NAYARA LOPES

ATIVIDADE ANTI-HERPÉTICA DE COMPOSTOS ORIGINÁRIOS DA ALGA MARINHA *Enteromorpha compressa* E DO FRUTO DE *Morinda citrifolia*

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor.

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Londrina, 30 de setembro de 2016.

Ao meu filho, meu marido e meus pais...
Bênçãos de Deus em minha vida!!!

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"Só sei que nada sei, e o fato de saber disso, me coloca em vantagem sobre aqueles que acham que sabem alguma coisa." (Sócrates)

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RESUMO

As doenças causadas pelo vírus herpes simplex (HSV) representam um importante problema médico e social, principalmente devido à sua transmissibilidade, estabelecimento de infecção latente e recorrência. Além disso, o HSV infecta 70-90% da população em geral. Portanto, a busca de novos compostos anti-herpéticos, se torna de extrema relevância. Neste contexto, as substâncias naturais capazes de apresentar mecanismos de ação alternativos podem ser potenciais candidatos para o controle de infecções virais. O objetivo deste estudo é investigar a atividade antiviral de sete polissacarídeos da alga verde (*Enteromorpha compressa*) (F1, SU1F1, SU1F2, SU2F1, SU2F2, SU3F1 e SU3F2) e quatro pectinas do fruto do noni (*Morinda citrifolia*) (PDN-A, PDN-B, PDN-N, PDN-S) para HSV-1 em culturas de células HEp-2. A citotoxicidade dos compostos foi avaliada pelo ensaio do MTT em células HEp-2. A atividade antiviral dos compostos foi investigada pelo ensaio de redução de plaques, utilizando os protocolos de tempo de adição (tratamentos antes, durante e após a infecção), além dos testes de inibição da adsorção e de atividade virucida. Dentre os polissacarídeos da alga, apenas o composto SU1F1 apresentou inibição viral satisfatória, sendo que esta atividade ocorreu principalmente nos estágios iniciais da replicação viral. Este composto foi, adicionalmente, avaliado pelo ensaio de imunofluorescência e RT-PCR (reação em cadeia da polimerase associada à transcriptase reversa), sendo confirmado o perfil de inibição viral, principalmente, na transcrição dos genes precoces imediatos. Dentre as pectinas do noni, três (PDN-A, PDN-B e PDN-N) demonstraram inibição viral com um desejável índice de seletividade, quando adicionadas concomitantemente à infecção viral. Porém, apenas a PDN-A apresentou inibição viral nos tratamentos pós-infecção. Diante da importância do desenvolvimento de novos antivirais, este trabalho exalta a eficácia dos compostos naturais no controle de infecções virais.

Palavras-chave: Herpesvírus. Antiviral. Algas marinhas. Noni.

LOPES, Nayara. **Antiherpetic activity of compounds from the seaweed *Enteromorpha compressa* and the fruit of *Morinda citrifolia*.** 2016. 90 p. Thesis (Doctoal Degree in Microbiology) – Universidade Estadual de Londrina, Londrina, 2016.

ABSTRACT

Diseases caused by herpes simplex virus (HSV) represent an important medical and social problem, mainly due to its transmissibility, the establishment of latent infection, recurrence, and infects 70-90% in the general population. Therefore, the search for new anti-herpetic compounds becomes extremely important. In this context, the natural substances capable to provide alternative mechanisms of action may be potential candidates for the control of HSV infections. The aim of this study is to investigate the antiviral activity of seven polysaccharides (F1, SU1F1, SU1F2, SU2F1, SU2F2, SU3F1 and SU3F2) of the green alga (*Enteromorpha compressa*) and four pectins (PDN-A, PDN-B, PDN-N and PDN-S) of the noni fruit (*Morinda citrifolia*) for HSV-1 in HEp-2 cell cultures. The cytotoxicity of the compounds in HEp-2 cells was determined by the MTT assay. Antiviral activity was assayed by plaque reduction test using protocols, such as, the time of addition (compounds added before, during and after infection), the inhibition of the attachment and virucidal assays. Among the seaweed polysaccharides, only SU1F1 showed satisfactory viral inhibition, and this activity occurred mainly in the early stages of viral replication. This compound was submitted to immunofluorescence assay and RT-PCR (reverse transcriptase-associated polymerase chain reaction) which confirmed the viral inhibition profile, showing primarily the inhibition of the immediate early genes transcription. Among the four noni compounds, three of them (PDN-A, PDN-B and PDN-N) inhibited viral replication with desirable selectivity index when treatment was carried out simultaneously to the infection. However, only PDN-A maintained the viral inhibition in treatments after infection. Given the importance of the development of new antivirals, this work strengthens the effectiveness of natural compounds in the control of viral infections.

Keywords: Herpesvirus. Antiviral. Seaweeds. Noni.

SUMÁRIO

1 INTRODUÇÃO

O vírus herpes simplex tipo 1 (HSV-1), um membro da família Herpesviridae e gênero Simplexvirus, é um vírus de DNA de cadeia dupla, envelopado e que possui a capacidade de se manter latente nos gânglios próximos do local da infecção. Estima-se que 40-80% da população mundial possui este vírus latente no organismo, sendo que grande parte dos indivíduos já tiveram pelo menos um episódio da doença e cerca de 90% das pessoas entre 20 e 40 anos têm anticorpos contra o HSV-1. Além disso, uma pequena fração de pacientes com infecção pelo HSV-1 desenvolve doenças mais graves no SNC, a mais notável das quais é a encefalite. A incidência anual de encefalite pelo HSV-1 foi estimada em 2-4 casos/um milhão de indivíduos. No Brasil, o HSV-1 é o agente mais comum de encefalite viral esporádica em adultos. Indivíduos imunocomprometidos e pacientes que se submeteram a transplante de órgãos são de alto risco para o aumento da gravidade deste tipo de infecção (ARDUINO; PORTER, 2008).

A maioria dos tratamentos para HSV utilizam análogos de nucleosídeos, como por exemplo, o aciclovir (ACV). No entanto, o uso difundido do ACV mostrou que o HSV desenvolve resistência por meio de mutações em genes que codificam para a timidina-quinase ou para a DNA polimerase. Portanto, é importante desenvolver novas drogas antivirais com diferentes mecanismos de ação que podem ser um substituto ou complemento para o aciclovir (ARDUINO; PORTER, 2008; LOPES et al, 2013).

Neste contexto, as substâncias naturais passíveis de apresentarem mecanismos de ação alternativos, ao contrário dos antivirais sintéticos, podem ser potenciais candidatos para o controle das infecções virais. As propriedades curativas nas plantas já são consideradas desde muito tempo atrás. Populações de diversas etnias aplicam emplastros e infusões embebidas em chás de milhares de plantas, um hábito que remete à pré-história. Os relatos históricos mostram muitos resultados terapêuticos envolvendo tanto a cura quanto o alívio de sintomas, porém envenenamentos também parecem ter ocorrido (CHATTOPADHYAY et al., 2009).

Além do desenvolvimento de medicamentos, há também a necessidade de novas substâncias com atividade virucida, para uso como antissépticos e desinfetantes de superfícies, visto que os existentes não tem se mostrado efetivo no combate aos vírus em ambientes de modo geral. Tais compostos seriam muito úteis

para diminuir a transmissibilidade dos vírus (VLIETINCK; BERGHE, 1991; CHATTOPADHYAY et al., 2010).

A realização deste trabalho constitui uma importante estratégia de triagem de compostos ativos de origem natural, direcionando a execução de estudos posteriores mais aprofundados, que possibilitaria a inserção de tais substâncias no arsenal disponível para o controle e tratamento de viroses.

2 OBJETIVOS

2.1 OBJETIVO GERAL

- Analisar a atividade antiviral de sete polissacarídeos obtidos da alga marinha *Enteromorpha compressa* e quatro pectinas do fruto de *Morinda citrifolia* para o HSV-1, *in vitro*.

2.2 OBJETIVOS ESPECÍFICOS

- Verificar a toxicidade dos compostos em células HEp-2 e determinar a concentração citotóxica de 50% (CC_{50}), utilizando o teste do MTT;
- Avaliar a atividade antiviral para o HSV-1, e determinar a concentração inibitória de 50% (CI_{50}), através do ensaio de redução de plaque;
- Determinar o índice de seletividade (IS) das substâncias testadas;
- Analisar, por ensaio de redução de plaque, a atividade dos compostos nas diferentes etapas da replicação viral: efeito virucida, adsorção, pré-tratamentos e pós-tratamentos;
- Analisar a influência das substâncias na síntese proteica viral, pelo ensaio de imunofluorescência (IF);
- Analisar o efeito das substâncias nas etapas de transcrição gênica do HSV-1, através da reação em cadeia da polimerase associada à transcriptase reversa (RT-PCR).

3 REVISÃO BIBLIOGRÁFICA

3.1 VÍRUS HERPES SIMPLEX

3.1.1 Histórico

Desde os primórdios da civilização humana, as infecções virais, supostamente, têm estado presentes afligindo a população humana. Registros históricos sugerem a ocorrência de lesões semelhantes às causadas pelo vírus herpes simplex (HSV) há milênios. Lesões genitais sugestivas da virose foram mencionadas nos mais antigos documentos farmacêuticos - Tábua Sumeriana e Papiro de Ebers, aproximadamente, nos anos 3000 e 1500 a.C. Hipócrates (Grécia, 460/377 a.C.) descreveu lesões vesiculares na pele, denominando-as *herpes* (do grego *herpein* = rastejar, réptil). O historiador grego Heródoto (484/425 a.C.) descreveu a infecção herpética como capaz de causar febre, pequenas vesículas na boca e ulcerações nos lábios, denominando-a *herpes febrilis.* Galeno (129/204 a.C.), farmacêutico e médico grego, sugeriu que as referidas lesões eram humores malignos, em forma de bolhas, chamando-as de *excretinas* herpéticas. A partir destes relatos históricos até o século XVII, o termo *herpes* foi aplicado a inúmeras enfermidades da pele, mesmo as não causadas pelo HSV. Somente na era moderna, 1873, Vidal comprovou que o herpes era infeccioso e poderia ser transmitido de pessoa a pessoa. Gruter demonstrou o isolamento e a transmissão do vírus em coelhos, entretanto, foi Lowenstein o responsável pelo primeiro isolamento do HSV reconhecido pela comunidade científica em 1919. Em 1930 as recorrências foram reconhecidas como um dos marcos da biologia da infecção herpética, por Andrews e Carmichael. Em 1968, Nahmias e Dowdle demonstraram a existência de dois sorotipos virais, HSV-1 e HSV-2, baseado nas características antigênicas e biológicas (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

As características do HSV de causar diferentes quadros clínicos, a capacidade de estabelecer latência por toda a vida do hospedeiro e a recorrência, no local inicial da infecção, fazem do vírus um dos mais intensamente estudados. O amplo conhecimento de sua organização genômica e biológica e a habilidade do vírus infectar diversos tipos celulares representam um campo de estudo bastante

atrativo. Destacam-se a translocação de proteínas, conexões sinápticas no sistema nervoso, estrutura de membrana, regulação gênica, terapia gênica, terapia contra o câncer, dentre outros (ROIZMAN; KNIPE; WHITLEY, 2007; SILVA; BOWERS, 2009).

Altas taxas de soropositividade ao HSV na população constituem uma preocupação sanitária, apesar das infecções graves serem, normalmente, raras. O HSV-1 está principalmente associado com a doença mucocutânea oral, encefalite esporádica, e é uma das principais causas de cegueira, pelo acometimento da córnea. Está, entretanto, despontando como uma causa importante da doença genital. O HSV-2 é o principal causador de doença ulcerosa genital, associada a sua elevada prevalência em regiões como na África sub-Sahariana. A infecção contribui significativamente para a propagação do HIV-1 nesta região (KOELLE; COREY, 2003; ROIZMAN; KNIPE; WHITLEY, 2007; FATAHZADEH; SCHWARTZ, 2007; BOLLAND; PIERCE, 2015; PETRO et al, 2015).

Os HSV pertencem a ordem *Herpesvirales* composta por 3 famílias: *Herpesviridae*, compreendendo os herpesvírus de mamíferos, pássaros e répteis; *Alloherpesviridae* incluindo vírus de peixes e anfíbios e *Malacoherpesviridae*, abrangendo os herpesvírus de moluscos (DAVISON, 2010).

A família *Herpesviridae* é caracterizada por vírus que codificam várias enzimas envolvidas na replicação (timidina-quinase, timidilato-sintetase, ribonucleotídeo-redutase) e na síntese do ácido nucleico viral (DNA polimerase, helicase, primase) e no processamento das proteínas virais (proteíno-quinase). Estabelecem também infecções latentes com reativações. É constituída pelas subfamílias: *Alphaherpesvirinae*, *Betaherpesvirinae* e *Gamaherpesvirinae*, de acordo com a sequência do gene conservado da glicoproteína H (gH), do envelope. Na subfamília *Alphaherpesvirinae* encontram-se o gênero *Simplexvirus* onde estão enquadrados os herpesvírus humano 1 e 2 (HHV-1 e HHV-2) ou HSV-1 e 2 e o gênero *Varicellovirus* com o herpesvírus humano 3 (HHV-3) ou vírus da varicela-zoster (VZV) e o herpesvírus bovino 1 e 5 (BoHV-1 e 5) (ROIZMAN; KNIPE; WHITLEY, 2007).

Os vírus da subfamília *Alphaherpesvirinae* apresentam um ciclo de replicação rápido e lítico, *in vitro* e *in vivo* e possuem a capacidade de infectar células epiteliais e nervosas, estabelecendo infecção latente em gânglios do sistema nervoso (ROIZMAN; KNIPE; WHITLEY, 2007).

Já os representantes da subfamília *Betaherpesvirinae,* de replicação lenta, são associados a quadro clínicos, principalmente, em imunocomprometidos, quais sejam, citomegalovírus humano (HCMV), herpesvírus humano 6 (HHV-6) e herpesvírus humano 7 (HHV-7). Na subfamília *Gamaherpesvirinae* são descritos o herpesvírus humano 8 (HHV-8) e o vírus Epstein-Barr (EBV), respectivamente, agentes etiológicos do sarcoma de Kaposi (KSHV) e mononucleose infecciosa. O EBV causa também o Linfoma de Burkitt. Ambos infectam linfócitos de forma lítica e latente com potencial oncogênico (SANTOS; ROMANOS; WIGG, 2008). Os representantes *Beta* e *Gamaherpesvirinae* são conhecidos por causarem manifestações sistêmicas (ROIZMAN; KNIPE; WHITLEY, 2007).

3.1.2 Estrutura Viral do HSV

O virion do HSV apresenta-se como uma partícula esférica com diâmetro aproximado de 190 nm a 225 nm (incluindo as espículas). É formado pelo capsídeo, uma estrutura protéica amorfa chamada tegumento e envelope lipoprotéico (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008; LACASSE; SCHANG, 2010). O genoma do virion é constituído de DNA de dupla fita linear, possui pelo menos 84 sequências abertas de leitura (*open reading frames* – ORF), que codificam aproximadamente 100 proteínas estruturais e não estruturais necessárias a replicação viral. A massa molecular do genoma do HSV é de aproximadamente 150 kbp, sendo 68% G/C para HSV-1 e 69% para HSV-2 (ROIZMAN; KNIPE; WHITLEY, 2007).

O genoma é constituído de dois componentes que são formados por sequências únicas (U) ligadas covalentemente, chamadas de L (*long*) e S (*short*), designadas pelo tamanho do fragmento genômico que será lido, utilizando as siglas U_L ou U_S. Pode ser feita uma divisão do genoma em seis importantes regiões: 1) **Sequências "a" –** As terminações da molécula linear, chamadas de sequências "a", são importantes na circularização do DNA viral e no empacotamento do DNA no vírion; 2) **R_L –** Sequência longa repetida (R_L) de 9kpb, codifica a proteína regulatória inicial α0 e contém também o promotor e a maioria dos genes relacionados aos transcritos associados à latência (LAT – *latency-associated transcripts*); 3) **U_L –** Região longa única (U_L) de 108kpb, codifica pelo menos 56 proteínas diferentes; 4) **R_S –** Região de repetições curtas (R_S) de 6,6 kpb, codifica a mais importante

proteína precoce imediata, que é uma poderosa ativadora transcricional; 5) **Ori_L –** Origem de replicação (Ori_L), está no meio da região U_L, enquanto a Ori_S está em R_S, sendo que todo o conjunto de Oris funciona durante a infecção para gerar um complexo de replicação; 6) **U_S –** Região curta única (U_S), codifica 12 ORFs, várias das quais são glicoproteínas importantes na formação do vírus e nos mecanismos de defesa do organismo (SANTOS; ROMANOS; WIGG, 2008).

O capsídeo é constituído por 162 capsômeros, sendo 150 hexaméricos e 12 pentaméricos. Quatro proteínas virais (VP - virion protein) formam o capsídeo: VP5 (U_L19), VP26 (U_L35), VP23 (U_L18), e VP19C (U_L38). A VP5, principal proteína do capsídeo, está presente em cinco cópias em cada capsômero pentamérico e seis cópias em cada capsômero hexamérico. A VP26 está presente em seis cópias como um anel na parte superior das subunidades VP5 em cada capsômero hexamérico. Uma molécula VP19C e duas moléculas de VP23 formam trios proteicos que ligam capsômeros adjacentes resultando em um pseudo-trimêro com cada subunidade interagindo com dois capsômeros. O capsídeo ainda contém mais duas proteínas, a U_L6, que forma um dodecâmero constituindo um portal, através do qual o DNA viral será encapsidado; e VP24 (U_L26), uma protease que processa um suporte durante a encapsidação do DNA. A proteína U_L16 também pode estar associada ao capsídeo, já que ela desempenha um papel importante na encapsidação do DNA (ROIZMAN; KNIPE; WHITLEY, 2007).

Entre o capsídeo e o envelope encontra-se o tegumento que contém 22 proteínas: U_L4, U_L11, U_L13, U_L14, U_L16, U_L17, U_L21, U_L36 (VP1/2), U_L37 (VP11/12), U_L41, U_L46, U_L47 (VP13/14), U_L48 (VP16), U_L49 (VP22), U_L51, U_L56, U_S2, U_S3, U_S10, U_S11, ICP0 (*infected cell protein*) e ICP4 (METTENLEITER, 2004, 2006). As principais proteínas constituintes do tegumento são: α-TIF (α-trans-inducing fator), também chamada VP16, envolvida na regulação da transcrição de genes precoces imediatos; a proteína VHS (*host shut-off protein* ou VP22), relacionada com a regulação da transcrição de genes da célula hospedeira (para a síntese de macromoléculas da célula infectada) e com a estabilização de certas proteínas virais, como a gE, gD e ICP0, além de estar relacionada a disseminação viral célula-a-célula durante a infecção lítica; e uma grande proteína, a VP1-2, que parece participar da liberação do DNA viral no poro nuclear após a penetração (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008; SILVA; BOWERS, 2009).

O envelope consiste em uma bicamada lipídica contendo 5 proteínas: U_L20, U_L43, U_L45, U_L49A e U_S9, e 11 glicoproteínas: U_L1 (**gL**), U_L10 (**gM**), U_L22 (**gH**), U_L27 (**gB**), U_L44 (**gC**), U_L53 (**gK**), U_S4 (**gG**), U_S5 (**gJ**), U_S6 (**gD**), U_S7 (**gI**) e U_S8 (**gE**). Estas glicoproteínas são importantes na interação com receptores celulares necessária a entrada do vírus na célula no início da infecção. Dentre estas, a glicoproteína B (gB), gC, gD, gH e gL são importantes para a adsorção, fusão com a membrana celular e internalização do vírus (METTENLEITER, 2004, 2006; SILVA; BOWERS, 2009).

3.1.3 Replicação Viral

O HSV estabelece infecções lítica e latente em células epiteliais e neurônios, respectivamente, sendo que na primeira, todos os seus genes são expressos e novas partículas virais são geradas. Na infecção latente, a expressão gênica viral é quase nula e apenas alguns transcritos (LAT) são sintetizados (LACASSE; SCHANG, 2010).

A infecção inicia-se (adsorção) pela ligação das glicoproteínas gC e gB do envelope às glicosaminoglicanas (GAG) presentes na superfície celular, tais como o sulfato de heparana (HS), sulfato de condroitina (CS) e sulfato de dermatana (DS). A participação das GAG é importante, haja vista que a sua remoção enzimática provoca uma diminuição de 10 a 20 vezes a formação de plaques (ROIZMAN; KNIPE; WHITLEY, 2007; AKHTAR; SHUKLA, 2009).

O passo seguinte (penetração/entrada) envolve a interação de gD com um receptor específico e a fusão do envelope com a membrana plasmática. A glicoproteína gD interage com, pelo menos, três tipos diferentes de receptores, os mediadores nectinas, os HVEM (mediadores da entrada do vírus herpes) e uma forma específica de sulfato de heparana, o 3-O-sulfatado heparana sulfato (3-OS HS). A gD em conjunto com gB, gH e gL permitem a fusão do envelope com a membrana plasmática, consolidando esta fase (ROIZMAN; KNIPE; WHITLEY, 2007).

Após a penetração no citoplasma, o nucleocapsídeo é transportado até o poro nuclear, onde o DNA viral é liberado no núcleo. A transcrição do genoma viral, a replicação do DNA viral e a montagem de novos capsídeos são realizadas no núcleo. Nestas condições, o núcleo é submetido a uma série de alterações estruturais que facilitam a replicação viral e bloqueiam a resposta da célula

hospedeira. A compartimentalização das proteínas virais é necessária para uma eficiente replicação do DNA viral e a transcrição de genes tardios. O genoma é acompanhado da proteína tegumentar α-TIF complexada com as proteínas do hospedeiro, HCF (*host cell factor*) e Oct-1 (octâmero 1), necessários para o início da transcrição gênica viral. No citoplasma, as proteínas virais precocemente sintetizadas desagregam os polirribossomas e degradam os RNA celulares, inibindo a síntese de proteínas celulares (ROIZMAN; KNIPE; WHITLEY, 2007).

O DNA viral é transcrito durante toda a infecção produtiva pela RNA polimerase II do hospedeiro, com a participação de fatores virais. A síntese dos produtos de genes virais é estreitamente regulada: a expressão é coordenadamente regulada e sequencialmente ordenada em cascata (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008). Ou seja, a expressão de genes ocorre de forma que os produtos de cada classe de genes são necessários para a expressão dos genes da classe subsequente. A primeira classe de genes a ser transcrito e traduzido são os precoces imediatos (genes *alfa*), ativados pelo complexo α-TIF/proteínas celulares, produzindo as proteínas precoces imediatas, ou polipeptídeos α, atingindo o pico máximo em três horas após a entrada do vírus na célula. Seis proteínas α são sintetizadas nos estágios iniciais da infecção produtiva: ICP0 (α0), ICP4 (α4), ICP22 (α22/U_S1), ICP27 (α27/U_L54), ICP47 (α47/U_S12) e U_S1.5. Com exceção de ICP47, todas as outras cinco proteínas estimulam a expressão dos genes sequenciais (genes precoces ou *beta*), e são regulatórias de toda a síntese proteica e genômica do vírus. As proteínas ICP4, ICP0 e ICP27 atuam para ativar a expressão dos genes virais durante a transcrição ou expressão de RNAm e interagem para formar complexos nucleares com o genoma viral. As outras duas proteínas, ICP22 e ICP47 são dispensáveis para a replicação em muitos tipos de culturas de células. A ICP47 parece participar na modulação da resposta do hospedeiro à infecção, interferindo especificamente com a apresentação de antígenos virais na superfície das células infectadas (SANTOS; ROMANOS; WIGG, 2008; BLOOM; GIORDANI; KWIATKOWSKI, 2010).

A ativação da maquinaria de transcrição da célula pelos produtos dos genes α resulta na expressão dos genes β. Os genes precoces codificam várias proteínas importantes na replicação do DNA viral, e são pré-requisitos para a expressão da terceira classe de genes (genes tardios ou genes *γ*). Sete genes são necessários e suficientes para a replicação do DNA viral: UL30 (codifica a DNA polimerase), UL42

e UL29 (codificam proteínas de ligação ao DNA), UL9 (codifica a proteína de ligação à origem) e UL5, UL8 e UL52 (codificam o complexo helicase/primase). Quando níveis suficientes das proteínas codificadas por estes genes se acumulam dentro da célula infectada, a replicação do DNA viral ocorre. As proteínas β são importantes para a síntese de novas moléculas de DNA e para a regulação de genes correlacionados com a sua expressão completa, como os genes da DNA polimerase, timidino-quinase e de proteínas de ligação à fita única de DNA, denominadas complexo primossoma ou helicase-primase. O pico máximo de síntese dessas proteínas ocorre em seis horas, podendo ser detectados em três horas pós-infecção (SANTOS; ROMANOS; WIGG, 2008; BLOOM; GIORDANI; KWIATKOWSKI, 2010).

O DNA genômico inicia sua replicação próximo ao domínio nuclear 10 (ND-10). Algumas proteínas são necessárias para a formação dos sítios pré-replicativos, necessitando de ICP8, complexo helicase/primase, assim como a proteína de ligação à origem. Em seguida, a DNA polimerase e seu fator de processamento (U_L42) se unem ao complexo. A medida que a síntese do DNA viral progride, o sítio pré-replicativo aumenta de tamanho para formar compartimentos de replicação que coalescem e tomam conta do núcleo. Além disso, ocorre o recrutamento de várias proteínas e fatores celulares para o compartimento de replicação, incluindo proteínas de replicação do DNA celular, p53, proteínas de recombinação e reparo do DNA, RNA polimerase II e o fator regulatório de interferon-3 (SANTOS; ROMANOS; WIGG, 2008).

Após a replicação do genoma, os genes tardios (*γ*) são transcritos. A expressão destes genes está relacionada à produção de proteínas estruturais, como as constituintes do tegumento, capsídeo e espículas glicoproteicas. A replicação do DNA viral representa um evento crítico no ciclo replicativo do vírus. Elevados níveis de replicação do DNA direcionam irreversivelmente a célula para produzir novos vírus, o que resulta na destruição desta. Os genes tardios podem ser divididos em duas classes: tardios γ1 (genes fugazes) e verdadeiramente tardios γ2. Os transcritos γ1 são expressos em baixos níveis antes da replicação do DNA e alcançam níveis elevados assim que a replicação se inicia. Já os transcritos γ2 são difíceis de detectar até que termine a replicação do DNA. Mais de 30 proteínas fazem parte da estrutura do vírus, e todas são expressas pelos genes tardios (SANTOS; ROMANOS; WIGG, 2008; BLOOM; GIORDANI; KWIATKOWSKI, 2010).

A montagem das novas partículas virais ocorre em várias etapas. O capsídeo é montado no núcleo, a partir de proteínas pré-sintetizadas, como um arcabouço vazio contendo em seu interior proteínas de suporte (*scaffolding*), as quais vão interagir com o DNA viral para permitir o empacotamento. Os envoltórios vazios contendo o suporte interno formado pelas proteínas *scaffolding* são montados primeiro, e mais tarde, quando o DNA é empacotado, esse suporte proteico se perde (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

O DNA viral empacotado forma os nucleocapsídeos, que vão sofrer maturação se tornando infecciosos e saem do núcleo por brotamento ou passagem pelo poro nuclear. A hipótese mais plausível para a aquisição do envelope nos alfa-herpesvírus é o processo de duplo envelopamento. No processo de maturação inicial no núcleo, os capsídeos parecem ser revestidos com a proteína do tegumento U_L31, que dirige o brotamento através da lamela interna nuclear, na região em que as proteínas fosforiladas U_L31 e U_L34 foram inseridas. Esse primeiro capsídeo envelopado brotaria da membrana do núcleo e perderia este primeiro envelope. Posteriormente, o capsídeo se associaria com várias proteínas do tegumento, incluindo α-TIF e VHS, que parecem auxiliar no envelopamento (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

A finalização do processo ocorreria com os capsídeos brotando em vesículas exocíticas que contem em sua membrana todas as glicoproteínas do envelope do vírion infeccioso. As partículas virais infecciosas poderiam permanecer associadas à célula dentro das vesículas, se espalhariam para outras células não-infectadas, via fusão das membranas citoplasmáticas e seriam liberadas da célula por vesículas exocíticas no espaço intercelular. A saída da célula é dependente de gD e gH. Em células permissivas, o ciclo de biossíntese do HSV dura aproximadamente 18 a 20 horas (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

3.1.4 Latência

A capacidade de estabelecer infecção persistente do tipo latente é uma característica de toda a família *Herpesviridae,* variando o sítio de latência de acordo com a subfamília. A principal característica da subfamília *Alfaherpesvirinae* é estabelecer latência nos nervos sensoriais (SANTOS; ROMANOS; WIGG, 2008).

Na infecção primária a progênie viral produzida localmente ganha acesso aos neurônios sensoriais através da membrana neuronal, nos terminais axonais, e o nucleocapsídeo é levado por transporte axonal retrógrado para o núcleo no corpo celular de um neurônio de um gânglio. O DNA viral é liberado para dentro do núcleo, presumivelmente, em um mecanismo semelhante ao utilizado em uma infecção lítica, persistindo no núcleo na forma episssomal circular. A infecção latente é caracterizada por um desligamento das funções de replicação do vírus e pela incapacidade de detecção de vírus infeccioso. Os únicos produtos gênicos expressos são os transcritos relacionados à latência (LAT*)* (KNIPE; CLIFFE, 2008; BLOOM; GIORDANI; KWIATKOWSKI, 2010).

Somente uma pequena quantidade de neurônios, no gânglio, é infectada e abrigam múltiplas cópias do genoma viral, mas nem todos expressam LAT. Este fato leva a constatação de que, uma vez estabelecida a infecção latente, mecanismos de repressão do hospedeiro ajudam na manutenção da latência, independentemente da expressão de LAT. Por outro lado, existem evidências de que a síntese dos LAT pode facilitar a reativação espontânea dos vírus latentes (SANTOS; ROMANOS; WIGG, 2008).

Um mecanismo proposto para a repressão dos genes líticos é a associação de heterocromatina com o genoma viral, apresentando histonas acumuladas nos promotores líticos, durante o estabelecimento da latência, e pelas histonas estarem na forma hipoacetilada e enriquecidas com heterocromatina marcada com histona H3 dimetil-lisina 9 (H3K9me2) (WAGNER et al, 1988; KUBAT et al, 2004; DU CHENE et al, 2007; KNIPE; CLIFFE, 2008).

Diversos mecanismos têm sido propostos para explicar a infecção latente nos neurônios. O primeiro deles seria a repressão da expressão dos genes líticos virais por influência celular. Aparentemente, o fator celular Oct-2 (octâmero 2) reprime a expressão dos genes α através do bloqueio de Oct-1. A segunda hipótese, que os neurônios não possuiriam fatores celulares necessários para a expressão dos genes α, provavelmente, pela não expressão do Oct-1. A terceira hipótese está relacionada à inibição da expressão dos genes líticos pelos genes LAT, que levariam a formação de heterocromatina, inibindo os promotores dos genes líticos por meio de RNA silenciadores. A quarta hipótese refere-se à inibição da replicação viral pela resposta imunológica do hospedeiro. Participação de células $TCD8^{+}$ e/ou interferon-γ que impedem a expressão de genes virais e a replicação (ROIZMAN; KNIPE;

WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008; ARDUINO; PORTER, 2008). A quinta hipótese seria a maior expressão da proteína Oct-2, que reprime a expressão de genes α porque não consegue se complexar a α-TIF, diferentemente do que ocorre nas células epiteliais. Supõe-se, entretanto, que o estabelecimento da infecção latente seja multifatorial (SANTOS; ROMANOS; WIGG, 2008).

Embora o mecanismo de reativação também seja pouco conhecido, em geral os estímulos têm ligação com a capacidade de causar estresse, tanto ao hospedeiro quanto diretamente ao neurônio. Alguns mecanismos desencadeantes incluem febre, exposição à luz ultravioleta, menstruação, desequilíbrio hormonal e estresse emocional. A reativação do herpes orofacial pode ocorrer também dentro de três dias após traumatismos gerados por tratamento de canal dentário ou extração de dente, ou ainda em tratamentos estéticos que causam abrasão da pele por laser ou ácido retinóico. É provável que a síntese local de prostaglandinas E e F por conta do trauma ativem a liberação de adenosina monofosfato cíclico (AMPc), com ativação da proteíno-quinases celulares, culminando na expressão de genes virais (SANTOS; ROMANOS; WIGG, 2008).

3.1.5 Patogênese da Infecção pelo HSV

A transmissão do vírus é dependente do contato íntimo e pessoal de um hospedeiro suscetível com doentes que estejam excretando ativamente o HSV. Os vírus penetram no organismo pelo contato direto com a pele, mucosa oral ou genital com solução de continuidade, podendo também ser transmitidos por meio de fluidos corporais como a saliva, sêmen e secreções cervicais, ou através do contato com o líquido das vesículas. O risco da infecção é maior quando ocorre o contato direto com o líquido das vesículas durante a presença da lesão herpética. Com a replicação do vírus no local da entrada, o vírion é transportado até o gânglio onde estabelecerá latência (MEHNERT; CANDEIAS, 2005; FATAHZADEH; SCHWARTZ, 2007; ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008; AKHTAR; SHUKLA, 2009).

A infecção sistêmica com disseminação viral aos órgãos internos ocasionando a doença generalizada acomete neonatos, gestantes e pacientes imunocomprometidos. O envolvimento de órgãos de forma generalizada é associado

à viremia em hospedeiros incapazes de limitar a replicação do vírus em superfícies mucosas (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

A degeneração celular pela replicação viral provoca alterações profundas em sua organização estrutural e bioquímica, resultando em sua morte. Como resultado da lise celular, o exsudato claro (líquido vesicular) é acumulado entre a epiderme e a derme. Esse fluido contém grande quantidade de vírus, restos celulares e células inflamatórias. As lesões são, portanto, a combinação de morte celular induzida pela replicação do vírus e a resposta inflamatória mediada pelas respostas específica e inespecífica do hospedeiro. Quando ocorre a remissão da lesão, o fluido torna-se purulento, com o recrutamento de mais células inflamatórias, seguido do aparecimento de crosta, e entra em remissão sem deixar cicatriz (KOELLE; COREY, 2003; SANTOS; ROMANOS; WIGG, 2008).

3.1.6 Quadro Clínico do HSV

O homem é o único reservatório do HSV-1 e -2. A sintomatologia provocada pelo HSV-1 é principalmente limitada as lesões bolhosas da boca e ceratite nos olhos. O HSV-2, em contraste, é em grande parte responsável por lesões genitais. No entanto, ambos são capazes de causar lesões nos dois locais do corpo e podem causar doenças potencialmente fatais em indivíduos imunocomprometidos, incluindo recém-nascidos, pacientes com HIV ou doentes submetidos a tratamento com imunossupressores. O HSV-1 e -2 podem causar infecções do sistema nervoso central, sendo uma das causas da encefalite esporádica aguda em adultos e crianças com mais de 6 meses de idade. Outras doenças neurológicas incluem meningite, encefalite e mielite. O HSV-2 foi mais frequentemente associado à meningite asséptica recorrente do que o HSV-1, e causa complicações neurológicas mais frequentemente do que a maioria dos outros vírus (KUO et al., 2008; AKHTAR; SHUKLA, 2009; AKYA et al, 2015). Existem dois picos de prevalência etária para as infecções - pessoas menores de 20 anos e maiores de 50 anos de idade.

As infecções herpéticas são comuns na população, sendo que a soroprevalência em adultos é cerca de 80-90% para o HSV-1 e varia entre 10 e 25% para o HSV-2. Indivíduos assintomáticos podem periodicamente eliminar partículas virais na saliva, sendo observado em 2-9% do total de casos, o que contribui para a alta prevalência do vírus. A disseminação viral é geralmente maior em pacientes

imunocomprometidos (aproximadamente 38%) ou nos submetidos à cirurgia oral (aproximadamente 20%). A disseminação assintomática do HSV ocorre principalmente durante a fase prodrômica da doença primária e ocorre em 60% dos pacientes que não desenvolvem vesículas após os sintomas iniciais da doença (ARDUINO; PORTER, 2008; REIL et al., 2008).

Após a infecção primária, o HSV latente é reativado periodicamente, com migração dos vírus dos gânglios sensoriais para causar a doença oral ou genital recorrente em células epiteliais. A suscetibilidade genética de um indivíduo, estado imunológico, idade, localização anatômica da infecção, dose inicial do inoculo e subtipo viral parecem influenciar a frequência de recorrência. Normalmente, a infecção primária é mais invasiva que a recorrente, devido à inexistência de imunidade celular e humoral. A infecção primária por HSV-1 ou HSV-2 dura de duas a três semanas, mas a dor pode persistir por até seis semanas. Os surtos recorrentes podem ocorrer em intervalos de dias, semanas ou anos. Para a maioria das pessoas, as recorrências são mais frequentes nos primeiros anos após o primeiro ataque. Durante esse tempo, a resposta imunológica do indivíduo faz com que progressivamente as recorrências sejam mais brandas e menos frequentes, entretanto, o sistema imunológico não é capaz de erradicar o vírus do organismo (FATAHZADEH; SCHWARTZ, 2007; SANTOS; ROMANOS; WIGG, 2008; AKHTAR; SHUKLA, 2009).

3.2 Controle e Tratamento das Infecções Herpéticas

3.2.1 Desenvolvimento de vacinas

As infecções pelo HSV impõe um enorme ônus para a saúde pública no mundo todo, sugerindo que o desenvolvimento de uma vacina contra o HSV é de grande relevância. No entanto, a prevenção da infecção por HSV apresenta problemas únicos por causa da recorrência, mesmo na presença de imunidade humoral. Em modelos animais, a proteção contra a infecção potencialmente fatal pôde ser alcançada com vacinas atenuadas, inativadas ou de subunidades glicoprotéicas (ROIZMAN; KNIPE; WHITLEY, 2007; BOLLAND; PIERCE; 2015).

A gD do HSV-2 (glicoproteína do envelope necessária para a entrada viral e disseminação célula-a-célula), tem sido uma potencial candidata para a produção da

vacina. Porém, os resultados de ensaios clínicos desta vacina têm sido pouco alentadores (KOELLE; COREY, 2003; ROIZMAN; KNIPE; WHITLEY, 2007; BOLLAND; PIERCE; 2015). Uma vacina produzida a partir de subunidades gD-2 com adjuvantes, não forneceu proteção contra a doença genital por HSV-2, mas surpreendeu com proteção cruzada à infecção por HSV-1 em 35% dos casos e proteção de 58% contra a doença já instalada (redução das recorrências) pelo HSV-1. Anticorpos neutralizantes foram detectados no soro, mas não em mucosas. A vacina induziu células T CD4+, mas não T CD8+, porém, as respostas celulares não se correlacionaram com a proteção ao HSV-1. Estes resultados sugerem a necessidade de desenvolvimento de mais imunógenos complexos para induzir respostas imunes, suficientemente protetoras contra ambos os sorotipos (BELSHE et al, 2012).

Atualmente, contrariando as evidências, Petro et al. (2015) desenvolveram uma nova vacina utilizando um vírus HSV-2 que não tem o gene que codifica a glicoproteína D. Os autores sugeriram que a deleção de gD-2, iria restringir a infecção à um único ciclo de replicação viral e, assim, proporcionar uma possível vacina efetivamente atenuada e segura. Como o HSV-2 requer a gD para infectar as células, foi desenvolvida uma cepa vacinal por meio do crescimento em cultura de células VD60, um tipo células Vero que expressa a gD do HSV-1. Isso permitiu que o vírus capture a gD a partir das células e se replique. O vírus resultante seria bastante seguro, pois não produziria progênie infecciosa.

Por que a glicoproteína D isolada não produz uma vacina eficaz e a remoção desta o faz é um enigma, entretanto, os desafios permanecem ao desenvolvimento de uma vacina eficaz contra as doenças causadas pelo HSV (BOLLAND; PIERCE; 2015).

3.2.2 Medicamentos anti-herpéticos

As drogas anti-herpéticas tópicas ou sistêmicas atualmente em uso apresentam eficácia comprovada na redução dos sintomas e no tempo da manifestação clínica localizada. Dada a ação terapêutica destas substâncias somente no momento da replicação do vírus, considerando a ocorrência do estado de latência do vírus, torna-se impossível a debelação completa da infecção herpética. O paciente pode ter recorrências mesmo sob tratamento e continua a

transmitir o vírus (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008; ARDUINO; PORTER, 2008).

A iododesoxiuridina (5-iodo-2'-desoxiuridina - IDU) é um análogo halogenado da timidina, sintetizada por Prusoff em 1959, e introduzido na terapia antiviral por Kaufmann e colaboradores, em 1962. O mecanismo de ação consiste na incorporação da forma IDU difosfatada ao DNA viral nascente e o sequente bloqueio de sua síntese, inibindo a replicação viral. Não tem ação seletiva por ser fosforilado por enzimas celulares e, portanto, compromete também a síntese do DNA celular. Apresenta elevada toxidade motivo pelo qual o seu uso era restrito somente à forma tópica. Foi o primeiro antiviral utilizado no tratamento de ceratite herpética, porém com efeitos colaterais importantes, como ceratite epitelial pontuada tóxica, demora na cicatrização epitelial, conjuntivite folicular, obstrução lacrimal e reação de hipersensibilidade. Com a concepção semelhante ao IDU, a trifluridina (5-trifluorometil-2-desoxiuridina F3T) foi desenvolvida e também desencadeava reações adversas em tratamentos prolongados, porém em menor intensidade (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

A vidarabina (9-beta-D-arabinofuranosiladenina), ou Ara-A, é uma análogo da adenosina sintetizada em 1977 e, posteriormente, verificou-se que é naturalmente produzida por *Streptomyces antibioticus*. Foi o primeiro antiviral licenciado para o tratamento de encefalite e infecções neonatais por HSV, bem como para as infecções por varicela-zoster. A Ara-A foi responsável pelo decréscimo de casos fatais de encefalite pelo HSV em neonatos, substituída mais tarde pelo aciclovir para o tratamento de todas as infecções pelo HSV (ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

O aciclovir (9-[2-hidroxietoximetil] guanina, ACV, Zovirax®) foi desenvolvido em 1970 por Gertrude Ellion e colaboradores e liberado para o tratamento de infecções de HSV e vírus varicela-zoster (VZV). É análogo da guanosina que apresenta uma cadeia acíclica substituindo a desoxiribose. O seu mecanismo de ação depende também de fosforilação à forma trifosfato e inibe etapas do ciclo de replicação viral, com afinidade aumentada pela polimerase viral em comparação a polimerases celulares. O ACV é, seletivamente, monofosforilado pela timidina quinase (tk) viral com muito maior afinidade do que pela enzima celular homóloga. A conversão às formas di- e trifosfatos é implementada pela enzima celular. A forma ACV-trifosforilada ativa encontra-se em concentrações 40 a 100 vezes maiores nas

células infectadas comparativamente às células sadias, produzindo poucos efeitos colaterais. A resistência ao ACV ocorre nas infecções com os vírus que não expressam (p. ex., vírus Epstein Barr) ou com mutantes deficientes na enzima. Desta forma, o ACV teve a primazia de ser o primeiro antiviral dotado de atividade seletiva, persistindo como referência mesmo após, aproximadamente, trinta anos do seu desenvolvimento. Possui o melhor índice terapêutico (dose tóxica/dose efetiva) de todos os antivirais sendo praticamente desprovido de toxicidade (SCHAECHTER et al., 2002; KUCHENBECKER, 2006; ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008).

O penciclovir (9-(4-hidroxi-3-hidroximetil-1-butil) guanina, Penvir®), assim como o aciclovir, é altamente seletivo contra o HSV e, juntamente com as respectivas pró-drogas, famciclovir ([2- (acetil-oxi-metil) -4- (2-aminopurin-9-il) butil] acetato) e valaciclovir (2- [(2-amino-6-oxo-3H-purin-9-il) metoxi] etil (2S)-2-amino-3-metilbutanoato), são os fármacos mais amplamente utilizados para o tratamento destas infecções. Visto que a absorção do aciclovir e penciclovir é extremamente pobre por via oral, o uso destas pró-drogas possibilita uma melhor eficácia terapêutica quando é necessária a utilização desta via de administração, sendo as duas primeiras mais eficazes por via tópica e endovenosa (HODGE, 1993; ROIZMAN; KNIPE; WHITLEY, 2007).

O fosfonoformato (Foscarnet®) é o sal sódico do ácido fosfonofórmico análogo anômalo do pirofosfato e inibe a DNA polimerase viral ao ligar-se diretamente ao sítio de ligação do pirofosfato. É ativo contra o HSV ou citomegalovírus (CMV) com mutações na timidino-quinase ou U_L97, respectivamente. Resistência ao fosfonoformato é rara, mas pode ocorrer como resultado de mutações na DNA polimerase viral (RANG et al., 2004; ROIZMAN; KNIPE; WHITLEY, 2007; SANTOS; ROMANOS; WIGG, 2008; WILSON; FAKIOGLU; HEROLD, 2009).

3.3 PESQUISA E DESENVOLVIMENTO DE NOVAS DROGAS

Ao longo dos séculos, os medicamentos à base de plantas correspondiam à única forma de tratamento de doenças em muitas civilizações. As plantas e os produtos naturais, portanto, têm sido importante base da medicina tradicional e fonte de informação para o desenvolvimento de medicamentos contemporâneos. Dentre

os vários objetivos que têm sido propostos no desenvolvimento de novos fármacos, um deles é a investigação de drogas cujos mecanismos de ação minimizem a seleção de agentes resistentes. Entretanto, esta abordagem é dificultada pela emergência de doenças, fruto de atividade humanas desordenadas e não sustentáveis, além, da extinção de potenciais espécies naturais (CHATTOPADHYAY et al, 2010).

Há o conhecimento prévio de que os metabólitos secundários de plantas são particulares de cada espécie e, em várias situações, são sintetizados principalmente para a defesa contra predadores. Estes produtos podem ser potenciais candidatos a serem utilizados em pesquisas de medicamentos. Isso nos faz pensar que quão maior for a gama de espécies de plantas exploradas, maior será a chance encontrar substâncias eficazes, visto a grande complexidade de etapas necessárias até a inserção de um novo medicamento no mercado (CHATTOPADHYAY et al, 2010).

Produtos naturais derivados de plantas já deram origem à cerca de 30-40% das drogas antimicrobianas ou antineoplásicas. Porém, admite-se que estas fontes naturais apresentam uma potencialidade inesgotável para o desenvolvimento de novos medicamentos. Nos últimos 20 anos, numerosos programas de triagem baseados em ampla fundamentação foram desenvolvidos, em diferentes partes do mundo, para a análise de atividade antiviral *in vitro* e *in vivo*. Grandes esforços estão concentrados em suprir as necessidades da humanidade, que vive exposta a uma gama de doenças, muitas delas sem qualquer opção profilática ou terapêutica (CHATTOPADHYAY et al, 2009; WANG et al, 2014).

3.3.1 *Enteromorpha compressa*

As algas verdes vêm sendo amplamente exploradas para obtenção de compostos naturais bioativos há aproximadamente 20 anos, principalmente devido a sua ampla distribuição e grande biomassa. Elas são cultivadas para consumo alimentar e também muito apreciadas pelo seu alto valor nutricional e benefícios para a saúde (WANG et al, 2014).

O interesse na utilização de algas verdes tem aumentado recentemente devido à grande quantidade de ingredientes ativos, especialmente aqueles que podem ser utilizados para fins médicos, como frações lipídicas, proteínas, peptídeos, polissacarídeos, carotenoides e compostos fenólicos, alcalóides, mucilaginosos.

Entre estes compostos, os mais intensivamente investigados para fins terapêuticos são os polissacarídeos, os quais se apresentam com elevado peso molecular, com sua atividade biológica determinada pela sua estrutura química, incluindo o grau de sulfatação, a composição de açúcar, conformação espacial e estereoquímica (RAY, 2006; HARDEN et al., 2009; WANG et al, 2014).

A *E. compressa* é uma delas, marinha, comestível, pertencente à família *Ulvaceae,* que produz uma variedade impressionante de compostos bioativos, os quais já provaram ser úteis como antioxidante, antialérgico e anti-inflamatório (RAMAN; RAO; RADHAKRISHNAN, 2004; CHO et al, 2010; SHANAB; SHALABY; EL-FAYOUMY, 2011).

Nas pesquisas de novos antivirais, os polissacarídeos sulfatados e outras substâncias obtidas a partir de algas também têm despertado bastante interesse (ZHU et al., 2006; BANDYOPADHYAY et al., 2011).

Diversos trabalhos têm relatado a atividade antiviral de substâncias extraídas de várias espécies de algas marinhas, contra diversos vírus animais, apresentando compostos com grande eficácia e baixa toxicidade (WITVROUW; DE CLERCQ, 1997; HUHEIHEL et al., 2002; MORI et al., 2005; PEREIRA et al., 2004; ZHU et al., 2004, 2006; IWASHIMA et al., 2005; MATSUHIRO et al., 2005; RODRÍGUEZ et al., 2005; TALARICO et al., 2005; LEE et al., 2006; TALARICO; DAMONTE, 2007; CASSOLATO et al., 2008; HIDARI et al., 2008; WANG et al., 2008; HARDEN et al., 2009; MAYER et al., 2009). Estas substâncias têm mostrado interagir com muitos alvos virais, variando desde a adsorção do vírus à célula hospedeira até a liberação das novas partículas virais para o meio extracelular. A exploração destas substâncias pode resultar em novos fármacos antivirais promissores, com mecanismos de ação complementares às drogas já existentes, o que muito contribuiria para o tratamento das doenças virais (VLIETINCK; ERGHE, 1991).

Figura 1 – *Enteromorpha compressa* (popularmente conhecida como “alface do mar”)

3.3.2 *Morinda citrifolia*

Morinda citrifolia, popularmente conhecida como noni, é uma árvore pequena com folhas brilhantes e formato elíptico. Seu fruto tem aparência distinta com formato ovoide de cores verde, amarela ou branca, de acordo com o estágio de maturação. Figura entre as plantas medicinais originariamente da Polinésia e tem sido utilizado por mais de 2000 anos (WANG et al, 2002; GUPTA; PATEL, 2013).

Segundo a tradição local, o suco do fruto tem sido muito utilizado na medicina popular na artrite, diabetes, hipertensão arterial, dores musculares, inflamações, problemas menstruais, dor de cabeça, doenças cardíacas, AIDS, úlcera gástrica, entorses, depressão, senilidade, má digestão, arteriosclerose, problemas dos vasos sanguíneos, toxicodependência e vários tipos de câncer. Atribui-se ao fruto também grande valor nutricional (WANG et al, 2002; GUPTA; PATEL, 2013).

Compostos como escopoletina, ácido octanóico, potássio, vitamina C, terpenóides, alcalóides, antraquinonas, ácido linoleico, alizarina, aminoácidos, caroteno, vitamina A, flavonas, entre outros, têm sido identificados nas folhas, raízes e frutos, em elevadas concentrações (GUPTA; PATEL, 2013).

As atividades anti-inflamatória, antibiótica, analgésica, antioxidante e imunomoduladora são corroboradas em trabalhos científicos (HIRAZUMI; FURUSAWA, 1994; WANG et al, 2002; AKIHISA et al, 2007; MA et al, 2013; GUPTA; PATEL, 2013). A atividade antiviral de substâncias obtidas da *M. citrifolia* já foi relatada para o HIV-1 e para o vírus da Hepatite C (SELVAM et al, 2009; RATNOGLIK et al, 2014)

Os atributos conferidos a alga *E. compressa* e a planta *M. citrifolia* os revelam como promissores ferramentas na exploração de princípios ativos com as mais variadas atividades, úteis para o desenvolvimento de novos medicamentos.

Figura 2 – *Morinda citrifolia* (“noni”)

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4 ARTIGO 1

Green seaweed *Enteromorpha compressa* (*Chlorophyta, Ulvaceae*) derived sulphated polysaccharides inhibit herpes simplex virus

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Abstract

The herpes simplex virus (HSV) diseases represent a relevant medical and social problem due to their communicability and recurrence following latency. The green algae are rich source of polysaccharides referred to as ulvans, reported as being biologically and pharmacologically active. In this work, we analyzed the activity of seven chemically modified polysaccharides from *Enteromorpha compressa* (*Chlorophyta, Ulvaceae*), against HSV. Only the derivative named SU1F1 showed satisfactory viral inhibition activity, with a high selectivity index, and, therefore, it was submitted to analysis of the probable mechanism of action and structure. SU1F1 is a sulphated (22% w/w) heteroglycuronan with an apparent molecular mass of 34 kDa. The antiviral activity was assayed by plaque reduction assay under the protocols of the time-of-addition (from 3h before infection to 16h after infection), the inhibition of virus adsorption and penetration, and the virucidal effects. SU1F1 showed a high viral activity at the time 0h. We demonstrated that its inhibitory effect was maintained until 4h post-treatment with 100% of viral inhibition at 100 µg/ml. No effect was observed in additional protocols (the pre-treatment, the inhibition of adsorption and penetration and virucidal assays). Reverse Transcriptase associated PCR (RT-PCR)

results were in accordance with plaque reduction assay and demonstrated the activity of SU1F1 at the initial stages of HSV replication.

Keywords: Antiviral; *Enteromorpha compressa* polysaccharides; Herpes simplex vírus

1. Introduction

Herpes simplex virus (HSV) is a neurotropic virus (family *Herpesviridae* and genus *Simplexvirus*) represented by two serotypes. In HSV oral or genital infection, the virus replicates in epithelial cells, travels by axonal transport to either to trigeminal or dorsal root ganglia where latency is established. In recurrent infections, virion returns by axonal retrograde transport to the primary sites where replication takes place [1, 2]. Immunocompromised generally experiences more severe and frequent recurrent illness and even fatal disease. Primary infection in newborn can be associated with meningitis, encephalitis and eye infection or to a severe disseminated disease [2]. Meningitis and encephalitis can account for 70% mortality in the absence of treatment [3-7]. However, long-term acyclovir (ACV) treatment may lead to the selection of HSV resistant being an additional concern for the immunocompromised. This scenery makes the search for new antiherpetic drugs relevant, apart from, a challenging task that the control of the disease represents. Antiviral agents of natural origin are thought to have easy acceptability because of their low toxicity and low price [8-10], besides, what has been merging from the preliminary experiments, their multi-step mechanism of action. Seaweeds are important source of biologically active natural substances, mainly large amounts of sulphated polysaccharides [11, 12]. The green alga *E. compressa* (*Ulvaceae*) are a rich source of polysaccharides referred to as ulvans [13]. These high molecular weight compounds have several biological and pharmacological activities, determined by their chemical structure, including the degree of sulfation, molecular weight, sugar constituent, spatial conformation and dynamic stereochemistry [14, 15]. Several studies have reported the antiviral activity of algae derivative compounds against animal virus with high efficacy and low toxicity [8, 11, 15-30]. So far, semisynthetic ulvan sulphates have not been studied, although structural patterns like, for example, the presence of sulphate groups can also be found in chemically derived products. Therefore, in this work we studied the activity of chemically modified polysaccharides from *E. compressa* against HSV in cell

culture. This is the first time the activity of a chemically modified polysaccharide of *E. compressa* is reported against HSV-1 replication.

2. Materials and Methods

2.1 General Procedures

The chemicals used were of analytical grade or the best available. Evaporations were carried out under reduced pressure <50 °C (N-1100 Rotary Evaporator; Eyela, Tokyo, Japan) and concentrated solutions were freeze-dried (Cool Safe 55-F freeze drier; Scanvac, Lynge, Denmark). Dialysis against distilled water was performed with continuous stirring, and toluene was added to inhibit microbial growth. Total sugar and uronic acids were determined by the phenol−sulfuric acid [31] using galactose and m-hydroxydiphenyl [32] assay using glucuronic acid as standard, respectively. Neutral sugars were analysed after hydrolysis with 1M H_2SO_4 (3 hours, 100 °C). The released monosaccharide residues were reduced ($NaBH_4$), acetylated (Ac_2O) and analyzed as their alditol acetate by GC and GC–MS [33]. GC was carried out with a Shimadzu GC-17A chromatograph (Shimadzu, Kyoto, Japan), fitted with a flame ionization detector, and a DB-225 column (30 m × 0.53 mm i.d.) and helium as gas vector. GC-MS was performed with a Shimadzu QP 5050A GCMS instrument (Shimadzu) at 70 eV. Conditions for GC-MS were as described previously [34]. UV−vis spectra were recorded on a UV-2450 spectrophotometer (Shimadzu, Japan). Infra-red (IR) spectra were recorded on a Fourier transform (FT) spectrophotometer (Spectrum RX1; PerkinElmer, CITY, ST, Singapore) using KBr discs containing finely powdered samples.

2.2 Source of materials

Samples (130 g) of *E. compressa*, collected from the Okha coast of Gujarat, India, were converted into the depigmented algal powder (DAP; 95 g) as described [13].

2.3 Generation of sulphated polysaccharides

Synchronized isolation and chemical sulfation of polysaccharides from the depigmented algal powder (DAP) of *E. compressa* was carried out using a one-step strategy [10]. Dry algal powder (2 g) was suspended in 30 mL of anhydrous N,N-dimethylformamide (DMF) and the suspension heated at 90 $^{\circ}$C for 8 hours (h) under vacuum. After cooling with freezing bath, the suspension was mixed with 30 ml of 2:1

oleum−DMF (v/v) under ice-cold conditions. The reaction mixture was maintained under inert atmosphere at 25−30 °C for 10 min. Following neutralization with 20% NaOH, the reaction mixtures were filtered, desalted using Sephadex G-10 column (2.6 cm × 90 cm; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Solutions eluted between K_{av} values 0 and 0.9 were collected and lyophilized to yield a chemically altered sulphated ulvan fraction, named SU1. Similarly, two other chemically modified fractions, namely SU2 and SU3 were generated by using 2 h and 6 h reaction periods, respectively. Fraction SU1 upon size exclusion chromatography (SEC) on Superdex 30 yielded two fractions: SU1F1 and SU1F2. Similarly, fraction SU2 upon SEC produced fractions: SU2F1 and SU2F2, whereas fractions SU3F1 and SU3F2 were obtained from SU3. The sulphated heteroglycan (F1) named as ulvan was isolated from the depigmented algal powder (DAP) of *E. compressa* as described [35].

2.4 Purification of chemically modified polysaccharides by SEC

SEC on Superdex 30 column (Amersham Biosciences AB, Uppsala, Sweden; 2.6 × 35 cm) was performed using 0.2 M NaOAc buffer (pH 5.0) as eluent at 20 ml/h. Fractions were collected using a Pharmacia Biotech RediFrac fraction collector and analyzed for total sugar content using the $PhOH–H_2SO_4$ reaction [31]. Elution of polysaccharides was expressed as a function of the partition coefficient K_{av} [K_{av}= {V_e – V_0}/{V_t – V_0}, where V_t and V_0 being were the total and void volume of the column determined using potassium hydrogen phthalate and dextran (500 kDa), respectively and V_e is the elution volume of the sample]. The column was calibrated with standard dextrans (500, 40, 10 and 1 kDa).

2.5 Sulphate estimation, desulfation and glycosidic linkage analysis

Estimation of sulphate by the modified barium chloride method was carried out as described [13]. The ulvan F1 was converted into its pyridinium salt and then desulphated by solvolysis using the method of Falshaw and Furneaux [36] as described [13]. The solution was dialyzed and lyophilized yielding the desulphated form F1D. Methylation was carried out by the method of Blakeney et al. [37]. In the methylation procedure, free hydroxyl groups in the carbohydrates were deprotonated (LiDMSO) and methylated (CH_3I), then the glycosidic linkages were hydrolyzed (2 M CF_3CO_2H, 3 h, 100 °C), and the partially methylated monosaccharides were reduced

($NaBD_4$), acetylated (Ac_2O) and analyzed by GC and GC-MS. The partially methylated alditol acetates (PMAAs) were identified by the measurement of relative retention times, methoxyl substitution pattern as obtained from GC-MS and glycosyl make-up of the nonmethylated polymers.

2.6 Cells and Virus

HEp-2 cells (human larynx epithelial cells carcinoma, ATCC CCL-23), used throughout, were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Invitrogen - Gibco, USA), 100 IU/ml penicillin (Novafarma Ind. Farm., BR), 100 µg/ml streptomycin (Gibco BRL, USA) and 2.5 µg/ml of amphotericin B (Meizler Biopharma S/A, BR). The clinical isolate HSV-1 was provided by the Departamento de Virologia (IMPPG/UFRJ, BR). The virus stock was titered by plaque assay and stored at –20 ºC in 10% glycerol.

2.7 Cytotoxicity Assay

The cytotoxicity of the compounds in HEp-2 cells was evaluated by MTT (dimethyl-thiazolyl-diphenyltetrazolium bromide) kit assay (Sigma Chem. Co., USA), according to manufacturer's specifications, and as in Faccin et al [38]. The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis, as the concentration of the substances capable of reducing the optical density of MTT product by 50% in comparison to control.

2.8 Antiviral Activity of polysaccharides

2.8.1 Plaque reduction assay (*PRA*): The antiviral activity of the seven polysaccharides was evaluated by plaque reduction assay according to Melo et al. [39]. Briefly, cell cultures grown at approximately 100% confluence in 24-well plates (TPP, Switzerland) were infected with HSV-1 (MOI of 1.0) and treated with varying concentrations of the compounds (200, 100, 50 and 25 µg/ml), simultaneously. Infected and treated cell cultures were overlaid with nutrient agarose (DMEM 2x/1.8% agarose [v/v]) containing 25 mM $MgCl_2$ and incubated for 40h at 37ºC. Cells were fixed with 10% formaldehyde PBS, pH 7.3, and stained with 0.5% crystal violet in 20% ethanol. The percent of viral inhibition (%VI) was calculated as: %VI = 1 – (PFU in treated cells/PFU in control cells)] x 100 [40], where, PFU is plaque forming unit. The 50% inhibitory concentration (IC_{50}) was determined, by regression analysis,

as the concentration of the substance capable of reducing the number of PFU in 50%. The selectivity index (SI) was expressed as the ratio of CC_{50}/IC_{50}. The activity was performed under the assays of the time-of-addition, the inhibition of adsorption assay, the inhibition of penetration and virucidal.

Time-of-addition assay: Varying concentrations of polysaccharide were added to cell cultures before (-3h), during (time 0h) and after (+1h, +2h, +4h, +8h and +16h) infection, followed by PRA, as in Faccin-Galhardi et al. [41].

Inhibition of adsorption assay: The cell cultures were pre-incubated at 4ºC for 1h and submitted to varying concentrations of polysaccharide simultaneously with viral infection. The cells were again incubated at 4ºC for 1h followed by PRA [42].

Inhibition of penetration assay: The cells were pre-incubated at 4ºC for 30 min, infected and maintained at 4ºC for further 90 min. The cultures were submitted to concentrations of polysaccharide and incubated at 37ºC for 10 min. The cells were washed with PBS (pH 3) for 1 min and immediately neutralized with PBS (pH 11) followed by PRA. This test performed as in Cheng et al. [43] with minor modifications.

Virucidal Assay: Virus dilutions were incubated at 37ºC for 1h with varying concentrations of polysaccharide (v/v), inoculated and followed by PRA [44].

Zynvir (sodium acyclovir - ACV) (Novafarma Ind. Farm., BR) was used as positive control for viral inhibitor.

2.8.2 Immunofluorescence assay (IFA): Briefly, cell cultures grown in 24-well plates with coverslips were infected either with HSV-1, in the presence of varying concentrations of polysaccharide. In the sequence, cells were collected, fixed and blocked with 2% powdered skim milk. Mouse anti-HSV-1 (Santa Cruz Biotechnol., USA) and goat anti-mouse IgG FITC conjugate (Sigma Chem. Co., USA) were used. UV-light microscope (Zeiss Axio Imager.A1) was used to score 100 cells/coverslips and the percentage of fluorescent cells inhibition calculated [38].

2.8.3 RT-PCR: The effect of polysaccharide in HSV-1 transcripts synthesis was performed as previously described [45, 46]. Viral transcripts of $2x10^6$ infected cells and treated with varying concentrations of the polysaccharide (50, 25, 12.5 and 6.25 µg/ml) were extracted with QIAamp® RNA Mini Kit at 4, 10 and 16 h after the infection to assay the effect in the three stages of virus transcription (immediate early, early and late genes). Appropriate controls were prepared. The reverse

transcription was performed initially with a final volume of 20 µl. A first reaction mixture consisting of 5 pmol of random primers (*Invitrogen**)**, 1 mM dNTP (*) and RNAse-free water up to a volume of 7 ml was prepared. Seven microliters of the first reaction was mixed with 5 µl of the extracted viral RNA and incubated for 5 min at 65ºC. Afterwards, a second reaction mix consisting of 1x M-MLV reaction buffer, 0.01 M DTT, 100 U M-MLV Reverse Transcriptase (*) and RNAse-free water up to a volume of 8 µl was prepared. Eight microliters of the second reaction mixture was added to each sample and sequentially incubated for 10 min at 25ºC, 50 min at 37ºC and 15 min at 70ºC. The PCR reaction was carried out separately for each transcript in a final volume of 30 µl containing 0.2 mM of each dNTP, 2 pmol of each primer, 2.5 U of *Taq* DNA polymerase with PCR buffer A (*) and cDNA. Specific primers for immediate early, early and late transcripts were 5' GGC GGG AAG TTG TGG ACT GG 3' and 5' CAG GTT GTT GCC GTT TAT TGC G 3' (ICP4), 5' CAT CGA AAC CCA CTT TCC CGA ACA 3' and 5' GCT GTC GCA TTT GGC GGC AA 3' (U_L52) and 5′ CGA TCG CCC GGG GGC AGT TT 3′ and 5′ ACG GGT TGG TGT GAC ACA GG 3′ (U_L13), resulting in a 138, 240 and 600 bp amplified product, respectively. The PCR program consisted of denaturation at 94ºC for 4 min, followed by 40 cycles of denaturation for 1 min at 94ºC, annealing at 60ºC for 1 min for ICP4, 55ºC for U_L52 and 60ºC for U_L13, and extension for 1 min at 72ºC. The final cycle of extension was 7 min at 72ºC. Ten-microliter aliquots of the PCR products were resolved in a 12% polyacrylamide gel.

2.9 Statistical analysis

Anova followed by Tukey's test (BioEstat 5.0 for Windows XP, 2007) were applied to determine the difference among experiments with the polysaccharides and control groups. The experiments were carried out in triplicate. Values of $p < 0.05$ were considered significant.

3. Results

3.1 Isolation and persulphation of ulvan

The marine alga *E. compressa* was used to obtain chemically altered ulvan sulphates with different sulphate content, sugar composition and molecular masses. The detailed structural characterization of the parental ulvan has previously been published [13]. Briefly, a ulvan (F1) with an apparent molecular mass of 55 kDa and

consisting of 49% rhamnose, 18% glucose, 13% xylose, 9% galactose and 14% glucuronic acid units has been reported [13]. This polymer, which contained 6% sulphate (w/w), had a branched structure. Herein, a one-step strategy was used to generate chemically altered sulphated polysaccharides from depigmented algal (*E. compressa)* powder (DAP) that contains a significant portion of naturally gifted ulvan scaffold. In particular, oleum−DMF reagent treatment of DAP for 10 min produced a brown colored transparent solution. Upon neutralization with aqueous 20% NaOH solution followed by desaltation and lyophilization, a sulphated ulvan containing fraction named as SU1 was obtained (11%, w/w). Notably, oleum−DMF reagent being polar in nature was competent for the extraction of polysaccharide as this class of biomacromolecule contains polar hydroxyl plus sulphate groups. However, the beauty of this method lies in the use of oleum−DMF as a "double agent" that efficiently extracts the polysaccharide and in unison decorates this polymer with sulphate groups. Similarly, using the same reagent, but with two other reaction periods fractions SU2 and SU3 were generated (Figure 1). Afterward, purified fractions SU1F1 and SU1F2 were isolated by SEC of SU1 fraction on Superdex 30 column (Figure 2). Based on calibration with standard dextran, the apparent molecular mass of SU1F1 was determined to be 34 kDa. The low molecular mass fraction SU1F2 has a molecular mass of <5 kDa. Similarly, using SEC on Superdex 30 four other fractions derived from SU2 and SU3 (Figure 1) were also obtained. SEC profile was characterized by single-peak elution, thus illustrating the homogeneity of SU1F1. This chemically altered fraction contained rhamnose, galactose, glucose, xylose and glucuronic acid residues in the molar ratios of 49, 9, 15, 13, and 14. Hence, the sugar composition of fraction SU1F1 is similar to that of parental ulvan [13]. It also contained sulphate (22% w/w). Indeed, the IR spectrum of this fraction showed a band at 1250 cm^{-1} related to the >S=O stretching of the sulphate group [47]. Another band at 810 cm^{-1} arising from primary sulphate group of polysaccharide [48] was also detected (Figure 3). Referring to this report, bands at approximately 810 cm^{-1} were tentatively ascribed to 6-*O*-sulphate groups of glucose and galactose residues as these residues have primary hydroxyl group. Structural changes were also demonstrated by the use of glycosidic linkage analysis, comparing the parental ulvan previously described as WE fraction [13], with the novel chemically modified ulvan sulphate SU1F1. Ulvan contains 1,4- and 1,2,4-linked rhamnose 3-sulphate, 1,4-linked glucose, 1,3- and 1,6- linked galactose, 1,4- and

terminally linked glucuronic acid and 1,4-linked xylose partially sulphated on *O*-2 [13], whereas the chemically modified product is highly sulphated in accord with the presence of various non-, mono- and di-*O*-methylated derivatives (Table 1).

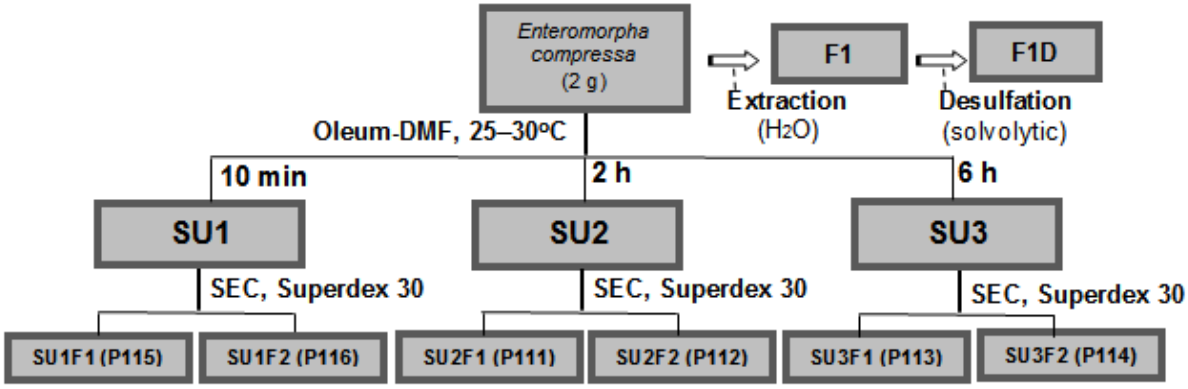

Figure 1 - Isolation of chemically modified polysaccharides (F1, SU1F1, SU1F2, SU2F1, SU2F2, SU3F1 and SU3F2) from *Enteromorpha compressa*.

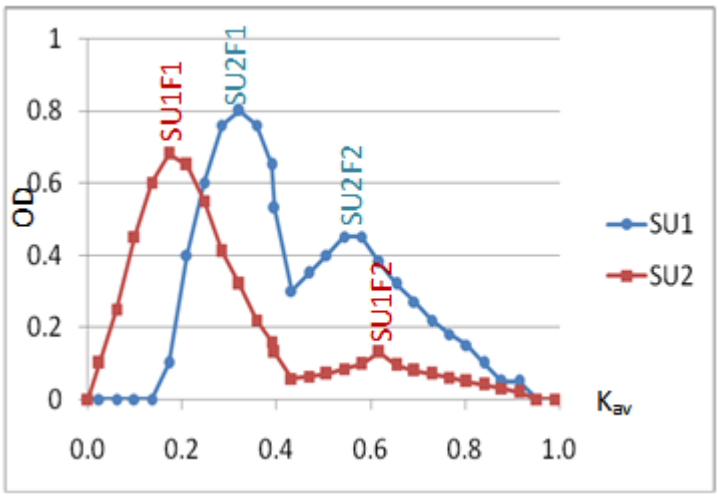

Figure 2 - Elution profiles of SU1 and SU2 from a Superdex 30 column equilibrated with 0.2 M NaOAc buffer (pH 5.0). The column was eluted with this buffer at 20 ml/h at 30−35 °C. Elution of polysaccharides was expressed as a function of the partition coefficient K_{av} [K_{av}= {V_e – V_0} / {V_t – V_0}, where V_t and V_0 being were the total and void volume of the column determined using potassium hydrogen phthalate and dextran (500 kDa), respectively and V_e is the elution volume of the sample]. The column was calibrated with standard dextrans (500, 40, 10 and 1 kDa).

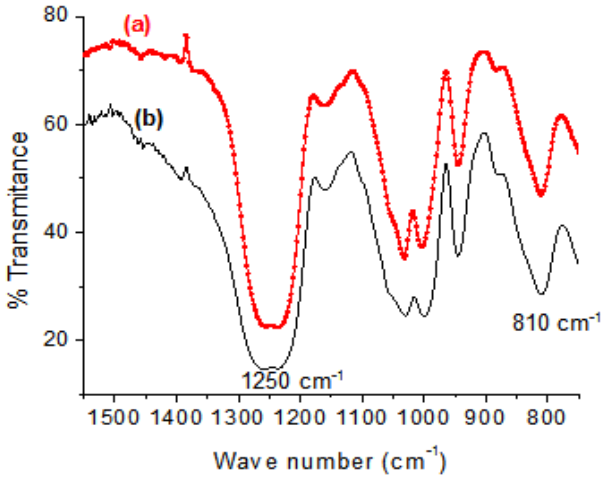

Figure 3 - FT-IR spectra of ulvan sulfates (a) SU3F1 and (b) SU1F1 chemically modified from *Enteromorpha compressa*. The presence of sulfate groups is indicated for all spectra (1250 cm^{-1}).

Partially methylated alditol acetates[a]	Deduced linkages	m/z values	SU1F1
3,4-Me_2-Rha	1,2-	43, 130, 131, 174, 175, 190, 234	Trace
2,3-Me_2-Rha	1,4-	118, 143, 163, 203	2
2,4-Me_2-Rha	1,3-	118, 131, 173, 174, 234	2
2-Me-Rha	1,3,4-	118, 215	29
3-Me-Rha	1,2,4-	130,143,190,203	Trace
Rha	1,2,3,4-	115, 129, 157, 171, 188, 231	32
2,4-Me_2-Gal	1,3,6-	43,118,174,189,234	1
2,3-Me_2-Gal	1,4,6-	118, 162, 201, 261	4
2,3-Me_2-Glc	1,4,6-	118, 162, 201, 261	12
2,3-Me_2-Xyl	1,4-	102, 118, 129, 189	1
3-Me-Xyl	1,2,4-	87, 88, 129, 130, 189, 190	5
Xyl	1,2,3,4-	115,116, 128, 145, 146, 217, 218	12

[a] 3,4-Me-Rha = 3,4-di-*O*-methyl-1,2,5-tri-*O*-acetyl rhamnitol etc.

Table 1 - Partially *O*-methylalditol acetates derived from the chemically modified sulfated polysaccharide (SU1F1) of *Enteromorpha compressa*.

3.2 Antiviral activity

Table 2 shows the IC_{50} and the SI values of polysaccharides derived from *E. compressa,* as well as, their CC_{50}. Despite the low toxicity presented by polysaccharides, only SU1F1 showed relevant antiviral activity, with a low IC_{50} (28.25 µg/ml) and with the highest SI, therefore, the only subjected to further testing. SU1F1 showed great antiviral activity when added concomitantly with viral infection (time 0h), with the following %VI, 100% for 100 and 200 µg/ml, 75.6% for 50 µg/ml and 38% for 25 µg/ml. For the times 1h, 2 h, 4h and 8h after infection, the %VI was maintained at 100% for the two highest tested concentrations (100 µg/ml and 200 µg/ml). For 50 µg/ml and 25 µg/ml, a significant viral inhibition was detected by the addition of SU1F1 1h and 4h after infection ($p > 0.05$), however, a decrease in the inhibition was observed when added 8h and 16h after infection. The pre-treatment showed no inhibition even when SU1F1 was added 3h before the infection, at concentrations eight times greater than that of the IC_{50} ($p < 0.05$). The preincubation of virus suspensions with SU1F1 showed an inactivating effect around of 15% at the concentrations of 100 µg/ml and 200 µg/ml. Along with the inhibition of adsorption, the inhibition of penetration was also investigated. However, no effect was observed in both assays ($p < 0.05$) at all tested concentrations. Figure 4 shows the SU1F1 profile of viral inhibition under all tested protocols.

By IFA we observed that HSV-1 antigen expression was repressed by SU1F1 at 12.5, 25 and 50 µg/ml with inhibition of 0%, 25% and 49%, respectively.

The effect of SU1F1 in HSV-1 transcripts synthesis could be detected for the immediate early gene even at the lowest concentrations of 6.25 µg/ml (Figure 5).

Table 2 - The 50% inhibitory concentration (IC_{50}) of chemically engineered polysaccharides from *Enteromorpha compressa* and acyclovir (ACV) for HSV-1 in HEp-2 cells, by plaque assay. The 50% cytotoxic concentrations (CC_{50}) and the selectivity index (SI) are also shown.

	CC_{50} (µg/ml)	IC_{50} (µg/ml)	SI
F1	1000	153	6.5
SU1F1	1000	28.2	35.3
SU1F2	1000	200	5
SU2F1	495	160	3.1
SU2F2	1000	274	3.6
SU3F1	845	186	4.5
SU3F2	1000	230	4.3
ACV	2500	2100	1.21

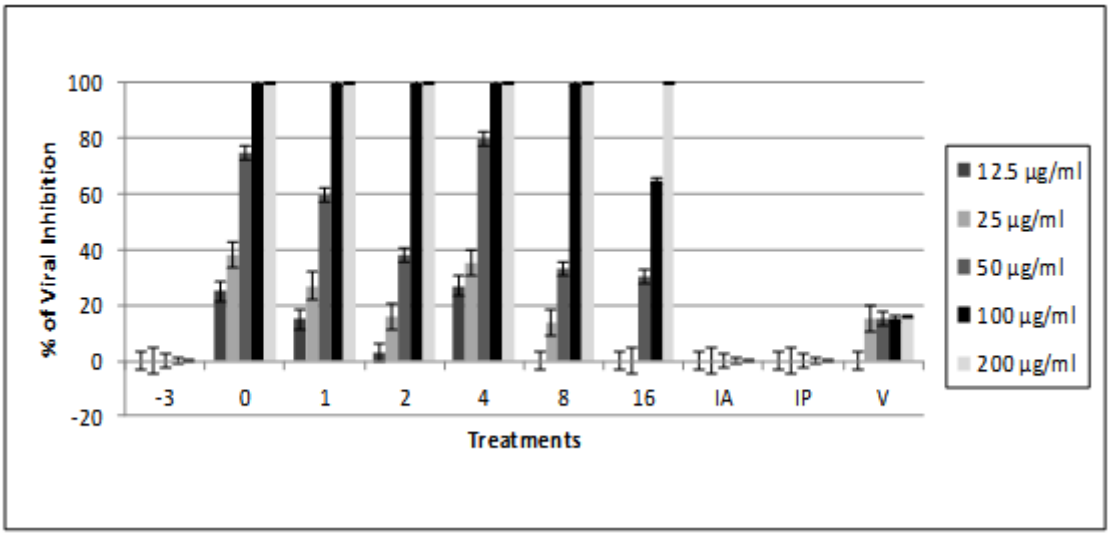

Figure 4 - The percent of HSV-1 inhibition by SU1F1 under the protocols: (a) Time-of-addition (−3 to +16); (b) Inhibition of adsorption (IA); (c) Inhibition of penetration (IP) and (d) Virucidal (V) in HEp-2 cell cultures monitored by plaque reduction assay, at the indicated concentrations. The percentage of viral inhibition (%VI) was determined in comparison to controls and the results are expressed as mean ± SD of triplicate independent experiments.

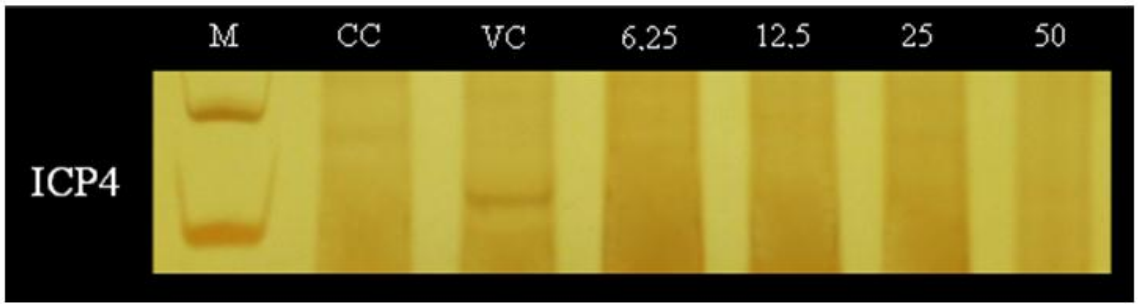

Figure 5 - The inhibition of HSV-1 transcript synthesis for the immediate early gene by SU1F1 at the indicated concentrations (µg/ml). Polyacrilamide gel electrophoresis of RT-PCR amplicon for HSV-1 ICP4 (138 bp). MW std. (M) and controls for virus (VC) and cell (CC).

4. Discussion

A wide range of compounds, such as, terpenes, polyphenols and steroids have been reported in various marine green algae [49, 50]. However, among all macroalgae, the green algae have less than 300 known compounds and are the least producers of natural compounds when compared to the red (Rhodophyta) and brown algae (Phaeophyta) [51, 52]. Many of these compounds have demonstrated biological activities, including the antiviral [53]. The capacity of sulfated polysaccharides obtained from green seaweeds to inhibit the replication of human and animal viruses has been reported many times. Several works suggest that one of the mechanisms of action is the electrostatic interference with viral attachment to the cell surfaces. However, more recent reports have increased the interest around the antiviral effects of the algal polysaccharides, which have a number of mechanisms of action that occur before and after virus penetration into the cell, involving specific structural features of the polysaccharides [8, 29] Our work isolated and evaluated the anti-herpetic activity of seven polysaccharides from green algae *E. compressa*.

Ray [54] identified several types of polysaccharides in *E. compressa* extract, including a sulphated heteroglycan similar to ulvan of *Ulva rigida*. The ulvan is a major sulphated polysaccharide found in the cell wall of green algae, composed mainly of rhamnose, glucuronic and iduronic acid, xylose, glucose, galactose and sulphate. Ulvan has been extensively investigated for development of novel drugs and functional foods [55-59]. A one-pot procedure has been utilized to produce chemically modified sulphated polysaccharides from *E. compressa* containing ulvan. This approach relies on the polar nature of oleum−DMF reagent and its ability to alter the structural elements of polysaccharides (ulvans) present in depigmented algal

powder. In particular, the directive decoration of hydroxyl functionality with bioactive sulphate group together with changes in the molecular mass during isolation by the used reagent yielded compounds with modified biological activity. Amongst the compounds generated from *E. compressa* in our study, only SU1F1 showed significant anti-HSV-1 activity, with the highest selectivity index. Remarkably, the antiviral activity of F1 significantly increased after chemical derivatization (SU1F1). In spite of SU1F1 fraction have a sugar composition similar to parental polysaccharide ulvan (F1), some factors, as the molecular mass may influence the antiviral activity. When various fractions of semi-synthetic glucan sulphates with molecular mass ranging from 1 to 500 kDa were examined for their antiviral activity, it was found that the higher molecular mass fractions were more effective than the lower ones [16]. Therefore, in many cases, the higher the molecular mass, the higher is the antiviral activity. We observed that the first fractions isolated from each reaction time (SU1F1), had IC_{50} less than the second purified fractions (SU1F2). For SU1F2, the largest detected IC_{50} value (200 µg/ml), compared to SU1F1 may be related to low molecular mass (<5 KDa). However, when we compare SU1F1 e F1, although the first has smaller molecular mass (34 KDa), the sulphate content is high, which ensures the strong antiviral activity. Thus, the higher anti-herpetic effect of SU1F1 can also be explained by the high degree of sulphation (DS) present in this molecule (22%) compared to F1 (6%), because the higher the DS, the better its antiviral potency [16]. The influence of sulphate groups for anti-herpetic activity was showed for several families of polysaccharides such as spirulan, agaran, fucan, xylomannan, and their desulphated and further sulphated derivatives [13, 35, 60-63]. Based on these results, further experiments were only performed with the SU1F1.

Many sulphated polysaccharides have a potent anti-HSV-1 activity when added simultaneously to viral infection (time 0h) by the virucidal effect or suppression viral attachment and penetration [8]. This could occur by the ability of these compounds to interfere electrostatically with the positively charged region of viral glycoprotein and the negatively charged HS chains of the cell receptor. SU1F1 fraction demonstrated weak virucidal effect even at high concentrations. The best SU1F1 activity was demonstrated after virus penetration. Similar results were found by Mendes et al [64] with macerated extract of *Ulva fasciata* against human metapneumovirus and by Lee et al [65] with two sulphated polysaccharides isolated from *Caulerpa brachypus* and *Codium latum* that showed strong anti-HSV-1 activities when treated 8 h post-

infection (p.i.). These experiments demonstrate that some sulphated polysaccharides from green seaweeds interfere with later steps of virus replication in accordance with our study. According to Su et al. [46], the transcription of HSV immediate-early (IE) mRNAs begin immediately after viral penetration and are detected at 1 – 2 h p.i. Subsequently, the transcription of early (E) mRNAs are detected at 2 – 3 h p.i., and, the later (L) mRNAs are synthesized with increasing rates until at least 12 h p.i. The synthesis of viral DNA begins after the appearance of E proteins and are detected in the first 3 h p.i., with peak at 7 - 10 h p.i. [66, 67]. Thus, the antiviral effect of SU1F1 until 8 h p.i. at high concentrations, suggests the inhibition of DNA replication and transcription with downregulation of HSV protein synthesis.

In order to confirm these hypothesis, the IF and RT-PCR were performed. The dose-dependence inhibition, found in these experiments occurred in both expression and protein synthesis. It has been also shown that sulphated polysaccharides extracted from *Eucheuma gelatinae* [68], *Caesalpinia ferrea* [69] and *Agaricus brasiliensis* mycelia [70] presented a broad inhibitory activity in the syntheses of viral protein and nucleic acid. However, the specific mechanism that SU1F1 exerts its antiviral effect needs to be further investigated. Some polysaccharides are able to bind to cell-surface receptors and induce intracellular signaling pathways. The replication efficiency of viruses is dependent on specific intracellular signaling. By the modulation of a particular signal by polysaccharides binding to the cell surface receptor one could expect the triggering of the antiviral activity. Interferon could not be ruled out as one of the possibilities [8]. The anti-cytomegalovirus effect of spirulan-like polysaccharides probably occurs by this mechanism [71]. Polysaccharides rich in glucan–protein complex, like α- and β-glucans have shown antiviral effect against several virus [72]. These polysaccharides decrease viral nucleic acid levels and stimulate the immune system by binding to Toll-like receptors and dectins and causes the induction of various cytokines, among them interferon.

In conclusion, we demonstrated that the SU1F1 fraction presents a potent antiviral activity, inhibiting HSV replication with a broad mechanism of action. Therefore, SU1F1 can be a promising substance for pre-clinical study in the drug development for the control of HSV related diseases.

Acknowledgements

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Conflicts of interest

The authors declare no competing financial interest.

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5 ARTIGO 2

The pectin from *Morinda Citrifolia*: characterization, the *in vivo* and *in vitro* toxicity evaluation and antiviral activity

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Abstract

Pectin is extracted from the lyophilized pulp from Noni (Morinda Citrifolia) in three different pH values (3, 7 and 10) using procedure to different extractors agents, the best performance sample was subjected to sulfation process was performed the following characterization by FT-IR, ^{1}H NMR, AFM and Elemental Analysis. Values FT-IR and ^{1} H NMR to calculate the degree of esterification (degree of methylation) were used. The PDN-a sample was subjected to toxicological testing in vivo (rats) and the PDN-s sample was determined their cytotoxicity in vitro (human neutrophils). The pectin was also evaluated for the *in vitro* anti-herpes simplex virus (HSV) activity, but only PDN-a successfully inhibited virus replication.

Keywords: Pectin, Sulfonation, Characterization, Toxicity, Cytotoxicity, HSV.

1 Introduction

Many plants are known to have biological potential as antiviral (Mukhtar et al, 2008), antibacterial (Ikeda et al, 2007) and also in the replication of HIV (Notka et al, 2004) infection process. Among these plants can highlight the Morinda citrifolia Linn, known as Noni, which originates in the southern Pacific islands.

According to studies, Chan-Blanco et al (2006), on their chemical composition, Noni consists of 90% water and the main components of the soluble solids are dry matter, fiber, protein and carbohydrates. Table 1 shows some of the bioactive assignments constituted morphological parts of the Noni.

The phyto-therapy treatment has only a few not desirable side effects when compared with the corresponding synthetic drugs treatment (Turolla; Nascimento, 2006), and with that in mind, the research of Morinda citrifolia in countries where the plantation and consumption of the Noni are addressed to its bioactive properties, namely: antibacterial, natural antiseptic, metabolic regulator, cellular regenerator, antioxidant, antiviral, antifungal, hypotension and immuno-stimulant (Wang et al, 2002); anticancer activities (Furusawa, 2003), its effect over the immunologic system (Palu et al, 2008), its action on hepatic protection (Wang et al, 2008), its antitumor potential (Furusawa et al, 2003), its activities on gastrokinetics (Nima et al, 2012), and its activities on anti-helminthic animals (Brito et al, 2009).

The Noni and all its biological properties from the active molecules in this plant has few research studies that are directed to the pectin of this fruit. The pectin from other sources have being studied with the goal of finding the biological properties as observed by Vladislav et al (2009), when the anti-metastase on prostate cancer treatment in rats was verified. Authors like Yan and Katz (2010) and Jackson et al (2007), have also verified the use of pectin in the treatment to prevent the metastasis of cancer in different organs (Nangia-Makker et al, 2002; Liu et al, 2008).

Another way to use the pectin from Noni is by modifying its chemical composition by sulfonation to improve its bioactivity, studies were done by Maas et al (2012), showing that a sulfacted polysaccharide has good effect on blood clothing, being anti-thrombotic. The substitution of carboxylic groups of the pectin by esters of sulfate, increases the pharmaceutical potential compared with the non-sulfactedpectins. In recent studies to confirm the improvement of sufacted pectin,

(Bae et al, 2009; Cipriani et al, 2009; Fan et al, 2011) verified the blood anti-clothing behavior, same property found the by Vityazev et al (2010), associated with the effect on the fibrinogen inhibition.

Table 1 – Some chemical constituents of the Noni plant.

Plantpart	Compound	Reference	Aplication[a]	Reference[b]
Root	Damnacanthal	Morton J.F. (1992)	Anti-microbial; Anti- HIV	Ali, A.M. *et al,.* (2000)
	Morindone	Sang, S. *et al.,* (2002)	Anti-microbial	Ali, A.M. *et al.,* (2000)
	Rubiadine	Cardon, D. (2003)	Atividadehepatoprotetora	Guntupalli M. Mohana Rao. *et al.,* (2006)
	Pentose andHexose	Dittmar, A. (1993)	Bioetanolproduction	Vera Novy*et al.,* (2013)
Seed	Ricinoleicacid	N. Solomon. (1999)	Anti-tickmite	André Arnosti *et al.,* (2011)
	Caproicacid	Dittmar, A. (1993)	Genes vector delivery (DNA)	BuddhadevLayek& Jagdish Singh (2013)
fruit	Ascorbicacid	Liu, G. *et al*., (2001)	Improve the antifungaland antioxidant effect	Omar Arafat Kdudsi Khalil. *et al.,* (2012)
	Scopoletine	Farine, J. P. *et al*., (1996)	Antitumoral	Wukun Liu. *et al.,* (2012)
	Caprílicoacid	Sang, S. *et al*., (2002)	Chemical hygienics	Su-sen Chang. *et al.,* (2010)
	Methionine	Dittmar, A. (1993)	Immuno nutriente effect	PanagiotisSakkas. *et al.,* (2013)
leaves	Glutamicacid	Dittmar, A. (1993)	Hipotensor effect	Stamler, J. *et al.,* (2009)
	Cisteine	Dittmar, A. (1993)	Cardioprotetoreffect	Chunhua Liu. *et al.,* (2011)
	β-sitosterol	Sang, S. *et al*., (2002)	cholesterol reduction effect	Cicero, Arrigo F.G. *et al.,* (2002)

[a]Effect of chemical compounds form other sources.

[b]Studies of chemical compounds from other sources.

2 Experimental

2.1 Extraction of polysaccharide

The citric pectin (PC) was obtained from Sigma-Aldrich (St. Louis, MO, EUA). All the other reagents were of analytic grade. The dry fruit pulp of *Morinda citrifolia Linn* was obtained by the dehydration of the ripe fruit, collected on the geographic coordinates of S 3.83111º W 38.55090°, in Fortaleza city, of the Ceará state, on the north east side of Brazil.

The extraction was accomplished following the method of Koubala et al (2008). Three portions of the dry pulp of Noni (20.0 g) were dispersed in 900 mL of the extractor agent that could be in each method: a solution of ammonia oxalate, pH 3.0; or distilled water, pH 7.0;or a solution of sodium hydroxide pH 10.0, respectively for the extraction in acid, neutral or basic medium. The suspension was kept under stirring for one hour and the temperature of 80 ^{o}C. The solution containing the extract was filtrated and then centrifuged at 800 rpm for 10 minutes. After concentration by evaporation under lower pressure to one third of the initial volume, the pH was adjusted to 7.0 with a solution of sodium hydroxide when using the acid extraction. Oxalic acid was used to neutralize when the basic extraction method was used. The polysaccharide was separated by precipitation after the addiction of ethanol (1:3 v/v) that cause the precipitation. After lyophilization the isolated material was named PDN-a, PDN-n and PDN-b (pH 3, pH 7 and pH 10, respectively).

2.2 Chemical modification by sulfonation (PDN-s)

The following experimental procedure is an adaptation by the method proposed by O'Neill (1995) in which 300.1 mg of pectin (pH=3) were added in a mixture of solvents composed by pyridine and N,N-dimethylformamide (50:10 v/v), under stirring at 25 ^{o}C by 12 hours. After this procedure, the mixture was placed in a close container at 4 ^{o}C where 4.0 mL of chlorossulfonic acid was added slowly under stirring in an ice bath. With the end of the acid addition, the solution was neutralized with a saturated solution of $NaHCO_3$. The product was dialyzed for 120 hours and lyophilized and tagged PDN-s.

2.3 Polysaccharide characterization

2.3.1 FT-IR analyses of (PDN-a), (PC) and (PDN-s)

The infrared analysis for the pectin (PC) sample, purchased from Sigma-Aldrich and the pectin from the acid extraction (PDN-a) and the sulfacted pectin (PDN-s) were obtained from a Wastakenon – Perkin Elmer 16 PC spectrometer, using the KBr pellet method, scanning the range from 600 to 2000 cm^{-1}, 20 times.

2.3.2 Noni pectin (PDN-a) analysis by NMR ^{1}H and the determination of the methylation degree

The NMR ^{1}H were obtained in a Brucker Advance-DMX 500, with the pectin samples dissolved in D_2O in a concentration of 20 mg/mL with the sample left in D_2O for 24 hours before analysis. The analysis was performed at 80 ^{o}C in order to displace the water signal to approximately δ = 4.2 ppm where its position do not interfere with the pectin signals (Rosenbohm et al*,* 2003). The degree of methylation, was obtained by measuring the areas of signals from protons H-5 adjacent to the esters groups (ICOOMe) and compared with the areas from the signals from protons H-5 next to the carboxylate groups (ICOO-). The proximity of the signals from protons H-1 and H-5 (COOMe) (IH1 + ICOOMe). The degree of methylation by equation 3 resolves the amount of protons H-5 that is equivalent to the sum of the anomeric protons H-1:ICOOMe + ICOO- = 1H1. By the main of simple mathematics the degree of methylation (DM) can be found (Rosenbohm et al, 2003).

$$
\begin{aligned}
DM &= (ICOOMe)/\,(ICOOMe + ICOO^{-}) \\
&= 2(ICOOMe)/\,2(ICOOMe + ICOO^{-}) \\
&= [ICOOMe + ICOOMe + (ICOO^{-} - ICOO^{-})]/\,[ICOOMe + ICOOM \\
&\qquad + (ICOO^{-} + ICCO^{-})] \\
&= [(ICOOMe + IH1) - (ICOO^{-})]/\,[(ICOOMe + IH1) + (ICOO^{-})]
\end{aligned}
\qquad (1)
$$

2.3.3 Atomic force microscopy (AFM)

AFM was obtained in a NanoScope III A Veeco Instruments, with an intermittent contact mode using a silicon probe of 228 μm, resonance frequency of

200KHz and force constant of 5-8 N/m. Approximately 10µL of the PDN-a and PDN-s samples were separately and placed in a mica holder. The moisture excess was extracted by a flow of nitrogen and the scan was done in a speed of 1 Hz with the resolution of 512 x 512 pixels.

2.3.4 Elemental analysis

The PDN-a and PDN-s samples were analyzed to find the C, H, S and N contends in a microanalyzer Carlo ERBA EA 1108. The degree of sulfonation (DS) was calculated according to the equation 2 (Bae et al, 2009).

$$DS = [162 * (\%S \,/\, 32)] \,/\, \{100 - [(80 \,/\, 32) * (\%S)]\} \quad (2)$$

2.4 Animals and housing conditions

Twenty conventional female mice of Swiss strain, three weeks old, were obtained from the Central Animal Facilities of the Federal University of Ceará (Fortaleza, Brazil). The animals were housed at the Department of Biology, at the same University, with temperature (23.0 ± 2.0 °C), photoperiod (12 h of light/12 h of dark) and humidity (45-55%) controlled. The mice were kept in adequate numbers in polypropylene cages with pine shavings as substrate and water and feed (Biobase, Bio-Tec, São Paulo, Brazil) ad libitum until they reach the approximate weight of 20 g.

All protocols with animals adopted in this work were submitted to the Ethics Committee on Animal Research of the Federal University of Ceará, which follows the Law nº 11.794 of October 2008 on the use of animals in the scientific research in Brazil.

2.5 Isolation of human neutrophils

Human leucocyte-rich blood from healthy adults was obtained from HEMOCE (blood bank), Fortaleza, CE, Brazil.

2.6 Acute oral toxicity assay

An acute oral toxicity study (single dose) of Mo-CBP3 was performed following the protocol n° 423 of the Organization for Economic Cooperation and

Development (OECD, 2001). Female mice (n = 6 per group) of Swiss strain, 5 weeks old and weighing 18-22 g were used. The PDN-a was resuspended in distilled water and administered orally at doses of 5, 50 and 300 $mg.Kg^{-1}$ of body weight, thus accounting for 3 test groups. A control group received only distilled water. All mice were observed within the first hours after administration of the PDN-a and then twice daily for 14 days. The possible intervention of the PDN-a on the natural behavior of mice was observed, as well as evidences of toxicity from the verification of the following symptoms/characteristics: piloerection, anesthesia, motor activity, vocal tremor, touch response, balance, writhing, tremors, ptosis and misshapen or dark feces. The body weight (g) of all mice was recorded on days 0, 4, 7, 10 and 14. On day 14, the animals were slightly sedated with halothane (Zeneca, São Paulo, Brazil) and exsanguinated via retro-orbital sinus for determination of haematological and serum biochemical parameters. Then, the animals were sacrificed by cervical dislocation and dissected for observation (monitored by a pathologist) of the anatomical and morphological condition of 16 organs, and then carefully weighed to obtain the relative wet weight.

2.7 Hematology and serum biochemistry

A part of the blood collected at the end of the acute oral toxicity assay was kept in heparinized tubes for determination of hematological parameters and another part was collected to obtain serum for the determination of biochemical parameters. For hematological analysis an aliquot of 15 µL of blood of each animal was applied into a veterinary hematology analyzer (Sysmex, model 100iV Poch - Diff, Kobe, Japan). The analyzed parameters were: number of white blood cells (WBC), number of red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), red cell distribution width measured as coefficient of variation (RDW-CV) and red cell distribution width measured as standard deviation (RDW-SD).

Blood samples for serum biochemistry analyses were kept during 12 h, at 4 ºC, until complete coagulation. Then, the coagulated blood was centrifuged at 600 x *g*, for 10 min, and the serum was collected. The following biochemistry parameters were measured: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase, creatinine, urea, total protein, albumin and total cholesterol. All

analyses of blood serum were performed using specific kits (Labtest, Minas Gerais, Brazil) for each parameter, according to the manufacturer's instructions.

2.8 Relative organ weights

At the end of the acute toxicity experiment, the animals were carefully dissected and the organs/tissues were checked for their appearance (color, presence of stains and/or anatomical irregularities) by a histopathologist, and then weighed in a precision balance (0.0001g). The organs/tissues dissected were: brain, thymus, heart, lungs, liver, spleen, pancreas, stomach, small intestine (duodenum, jejunum and ileum), large intestine, kidneys, bladder, oviduct + uterus and ovaries.

2.9 Statistical analysis

Statistical comparisons were designed to determine whether differences in the response variables described above (body weight, hematology, serum biochemical parameters and relative organ wet weights) among groups were attributable to the test PDN-a compared to the control group, water. The homogeneity of variance was analyzed by simple analysis of variance (One-way ANOVA) with the GraphPad Prism 6.0 software (San Diego, CA, USA). Differences were considered significant when $p < 0.05$)

2.10 Cytotoxic studies on human neutrophil

The cell suspension containing predominantly neutrophils (about 92 %) were isolated by Lucisano and Mantovani's method (1984) with slight modifications (Kabeya et al, 2002). The cell viability determined by the trypan blue test was 89 ± 2.0 %.

2.11 Lactate dehydrogenase (LDH) assay

Human neutrophils (2.5×10^6 cells/mL) in a suspension were incubated with the pectin (PDN-s) concentration in 10, 50, 100 and 200 µg/mL, DMSO (1% v/v, control/vehicle), HBSS (not treated cells) or Triton X-100 (Tx, 0.2% v/v, standard drug) for 15 min at 37 °C. The LDH activity was determined by a commercially available method (LDH liquiform of Labtest Diagnosis, Lagoa Santa, MG, Brazil).

2.12 Antiviral activity

2.12.1 Cells and Virus

HEp-2 cells (human larynx epithelial cells carcinoma, ATCC CCL-23) were grown in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (Invitrogen/Gibco, USA), 100 IU/ml penicillin (Novafarma Ind. Farm., BR), 100 µg/ml streptomycin (Gibco BRL, USA) and 2.5 µg/ml of amphotericin B (Meizler Biopharma S/A, BR). The clinical isolate HSV-1 was provided by the Departamento de Virologia (IMPPG/UFRJ, BR).

2.12.2 Cytotoxicity Assay

The cytotoxicity of the PDN-a and PDN-s was evaluated by MTT (dimethyl-thiazolyl-diphenyltetrazolium bromide) in HEp-2 cells (Faccin et al., 2007). The 50% cytotoxic concentration (CC_{50}) was calculated by regression analysis, as the concentration of the substance capable of reducing the optical density of MTT product by 50% in comparison to control.

2.12.3 Plaque reduction assay (*PRA*)

The antiviral activity of PDN-a and PDN-s was evaluated by plaque reduction assay according to Melo et al (2008). Briefly, cell cultures were infected with HSV-1 and simultaneously treated with varying concentrations of the compounds (100, 50, 25 and 12.5 µg/ml). The percent of viral inhibition (%VI) was calculated as previously described (Nishimura; Toku; Fukuyasu, 1977). The 50% inhibitory concentration (IC_{50}) determined by regression analysis is the concentration capable of reducing virus activity in 50% and the ratio CC_{50}/IC_{50} defined as the selectivity index (SI). The activity was performed under the protocols of the time-of-addition, the inhibition of adsorption and virucidal assay.

Time-of-addition assay: Varying concentrations of the compounds were added to cell cultures before (-1h), during (time 0h) and after (+1h and +2h) infection (Faccin-Galhardi et al, 2012).

Inhibition of adsorption assay: Before submitting cell cultures to varying concentrations of the compounds, simultaneously, with viral infection, cell were incubated at 4ºC for 1h and further incubated at the same conditions (4ºC for 1h) after the treatment and infection (Minari et al, 2011).

Virucidal Assay: Virus dilutions were incubated with varying concentrations of the substances before infection (Rincão et al, 2012).

Zynvir (sodium acyclovir - ACV) (Novafarma Ind. Farm., BR) was used as positive control.

For statistical analysis Anova followed by Tukey's test (BioEstat 5.0 for Windows XP, 2007) were used and $p < 0.05$ was considered significant.

3 Results

3.1 Extractions

During the procedure of extraction of pectin was observed that the influence of the pH for the solution of basic aqueous extraction (pH 7 and 10, respectively) was the main factor for the low income, according to McCready in 1970 and other more recent researchers, such as Levigne et al (2002) reported that extraction condition has effects not only on the extraction itself, but also on the yield and chemical structure of the extracted material. It is particularly recognized that reducing the initial pH extraction allows for better yields and extreme reduction may be disadvantageous, since it may accelerate the degradation of the polymer and esterification of the pectin (Yapo et al, 2007). Extraction in acidic pH value approximately to 3.5, very close to the value found by Chunhieng (2003) in his work on the development of new foods, nuts directed to Brazilian origin and *Morinda citrifolia* origin increased from 3.0 Cambodian, where the pH value was 3.72 and confirming what was found in 2002 by researchers from the European Commission, found that a pH between 3.4-3.6. Through the extraction and purification procedures performed obtained an average yield according to Table 2. very similar to those obtained results by Bui et al (2006), and 16% was also consistent with the results found by Hirazumi and Furusawa (1999), which earned a 13% return on their work.

Table 2 – The percentage of yield from different pH values during extraction.

Extraction	Mass (g)	Yield (%)
Acid	3,8789	19,39
Neutral	2,9681	14,84
Basic	2,4582	12,29

3.2 Pectin Sulfonation

Because the pectin biological activities are related with the structural characteristics, it would be better to do the chemical modification to improve its properties (Bae et al, 2009). The sulfonate groups' presence in the PDN-s structure was confirmed by FT-IR spectroscopy. Absorption bands at 825 cm^{-1} and 1250 cm^{-1}, where found to be very similar to those found by Bae et al, (2009), where the band at 810 cm^{-1}, is assigned to the stretching mode for the S=O bond and the one at 1250 cm^{-1}, assigned to the stretching mode for the C–O–S bonds fragment, figure 1.

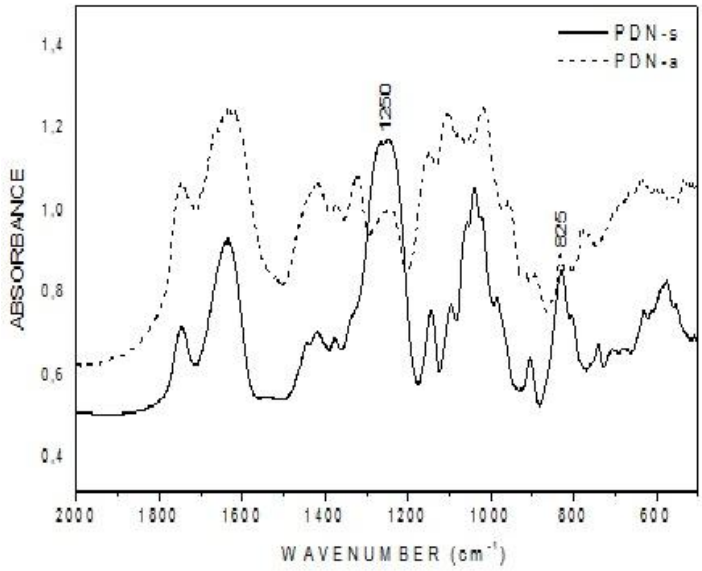


Figure 1 – FT-IR spectra for (PC, PDN-a and PDN-s) pectins.

3.3 Infrared spectroscopy (FT-IR) and Determination of degree of esterification

The FT-IR results for the sample are shown in the figures 1 and 2, where the bands at 3429 cm^{-1} corresponds to the stretching mode vibration of the hydroxyl groups, the band at 2936 cm^{-1} corresponds to the stretching of the C-H bond, and the band at 1746 cm^{-1} corresponds to the stretching modes of the esterified carboxylic groups, the other two bands at 1637 and 1422 cm^{-1} corresponds to the vibrations of the O=C-O bond and other two at 1106 and 966 cm^{-1} that according to Manrique and Lajolo (2002), corresponds to the digital impression, specific to polysaccharides.

The part of the IR spectra from 1500 to 1800 cm^{-1} is of special interest due to the esterification degree evaluation, once it has the absorption bands associated with the carboxylic acid and its esters in the pectin molecule (Fellah et al, 2009).

The results on the esterification degree by the integration method are related on table 3. There is a correlation between values found for the pectin commercially valuable and the pectin studied in this research, both presented a degree of esterification lower than 50%, being so, characterized as low degree of esterification. Marcon et al (2005) extracted pectin with low degree of esterification (14.3 to 29.4%) from apple bagasse using the method of citric acid 5%, time of extraction of 30 to 80 minutes and temperature from 50 to 100 ^{o}C. Koubala et al (2008) extracted pectin with the esterification degree of 49%, extract from lemon, in HCl solution pH 1.5 at 85 ^{o}C for one hour.

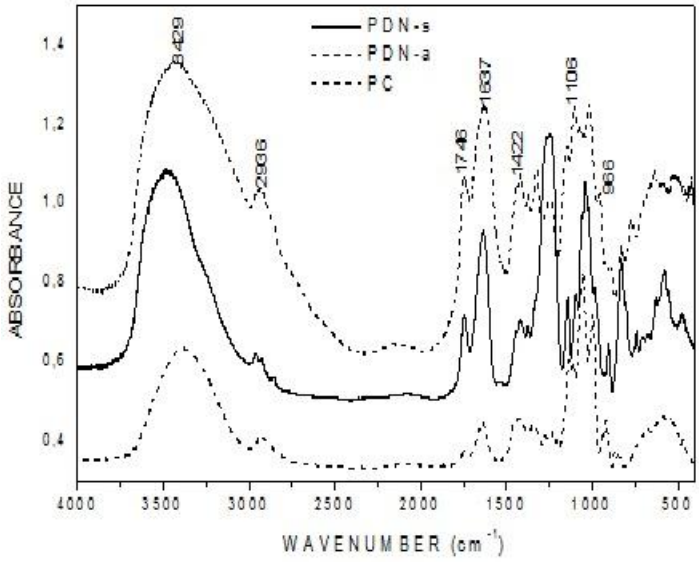


Figure 2 – FT-IR spectra for (PC, PDN-a e PDN-s).

Table 3 – Chemical physics properties.

Sample	*(DM)* NMR ^{1}H	*(DE)* FT-IR
PDN-a	30,71	30,28
PC	-	34,15

3.4 The NMR ^{1}H spectra results for PDN-a: the determination of the degree of methylation (DM)

The proton signals H-1 and H-5 adjacent to the esters groups can be seen in the graph of figure 3, as bands at 5.5 – 5.4 ppm and the signal from H-5 next to carboxylate groups (ICOO-) used to be around 4.8 ppm. These signals (H-1 and H-5) should appear around 5.2 and 4.9 ppm respectively. The displacement observed is caused by the conditions of storage and conservation of the sample prepared before

analysis. The characterization of the Noni by NMR ^{1}H, allowed the calculation of the methylation degree. According to the method proposed by Rosenbohm et al (2003), the degree of methylation was found to be around 30% (Figure 3). The value is in close accordance to those found by FT-IR and potentiometric analysis.

Five signals could be observed in the NMR ^{1}H spectra (Figure 4) and can be attributed to H-1 at 5.50 ppm; H-2 ppm, 3.74 ppm; H-3, 3.92 ppm; H-4, 4.43 ppm and H-5, 4.82 ppm. The values are in agreement to those found by Tamaki et al 2008.

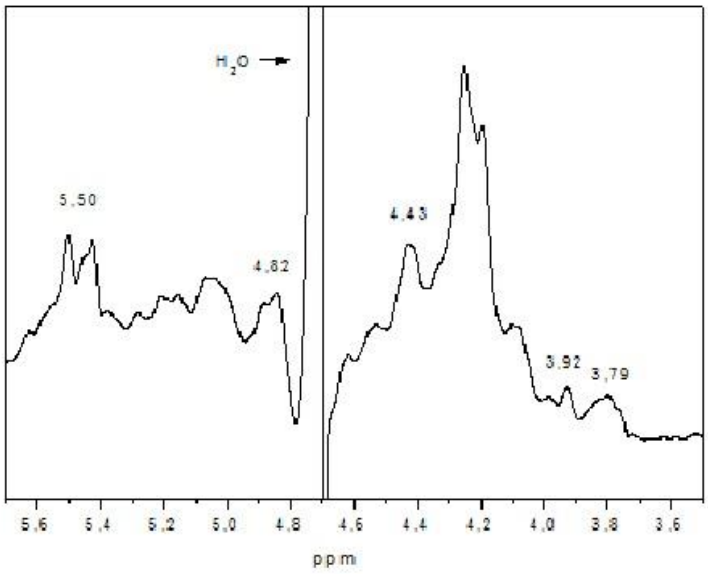


Figure 3 – The values for H-1; H-5; H-4; H-3 e H-2, respectively for sample PDN-a.

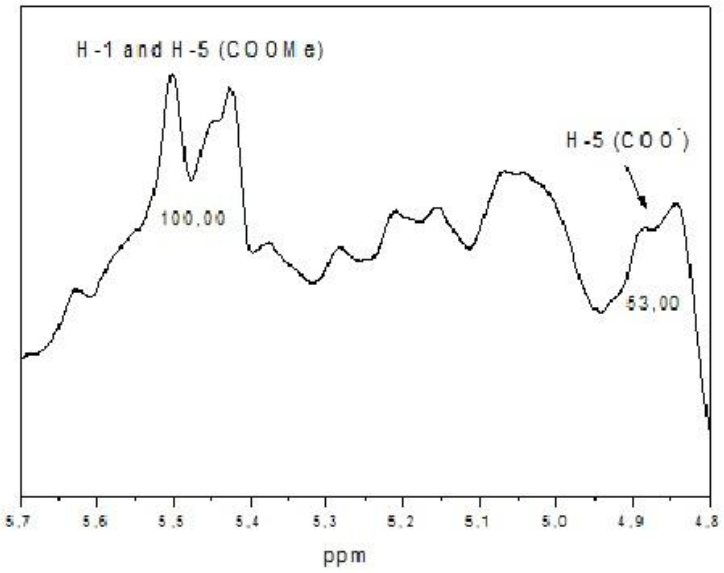


Figure 4 – The degree of methylation determination for PDN-a sample.

3.5 Atomic force microscopy

The AFM can effectively attain the image at the atomic level (figures 5c, 6c, 7 and 8). In the figures 5 and 6, shown the transversal sections of the pectin chain (PDN-a and PDN-s) respectively, the transversal line showing a cut was draw thought the AFM image and the high profile of the transversal section that are enhanced (figure 5a and 6a). The results in the part of the transversal section between the two selected cursors are presented in the superior part to the left in figures 5b and 6b. The potential spectrum in the transversal section is presented in the central part to the inferior of the figures 5d and 6d. Up to three pairs of the cursors can be applied in the line section and the results are in red, green and white in the lower part to the right in figures 5e and 6e.

Many factors can interfere in the width of the pectin images from AFM, as an example: the sample shape, the size, its properties and the possible interactions between the cantilever point and the sample surface (Ikeda; Shishido, 2005), as well as the conditions of extractions that favors the pectin degradation. Analyses from AFM of the pectin of same source in different stages of maturation, presented different chain size, due to the rigidity of the polymeric chain. That can be related to the process of maturation, where there is an increase of the soluble pectin, pectin acids and calcium pectate. The presence of these substances is associated with the diminution of the protopectin, indicating that the soluble pectins are originated from polymers more strongly attached to the cellular walls and possibly to the hemicellulose. The increase in the solubilization and despolimerization is generally correlated with the diminution of the strength on the tissue (Wakabavashi, 2000).

All these factors are justified when the samples are from the same source at different states of maturation. However on the present study the samples PDN-s that came from PDN-a, where subjected to the same extraction process with the PDN-s obtained after sulfonation, justifying the small size of the polymer chain and its fragmentation.

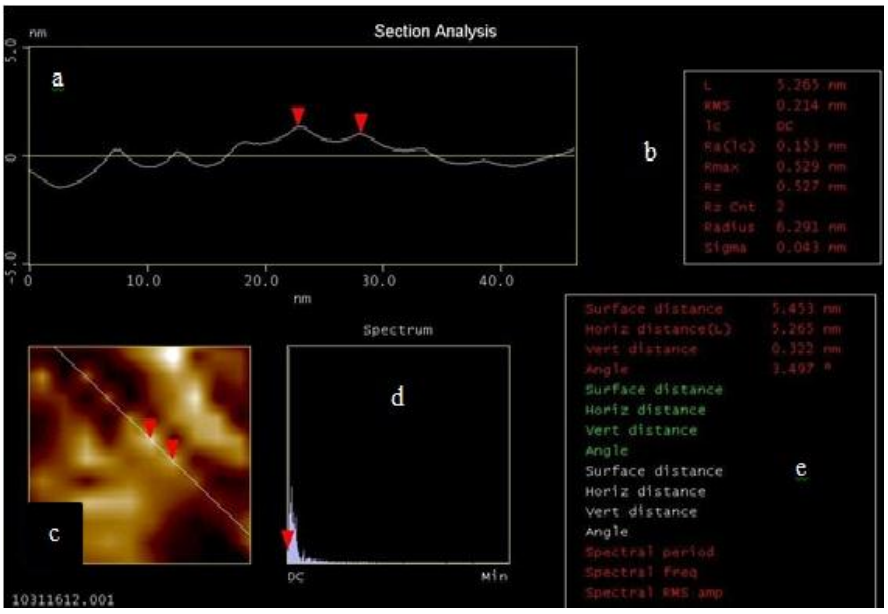


Figure 5 – Transversal analysis of the pectin chain (PDN-a): (a) the height profile in a transversal line; (b) the measurements in the transversal section dimension between two colored cursors; (c) an AFM image; (d) the energy spectrum of the transversal section; (e) dimension measures of three cursors pairs placed at the section line.

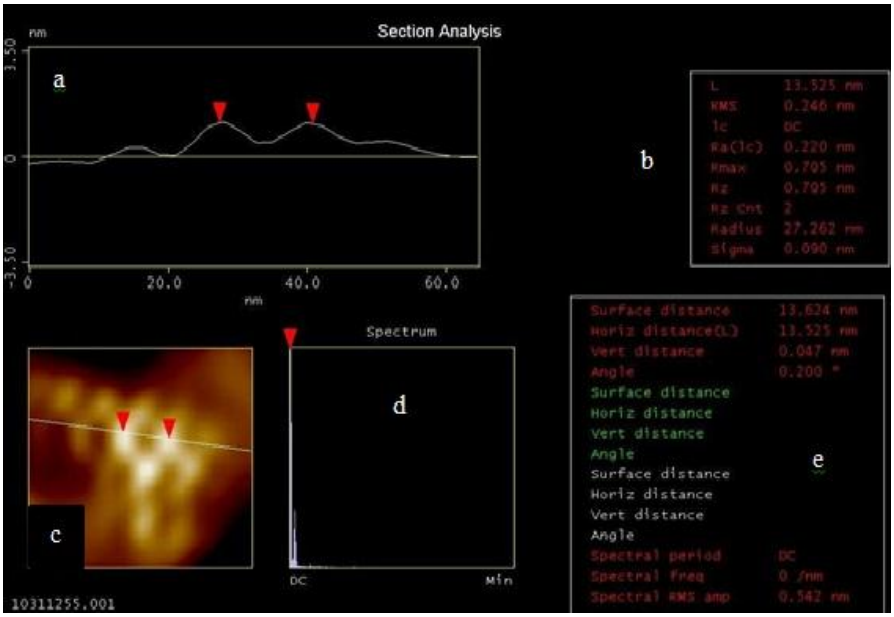


Figure 6 – Transversal analysis of the pectin chain (PDN-a): (a) the height profile in a transversal line; (b) the measurements in the transversal section dimension between two colored cursors; (c) an AFM image; (d) the energy spectrum in the transversal section; (e) dimension measures of three cursors pairs placed at the section line.

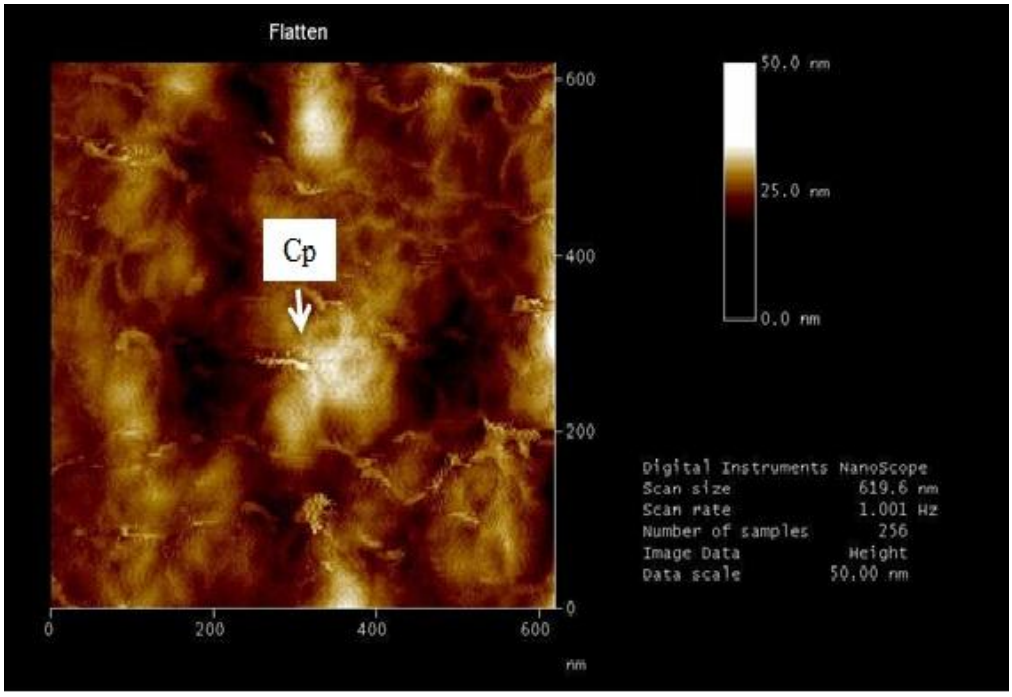


Figure 7 – AFM image for the pectin (PDN-A). Note: Cp (pectin chain).

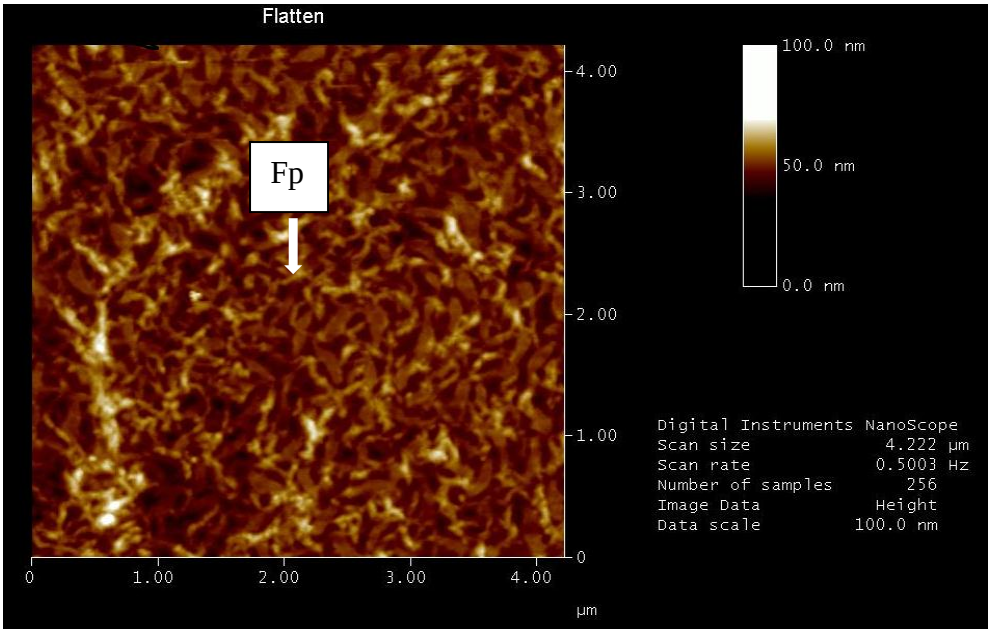


Figure 8 – The AFM image for the pectin (PDN-s). Note: Fp (pectin fragment).

3.6 Elemental analysis

The observed values in table 4, shows that for the PDN-s sample the amount of carbon, nitrogen and hydrogen is reduced, due to the sulfonation that produces the polymer chain degradation (Zhang et al, 2010; Mass et al, 2012). The sulfur content

for the samples PDN-s confirms the sulfonation, presenting according to equation 4 the value for DS of 0.65.

Table 4 – Elements found in the samples of PDN-a and PDN-s.

Samples	Carbon (%)	Sulfur (%)	Nitrogen (%)	Hidrogen (%)
PDN-a	30,11	Trace	6,35	5,50
PDN-s	9,46	9,72	0,66	2,40

3.7 Toxicity and pharmacology

The acute oral administration of PDN-a in single doses of 5, 50 and 300 and mg.Kg^{-1} resulted in no mortality or any apparent symptoms of systemic toxicity or behavioral changes in mice. In addition, mice in the test groups showed no significant ($p > 0.05$) changes in body weight gain when compared to the control group (water) over 14 days (Figure 9). Any significant reduction in body weight of the animals in the test groups could be indicative of toxic and/or anti nutritional effects inherent to the samples tested (Carvalho et al, 2011).

The results of hematological and biochemical parameters measured at the end of the experiment (14th day) are listed in Table 5 and 6, respectively. For the hematological parameters, just the platelet counting had a significant ($p < 0.05$) increase in the group administered with the PDN-a, at 50 mg.Kg^{-1}. However, this result seems not to be significant since in the highest dose tested (300 mg.Kg^{-1}) the platelets number did not change ($p > 0.05$).

As to data on serum biochemical parameters (Table 6), differences ($p < 0.05$) in ALT, AST, creatinine and urea between the PDN-a, and control group were observed. Nevertheless, these differences were not in a dose-dependent manner for any one of the parameters analyzed. For albumin, total protein and cholesterol no changes related to the control group (water) were detected in the test groups.

The sample did not affect the relative organs weight of mice, except of the thymus and pancreas. For the thymus was not possible to establish a crescent correlation between the sample tested and increasing/decreasing of this organ. As for the relative wet weight of pancreas, is difficult to correlate any toxic effect to PDN-a since the larger concentrations tested (50 and 300 mg.Kg^{-1}) did not present adverse effects. Changes in organ weights (increase or decrease), especially in organs involved in detoxification processes, may represent inherent toxic effects of

the administered substances when compared to control samples (Carvalho et al, 2011).

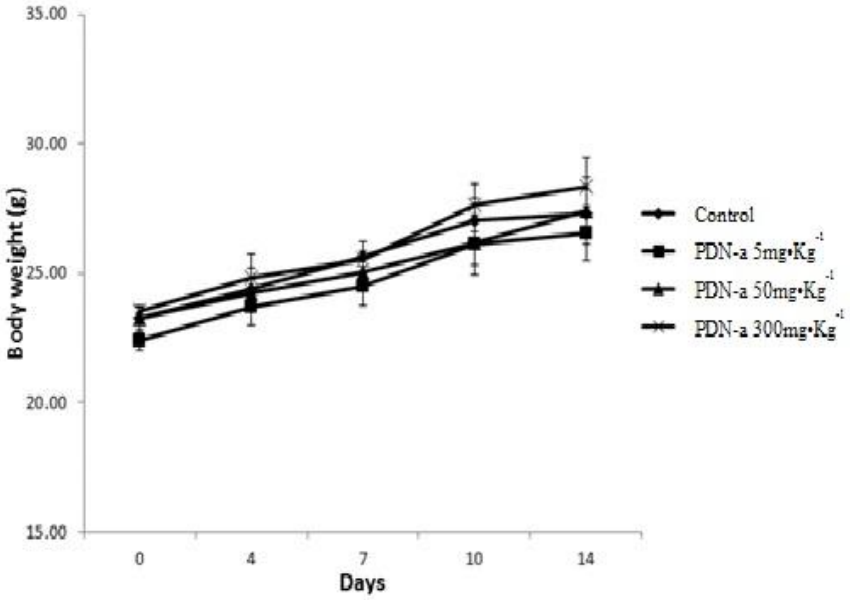


Figure 9 – Body weight gain (g) in mice (n = 6/group) subjected to a single oral dose of PDN-a at concentrations of 5, 50 and 300 mg.Kg^{-1} compared to the control, distilled water, during 14 days. Values are means ± standard deviations for each day of weighing, and all standard deviations ≤ 5%. Measurements of body weight on days mentioned showed no significant difference ($p > 0.05$; One-way ANOVA) between groups.

Table 5 – Hematological parameters of mice (n = 5/group) subjected to a single oral dose of PDN-a, at concentrations of 5, 50 and 300 mg.Kg^{-1} compared to the control, distilled water.

Parameters	**PDN-a (mg.Kg^{-1})**			
	Water	**5**	**50**	**300**
WBC (10^3/µL)	3,80 ± 0,33	4,20 ± 0,50	4,53 ± 1,02	4,90 ± 0,41
RBC (10^{12}/µL)	9,02 ± 0,08	8,57 ± 0,29	9,44 ± 0,44	9,20 ± 0,44
HGB (g/dL)	13,78 ± 0,69	13,16 ± 0,74	14,63 ± 0,81	14,08 ± 0,81
HCT (%)	38,20 ± 1,43	38,33 ± 1,78	41,25 ± 1,77	39,40 ± 2,07
MCV (fL)	43,34 ± 1,45	43,84 ± 1,21	44,40 ± 1,71	43,60 ± 0,61
MCH (ρg)	15,32 ± 0,64	15,34 ± 0,44	15,60 ± 0,43	15,28 ± 0,31
MCHC (g/dL)	35,32 ± 0,60	35,00 ± 0,34	35,18 ± 1,07	35,06 ± 0,75
PLT (10^3/µL)	876,30 ± 65,18	1027,0 ± 84,18	1120,0 ± 36,69[a]	989,70 ± 103,50
RDW-SD (fL)	26,56 ± 0,70	26,98 ± 0,92	26,83 ± 1,17	26,38 ± 0,81
RDW-CV (%)	14,54 ± 1,23	14,54 ± 0,61	14,40 ± 1,06	14,22 ± 0,53

The values are mean ± SD.

[a]$p < 0.05$ (ANOVA) experimental groups compared to blank control group.

[b]p< 0.05 (ANOVA) experimental group 5 mg.Kg^{-1} compared to experimental groups 50 and 300 mg.Kg^{-1}.
[c]p< 0.05 (ANOVA) experimental group 50 mg Kg^{-1} compared to experimental group 300 mg.Kg^{-1}.

Table 6 – Biochemical parameters of mice (n = 5/group) subjected to a single oral dose of PDN-a at concentrations of 5, 50 and 300 mg.Kg^{-1} compared to the control, distilled water.

Parameters	PDN-a (mg.Kg^{-1})			
	Water	5	50	300
ALT (U/mL)	99,67 ± 18,56	33,00 ± 3,33[a]	54,11 ± 10,72[a]	56,33 ± 4,71[a]
AST (U/mL)	118,60 ± 12,38	93,23 ± 5,32[a]	105,30 ± 2,35	76,56 ± 5,45[a, c]
Alkalinephosphatase (U/L)	184,60 ± 14,65	185,60 ± 8,45	184,00 ± 25,56	161,30 ± 9,99
Creatinine (mg/dL)	0,268 ± 0,04	0,427 ± 0,06[a]	0,297 ± 0,06	0,471 ± 0,01[a,c]
Urea (mg/dL)	47,34 ± 3,69	41,64 ± 3,38	57,07 ± 3,16[a, b]	49,20 ± 4,60
Total proteins (g/dL)	4,72 ± 0,35	4,90 ± 0,45	4,46 ± 0,20	4,63 ± 0,29
Albumin (g/dL)	2,97 ± 0,27	3,13 ± 0,44	3,17 ± 0,23	3,18 ± 0,32
Cholesterol (mg/dL)	127,6 ± 12,25	111,40 ± 6,68	133,4 ± 8,56[b]	121,40 ± 12,83

The values are mean ± SD.
[a]p< 0.05 (ANOVA) experimental groups compared to blank control group.
[b]p< 0.05 (ANOVA) experimental group 5 mg.Kg^{-1} compared to experimental groups 50 and 300 mg.Kg^{-1}.
[c]p< 0.05 (ANOVA) experimental group 50 mg.Kg^{-1} compared to experimental group 300 mg.Kg^{-1}.

3.8 Cytotoxicity

3.8.1 Cytotoxicity studies on human neutrophils

The addition of PDN-s at concentrations ranging from 10 to 200 μg/ml produced no significant effect on neutrophil viability, and the LDH activity at higher concentration of pectin (LDH activity: 4.08 ± 0,51 %) was not reduced significantly as compared to controls (LDH activity: 6.86 ± 1.16 %), which is indicative of a greater number of viable cells present.

3.9 Antiviral activity

The CC_{50} for PDN-a and PDN-s were 605 μg/ml and 44 μg/ml, respectively. Both compounds showed IC_{50} approximately of 46 μg/ml, and, therefore, PDN-s was excluded (CC_{50} almost equal to IC_{50}) (Table 7). Alternatively, the PDN-a demonstrated significant antiviral activity when used concomitantly with viral infection, reaching 100% of viral inhibition at 100 μg/ml ($p < 0.05$). This activity was maintained when the compound was added 1 and 2 hours after infection ($p < 0.05$).

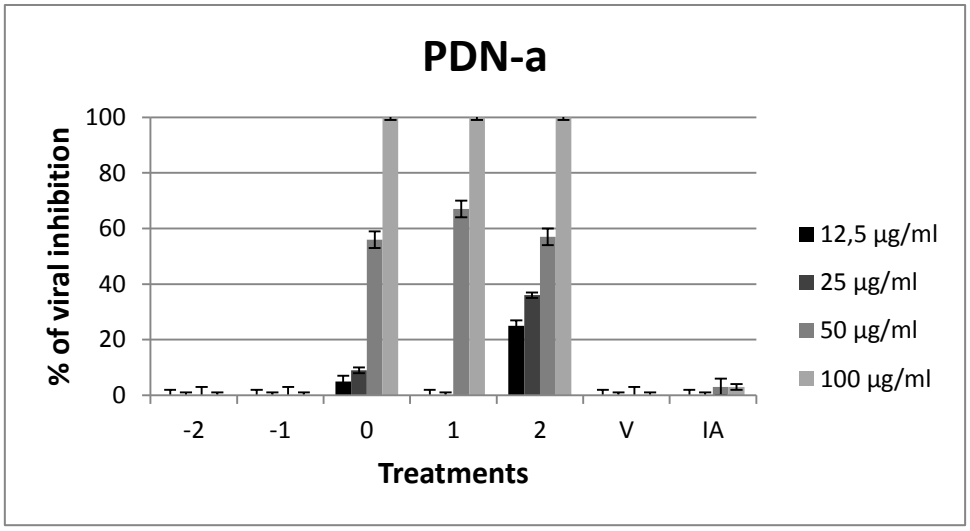
No inhibition was observed when cells were treatment before infection or in virucidal and inhibition of adsorption assays (Figure 10).

Table 7 – The 50% inhibitory concentration (IC_{50}) of Noni pectins PDN-a and PDN-s and acyclovir (ACV) for HSV-1, and their 50% cytotoxic concentrations (CC_{50}) and the selectivity index (SI).

	CC_{50}	IC_{50}	SI
PDN-a	605*	43*	14
PDN-s	44.06	49	0
ACV	2500	2100	1.21

* µg/ml

Figure 10 – The percentage of HSV-1 inhibition (%VI) by PDN-a under the protocols: (a) Time-of-addition (−2 to +2); (b) Inhibition of adsorption (IA) and (c) Virucidal (V) at the indicated concentrations.



4 Conclusion

Extraction with oxalate of ammonia, it seems appropriate for the recovery of pectins Noni relative to income, on certain conditions (pH 3.0 / 80°C / 60 min) compared to other pH values (7 and 10).

The analyzes of FT-IR and 1 H NMR showed very efficient characterization of pectin Noni, enabling the determination of the degree of esterification, the elementary analysis confirmed the procedure of sulfation of the sample, since the

AFM analysis confirmed the structural differences between the sample PDN-a and PDN-s.

Regarding all changes toxicity could not be detected consistently assigned to the tested sample. Such variations presented within the specific groups are commonly identified in mammalian toxicity studies, especially when heterogeneous strains and the results of cytotoxicity tests with the addition of PDN-s are employed in concentrations tested did not produce any significant effect on the viability of neutrophils and LDH activity was not significantly reduced compared with the controls, indicating a greater number of viable cells present.

The PDN-a demonstrated anti-HSV activity when added during the infection and post-infection treatment was also effective even at lower concentrations. PDN-s was excluded for its toxicity. Therefore, a potential antiviral activity is foreseen for PDN-a and further studies have to be carried out to get insight into the step pectin acts in the replication of HSV-1.

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6 CONCLUSÃO

Neste trabalho, investigamos a atividade antiviral de substâncias originadas de duas plantas, *Enteromorpha compressa*, uma alga marinha verde, e *Morinda citrifolia*, uma árvore frutífera popularmente conhecida como noni. O quadro atual das infecções virais é o que nos impulsiona a buscar novas opções de tratamento alternativas aos medicamentos já existentes ou como fármacos adicionais ao tratamento convencional. As substâncias naturais são capazes de agir em diversas etapas do ciclo replicativo viral, o que desempenha um importante papel, ampliando a eficácia dos tratamentos e dificultando o surgimento de vírus resistentes.

Além disso, ambas as substâncias testadas neste estudo demonstraram atividade antiviral em baixas concentrações e distantes de sua concentração tóxica, o que gera índices de seletividade extremamente altos, possibilitando a inserção de opções terapêuticas efetivas e seguras.

É importante salientar que a enorme gama de substâncias presentes na ampla variedade de plantas disponíveis para a exploração científica aumenta as esperanças de serem revelados novos compostos úteis no combate às infecções causadas por vírus.

A realização deste trabalho constitui uma importante estratégia de triagem de compostos ativos em plantas, direcionando a execução de estudos posteriores mais aprofundados, que possibilitaria a inserção de tais substâncias no arsenal de controle e tratamento de viroses.