas não exclusivamente, em neurônios (MUYLKENS *et al.,* 2007).

O vírion apresenta capsídeo de simetria icosaédrica e envelope lipoprotéico com glicoproteínas. Entre o capsídeo e o envelope encontra-se uma camada amorfa, denominada tegumento, constituído principalmente proteínas que são importantes na regulação do ciclo replicativo viral (ROIZMAN *et al*., 2007). O genoma consiste em uma grande molécula linear de DNA fita dupla, empacotado na forma toróide (MADIGAN; MARTINKO; PARKER, 2004). O RNA, transcrito no núcleo da célula hospedeira, possui três classificações: precoce imediato (α), precoce tardio (β) e tardio (γ) que codificam, respectivamente, proteínas regulatórias da replicação, proteínas da replicação, incluindo a timidino quinase e a DNA polimerase e proteínas estruturais como gB, ICP5 e gC (MADIGAN; MARTINKO; PARKER, 2004; KUO *et al*., 2008). De acordo com Su *et al*. (2008), os RNAs precoce imediato são detectados na primeira meia hora pós-infecção (pi), os precoce tardio de 2-3 horas pi e o tardio, pelo menos até 12 horas pi.

O HSV-1, também referido como herpesvírus humano tipo 1 (HHV-1), é um dos patógenos humanos mais comuns, sendo um problema de saúde pública que estima-se estar presente em cerca de 40-80% da população mundial (JONES, 2003; ROIZMAN *et al*., 2007). Pertence ao gênero *Simplexvirus* e se apresenta sob duas formas sorológicas, sorotipos 1 (HSV-1) e 2 (HSV-2). O HSV-1 causa uma variedade de infecções em humanos, que dependem da porta de entrada do vírus, da competência imunológica do hospedeiro e da natureza da doença (primária ou secundária), variando desde infecções assintomáticas a

infecções mucocutâneas orolabiais, oculares, genitais, gastrointestinais, esofageais, eczemas e, em casos raros, encefalites esporádicas, além de, aproximadamente, 25% das infecções genitais herpéticas. Também existem evidências de que infecções pelo HSV-1 no sistema nervoso central em combinação com o fator genético do hospedeiro possam levar ao desenvolvimento da doença de Alzheimer (JONES, 2003; BRADY; BERNSTEIN, 2004; ITZHAKI; WOZNIAK, 2008). Além disso, contribuem na propagação do vírus da imunodeficiência humana (KUO *et al*., 2008).

O BoHV-1 pertence à mesma família e subfamília do HSV-1, porém classificado no gênero *Varicellovirus* e compartilhando uma variedade de propriedades biológicas (ROIZMAN; KNIPE; WHITLEY, 2007). Antigamente era denominado Vírus da rinotraqueíte infecciosa bovina (IBR), da vulvovaginite pustular infecciosa (IPV), ou ainda, da balanopostite pustular infecciosa (IBP), por ser o agente etiológico destas enfermidades, além de causar conjuntivites, abortos, infertilidade, imunossupressão, encefalites e infecção sistêmica em neonatos (JONES; CHOWDHURY, 2007; MUYLKENS *et al.,* 2007; NANDI *et al.,* 2009). Tem ampla distribuição mundial, inclusive em países de bovinocultura expressiva (GIBBS; RWEYEMAMU, 1977), impondo perdas significativas causadas tanto pela doença como pela restrição comercial (BOWLAND; SHEWEN, 2000; NANDI *et al.*, 2009). Esse patógeno mostra diferenças significativas de incidência e prevalência em cada região, de acordo com a localização geográfica e o plano de manejo das regiões consideradas (ACKERMANN; ENGELS, 2006). Nos Estados Unidos, infecções pelo BoHV-1 causam um prejuízo anual de cerca de 500 milhões de dólares para a indústria de gado (JONES, 2003).

A infecção se dá por penetração no hospedeiro através de microfissuras ou escarificações da pele e mucosas e a primeira multiplicação ocorre nas células epiteliais locais, seguida de disseminação pelas vias hematogênica e neurogênica, para os órgãos alvo (MEHNERT; CANDEIAS, 2005).

A capacidade do BoHV-1 estabelecer latência nos neurônios sensoriais e motores faz com que este seja o principal obstáculo às campanhas de erradicação da doença através de vacinação. Além disso, nenhuma vacina é capaz de prevenir a infecção e o estabelecimento de latência, apenas reduz o impacto clínico da infecção. No caso de uso do vírus vacinal atenuado há a possibilidade deste estabelecer infecções latentes, podendo, durante a reativação, sofrer mudança para a forma patogênica (TIKOO *et al.*, 1995; MUYLKENS *et al.*, 2007).

Várias proteínas e peptídeos de origem natural inibem a infecção viral por bloquear a entrada do vírus na célula hospedeira ou nas fases posteriores da replicação viral

(JENSSEN, *et al*., 2008). A terapia administrada na fase inicial contribui no prognóstico da doença mas é essencial a detecção precoce dos casos (MADHAVAN *et al*., 1999).

A terapia antiviral ainda se encontra em evolução. Os primeiros agentes desenvolvidos foram os análogos de nucleosídeos tendo o Aciclovir como medicamento de referência mesmo trinta anos após sua descoberta (SCHAECHTER *et al*., 2002). O Aciclovir é um inibidor das DNA-polimerases de alguns herpesvírus. Dentro da célula, recebe um fosfato passando a forma monofosfato por intermédio de uma timidino-cinase viral e posteriormente, por ação de enzimas celulares é novamente fosforilado passando as formas di (DP) e trifosfatada (TP). Dessa forma, compete com a guanosina-TP, inibindo a DNA-polimerase viral e interrompendo a formação da cadeia de DNA do vírus. Mutações na timidino-cinase ou DNA polimerase virais podem produzir mutantes resistentes ao aciclovir (MORFIN; THOUVENOT, 2003).

A longo prazo, o uso dos análogos de nucleosídeos, principalmente em imunocomprometidos podem levar a seleção natural de mutantes resistentes (SU *et al*., 2008), destacando-se a necessidade do desenvolvimento de novas drogas antiherpéticas.

Ao longo dos últimos anos, grandes esforços têm sido feitos para criar um leque de estratégias para a identificação de novos e potentes antivirais e que também sejam ativos sobre os vírus latentes (BILLAUD; THOUVENOT; MORFIN, 2009).

POLIOVÍRUS

O poliovírus pertence à família *Picornavirida* e gênero *Enterovirus*. Como o próprio nome diz, os picornavírus são vírus pequenos 'pico' e de genoma de ácido ribonucléico 'rna'. Esta é uma das maiores e mais importantes famílias para o homem e a agropecuária. Além do vírus da poliomielite, se encontram os da hepatite A e da febre aftosa (GONÇALVES *et al.,* 2008).

Os enterovírus se replicam no trato alimentar e incluem também neste gênero os coxsackievirus, echovirus, enterovirus humano, alem de vários vírus entéricos não humanos (RACANIELLO, 2007). Possuem forma esferoidal, com 25-30nm de diâmetro, sem envoltório lipídico, com seu capsídeo apresentando simetria icosaédrica. O genoma é constituído por uma molécula de RNA de filamento simples e polaridade positiva sendo, portanto, infeccioso, funcionando diretamente como RNA mensageiro. O RNA genômico é ligado covalentemente a uma proteína chamada vpg (provavelmente iniciador da síntese do RNA), no terminal 5', e resíduos de adenina, chamada cauda poli(A), no terminal 3' (GONÇALVES *et al.,* 2008; PALLANSCH; ROSS, 2007).

A doença sintomática mais comum causada pelo PV é conhecida como poliomielite abortiva, caracterizada por uma ligeira febre, com ou sem sinais gastrointestinais, embora, menos frequentemente, infecções pelo PV resultem em meningite asséptica (não paralítica). Em média, apenas 0,5% das infecções evoluem para poliomielite paralítica, caracterizada por um quadro de paralisia flácida de início súbito, acometendo, geralmente, membros inferiores (PALLANSCH; ROOS, 2007). Existem três sorotipos de poliovírus, tipos 1, 2 e 3, todos com capacidade de provocar a doença paralítica (RACANIELLO, 2006). Não existe medicamento disponível para uso clínico nem cura para a doença, apenas tratamento para aliviar os sintomas. Calor e fisioterapia são utilizados para estimular os músculos e drogas antiespasmódicos são dadas para relaxá-los, melhorando a mobilidade do paciente (POLIO ERADICATION, 2010).

A vacinação é o principal método para levar à erradicação e ao controle da poliomielite no mundo. Programas e campanhas de vacinação têm sido cada vez mais reforçados. Em 1988, quando a iniciativa global de erradicação ao PV começou, o vírus causou paralisia em mais de 1000 crianças por dia, no mundo todo. Em 2009, pouco menos de 2000 casos foram reportados o ano todo. Atualmente, o PV foi eliminado na maior parte do mundo, e apenas alguns países da África e Ásia são endêmicos (POLIO ERADICATION,

2010). O PV-2 selvagem não é isolado no mundo desde 1999 e, portanto, este tipo de poliovírus pode já ter sido erradicado (MUELLER; WIMMER, CELLO, 2005).

O poliovírus é um dos modelos virais mais estudados e bem conhecidos. A estabilidade do capsídeo, a fácil purificação do vírion, a obtenção de altos títulos virais e a segurança para trabalhar em nível laboratorial (cepas vacinais) fazem com que seja alvo de várias pesquisas, sendo utilizado como modelo de vírus de RNA para estudos de replicação, patogênese e, inclusive, investigação de novas drogas antivirais (MUELLER; WIMMER, CELLO, 2005; RACANIELLO, 2007).

OBJETIVOS

GERAL

- Avaliar a atividade antiviral de polissacarídeo e derivados de *A. brasiliensis* e *A. indica* na replicação do herpesvirus humano e bovino tipo 1 e poliovirus tipo 1 em cultura de células HEp-2.

ESPECÍFICOS

- Avaliar a citotoxicidade do polissacarídeo, isolado do corpo de frutificação de *A. brasiliensis,* bem como de suas frações F1, F2 e F3 e seus derivados sulfatado e carboximetilado, em células HEp-2, além de avaliar a atividade antiviral destas substâncias contra o herpesvírus humano e bovino tipo 1, utilizando diferentes tratamentos pelo ensaio de plaque e imunofluorescência indireta;
- Avaliar a citotoxicidade do polissacarídeo P1, isolado da folha de *A. indica*, e seu derivado sulfatado P4, em células HEp-2, além de avaliar a atividade antiviral destas substâncias, utilizando diferentes tratamentos pelo ensaio de plaque e imunofluorescência indireta, na replicação do poliovírus tipo 1 e herpesvírus bovino tipo 1.

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ARTIGO 1: ANTIHERPETIC ACTIVITY OF FRACTIONS F1-F3 AND AN ISOLATE POLYSACCHARIDE FROM *AGARICUS BRASILIENSIS*.

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ABSTRACT: *Agaricus brasiliensis* is an edible, medicinal mushroom, traditionally used for the treatment of several diseases. In this paper, a polysaccharide (PLS) extracted from *A. brasiliensis*, its carboxymethylated (CPLS) and sulfated (SPLS) derivatives, as well as, fractions (F1-F3) obtained from the PLS were investigated for their antiviral effect in the replication of herpes simplex virus (HSV-1) and bovine herpes virus (BoHV-1) in HEp-2 cell cultures. The compounds were not cytotoxic for HEp-2 cells even at high concentrations (CC_{50} > 2500 μg/ml). The antiviral activity evaluated by plaque reduction assay and immunofluorescence assay demonstrated that PLS, SPLS and F3 inhibited the replication of both viruses in a dose-dependent profile, however, fractions F1, F2 and CPLS did not show significant effect up to 800 μg/ml. The lack of antiviral activity of these compounds in the virucidal and adsorption inhibition protocols, as well as, when added before infection suggests an action at initial steps of replication.

Key words: *Agaricus brasiliensis.* Antiviral. Bovine herpesvirus. Herpes simplex virus.

1 INTRODUCTION

Herpes simplex virus (HSV) and bovine herpesvirus (BoHV) are enveloped double-stranded DNA viruses belonging to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, responsible for mild to severe diseases in human and bovine. HSV-1 also referred to as human herpesvirus type 1 (HHV-1), a member of the *Simplexvirus* genus, is one of the most regular human pathogens, being a public health problem, and the causal agent of several diseases estimated to occur in approximately 40-80% of world population. The variety of infections is manifested with different degrees of severity, especially in immunocompromised patients. BoHV-1, classified under the *Varicellovirus* genus, shares many biological properties with HSV-1 (Jones, 2003). The virion is involved with several syndromes in cattle

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and buffaloes, including infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB). Occurs throughout the world and is responsible for significant losses in the cattle industry.

The infections caused by both agents are characterized by a relatively large host range, a short replication cycle and the ability to induce latent infection, mainly, in sensory neurons (Muylkens, Thiry, Kirten, Schynts; Thiry, 2007). These viruses establish a persistent infection and can be life threatening and affects greatly the quality of the host life. No proved effective vaccines are presently available for HSV infections and, although, vaccines for BoHV-1 are available, none is able to prevent the infection and the establishment of latency by challenging and wild strains (Roizman, Knipe; Whitley, 2007)

Acyclovir continues to be the reference drug for HSV diseases treatment even after thirty years of its development (Straus, 2002). However, in the long-term treatment, and especially in immunocompromised, it can lead to the selection of resistant mutants (Su et al., 2008). This highlights the need to develop new antiherpetic drugs. Over the past few years great efforts have been made to identify new and potent antiviral drugs which are also active against latency establishing virus (Billaud, Thouvenot; Morfin, 2009). In order to find new classes of antiviral drugs, natural products line research has been extensively studied.

Agaricus brasiliensis (*Agaricus blazei* Murill ss. Heinemann), commonly known as the Royal Sun *Agaricus*, ABM, Himematsutake, and Cogumelo de Deus is a native Brazilian basidiomycete (Wasser, 2002). It is often consumed as food and tea worldwide, because of its medicinal effects, such as, relief of physical and emotional stress, treatment for high cholesterol, diabetes, gastric disorders and osteoporosis. It is also used as antimutagenic, antioxidant, and as immune stimulator (Menoli, Mantovani, Ribeiro, Speit; Jordão, 2001).

Studies of the antiviral effects of *A. brasiliensis* are scant. Sorimachi et al. (2001) found that fractions obtained by ethanol precipitation of the mycelium aqueous extract inhibited the cytopathic effect of Western Equine Encephalitis virus (WEE) in vero cells. Faccin et al. (2007) showed that an polysaccharide, ethanol and aqueous fractions obtained from *A. brasiliensis* fruiting bodies inhibited significantly poliovirus 1 in HEp-2 cells. In addition, Hsu, Hwang, Chiang e Chou (2008) reported that the extract of *A. brasiliensis* can normalize liver function in chronic hepatitis B patients.

The aim of the present study was to investigate the antiviral activity of an *A. brasiliensis* polysaccharide, its fractions, as well as, carboxymethylated and sulfated derivatives in the replication of HSV and BoHV.

2 MATERIALS AND METHODS

2.1 CELLS AND VIRUSES

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

2.2 TEST COMPOUNDS

A. brasiliensis PLS and F1-F3 were obtained as previously described (Gonzaga, Ricardo, Heatley; Soares, 2005) and these compounds, including the sulfated (SPLS) and carboxymethylated (CPLS) PLS derivatives were supplied by the DQOI/UFC, Brazil.

2.3 ANTIVIRALS

Two commercial antivirals were used as positive control, acyclovir (Zynvir, Nova Farma, Brazil) and human alfa-2B interferon (Meizler Com. Intern. SA, Brazil).

2.4 CYTOTOXICITY ASSAY

Cell viability was assayed by MTT method (Sigma Chem, Co., USA), according to manufacturer's instructions. Briefly, 70% confluent monolayer cultures in 96-well microplates (Nunc A/S, Denmark) were treated with 500, 1000, 1500, 2000 and 2500 µg/ml of the compounds in maintenance medium (free of fetal bovine serum) in triplicate with appropriate cell control at 37° C with 5% CO_2 incubation. After 72 hour-incubation the medium was replaced with 10 µl of the medium containing 1,25 µg/ml MTT reagent (dimethyl-thiazolyl-diphenyl tetrazolium bromide). The dissolved formazan crystals were read at 570 and 690 nm. The 50% cytotoxic concentration (CC_{50}) was calculated as the

compound concentration capable of reducing the optical density of the MTT product by 50% in relation to the control, by regression analysis.

2.5 PLAQUE REDUCTION ASSAY (PRA)

Antiviral activity was evaluated by plaque reduction assay, according to Melo, Benati, Roman Jr, Mello, Nozawa e Linhares (2008)**.** Briefly, HEp-2 cell monolayers grown in 24-well plates (TPP, Switzerland) were infected with 50-100 PFU and treated with 200, 400, 600 and 800 µg/ml of the compounds, according to the protocols. Infected and treated cells were washed and overlaid with nutrient agarose (2 x DMEM/1,8% agarose) added of 25 mM of $MgCl_2$ (Yamamoto, Rincão, Linhares; Nozawa, 2009). After 40h cell were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), pH 7.3, and stained with 0,5% violet crystal in 20% ethanol. Plaques were counted and the percentage of viral inhibition (%VI) was calculated as follows: %VI = [1-(V*d*/V*c*)]x100, where V*d* and V*c* refer to the number of plaques in the presence and absence of the compounds, respectively (Nishimura, Toku; Fukuyasu, 1977). The inhibitory concentration 50% (IC_{50}) was calculated as the concentration of the compounds required to reduce in 50% the number of plaques . The selectivity index (SI) was calculated as the ratio of CC_{50} and IC_{50}. HSV-1 and BoHV-1 treated, respectively, with acyclovir (2500 µg/ml - Zynvir, Nova Farma, Brazil) and human interferon alfa-2B (10000 U/ml – Meizler Com. Intern. SA, Brazil) were used as positive control.

For the time-of-addition assay, the concentrations of the compounds, as before, were tested, according to Yang, Cheng, Lin, Chiang e Lin (2005). Briefly, 1) For cell pre-treatment protocol, the compounds were included in the cell culture medium for 1 h and 2 h and removed prior to infection; 2) Cell treatment with the compounds simultaneously to infection (0 h) and compounds left throughout; and 3) Cell treatment with compounds 1 h and 2 h post-infection and compounds left throughout.

The inhibitory effect of the compounds on virus adsorption was performed as previously described (Zhu, Chiu, Ooi; Ang Jr., 2004). Briefly, cell cultures were maintained at 4° C for 1 h followed by the infection in the presence of the compounds, at cited concentrations, during 80 min at 4° C, for viral adsoption. The cell culture was washed with cold PBS to remove the non-adsorbed virus and treated as describe for PRA.

The virucidal assay was performed according to Cheng, Lin e Lin (2002). The virus suspension was pre-incubated with varying concentrations of the compounds, as

previously (v/v), for 1 h at 37° C in water bath. Treated virus suspension was diluted to the tenth and its residual infectivity was determined by plaque assay.

2.6 IMMUNOFLUORESCENCE ASSAY (IFA)

Immunofluorescence assay was performed with HEp-2 cells grown in glass cover slips (Glasstecnica Import, Brazil) in 24-well plates infected with 100 µl of HSV-1 or BoHV-1 (MOI = 1) and treated with the compounds at 200, 400, 600 and 800 µl/ml, at the time of infection (0 h). At 24 h post-infection, the cells were washed with 0.05% tween-20 PBS, fixed with cold acetone (-20° C) for 20 min and blocked with powdered skimmed milk PBS for 30 min. The cells were incubated for 30 min at 37° C in dark moist chamber with mouse anti-HSV-1 (Santa Cruz Biotechnology, USA) or bovine anti-BoHV-1 antibodies (DMVP/UFSM, Brazil) and further washed three times with 0.05% tween-20 PBS, then incubated with goat anti-mouse IgG (Sigma Chem. Co., USA) and rabbit anti-bovine IgG conjugated with FITC (Sigma Chem. Co., USA), respectively. After washing, the cells were examined in a Zeiss fluorescence microscope (Imager A1 with Axio Cam MRc5) and 100 cells/coverslip were counted and the percentage of fluorescent cells calculated in comparison to control infected and nontreated cells. The experiments were carried out in duplicate.

2.7 STATISTICS

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

3 RESULTS

3.1 CITOTOXICITY ASSAY

The citotoxic concetration of PLS, SPLS, CPLS and fractions F1- F3 for HEp-2 cells was greater than 2500 µg/ml.

3.2 ANTIVIRAL ACTIVITY FOR HSV-1

The significant inhibitory effects of *A. brasiliensis* PLS and SPLS derivative, and F3 are shown in fig. 1. These compounds did not inhibit HSV-1 when added at 2 h and 1 h before infection and did not demonstrate any direct effect on virus particle or virus adsorption either, at the indicated concentrations. However, when PLS was added at time 0 h, 1 h and 2 h after infection, the percentages of inhibition were 77.5%, 83.6% and 2%, respectively. For the same time of treatment, F3 inhibited HSV-1 by 35.9%, 32.3% and 15%, respectively, at 800 µg/ml. The SPLS (at 800 µg/ml) and CPLS (at 2000 µg/ml) showed antiviral activity only when added at the time 0 h, with maximum inhibition of 83.7% and 37.5%, respectively. F1 and F2 showed no significant inhibition in none of the assays. The IC_{50} for PLS was 454 µg/ml with a SI >5.5, while SPLS IC_{50} was 346 µg/ml with SI >7.2. The IC_{50} for F3 was greater than 800 µg/ml.

The effect of PLS, SPLS and F3 in the synthesis of HSV-1 proteins by IFA is shown in table 1. These compounds showed reduction of fluorescent cells number up to 53.7%, 85.2% and 44.5% for PLS, SPLS and F3, respectively, at the highest tested concentration, in comparison to infected nontreated cell control, when the compounds were added during viral infection (time 0 h).

3.3 ANTIVIRAL ACTIVITY FOR BOHV-1

The antiviral activity of *A. brasiliensis* compounds for BoHV-1 was similar to that of HSV-1. Significant inhibition was only detected when PLS, SPLS and F3 were added at the time (0 h) and after (1 h, 2 h) infection . Fig. 2 shows the results of time-of-addition assay of PLS, SPLS and F3 at the indicated concentrations. When the compounds were added at the highest concentration at 0 h, 1 h and 2 h post-infection, the inhibition rates were 29.3%, 69.2% and 23.4%, respectively, for PLS. For SPLS, 53.6%, 28.6% and 41.5%, and, for F3, 28.0%, 77.4% and 37.3%. The CPLS presented low activity, with maximum of 26.1% inhibition, at the time 0 h, at 2000 µg/ml, while, F1 and F2 showed no significant inhibition. None of the compounds showed direct effect on BoHV-1 or at viral adsorption step either. For PLS the IC_{50} was 634 µg/ml and SI > 3.9. For SPLS the was 830 µg/ml and SI > 3, while, for F3 the IC_{50} was 674 µg/ml and SI > 3.7.

The results of IFA for PLS, SPLS and F3 are shown in the table 1. The compounds were added at the moment of infection (time 0 h) and showed reduction of

fluorescent cells up to 59.1%, 44.9% and 50.5%, respectively, for PLS, SPLS and F3, at 800 µg/ml, in comparison to infected nontreated cells.

3.4 POSITIVE CONTROL

Acyclovir at 2500 µg/ml inhibited HSV-1 by 67%, while, interferon at 10000 U/ml inhibited BoHV-1 by 100%.

4 DISCUSSION

Currently, an extensive number of natural compounds have been shown to inhibit several animal viruses experimentally, including herpesvirus. The antiviral activity of these compounds may be related to the inhibition of virus particle binding to receptor sites in the host cell or by inhibiting the replication process by interfering with the stages of penetration, uncoating, synthesis, assembly, maturation or release of the virus.

We tested the antiviral activity of a polysaccharide and its derivatives sulfated and carboxymethylated, as well as, fractions of *A. brasiliensis* against human and bovine herpesvirus.

The compounds tested showed similar dose-dependent antiviral activity for both virus. The inhibitory effect was found mainly in treatments at 0 h and 1 h after viral infection for compounds PLS, SPLS and F3, at the highest concentrations (800 µg/ml). These results are similar to those found by Faccin et al. (2007) that demonstrated the greatest inhibitory effect of the crude and aqueous extracts, as well as, for ethanol fraction of *A. brasiliensis,* at the time 0 h, for poliovirus.

When PLS, SPLS and F3 were added 1 h and 2 h before infection, no significant antiviral activity was observed. Moreover, the three compounds neither inhibited virus adsorption nor presented virucidal activity. These results suggest that these compounds act mainly at the early stages of viral replication, but, did not interfere directly in virus attachment or in the cell receptors. Amongst the fractions studied only F3 showed antiviral effect suggesting that this property was restricted to this fraction, although, F1 and F2 were similar to F3. The carboxymethylated derivative showed low viral activity, even at highest concentrations. The carboxymethylation has often been used to provide better water solubility of the polysaccharides (Wang, Yu; Mao, 2009), but, this change was not favorable for the antiviral property, as we demonstrated in our study.

The sulfated PLS increased the antiviral activity when we added concomitantly with the virus (time 0 h). The antiviral activity of sulfated polysaccharides has been reported in several studies mainly by interfering in the initial stages of viral replication (Zhu, Chiu, Ooi, Chan; Ang Jr, 2006). The mechanism of action can be attributed mainly for enveloped viruses by the interaction of the negatively charged sulfated PLS with the positive charges of the envelope glycoproteins, involved not only in recognition and binding to receptors on the cell surface, but, also in the penetration of the virus. We demonstrated that the antiviral activity of SPLS at time 0 h suggests an action during the early steps of the infection, after adsorption. Huang et al., (2008) showed that polysaccharides extracted from astragalus, a chinese medicinal herb, had its antiviral activity against Infectious Bursal disease virus increased after being sulfated. Hasui, Matsuda e Okutani (1995) showed that sulfated polysaccharides extracted from marine microalgae showed antiviral activity for many viruses, including HSV-1 in the early stages of replication, after viral adsorption, possibly by activation of intracellular signaling pathways. According to Ghosh, Chattopadhyay, Marschall, Karmakar, Mandal e Ray (2009), stimulation of intracellular signaling pathways by polysaccharides would induce interferon and, consequently, antiviral state.

It has been shown that increased antiviral activity of polysaccharides can be related to degree of sulfation and distribution of sulfate groups in the molecule (Huang et al., 2008; Karmakar, Pujol, Damonte, Ghosh e Ray, 2010; Saha et al., 2010; Wang et al., 2010). The neutral polysaccharides have also been found showing inhibition of viral infection, indicating that this property of polysaccharides is related to other characteristics of the molecule, such as molecular weight, constituent sugars, stereochemistry and molecular conformation (Marchetti, Pisani, Pietropaolo, Seganti, Nicoletti; Orsi, 1995; Lüscher-Mattli, 2000; Damonte, Matulewicz, Cerezo, 2004).

The immunofluorescence assay findings corroborated with the plaque reduction assay results, demonstrating also a dose-dependent response and reinforcing the hypothesis of the action of *A. brasiliensis* compounds in the initial steps of the replication of HSV-1 and BoHV-1.

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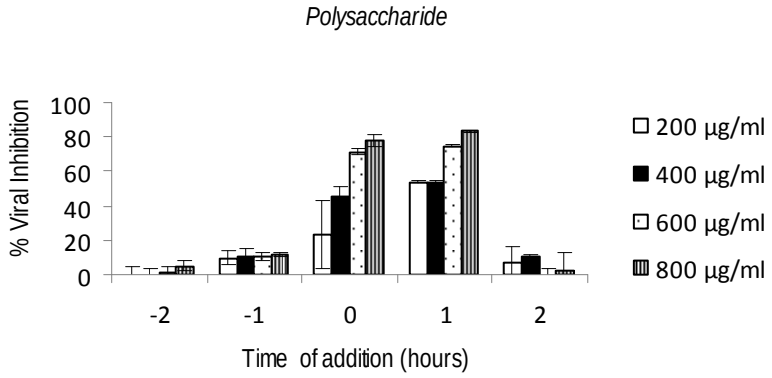
Figure 1 – The time-of-addition assay of polysaccharide (PLS) **(a)**, the sulfated derivative (SPLS) **(b)** and fraction F3 **(c)** of *Agaricus brasiliensis* in the replication of herpes simplex virus-1 in HEp-2 cells by plaque assay. The substances were added at varying concentrations (200-800 µg/ml)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

(a)



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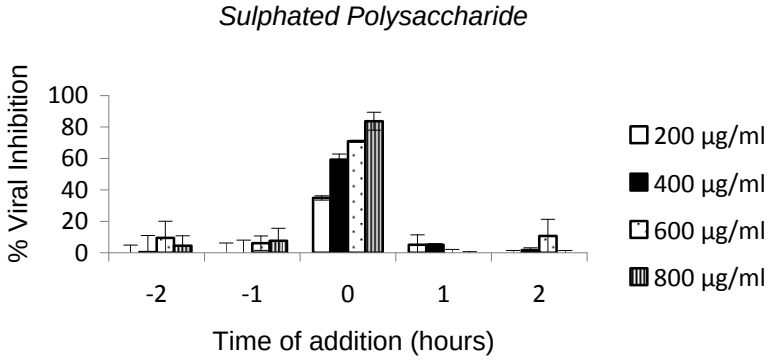

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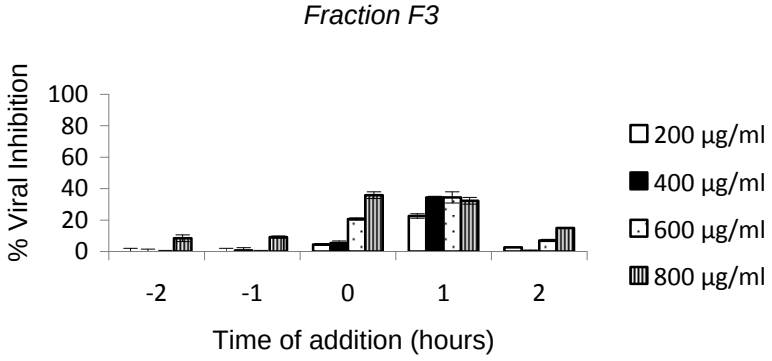


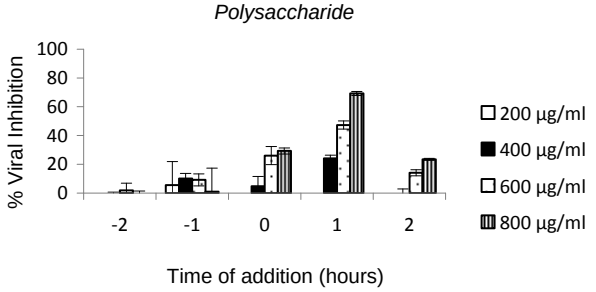
Table 1 – The effect of polysaccharide (PLS), the sulfated derivative (SPLS) and fraction F3 of *Agaricus brasiliensis* in the replication of HSV-1 and BoHV-1 monitored by immunofluorescence assay in HEp-2 cells. The compounds were added at varying concentrations (200-800 µg/ml) at zero hour of infection (simultaneously).

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

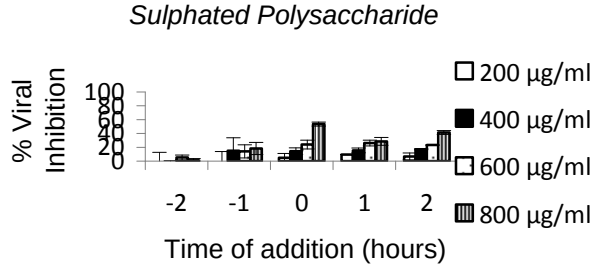
[a] µg/ml.
[b] Percent of fluorescent cells reduction in comparison to infected non-treated cells.

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

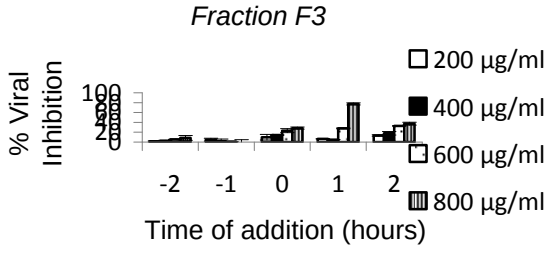
(a)



(b)



(c)



ARTIGO 2: ANTIVIRAL ACTIVITY OF POLYSACCHARIDE AND SULPHATED DERIVATIVE FROM *AZADIRACHTA INDICA* IN THE REPLICATION OF POLIOVIRUS AND BOVINE HERPESVIRUS.

Kristie Aimi Yamamoto[a], Lígia Carla Faccin[a], Bimalendu Ray[b], Carlos Nozawa[a], Rosa Elisa Carvalho Linhares[a].

ABSTRACT: *Azadirachta indica*, popularly known as neem, has been extensively used in Ayurvedic and homeopatic medicine due to pharmacological properties. Studies demonstrated the antiviral activity of neem extracts against some viruses. The antiviral and cytotoxic activities of polysaccharide P1 and its sulfated P4 obtained from *A. indica* against poliovirus (PV-1) and bovine herpesvirus type 1 (BoHV-1) were evaluated in HEp-2 cells. The compounds were not citotoxic until the higher tested concentration (1600 µg/ml). The evaluation of time-of-addition effect showed that polysaccharide P4 was more effective to PV-1 than P4, while polysaccharide P1 showed higher activity against BoHV-1. These substances were more effective when added during the infection (time 0h) for both viruses. For PV-1, P1 and P4 presented the 50% inhibitory concentrations (IC_{50}) of 77.5 and 12.1 µg/ml, and selectivity index (SI) of >20.6 and >131.96, respectively. BoHV-1 IC_{50} results were not satisfactory, demonstrated that these compounds could be used as an antiviral to non-enveloped viruses.

Key words: *Azadirachta indica*. Antiviral. Poliovirus. Bovine herpesvírus.

1 INTRODUCTION

In recent years, many compounds having potent anti-viral activity in cell cultures have been detected and some of these compounds are currently undergoing either preclinical or clinical evaluation. About 40 compounds are approved for clinical use and targeting only a small number of viruses. Moreover, the emergence or re-emergence of new viruses and selection of mutants resistant to antiviral drugs available has encouraged the search for new antiviral agents, mainly from natural products. Polysaccharides are a complex group of macromolecules possessing a wide range of therapeutically important biological properties and known to affect the growth of animal viruses (CHIU *et al*., 2004). The antiviral properties of sulfated polysaccharides have been known for almost 50 years. The anionic or sulfated polysaccharides from natural sources and of synthetic origin are safe and acceptable, and seem to offer improved prospects for efficacy. Therefore, sulfated modification could be

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used to improve the biological activities of polysaccharides and produce drug candidate with higher potency (SINHA *et al*., 2010).

Azadirachta indica A. Juss, popularly known as neem, has been extensively used in Ayurvedic and homeopatic medicine as an immunomoduladory, antiulcer, anti-inflamatory, antifungal, antibacterial, antiviral, antimalarial, antimutagenic and antioxidant properties, among other medicinal uses (SUBAPRYA; NAGINI, 2005; VEITCH; BOYER; LEY, 2008; ANAYAEHIE, 2009). Some studies demonstrated a substantial antiviral activity of neem extracts against some viruses. Badam, Joshi e Bedekar (1999) showed that a methanol extract fraction of neem leaves inhibited plaque formation of 6 serotypes of coxsackievirus B. According to SaiRam *et al*., (2000), neem oil can suppress the replication of poliovirus. Parida *et al*., (2002) showed the inhibitory effect of neem leaves aqueous extract on dengue virus type 2 either *in vivo* and *in vitro*. Vaibhav *et al*., (2010) suggested a direct anti HSV-1 property of neem bark aqueous extract, besides, it inhibited HSV-1 glycoprotein-mediated cell-cell fusion and polykaryocytes formation in cell culture. In addition, Saha *et al*., (2010) suggested that polysaccharide isolated from neem leaves inhibited early process of bovine herpesvirus (BoHV-1) replication.

Poliovirus, the etiologic agent of poliomielitis, is a member of the genus *Enterovirus* of the *Picornaviridae*, a family of small, non-enveloped and positive single-stranded RNA viruses (KIM; RACANIELLO, 2007). This is one of the largest and most important families for man and animals, which includes the hepatitis A and foot-and-mouth disease viruses (GONÇALVES *et al.,* 2008). Currently, poliovirus is under control in most part of the world, but, despite extensive efforts to eradicate the virus, the disease still occurs in some countries in Africa and Asia (POLIO ERADICATION, 2010). Although the disease represents no longer a major public health threat in the developed world, poliovirus is one of most thoroughly studied and best understood model of virus (MUELLER WIMMER; CELLO, 2005).

Bovine herpesvirus type 1 (BoHV-1), a DNA virus, member of the family *Herpesviridae*, is responsible for infections, such as, those involved with the upper respiratory (rhinotracheitis) and genital tracts (vulvovaginitis and balanopostitis) and is resistant to acyclovir (MELLO, 2008). It has similar biological properties to human herpes simplex virus (HSV) and also establishes latency in ganglionic neurons (JONES, 2003).

In this work we reported the antiviral activity of *A. indica* polysaccharide P1 and its sulfated form P4, at various stages of the replication of poliovirus and bovine herpesvirus, in cell culture.

2 MATERIALS AND METHODS

2.1 CELLS AND VIRUS

HEp-2 cells (epithelial cells of human larynx carcinoma, ATCC CCL-23) were grown at 37° C in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA), supplemented with 10% fetal bovine serum (Invitrogen/Gibco, USA) and treated with 100 µg/ml streptomycin (Gibco BRL, USA), 100 IU/ml of penicillin (Novafarma Pharmaceutical Industry, Brazil) and 2.5 µg/ml of fungizone (Meizler Biopharma SA, Brazil).

Poliovirus type 1 (PV-1) Sabin vaccinal strain (ATCC, VR-58) and Bovine herpesvirus-1 (BoHV-1), supplied by DMVP/CCA/UEL, Brazil, were propagated in HEp-2 cell cultures and stored at −20°C with 10% glycerol, before further analysis. The virus titer was determined by plaque assay.

2.2 COMPOUNDS

A. indica polysaccharide identified by P1 and its sulfated form P4 were used. The polysaccharides were kindly provided by the University of Burdwan, Burdwan, India. To determine the antiviral activity of the compounds, they were dissolved in DMEM treated with antimicrobials described and stored at -20°C.

2.3 CYTOTOXICITY ASSAY

The cytotoxicity of the compounds in HEp-2 cell was determined by MTT assay kit (Sigma Chem. Co., USA) according to the manufacturer's instructions. Briefly, growth medium of cell cultures grown in 96-well microplates (Nunc A/S, Denmark), at 70% confluence, was replaced by maintenance medium containing different concentrations of the compounds (100–1600 µg/ml). After 72 hours of incubation, 0.125 mg/ml of MTT reagent (dimethyl-thiazolyl-diphenyltetrazolium bromide) was added. After 3-hour incubation at 37°C, MTT was solubilized with 0.1 N HCl in anhydrous isopropanol for 15 min and the absorbance was read at 570 and 690 nm. The percentage of inhibition was calculated using the formula - Percent of viability = [100 – (A*t*/A*c*) x 100], where A*t* and A*c* refer to the absorbance of test substance and control (untreated cells), respectively. The 50% cytotoxic

concentration (CC_{50}) (the concentration of the test substance capable of reducing cell viability by 50% in comparison to cell control) was calculated by regression analysis.

2.4 ASSAYS FOR ANTIVIRAL ACTIVITY

Plaque reduction assay – Antiviral activity was determined by plaque reduction assay (PRA). Briefly, cell cultures grown at 95-100% confluence in 24-well plates (TPP, Switzerland) were inoculated with PV-1 or BoHV-1 (50 to 100 PFU) and incubated at 37°C for 1 h. The infected cell cultures were washed and overlaid with DMEM containing serial concentrations of P1 and P4 (25–200 µg/ml) added at different times (time-of-addition assay): before (−1 hour and −2 hours), during (0 h) and after (1 hour and 2 hours) viral infection (Yang et al. 2005). Cultures were overlaid with nutrient agarose (DMEM 2x/1.8% agarose [v/v]) containing 25 mM of $MgCl_2$. After 40 h incubation, cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), pH 7.3, for 24 h and stained with 0.5% crystal violet in 20% ethanol. Plaques were counted and the percentage of viral inhibition (%VI) was calculated as $[1-(Vd/Vc)]\times100\%$, where V*d* and V*c* refer to the number of plaques in the presence and absence of the compounds, respectively (NISHIMURA; TOKU; FUKUYASU, 1977). The minimal concentration of compounds required to reduce 50% of plaque numbers (IC50%) was calculated by regression analysis of the dose–response curves generated from plaque assays. The selectivity index (SI) was expressed by the ratio $CC_{50\%}/IC_{50\%}$.

Virucidal assay – The direct effect of P1 and P4 on PV-1 and BoHV-1 was determined by the incubation for 1 h at 37°C of virus suspension (10^5 PFU ml^{-1}) and DMEM containing the same concentrations of the test substances, as before, followed by cell inoculation and viral titer determination.

Adsorption inhibition assay – P1 and P4 were examined for their inhibitory effect of virus adsorption on HEp-2 cells, according to Zhu *et al*., (2004) with minor modifications. Briefly, cell monolayer was pre-chilled at 4 ◦C for 1 h and inoculated with virus strains in the presence of the test substances at the same concentrations used before. After 80 min of adsorption at 4°C, the cells were washed three times with cold PBS to remove the non-adsorbed virus followed by PRA.

Positive control – Interferon was used as positive control also monitored by PRA. PV-1 and BoHV-1 were treated with human alfa-2B interferon (Meizler Com. Intern. SA, Brazil) at 1000 U/ml.

Immunofluorescence assay (IFA) – The immunofluorescence assay was performed according to Faccin et al., (2007). HEp-2 cells grown in 24-well plates with circular glass cover slips (Glasstecnica Import, Brazil) were inoculated with 500 µl of PV-1 or BoHV-1 (MOI = 1) and treated with test substances at the concentrations of 25 – 200 µg/ml, at time zero of infection (0 h). Appropriate controls were used. At 24 h post-infection, the cells were washed with PBS with 0.05% tween 20, fixed with cold acetone (-20°C) for 20 min and blocked with 2% powdered skim milk in PBS during 30 min. The cells were incubated for 30 min at 37°C with rabbit anti-PV-1 (supplied by INCQS/ Fiocruz, Brazil) and bovine anti-BoHV-1 antibodies (DMVP/UFSM, Brazil), further washed three times with PBS/tween 20 and incubated with sheep anti-rabbit IgG and rabbit anti-bovine IgG conjugated with FITC (Sigma Chem. Co.,USA), respectively. The cells were examined in a Zeiss fluorescence microscope (Imager A1 with Axio Cam MRc5) and 100 cells/cover slips were scored and the percentage of fluorescent cells inhibition calculated. The experiments were carried out in duplicate.

2.5 STATISTICS

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

3 RESULTS

3.1 CYTOTOXIC ASSAY

The 50% cytotoxic concentration (CC_{50}) of the polysaccharide P1 extracted from *A. indica* and its sulfated derivative P4 on HEp-2 cells was determined by MTT assay. The CC_{50} was higher than 1600 µg/ml for both compounds (Table 1) and no effect on cell morphology was detected.

3.2 ANTIVIRAL ACTIVITY

The inhibitory effects of the tested substances by plaque formation in HEp-2 cells are shown in Table 1. For PV-1, the inhibitory concentrations (IC_{50}) of the polysaccharide P1 and its sulfated derivative (P4) were 77.5 and 12.1 µg/ml, respectively with

selectivity index (SI) >20.6 and >131.96, respectively. The polysaccharide P1 showed no significant inhibition at all tested concentrations, when added before viral infection. However, when added during and 1 and 2 hours after infection showed inhibition of 79, 44 and 27%, respectively, at the highest tested concentration (200 µg/ml) (Fig. 1a). Its sulfated derivative (P4) was more effective, acting in all times of viral infection. It presented 18.5, 25, 78, 56 and 67% of viral inhibition at -2, -1, 0, +1, +2 hours of infection, at 200 µg/ml, respectively (Fig. 1b). These polysaccharides were more effective against PV-1 compared to BoHV-1 in all assays. For BoHV-1, was not possible to calculate the IC_{50}, because the better activity at time-of-addition assay was at time 0h, at the highest tested concentration, with maximum inhibition rates of 44 and 26% to P1 and P4, respectively (Fig 2).

The compounds showed direct effect (virucidal activity) on PV-1 and the inhibition was dose-dependent. The percentages of inhibition were 87% and 32%, at 200 µg/ml, for P1 and P4, respectively (Fig. 3a). Moreover, P1 and P4 inhibited the adsorption of poliovirus in 52% and 46%, respectively. The inhibition of the adsortion and the virucidal assay to BoHV-1 showed 21% of viral inhition to P1 and no effect to P4 (data not shown).

Positive control made by PRA showed that interferon inhibited 100% of PV-1 and BoHV-1 replication at 1000 U/ml.

3.3 IMMUNOFLUORESCENCE ASSAY

The effect of P1 and P4 in the synthesis of viral proteins evaluated by IFA showed a dose-dependent reduction. At the highest concentration, P1 inhibited PV-1 and BoHV-1 in 100% and 61%, respectively, and P4 inhibited PV-1 and BoHV-1 in 89% and 52% respectively (Table 2).

4 DISCUSSION

In the present study, anti-PV and anti-BoHV activity of polysaccharides P1 and P4 from *A. indica* were described. The best antiviral effect of P1 and P4 was against PV-1, a non-enveloped enterovirus. The maximal antiviral action was observed when the compounds were added simultaneously with the virus and was decreasing after the time of infection (Fig. 1). Besides, they were able to inhibit the virus adsorption and showed direct effect at the particle. This may indicate that the mechanisms of action of these

polysaccharides are not limited only to inhibition of viral adsorption/penetration but may be also active during viral multiplication.

The compounds with antiviral activity to naked virus may act on some of the following steps and present the possible mechanisms of action: hydrophobic interactions with viral capsid proteins; interference with/saturation of viral entry sites on the cellular membranes; binding to viral RNA inhibiting its replication and transcription; perturbation of viral RNA-protein interactions, hence inhibition of the translation of viral proteins (SITOHY *et al*., 2008).

Sulfated derivative showed a greater inhibition compared to the original polysaccharide to PV-1, by plaque assay, which may suggest that the sulfation improves the action of substances in all times of viral replication. Not so for the virucidal activity and inhibition of adsorption, in which there was a decrease in the antiviral effect, possibly by this chemical modification reduce direct interaction with the viral particle or cell.

The activity of sulfated polysaccharide strongly depended on the degree of sulfation (DS). In certain scope, the higher DS, the better biological activity is. In addition to the well documented DS dependence, the specific position of the sulfate ester group also appears to be additionally important for the antiviral activity of sulfated polysaccharide (COPELAND *et al*., 2008; GHOSH *et al*., 2009). As for antiviral mechanism of sulfated polysaccharide, it is considered that $SO4^{2-}$ polyanions of sulfated polysaccharide can combine with virus or cells (HUANG *et al*., 2008). This antiviral effect may be due to distinct mechanisms: (i) direct interaction, (ii) alteration of the adsorption phenomenon, (iii) inhibition of penetration into the host cell or (iv) inhibition of the multiplication of the viruses in the cells (ZHU *et al*., 2006).

González and Carrasco (1987) and Biesert *et al*., (1990) proposed a mechanism of antiviral activity of carrageenan and other sulfated polyanions apparently different from that of viral adsorption inhibition. These compounds do not interfere with virus attachment or penetration but exert their action by preventing viral protein synthesis. It is possible that this latter mode of action is indirect, resulting from a change in cell membrane properties that affects the transmembrane and intracellular signaling processes involved in regulating virus expression. Their results suggested that the inhibition step occurred after viral internalization, but before the onset of late viral protein synthesis (ONO *et al.,* 2003). Protein synthesis of picornavirus usually happens during the first three hours of infection and leads to the accumulation of viral proteins in the cytoplasm of the infected cell (FERREIRA *et al*., 2008). Our results showed high percent of viral inhibition for P1 and P4 at time 0 hour

corroborating those obtained by plaque assay suggesting that the greater inhibitory activity in the early stages of viral replication is also due to inhibition of viral protein.

Our results showed low effect of polysaccharides on the enveloped viruses tested (BoHV-1) replication, about 20%, unlike of results of Saha *et al*., 2010. This slight inhibition can be due the weak direct interaction with virus particle or cell receptor.

Table 1 – Antiviral activities of the substances P1 and P4 from*Azadirachta indica* against poliovirus (PV) in HEp-2 cells by the plaque assay.

	PV	
	P-1	**P-4**
CC_{50} [a]	>1.600	>1.600
IC_{50} [b]	77.5	12.1
SI[c]	>20.6	>131.96

[a] - The 50% cytotoxic concentration in HEp-2 cells in µg/ml.
[b] - The 50% inhibitory concentration of viral replication in µg/ml.
[c] - Selectivity Index = CC_{50}/IC_{50}.

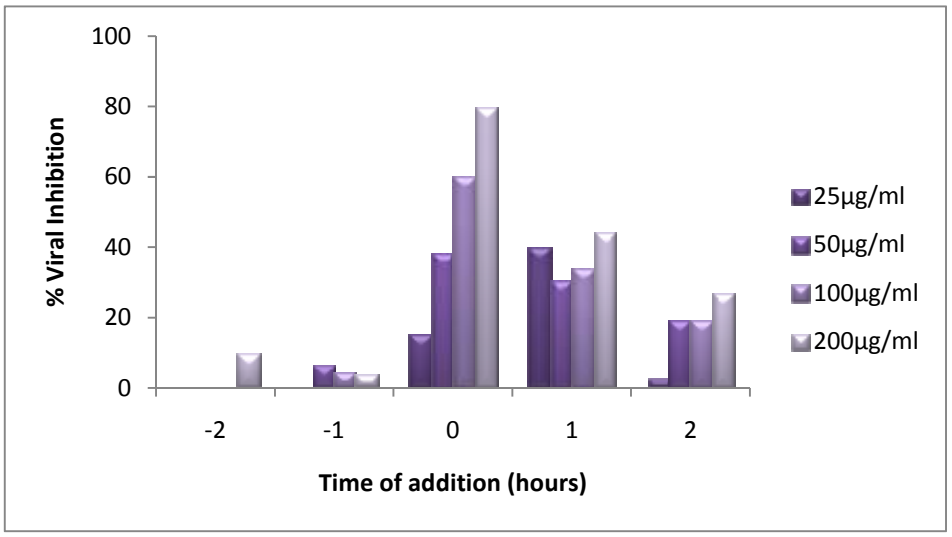
Table 2 – Immunofluorescence assay. Effect of P1 and P4 from *Azadirachta indica* on PV-1 and BoHV-1 replication monitored by immunofluorescence assay in HEp-2 cells.

	PV-1		**BoHV-1**	
	P1	**P4**	**P1**	**P4**
25[a]	15,3[b]	74,0	41,14	16,8
50	74,7	80,9	37,5	38,2
100	89,1	86,7	36,4	45,0
200	100,0	89,1	61,0	52,3

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

Fig. 1 – Time-of-addition effect of fraction P1 **(a)** from *Azadirachta indica* and its sulfated polysaccharide P4 **(b)** on Poliovirus replication in HEp-2 cells by the plaque assay. The substances were added at various concentrations before (-2 and –1 h) during (0 h) or after (1 and 2 h) virus infection.

(a) P1



(b) P4

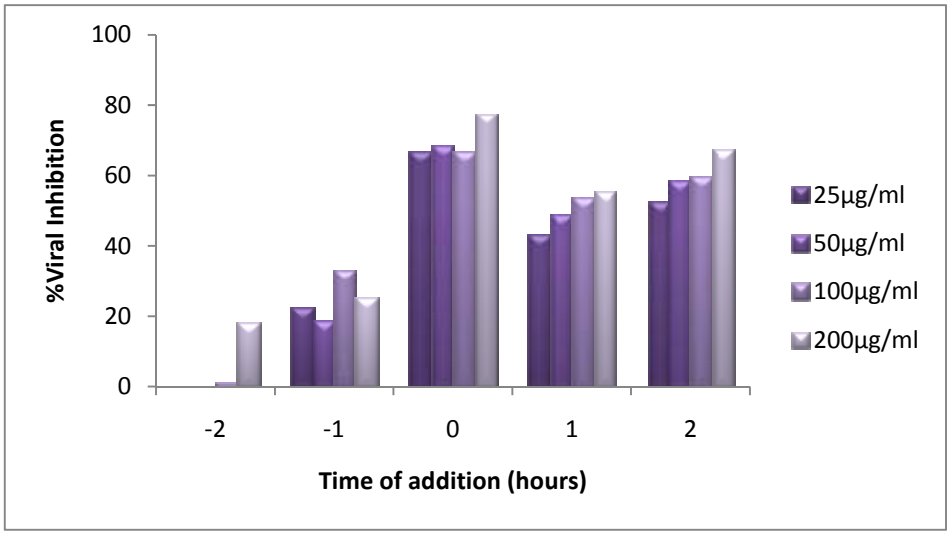
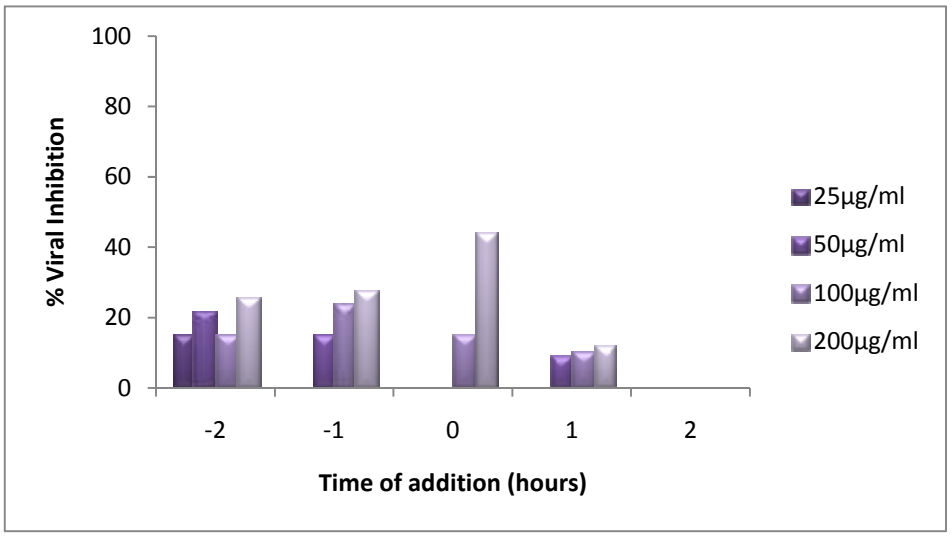


Fig. 2 – Time-of-addition effect of fraction P1 **(a)** from *Azadirachta indica* and its sulfated polysaccharide P4 **(b)** on Bovine herpesvirus (BoHV) replication in HEp-2 cells by the plaque assay. The substances were added at various concentrations before (-2 and –1 h) during (0 h) or after (1 and 2 h) virus infection.

(a) P1



(b) P4

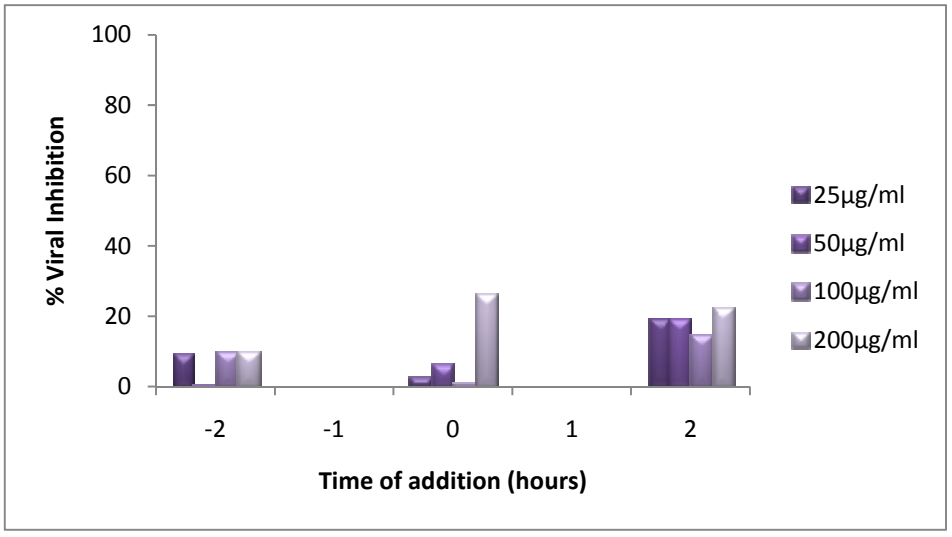
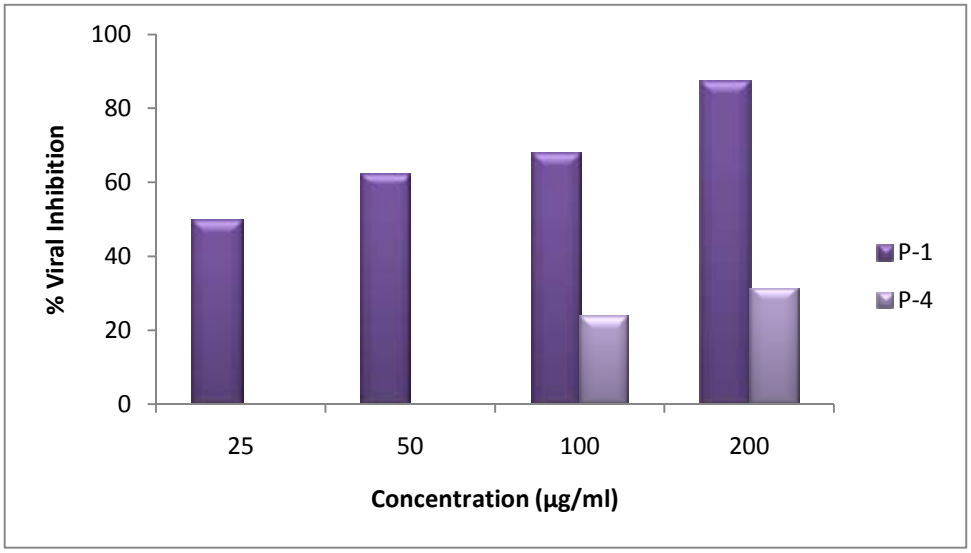
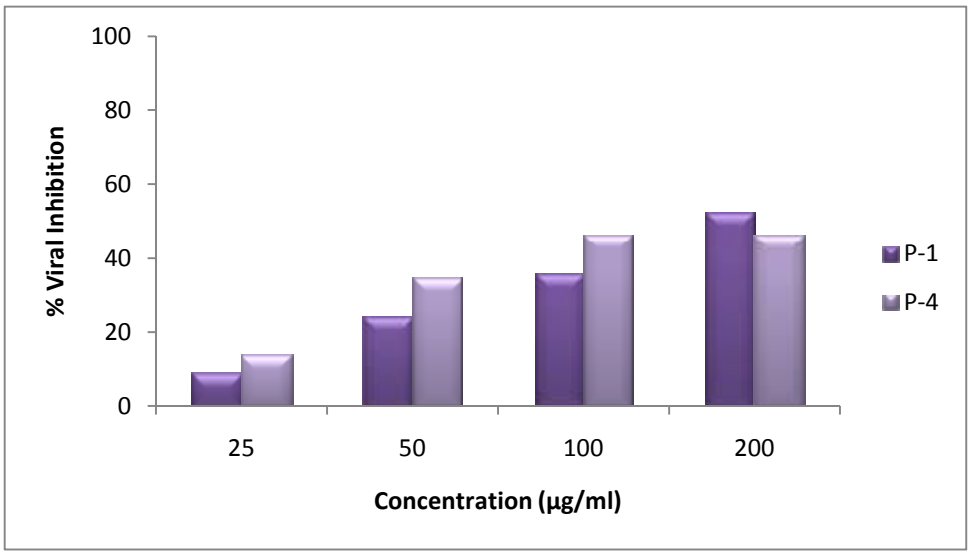


Fig. 3 – Virucidal activity **(a)** and adsorption inhibition **(b)** of P1 and P4 from *Azadirachta indica* on poliovirus replication in HEp-2 cells by the plaque assay.

(a)



(b)



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