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Romero Marcos Pedrosa Brandão Costa

Purificação, Caracterização Parcial e Aplicações Biomédicas de uma Lectina de Folhas de *Phthirusa pyrifolia* (H.B.K) Eichl

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ROMERO MARCOS PEDROSA BRANDÃO COSTA

"Purificação, caracterização parcial e aplicações biomédicas de uma lectina de folhas *Phthirusa pyrifolia* (H.B.K.) Eichl"

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Aprovado por:

Profa. Dra. Maria das Graças Carneiro da Cunha – Presidente

Prof. Dr. Eduardo Isidoro Carneiro Beltrão – Examinador interno

Profa. Dra. Maria Tereza dos Santos Correia – Examinador interno

Profa. Dra. Márcia Vanusa da Silva – Examinador externo

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SUMÁRIO

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RESUMO

Phthirusa pyrifolia é uma planta hemiparasita conhecida como Erva-de-passarinho e muito utilizada na medicina popular. Visando conhecer melhor as substâncias contidas nas folhas desta planta, o presente trabalho teve como objetivos, a Purificação e Caracterização parcial de uma lectina, bem como Aplicações Biomédicas envolvendo esta proteína. As folhas da planta foram coletadas no campus da Universidade Federal de Pernambuco, lavadas, secas, trituradas e o Extrato Bruto (EB) a 10% (p/v) em solução de 0,15M NaCl foi obtido por agitação a 4ºC durante 4h, seguido de filtração e centrifugação. No período de dois anos, novos extratos foram obtidos para avaliação do ciclo sazonal na Atividade Hemaglutinante Específica (AHE) da lectina. Este ensaio evidenciou que o EB obtido no mês de março apresentou maior AHE, sendo submetido, então, à precipitação salina com sulfato de amônio. Do fracionamento salino resultou a $F_{20\text{-}40\%}$, com maior AHE. Inicialmente, uma alíquota da $F_{20\text{-}40\%}$ foi cromatografada por afinidade em Sephadex G-100 utilizando 0,3M glicose como eluente. Em seguida os picos que apresentaram Atividade Hemaglutinante (AH) foram reunidos, dialisados, liofilizados e aplicados em cromatografia de troca iônica de CM-cellulose. A amostra adsorvida na coluna foi eluída com solução 0,5M Tris-HCl, pH 8,5. O pico que apresentou AH foi dialisado, liofilizado, submetido à SDS-PAGE e a banda de protéica visualizada foi denominada PpyLL. O gel eletroforético na ausência do agente redutor mostrou a lectina com uma banda de 15,6 kDa e em presença de agente redutor, duas bandas com massas moleculares de 15,6 kDa e 7,8 kDa. PpyLL é uma glicoproteína ácida com pH ótimo 7,5, termoestável até 70ºC, apresenta afinidade por eritrócitos humanos tipo O^+ e não foi inibida por açúcares simples, mas por frutose-1,6-bifosfato e pelas glicoproteínas caseína, azocaseína e albumina de soro bovino. A Fluorimetria mostrou que a 70ºC, a lectina apresentou uma alta intensidade de fluorescência do aminoácido Triptófano, alterando assim a sua conformação. No Teste de Avaliação da atividade antimicrobiana, a PpyLL mostrou atividade contra as bactérias *Staplylococcus epidermidis*, *Streptococcus faecalis*, *Bacillus subtilis* e *Klebsiella pneumoniae*, e contra os fungos *Fusarium lateritium* e *Rhizoctonia solani*. A nova lectina obtida através de um protocolo de purificação envolvendo cromatografias de afinidade e troca iônica apresenta características de grande relevância, as quais permitem a sua aplicação em processos biotecnológicos.

Palavras-chave: Lectina; *Phthirusa pyrifolia*; Purificação; Antimicrobial activity; Fluorimetria.

ABSTRACT

Phthirusa pyrifolia is a hemi-parasite plant, well-known as *erva-de-passarinho* in Brazil and commonly used in traditional medicine in developing countries. To aim research the compounds present in this plant leaves, the goals of the present work were Purification and Partial Characterization of a lectin and its uses in Biomedical applications. The plant leaves were collected on the campus of Universidade Federal de Pernambuco, dried, triturated and the crude extract (CE) [10% (w/v) solution of 0.15 M NaCl] was obtained by stirring at 4ºC for 4h, followed by centrifugation at 11,500 xg. Over two years, new samples of leaves were collected and the extracts were prepared to evaluate the seasonality interference on specific hemagglutinating activity (SHA) of the lectin. This test revealed that the CE obtained in March showed higher SHA than others, being then submitted to salt precipitation with ammonium sulfate. Salt fractionation $F_{20\text{-}40\%}$, presented the highest SHA. For chromatography procedures, initially, an aliquot of the $F_{20\text{-}40\%}$ was applied in an affinity chromatography column with Sephadex G-100 using 0.3 M glucose as eluent. Afterwards, the peaks that showed hemagglutinating activity (HA) were collected, dialysed in water, lyophilized and applied to ion exchange chromatography on CM-cellulose. The sample adsorbed in the column was eluted with a solution of 0.5 M Tris-HCl, pH 8.5. The peaks that showed HA were dialysed, lyophilized and subjected to SDS-PAGE, which revealed the lectin termed PpyLL. Two bands of proteins were observed with molecular weights of 15.6 kDa and 7.8 kDa in the gel in presence of a reducing agent. PpyLL is an acid glycoprotein with optimum pH at 7.5, thermostable at 70ºC, with affinity for human erythrocytes type O^+ and not inhibited by simple sugars but by fructose-1,6-biphosphate and glycoproteins (casein, azocasein and bovine serum albumin). Fluorimetry techniques revealed that at 70°C, the lectin produce high intensity of fluorescence of the amino acid tryptophan, thereby changing its conformation. Its optimum pH was observed in buffer 10 mM Tris-HCl, pH 7.5. With regards to antimicrobial activity, PpyLL revealed activity against bacteria *Staplylococcus epidermidis*, *Streptococcus faecalis*, *Bacillus subtilis* and *Klebsiella pneumoniae* and against the fungi *Rhizoctonia solani* and *Fusarium lateritium*. The novel lectin obtained by a purification protocol involving affinity chromatography and ion exchange chormatography presents its application in biotechnological processes.

Keywords: Lectin; *Phthirusa pyrifolia*; Ion-Exchange Chromatography; Antimicrobial activity; Fluorimetry.

LISTA DE ABREVIATURAS

AH: Atividade hemaglutinante

AHE: Atividade hemaglutinante específica

AHT: Atividade hemaglutinante total

BSA: Albumina sérica bovina

EB: Extrato bruto

F0-20: Fração 0-20% do extrato

F20-40: Fração 20-40% do extrato

F40-60: Fração 40-60% do extrato

F60-80: Fração 60-80% do extrato

F0-60: Fração 0-60% do extrato

F0-80: Fração 0-80% do extrato

SDS: Dodecil sulfato de sódio / Lauril sulfato de sódio

PAGE: Eletroforese em gel de poliacrilamida

PBS: Tampão fosfato de Sódio

PpyLL: *Phthtirusa pyrifolia* Leaf Lectin

SDS -PAGE: Eletroforese em gel de poliacrilamida contendo dodecil sulfato de sódio

TCF: Tampão citrato-fosfato

Tris: Tris (hidroximetil) aminometano

SHA: Specific Hemagglutinating Activity

HA: Hemagglutinating Activity

PAS: Periodic Acid Schiff

MALDI TOF: Matrix Assisted Laser Desorption/Ionization – Time of Flight

GluNAc: N-acetil-glicosamina

GlcNAc: N-acetil-galactosamina

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1. INTRODUÇÃO

1.1 Lectinas

As lectinas são proteínas ou glicoproteínas que, embora identificadas primariamente em plantas, encontram-se de forma ubíqua na natureza. Pertencem a uma classe de proteínas de origem não imune que apresentam uma característica muito importante: o reconhecimento através de ligações não covalentes aos carboidratos e/ou glicoconjugados, geralmente, presentes em superfícies de células eucariontes e procariontes, promovendo ou não a reação de aglutinação (Correia, 2008; Sharon, 2008). As lectinas têm sido isoladas a partir dos mais variados tecidos vivos, dentre eles: plantas (Oliveira et al., 2008 Cheung, 2009), anelídeos (Molchanova et al., 2007), insetos (Chai et al., 2008; Jiang et al., 2009) peixes (Singha, 2008), crustáceos (Sun et al., 2008; Wang et al., 2009), moluscos (Takahashi, 2008; Adhya, 2009), cogumelos (Zhao, 2009; Pohleven et al., 2009), protozoários (Marcipar et al., 2003) e fungos (Khan, 2007).

1.2 Ocorrência de Lectinas

De acordo com Wang et al (2004), por cerca de 20 anos acreditou-se que as lectinas, em geral, seriam encontradas apenas no meio extracelular. O seu artigo de Revisão tinha como objetivo desmistificar estes dados identificando a presença de lectinas na região núcleo-citoplasmática das células. No estudo em questão, foi observado que grande parte das galectinas (I, II e III), lectinas que apresentam afinidade ao monossacarídeo galactose, eram proteínas exclusivamente intracelular. Segundo Funakoshi e Suzuki (2009), a ocorrência de proteínas N-glicosiladas, ou seja, glicoprotéinas é maior no meio intracelular comparado com o meio extracelular. Alguns estudos recentes apontam para a perspectiva de que a presença de glicoproteínas no citosol provém uma evidência convincente de que o processo de glicosilação ocorre sob condições específicas, ou seja, correspondente as necessidades da célula eucariótica (Wang et al., 2004; Lanno, 2009). Segundo Van Damme (2008), dentre as famílias de lectinas existentes, seis são originárias de plantas, e estas são encontradas no núcleo ou no citoplasma da célula (Tabela 1).

Tabela 1. Ocorrência de lectinas de plantas segundo Van Damme (2008).

Família	***Característica***	***Especificidade***	***Localização***	***Exemplos***
Agaricusbisporus aglutinina	Homotetramérica	T-antigeno	Núcleo-citoplasmático	MarpoABA
Amaranthins	Homodimérica	GalNAc	Núcleo-citoplasmático	Amarantina
Euonymus europaeus aglutinina	Homodimérica	galactosideos	Núcleo-citoplasmático	EEA
Glanathus nivalis aglutinina	β-barril	manose	Forma vacuolar	GNA
Jacalina	β-prisma	manose	Forma vacuolar	Jacalina
Nictaba-like lectins	Homodimérica	GlcNAc	Núcleo-citoplasmático	Nictaba

A importância de se estudar a localização das glicoproteínas, em especial as lectinas, nos seres vivos, advém de que muitas preparações de extratos são realizadas, apenas, com o objetivo de extrair as proteínas do meio extracelular. No entanto, sabendo-se que grande parte das glicoproteínas são sintetizadas e encontradas no lúmen da célula, um experimento que visa promover a extração de lectinas seria afetado, e supondo que o meio extracelular fosse pobre em concentração de lectinas, a amostra de tecido escolhido seria descartada.

1.3 Divisões de lectinas vegetais de acordo com a estrutura

Segundo Van Damme et al (1998), as lectinas de plantas podem ser classificadas de acordo com a característica estrutural em:

- Merolectinas – Aquelas que são monovalentes e, por isso, não precipitam glicoconjugados ou aglutinam células. São representadas pela família da heveína;

- Hololectinas – Aquelas que possuem dois ou mais sítios de ligação a carboidratos que são idênticos ou muito semelhantes; este grupo compreende as lectinas que são capazes de aglutinar células e/ou precipitar glicoconjugados;

- Quimerolectinas - São proteínas que apresentam um ou mais sítios de ligação a carboidratos e um domínio não relacionado. Este domínio diferente pode ter atividade enzimática bem definida, ou outra atividade biológica, mas age independentemente do domínio ligante a carboidratos. Assim representadas pelos grupos RIPs tipo 2 e quitinase de plantas tipo I;

- Superlectinas - São proteínas com dois domínios de ligação a carboidratos. Este pode ser considerado um grupo especial de quimerolectinas, consistindo de dois

domínios estrutural e funcionalmente diferentes de ligação a carboidratos, como a lectina TGL da *Tulipa gesneriana L.,* que são formados por dois domínios de ligação a carboidratos, que reconhecem D-manose e L-fucose, respectivamente.

Entretanto, as lectinas vegetais podem ainda ser subdivididas de acordo com a afinidade aos carboidratos. Segundo Goldstein et al (1997), as lectinas de plantas podem ser subdivididas em cinco grupos, de acordo com o carboidrato que estas exibem uma maior afinidade: D-manose/D-glicose (Tian et al., 2008; Wong, 2008), D-galactose/N-acetil-D-galactosamina, (Rameshwaram e Nadimpalli, 2007), N-acetil-D-glicosamina (Adhya, 2009), L-fucose (Wu et al., 2009) e Ácido N-acetil-neuramínico (Chen et al., 2009). Recentemente outras lectinas têm sido isoladas apresentando afinidade com outros carboidratos, antes não relacionadas: frutose (Cheung, 2009) e rafinose, D-melibiose, lactose e galactose (Zhao, 2009).

1.4 Isolamento e Purificação

O isolamento de lectinas, em grande parte, inicia-se com a preparação do extrato bruto utilizando diversas soluções, onde as proteínas de maior afinidade com o solvente serão extraídas. As lectinas têm sido purificadas por métodos convencionais que se baseiam nos seus aspectos moleculares gerais, tais como carga elétrica, tamanho, solubilidade, e seus aspectos peculiares, como um grupo de proteínas com afinidade por carboidratos e glicoconjugados. Os principais métodos empregados na purificação de proteínas em diversas áreas como na biotecnologia ou no estudo dos poluentes ambientais são: precipitação salina (Silva et al., 2009), cromatografia de afinidade e gel filtração (Takahashi, 2008), cromatografia de troca iônica (Watanabe et al., 2008), extração líquido-líquido com micelas invertidas (Nascimento et al., 2008), entre outros.

A maioria das proteínas é inicialmente purificada por procedimentos de fracionamento a partir da adição de um sal. Esta técnica baseia-se no fato de que as proteínas em solução estão associadas a moléculas de água na sua superfície, camada de solvatação, que impedem as interações proteína-proteína. A adição de concentrações elevadas de sais remove a água adsorvida, permitindo interações hidrofóbicas proteína-proteína e a sua precipitação. Em função das proteínas possuírem muitos grupos carregados, a sua re-solubilidade depende da concentração dos sais dissolvidos, aumentando à proporção que os sais são adicionados (*salting in*) voltando a diminuir a medida que mais sais são adicionados (*salting out*). O sulfato de amônio, $(NH_4)_2SO_4$, é

o sal mais utilizado para precipitar proteínas, porque a sua alta solubilidade permite a precipitação protéica em soluções com elevada força iônica (Heu *et al*., 1995). As lectinas parcialmente purificadas pelo procedimento salino são geralmente submetidas ao processo de diálise em membranas semipermeáveis, método baseado na separação de moléculas por diferenças de peso molecular. As proteínas ficam retidas dentro da membrana enquanto que as moléculas menores tais como os carboidratos ou sais presentes na amostra passam para a solução solvente.

A cromatografia é uma técnica de separação diferencial dos componentes de uma amostra, entre uma fase móvel e uma fase estacionária (partículas esféricas empacotadas numa coluna). A mistura de proteínas ou outros produtos biológicos a separar é aplicada na fase estacionária e migra através da coluna. A maior ou menor interação para a fase afeta a sua separação. Geralmente a separação dos componentes da mistura é conseguida por eluição, em que as forças de ligação à matriz são pertubadas através da alteração da composição da fase móvel de um modo contínuo (gradiente linear) ou de um modo descontínuo (gradiente em degrau). Os processos cromatográficos normalmente conduzem a seletividades elevadas. Os fatores que influenciam a eficiência deste processo são a qualidade do suporte cromatográfico, a dispersão axial e a dificuldade de estabelecimento de equilíbrio entre as fases móvel e estacionária. Diferentes suportes são utilizados em cromatografia, incluem-se polissacarídeos (dextrana, agarose e celulose), polímeros sintéticos (poliacrilamida, poliestireno), materiais inorgânicos (sílica porosa, hidroxiapatita e vidro poroso) e materiais compósitos (poliacrilamida-agarose, dextrana-bisacrilamida, sílica porosa-dextrana). Dependendo do tipo de interações envolvidas, os processos cromatográficos podem ser classificados em gel filtração, troca iônica, interação hidrofóbica, fase reversa e afinidade (Aires-Barros e Cabral, 2003).

O método cromatográfico realizado em gel filtração utiliza a matriz para promover a separação das proteínas por peso molecular. Usualmente, o Sephadex (Figura 1), polímero de glicose formado por “cross-linked” com epicloridrina, é o gel de escolha utilizado para esses fins. No entanto, o mesmo pode ser empregado também como matriz de afinidade, devido aos radicais hidroxilas livres nas cadeias de dextranas.

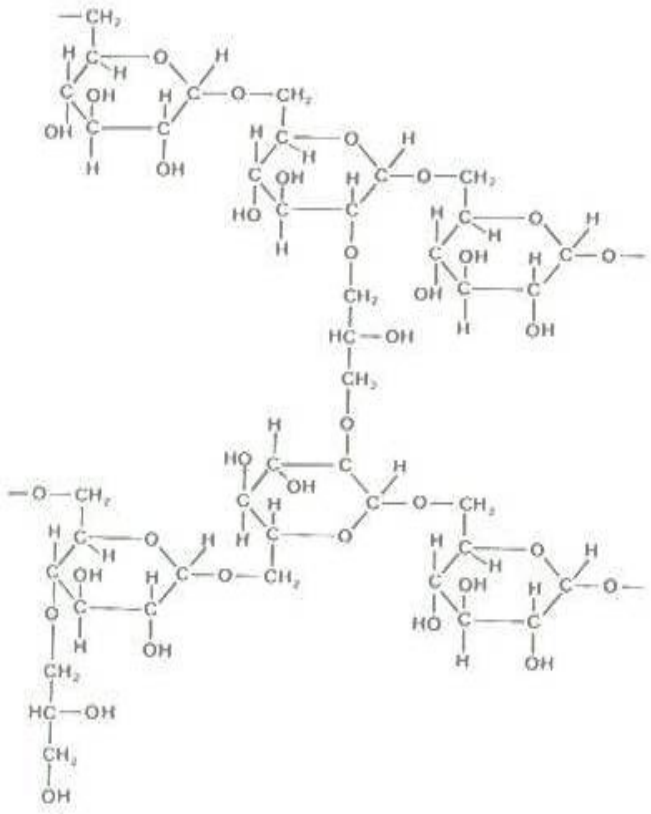

Figura 1. Estrutura parcial do Sephadex apresentando o polímero de glicose conectado por moléculas de epicloridrina.

A cromatografia de troca iônica caracteriza-se por promover a ligação da proteína com os grupos de carga de sinal contrário imobilizados na matriz (Datta et al., 2001). A fase estacionária é altamente carregada, sendo que os componentes com cargas de sinais contrários a estas são seletivamente adsorvidos da fase móvel. Os componentes adsorvidos podem se subseqüentemente eluídos, por deslocamento com outros íons, com o mesmo tipo de carga, porém com maior força de interação com a fase estacionária. A afinidade entre íons da fase móvel e a matriz podem ser controlados utilizando fatores como pH e a força iônica.

Dentre as matrizes mais amplamente utilizadas destacam-se aquelas derivadas da celulose (Fig 2A). A figura 2B apresenta a matriz de Dietilaminoetil-celulose (DEAE-Celulose), um trocador aniônico carregado positivamente, e a figura 2C apresenta a matriz de Carboximetil-celulose (CM-Celulose), um trocador catiônico carregado negativamente.

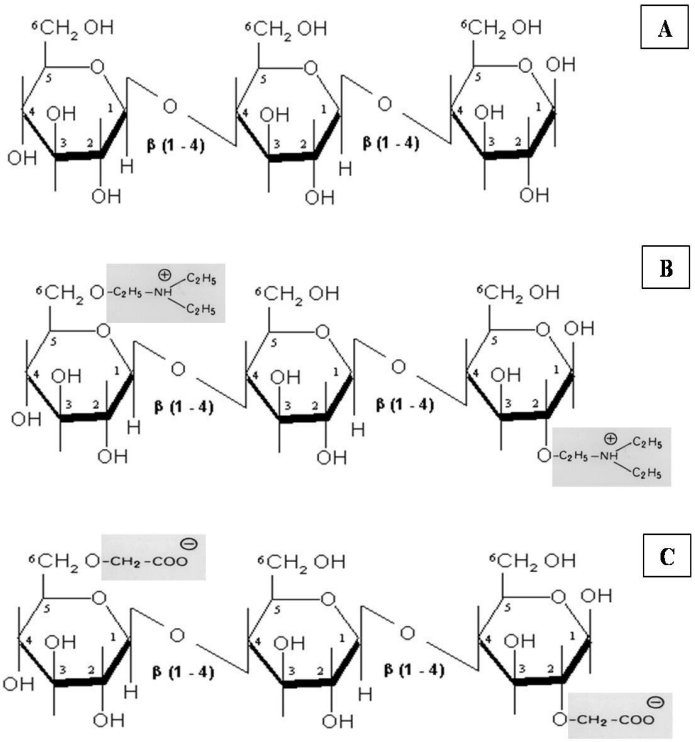

Figura 2. (A) Celulose; (B) Dietilaminoetil-celulose (DEAE-Celulose); (C) Carboximetil-celulose (CM-celulose).

1.5 Detecção de lectinas

A presença de lectinas em uma amostra pode ser facilmente detectada a partir de ensaios de aglutinação, geralmente utilizando eritrócitos humanos ou de animais, onde interagem com os carboidratos da superfície celular, através de seus sítios de ligação, formando diversas ligações reversíveis entre células opostas (Santos et al., 2005). Para assegurar que o agente aglutinante é uma lectina, são necessários ensaios subseqüentes de inibição da atividade hemaglutinante (AH), utilizando soluções de carboidratos distintos (Kawagishi et al., 2001; Jayati et al., 2005).

Recentemente, outro método de detecção da presença lectínica em uma amostra foi proposto por Tao et al (2009) avaliando a lectina obtida de *Viscum álbum* (ML-1), conhecida como Erva-de-passarinho européia. Segundo o autor, os métodos utilizados para detectar ML-1 incluem análises cromatográficas, onde a cromatografia em HPLC necessita de vários passos complexos para ocorrer reprodutibilidade do processo de

purificação e obtenção, bem como ensaios enzimáticos, nos quais são utilizados um grande número de reagentes. Segundo estes autores, o ensaio designado “surface plasmon resonance (SPR)" não requer técnicas refinadas e demora menos tempo para confirmação da presença lectínica na amostra (Soro humano, drogas injetáveis e soluções). A reação emprega interações termodinâmicas entre a ligação lectina-carboidrato, bem como imunoensaios e a visualização da reação ocorre em “real time”.

1.6 Caracterização de lectinas

A eletroforese é uma técnica importante para a separação de biomoléculas, baseada na migração de moléculas carregadas em um campo elétrico. A sua vantagem para as proteínas é que podem ser visualizadas e separadas, permitindo estimar rapidamente o número de proteínas distintas em uma mistura ou o grau de pureza de uma preparação protéica particular. Além disso, permite a determinação de propriedades importantes de uma proteína, como o seu ponto isoelétrico e a massa molecular aproximada. A eletroforese de proteínas é geralmente executada em géis de um polímero que apresenta ligações cruzadas, a poliacrilamida, agindo como uma peneira molecular, na proporção aproximada de sua razão entre carga e massa.

Um método eletroforético comumente usado para estimar a pureza e o peso molecular utiliza o detergente Dodecil-Sulfato de sódio (SDS). O SDS liga-se à maioria das proteínas, e esta ligação contribui com uma grande carga negativa líquida, fazendo com que a carga da proteína se torne insignificante e conferindo a cada proteína uma razão entre carga e massa semelhante. A eletroforese na presença de SDS separa, portanto, as proteínas quase que exclusivamente com base em sua massa molecular. A focalização isoelétrica é um procedimento utilizado para determinar o ponto isoelétrico (pI) de uma proteína. Quando uma mistura protéica é aplicada, cada proteína irá migrar até alcançar a região de pH igual ao seu pI. Combinando-se a focalização isoelétrica com a eletroforese na presença de SDS de modo seqüencial em um processo denominado Eletroforese bidimensional, podem-se resolver misturas complexas de proteínas. Este é um método analítico mais sensível do que qualquer outro método eletroforético aplicado de forma isolada.

Outra técnica envolvendo caracterização de lectinas detém-se a especificidade das lectinas para distintos eritrócitos e inibição por carboidratos e/ou glicoproteínas. Testes de inibição da Atividade Hemaglutinante (AH), fazendo uso de monossacarídeos,

dissacarídeos ou carboidratos complexos são freqüentes na caracterização de lectinas, desde que a especificidade é um critério para classificar lectinas de plantas em grupos de especificidades (Peumans, 1998).

As estruturas protéicas adquirem a sua função em um meio celular específico e, diferentes condições daquelas presentes no meio podem promover a desnaturação da biomolécula. Em questão estão os fenômenos físico-químicos relacionados à estabilidade das lectinas em diferentes condições de pH e temperatura. O estado desnaturado não necessariamente corresponde a um desenovelamento completo da proteína. A maioria das proteínas pode ser desnaturada pelo calor que afeta as interações fracas. Sendo assim, as proteínas podem ser consideradas termossensíveis (a maioria) e termoestáveis. O processo de desnaturação pode ocorrer também, por extremos de pH, por certos solventes orgânicos miscíveis com a água, como o álcool ou a acetona, por certos solutos como uréia e cloridrato de guanidino ou por detergentes. Os solventes orgânicos, a uréia e os detergentes atuam principalmente promovendo o rompimento das interações hidrofóbicas que estabilizam as proteínas globulares. Os extremos de pH alteram a carga líquida provocando a repulsão eletrostática e o rompimento de algumas ligações de hidrogênio.

1.7 Influência do Ciclo Sazonal nas propriedades físico-química da planta

As plantas são sésseis e como na maioria dos casos vivem em ambientes sazonais, estocar nutrientes é um processo importante e decisivo na sua sobrevivência. Segundo Martins *et al.* (2007), podem-se distinguir algumas classes de estocagem, como por exemplo: acúmulo de compostos que não promovem crescimento direto; compartimentação regulada metabolicamente ou síntese de dois compostos de reserva a partir de recursos que podem levar ao crescimento direto; reutilização de compostos que possuem função fisiológica imediata e contribuem para o crescimento ou defesa, e podem ser quebrados para sustentar um crescimento futuro.

Os vegetais acumulam matéria seca na forma de carboidratos, proteínas e lipídeos, visando assegurar o suprimento de esqueletos de carbono e energia química para o crescimento ou manutenção, quando não há produção de fotoassimilados (Buckeridge *et al.,* 2004). Este acúmulo pode ocorrer em diversos tecidos e órgãos, incluindo brotos, folhas, galhos, caules, raízes, sementes e frutos. Ao longo de seu ciclo

de vida, o crescimento e desenvolvimento da planta são influenciados pelo ciclo sazonal, implicando em uma mudança regular no ambiente e em respostas biológicas condicionadas por este. Variações sazonais na disponibilidade de água, luz e nutrientes em florestas tropicais possuem o potencial para limitar a produtividade das plantas (Newell & Mulkey, 2002). Uma resposta comum à variação temporal na disponibilidade de recursos é a remobilização de compostos de carbono e nutrientes internos, acumulados quando abundantes e estocados até que sejam necessários. Isto permite às plantas serem parcialmente independentes da disponibilidade externa de nutrientes (Cherbuy *et al.,* 2001). A remobilização interna inclui tanto a recirculação de reservas após a estocagem quanto a reciclagem dos tecidos senescentes, acarretando no re-uso de compostos. A captura de carbono é feita pelas folhas enquanto a de água e nutrientes minerais é feita pelas raízes, implicando no favorecimento da alocação de biomassa nas folhas quando houver limitação de luz e no favorecimento das raízes quando nutrientes minerais se tornarem limitantes para o crescimento (Shipley & Meziane, 2002).

1.8 Lectinas: Ferramentas nas áreas da Medicina e da Biotecnologia

Meados do século XIX, e mais precisamente no século XX, dezenas de lectinas foram purificadas dos mais variados tecidos vegetais (Van Damme et al., 1991) e o uso na área da medicina e biotecnologia demonstrou ser uma ferramenta importante no tratamento e diagnóstico de doenças. No ano de 1995, Mody e colaboradores publicaram a grande diversidade do uso de lectinas no campo da cancerologia, e atualmente, novos avanços relacionados a este tema têm repercutido de forma esperançosa para pacientes acometidos por diversos tipos de neoplasias (Tabela 2). Segundo Jemal et al (2006), o câncer Colo-retal é a principal causa de mortalidade e morbidade nos países desenvolvidos, e métodos como a colonoscopia, apesar de avançados, não garantem 100% o diagnóstico. O uso da lectina obtida de amendoim (Peanut aglutinina, PNA) imobilizada em nanoesferas, obteve um efeito positivo na identificação de células cancerígenas nessa região do intestino, através de um método fluorescente para atuar como adjuvante no diagnóstico do câncer Colo-retal (Sakuma et al., 2009). Outra freqüente atuação das lectinas como marcado histoquímico de tecidos que sofreram transformações neoplásicas foi constatado por Beltrão et al (2003) no uso da lectina purificada de *Parkia pendula*, onde a lectina atuou como uma ferramenta para o diagnóstico clínico-patológico e caracterização do tumor meningocelial.

As lectinas apresentam consideráveis diferenças em suas estruturas protéicas, características e conseqüentemente em suas propriedades biológicas. Devido a sua capacidade em detectar diferenças sutis entre os complexos de carboidratos presentes no meio extracelular e na superfície de células, e a sua estabilidade (boa resistência aos extremos de pH e degradação por enzimas proteolíticas presentes no trato gastrointestinal), as lectinas tornaram-se proteínas valiosas para detecção, isolamento e caracterização de glicoconjugados; para a citoquímica e a histoquímica; para identificação das mudanças ocorridas nas superfícies das células durante os processos fisiológicos e patológicos; para diferenciação celular em processos cancerígenos e para o estudo de processos imunológicos e inflamatórios (Sharon, 2007).

Tabela 2. Evolução do uso das lectinas como ferramentas no diagnóstico e terapia do Câncer.

Lectinas
Marcador de superfície de tumor (Lotan e Raz, 1988)
Terapia alvo (Monsigny et al., 1988)
Sondas em anatomia, histoquímica e citoquímica (Gabius, 1991)
Imunomodulação (Kery, 1991)
Terapia mitogênica (Ryder et al., 1992)
Terapia do cancer (Kannan et al., 1993)
Indução de atividade macrofágica em tumor necrótico (Boccil, 1993)
Liberação de superóxido em pacientes com carcinoma (Timoshenko et al., 1993)
Histoquímica de Lectinas: Progressão Tumoral (Pillai et al., 1996)
Interação Lectina-PSA como screening em câncer de próstata (Basu, 2003)
Bioreconhecimento *in vivo/in vitro* de células do câncer colo-retal (Sakuma et al., 2009)
Indução de apoptose e autofagia em melanoma humano A375 (Liu et al., 2009)
Indução de apoptose em Fibrosarcoma L929 (Liu et al., 2009)
Ação antiproliferativa em células de carcinoma cervical humano (Yan et al., 2009)
Indução na migração de neutrófilos (Figueiredo et al., 2009)
Indução na atividade de citocinas (Cheung, 2009)
Potencial Antineoplásico (Liu, 2009)
Indução de Neutropenia (Frakking et al., 2009)

Mediando os fatores imunológicos a quimiotaxia de neutrófilos é a primeira linha de defesa do organismo no processo infeccioso. Quando ocorre inflamação e infecção, moléculas de adesão aparecem na superfície das células endoteliais e se ligam as moléculas presentes na superfície dos leucócitos. Os polimorfonucleares (neutrófilos), então, migram através dos vasos sanguíneos e infiltram-se nos tecidos, processo conhecido por quimiotaxia. Segundo Toledo et al (2009), a lectina de *Artocarpus integrifolia* conhecida como ArtinM, apresentou uma potente atividade imunológica por induzir a migração de neutrófilos através de interações simultâneas de seus domínios de reconhecimento a carboidratos com glicanos expressados na matriz extracelular e na superfície de neutrófilos. Além disso, observou-se ainda que a lectina em contato com os neutrófilos aumentou o processo de fosforilação do aminoácido tirosina das proteínas intracelulares estimulando o processo de fagocitose. Outras lectinas estão incluídas entre os múltiplos fatores quimiotáticos desempenhados na ativação de neutrófilos: concanavalin-A (Con A), lectina de gérmen de trigo (WGA), selectinas, e algumas lectinas pertencentes à família das galectinas.

As lectinas já foram identificadas nos mais variados tecidos vegetais incluindo folhas (Sisenando et al., 2009), raízes e caules (Rameshwaram e Nadimpalli, 2008; Yan et al., 2009), rizomas (Tian et al., 2008), entrecascas (Nascimento et al., 2008), frutos (Cheung, 2009) e sementes (Santos et al., 2009; Chen et al., 2009). Esta larga distribuição sugere um importante papel que estas possam desempenhar no que diz respeito ao sistema de defesa da planta contra fitopatógenos, insetos, fungos e bactérias. Uma característica marcante observada nessa classe de proteínas encontrada em plantas está relacionada com sua habilidade em ”sobreviver” ao processo digestivo no trato gastrointestinal. Essa característica permite que as lectinas possam reconhecer e se ligar aos grupos glicosil presentes na superfície das células que compõem o aparelho digestivo. No local, as lectinas podem provocar distúrbios nos processos de digestão e absorção, e assim alterar a microbiota existente, ativando de forma indireta o sistema imune do consumidor, e por sua vez tornando-se um fator antinutricional. Por exemplo, os efeitos tóxicos de PHA, lectina de *Phaseolus vulgaris*, no sistema digestivo de alguns animais, insetos e nematódeos, são bem conhecidos, resultando em proteção para a planta (Vasconcelos e Oliveira, 2004). Outro dado interessante, relacionado ao fator antinutricional envolvendo as lectinas, foi observado por Gilbert (1988), onde 31 pessoas passaram mal, após almoçarem em um restaurante, no qual foi servido feijão vermelho. Os testes bioquímico-microbiológicos não identificaram a presença de

patógenos (fungos e bactérias), no entanto evidenciaram uma concentração alta de PHA, lectina presente no feijão.

Segundo Silva et al (2009), as lectinas têm apresentado efeitos em diferentes estágios de crescimento de muitos insetos: *Coleoptera* (Macedo et al., 2007), *Diptera* (Sa´ et al., 2008b), *Lepidoptera* (Coelho et al., 2007), *Nasutitermes corniger* (Sa´ et al., 2008a). Em seu estudo, a lectina obtida de *Cladonia verticilaris*, líquen, demonstrou atividade inseticida frente a *N. corniger* (cupim). Apesar de não se conhecer precisamente o mecanismo de ação inseticida das lectinas, tem-se sugerido que os modos de ação possam incluir toxicidade celular, e ainda alteração na função digestiva, uma vez que as lectinas podem atuar como inibidores de proteases, reduzindo, assim, os processos de digestão e absorção, ocasionando a morte do inseto (Coelho et al., 2007; Sa´ et al., 2008b). O uso de lectinas de plantas para o controle biológico de insetos torna-se viável diante do vasto potencial encontrado na flora mundial, bem como na redução de produtos químicos utilizados para esses fins.

As plantas requerem uma vasta quantidade de mecanismos de defesa que sejam eficazes no combate a patógenos microbianos, fungos e bactérias, promovendo assim resistência a invasão. Algumas respostas são constitutivas e não específicas, mas a maioria delas induz a um reconhecimento do patógeno. Moléculas liberadas pelo agente invasor promovem uma resposta da planta causando reforço na estrutura da parede celular. Entretanto, importantes grupos de proteínas antimicrobianas responsáveis, em parte, pelo sistema de defesa da planta, não são induzidas pelo ataque do patógeno, são elas: as lectinas e os peptídeos ricos em cisteína (Ghosh, 2009). Sendo assim, artigos relatando ação antimicrobiana de lectinas de plantas, independente do material escolhido, ou seja, folha, raiz e entrecasca, tornam-se facilmente encontrados na literatura (Sitohy, 2007; Tian et al., 2008; Ghosh, 2009; Chen et al., 2009).

1.9 Família Loranthaceae

A família Loranthaceae reúne, aproximadamente, 70 gêneros e 950 espécies, sendo encontrada de forma abundante em regiões tropicais (Ribeiro *et al*, 1999). Estas plantas são popularmente conhecidas no Brasil como "Erva-de-Passarinho", pois dependem dos pássaros para se dispersarem. Há, somente, uma espécie conhecida que se dissemina utilizando os Marsupiais como vetor (Amicoe & Aizen, 2000). Loranthaceae são espécies hemiparasitas que vivem no alto ou em torno do tronco das

árvores tropicais, por exemplo, *Bauhinia monandra* (Norton & Carpenter, 1998). Estes parasitas invadem o xilema do hospedeiro usando uma estrutura especial, o haustório, para obter água e sais minerais (Calvin & Wilson, 2006) de forma similar como descrito em Viscaceae (Zuber, 2004). Contudo, em algumas espécies de Loranthaceae há pequena quantidade de corpo vegetal (e.g. Tristerix aphyllus) e a penetração no hospedeiro ocorre somente na região do floema (Medel et al., 2002). Apesar de possuírem a propriedade de fotossíntese, a presença da Erva-de-passarinho pode causar danos à planta hospedeira afetando a qualidade e a quantidade de produção de frutos e até ocasionar a morte da planta (Sinha & Bawa, 2002). As espécies de Loranthaceae têm sido identificadas como pestes na agricultura e em sistemas agro-econômicos em todo o mundo (Norton & Carpenter, 1998). No entanto, vários tipos podem ser considerados "espécies chave" para animais que se alimentam do néctar e frutos (Aukema, 2003). Em 1920, Rudolf Steiner fez uma extraordinária predição sobre o uso de plantas hemiparasitas, acreditando que através da forma parasitária, o seu uso proveria algum valor medicinal contra patologias em especial o câncer. Desde então, considera-se o uso da Erva-de-Passarinho na medicina convencional, com muitos produtos e extratos agora utilizados na terapia do câncer, bem como na hipertensão, arteriosclerose e artrite. Compostos e produtos (viscotoxinas e lectinas) da Erva-de-Passarinho *Viscum album* têm sido avaliados nas propriedades contra o câncer, atividade citotóxica e imuno-modulatória incluído em Bussing (2000).

Phthirusa pyrifolia

Há poucos relatos na literatura sobre o gênero *Phthirusa.* A espécie *Phthirusa pyrifolia* (H. B. K.) Eichl, planta hemiparasita que pertence à família Loranthaceae (Erva-de-Passarinho), originária do Brasil, tem o seu uso na medicina popular recomendada em tratamento de afecções respiratórias como bronquite, tosse, pneumonia, e também para coqueluche, hepatite e atividade antiulcerogênica.

Uma característica marcante desta espécie é observada em seus haustórios, que apresentam plasticidade em suas dimensões e inserções, facilitando a sua adaptação em hospedeiros. Além disto, a grande quantidade de haustórios (Figura 3A), inflorescências (Figura 3B) e frutos (Figura 3C e 3D) gerados permitem uma ampla dissipação da espécie. Estas respostas adaptativas permitem que *P. pyrifolia* parasite uma grande variedade de plantas (Montilla, M.; A. A Zocar & G. Goldstein, 1989).

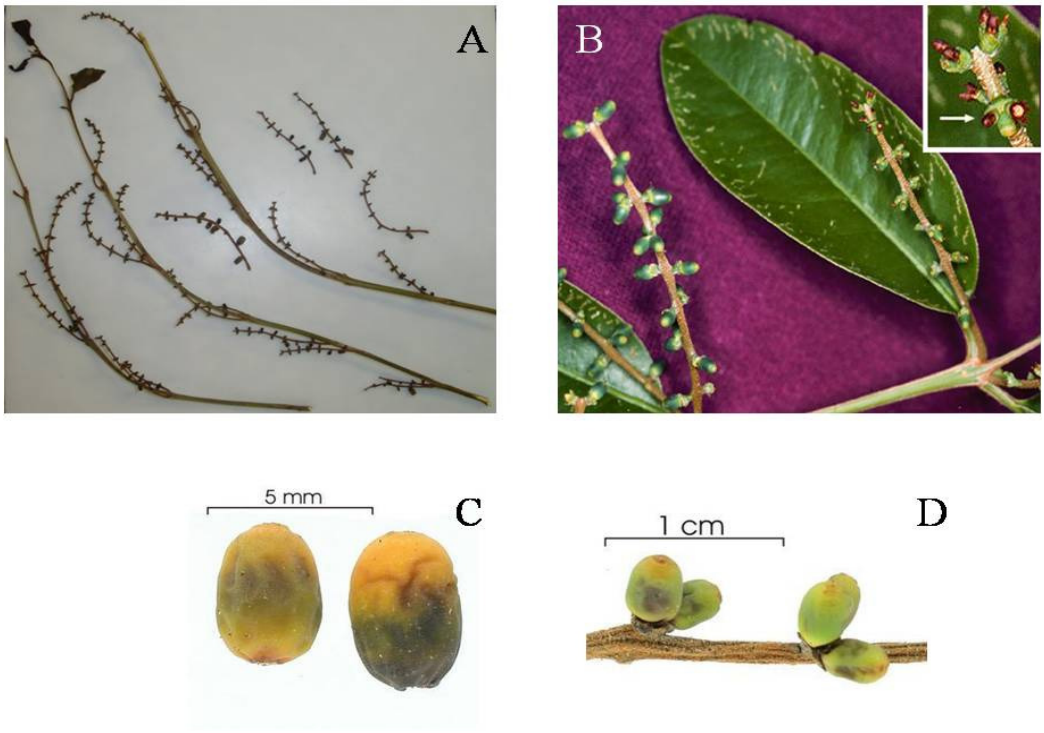

Figura 3. ***Phthirusa pyrifolia*****. (A)** Haustórios; Inflorescências (**B**); Fruto imaturo (**C**) e Fruto maduro (**D**).

2. RELEVÂNCIA

A investigação das possíveis atividades biológicas das lectinas aumentou nos últimos anos diante dos diversos resultados positivos que estas têm apresentado à comunidade científica. Extratos aquosos de uma espécie de Erva-de-passarinho européia têm sido largamente utilizados como uma terapia alternativa de doenças malignas em pacientes com mais de 70 anos. A pesquisa dos compostos presentes nos extratos aquosos de Ervas-de-Passarinho, bem como a atividade biológica dos seus constituintes, dentre eles, as lectinas, ganharam uma atenção especial pela sua capacidade imunomodulatória por aperfeiçoar a citotoxicidade das células Natural Killer e estimular a secreção de citocinas. Além disto, as lectinas de Erva-de-Passarinho demonstraram ter citotoxicidade e propriedades de indução na apoptose de uma grande variedade de células tumorais *in vit*ro e ainda ser adjuvante no tratamento do câncer de bexiga. Visando conhecer melhor as substâncias contidas nesta planta, o presente trabalho teve como objetivos a Purificação, Caracterização parcial e Aplicações Biomédicas de uma lectina extraída de folhas de *Phthirusa pyrifolia* (H. B. K.) Eichl com vistas em inserir esta nova lectina como ferramenta futura no campo biotecnológico.

3. OBJETIVOS

Objetivo geral

Isolar, Purificar e Caracterizar lectinas de folhas de *Phthirusa pyrifolia* (H.B.K.) Eichl.

Objetivos específicos

Obter o Extrato Bruto (EB) utilizando homogeneizado de folhas;

Avaliar a influência do Ciclo sazonal na Atividade Hemaglutinante;

Purificar parcialmente a lectina por fracionamento com sulfato de amônio;

Avaliar a inibição da Atividade Hemaglutinante (AH) da lectina do Extrato Bruto e da Fração parcialmente purificada utilizando carboidratos e glicoproteínas;

Purificar a lectina por diferentes processos cromatográficos: Gel filtração, Afinidade, Troca iônica, HPLC e FPLC;

Caracterizar parcialmente a lectina quanto à especificidade a carboidratos ou glicoproteínas, estabilidade térmica, especificidade para eritrócitos, estabilidade frente a variações de pH, influência de íons bivalentes;

Caracterizar a lectina sobre o aspecto molecular através de SDS-PAGE, PAGE em condição nativa, PAGE sob diferentes colorações (Schiff, Prata e Azul de Comassie);

Avaliar o efeito da temperatura sobre o Espectro de fluorescência dos aminoácidos Triptofano e Tirosina presentes na Lectina;

Avaliar a Atividade Antibacteriana (Concentração Mínima Bactericida e Concentração Mínima Bacteriana);

Avaliar a Atividade Antifúngica (Concentração Mínima Fungicida e IC_{50}).

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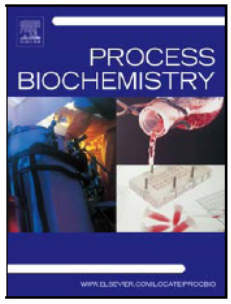

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A New Mistletoe *Phthirusa pyrifolia* Leaf Lectin with Antimicrobial Properties

Romero M.P.B. Costa [a, b], Antônio F.M. Vaz [a], Maria L.V. Oliva [c], Luana C.B.B. Coelho [a], Maria T.S. Correia [a], Maria G. Carneiro-da-Cunha [a, b (*)].

[a] Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego s/n, CEP 50.670-420 Recife, PE, Brazil

[b] Laboratório de Imunopatologia Keizo Asami–LIKA/UFPE, CEP 50.670-901, Recife, PE, Brazil

[c] Departamento de Bioquímica, Universidade Federal de São Paulo, CEP 04.044-020, São Paulo, Brazil

(*) Author for correspondence (Tel: +55.81.2126.8540; Fax: +55.81.21268576; E-mail: mgcc@ufpe.br).

Abstract

Phthirusa pyrifolia leaf lectin (PpyLL) was obtained from the hemi-parasitic medicinal plant mistletoe through saline saturation and two consecutive chromatographic steps on Sephadex G100 and ion exchange on CM-cellulose. SDS-PAGE of the protein under non-reducing conditions revealed a monomeric protein with a molecular weight of 15.6 kDa. Under reducing conditions in the presence of 2-mercaptoethanol, the protein showed two bands with molecular weights of 15 kDa and 7 kDa. PpyLL, an acidic glycoprotein with 19% sugar content, was not dependent on divalent cations. It was stable up to 70^{o}C and exhibited maximum hemagglutination at pH 7.5. Lectin fluorescence emission spectra at different temperatures showed that the lectin fluorescence increased when the temperature increased. PpyLL showed a unique affinity for the phosphate derivative of fructose, fructose-1-6-biphosphate. PpyLL showed effective antimicrobial activities against bacteria (Gram-positive: *Staphylococcus epidermidis*, *Streptococcus faecalis* and *Bacillus subtilis*; Gram-negative: *Klebsiella pneumoniae*) and fungi (*Fusarium lateritium* and *Rhizoctonia solani).* Therefore, PpyLL specificity, as determined by a new sugar affinity, may be significant to determine its biological potential.

Key-words: Lectin; *Phthirusa pyrifolia*; mistletoe; chromatography; fluorescence; antimicrobial activity.

1. Introduction

Lectins are naturally occurring proteins/glycoproteins with substantial structural diversity; they bind carbohydrate residues selectively and non-covalently and are involved in various biological processes. Lectins recognize sugar determinants in the wall or in the capsule of bacteria and have been suggested to participate in the innate immune response. This may be accomplished by inducing bacterial agglutination or acting as opsonins, which enhances the phagocytosis rates of microorganisms [1]. Some lectins may have affinity for carbohydrates but are not specific; it is not clear how lectins can identify several sugars present in different positions in bacteria, fungi, enzymes and other glycoproteins. The usual methods applied to purify lectins are saturation with ammonium sulfate, affinity chromatography [2], ion-exchange chromatography [3] and liquid-liquid extraction using reversed micelles [4]. Recently, lectin biology has applied these tools in the biomedical field and also in the treatment of diseases, including cancer [5]. Another application reported in the literature involving lectins is their antimicrobial activity [6], where lectins may act against microorganisms by interfering with their growth and playing a role in defense systems [7].

Mistletoes comprise about 900 species in 70 genera that are mainly distributed in tropical areas of Africa, Southwest Asia and South America [8]. *Phthirusa pyrifolia*, well known in Brazil as “erva-de-passarinho”, belongs to the Loranthaceae family and is a hemi-parasite plant that parasitizes a broad range of gymnosperms and angiosperms. The traditional medicine in developing countries uses a wide variety of natural products in the treatment of common infections. Research on the compounds present in aqueous extracts from mistletoe, including lectins, and their biological properties gained special attention due to their immune modulated capacity for improving natural killer cell

activities. Moreover, mistletoe lectins participate in the apoptosis of tumor cells *in vitro* and act as adjuvants in bladder cancer treatment [9]. However, not much is known about mistletoe lectin. The European mistletoe *Viscum album* is a hemi-parasitic plant that is widely spread over Europe and parts of Asia and contains at least three lectin isoforms (ML-I, ML-II and Ml-III); it has been suggested that ML-I is part of a defense system against insects, bacteria and fungi [10]. Mistletoes usually have slower rates of photosynthesis than their hosts. Seasonal variations in the availability of water, light and nutrients in tropical forests have the potential to limit the productivity of the plant components [11].

In this report, we described the purification and characterization of a novel lectin from *P. pyrifolia* leaves with the intention of expanding the understanding of mistletoe lectins.

2. Materials and Methods

2.1. Chemicals

Sugars (N-acetyl-D-glucosamine, D-arabinose, D-mannose, L-fucose, L-raffinose, D-galactose, D-fructose, D-glucose, sucrose, lactose, L-threalose, D-xylose, L-rhamnose, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, glucose-6-phosphate, fructose-1,6-biphosphate), glycoproteins (casein, azocasein, asialofetuin, fetuin, albumin from chicken egg white and bovine serum albumin) and gel matrices (CM-cellulose and Sephadex G100) were purchased from Sigma Chemical Company (USA). All reagents were of analytical grade.

2.2. Extract preparation

To evaluate the seasonal influence on the lectin content, once a month for two years the green leaves of *P. pyrifolia* L. were harvested from the top of the host plant, the *Bauhinia monandra* tree, at the campus site of Federal University of Pernambuco, Brazil. After that, the stalks were removed, and the leaves were briefly washed with distilled water and left to dry at 25^0C for 3 days. Dried leaves were then powdered, homogenized in 10% (w/v) 0.15 mol L^{-1} NaCl and maintained under agitation for 4 h at 4^oC. Afterwards, the mixture was filtered through gauze and centrifuged at 11,180 x *g* for 15 min. From all supernatants (extracts) obtained in the research period, the one that demonstrated the higher value of specific hemagglutinating activity (SHA) was termed the crude extract (CE).

2.3. Hemagglutinating activity and hemagglutinating activity inhibition

Hemagglutinating activity *(*HA) was assessed in microtiter plates according to Correia and Coelho [12]. Briefly, lectin preparations (50 μL) were two-fold serially diluted with 0.15 mol L^{-1} NaCl before the addition of 2.5% (v/v) suspension (50 μL) of rabbit, chicken or rat erythrocytes treated with glutaraldehyde or fresh human erythrocyte suspensions (A, B, O and AB). HA was defined as the inverse of the titer; this corresponded to the lowest sample dilution showing full hemagglutination and was examined visually through the absence of erythrocyte precipitation after incubation for 45 min. The SHA was determined by the ratio of HA by protein concentration. Rabbit erythrocytes were chosen for subsequent assays. HA inhibition in the presence of several sugars or glycoproteins was used to determine the lectin carbohydrate binding specificity.

2.4. Purification of Phthirusa pyrifolia leaf lectin

CE was 20%-saturated with ammonium sulfate for 4 h and centrifuged at 11,180 x *g* for 15 min. The precipitate was stored, and the supernatant was adjusted to 20-40% saturation. This procedure continued until 100% saturation. The highest SHA saline fraction was chosen, dialyzed against water, and used for the chromatographic procedures. Protocol purification comprised two steps. Initially, sample (1 mL) containing 24 mg mL^{-1} of protein was applied to a Sephadex G100 column (40 x 2 cm) previously equilibrated with 0.15 mol L^{-1} NaCl at a flow rate of 10 mL h^{-1}. After collecting unabsorbed samples (less than 0.03 absorbance at 280 nm), the column was washed with 0.3 mol L^{-1} glucose solution, and adsorbed fractions with HA (Sephadex Active Peak – SAP) were pooled, dialyzed against water, lyophilized, suspended in 0.15 mol L^{-1} NaCl (2 mL) and submitted (7 mg mL^{-1}) to ion-exchange chromatography on CM-cellulose. This column (10 mL) was equilibrated with 0.15 mol L^{-1} NaCl, and the lectin was eluted with 0.5 mol L^{-1} Tris-HCl, pH 8.5 buffer solution at a flow rate of 20 mL h^{-1}. Once again, adsorbed fractions with HA were pooled, dialyzed against water and termed *P. pyrifolia* leaf lectin (PpyLL). To analyze the purification protocol efficiency, PpyLL was chromatographed on a Superdex 75 HR 10/30 column followed by C-18 column. Lyophilized PpyLL suspended in 0.15 mol L^{-1} NaCl (1 mg mL^{-1}) was loaded onto a Superdex 75 HR 10/30 column coupled to a ÄKTA purifier system, equilibrated and eluted (1 mL of fractions) with 0.3 mol L^{-1} NaCl at 0.5 mL min^{-1}. The fractions with HA (1 mL) were pooled (0.6 mg mL^{-1}) and submitted to reverse phase chromatography in a C-18 column HPLC system. The column was equilibrated with solvent A [0.1% trifluoroacetic acid (TFA) in H_2O] and eluted using solvent B (90% acetonitrile: 10% H_2O: 0.1% TFA) in non-linear gradient, where B = 5% at t = 5 min; B = 70% at t = 27 min; B = 80% at t = 60 min and B = 100% at t = 69 min.

2.5. Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed according to Laemmli [13] using 10% (w/v) gel for SAP and 12% (w/v) gel for PpyLL. Protein samples were submitted to reducing and non-reducing conditions in the presence or absence of 2-mercaptoethanol reducing agent, respectively. Standard marker proteins [bovine serum albumin (BSA), 66 kDa; albumin from egg white, 45 kDa; carbonic anhydrate, 29 kDa; lysozyme, 14.3 kDa, purchased from Sigma (USA)] were used. Polypeptide bands were stained with Coomassie Brilliant Blue (CBB) R-250, silver stained (sensitive method for proteins) and periodic acid Schiff stained (method to identify glycoprotein). PAGE for native basic [7.5% (w/v) gel] and acidic [10% (w/v) gel] proteins were performed according to Reisfeld et al. [14] and Davis [15], respectively.

2.6. Protein assay and neutral carbohydrate analysis

The protein content was spectrophotometrically determined according to Lowry et al. [16] using a BSA calibration curve as the standard (0 - 500 μg mL^{-1}). The neutral carbohydrate contents of the purified lectin were determined by the phenol–sulfuric acid method in microplate format according to Masuko et al. [17] using a mannose calibration curve as the standard (0 – 100 μg mL^{-1}).

2.7. Effect of mono and divalent ions, pH and temperature

Samples of PpyLL (250 μg mL^{-1}) with 512 HA were used to evaluate the effect of metal ions, pH and temperature on PpyLL HA.

The effect of mono (Na^+ and K^+) and divalent metal ions (Ca^{2+}, Mg^{2+} and Mn^{2+}) on HA was performed according to Pajic et al. [18]. The purified lectin solution was

previously dialyzed against 0.025 mol L^{-1} EDTA (16 h at 4°C) and then dialyzed against deionized water (8 h at 4°C). Afterwards, the HA was performed in microtiter plates with dialyzed lectin preparation (50 µL) serially two-fold diluted in 0.02 mol L^{-1} of the different ions (50 µL) prepared in 0.15 mol L^{-1} NaCl. The microtiter plate was standing at room temperature (25°C) for 45 min before the addition of a 2.5% (v/v) erythrocyte suspension (50 µL).

The effect of pH on the HA of purified lectin was carried out in 0.01 mol L^{-1} buffers that differed in terms of their pKa value (citrate phosphate, pH 3.5-6.5; sodium phosphate, pH 7.0; Tris-HCl, pH 7.5-9.5; glycine-NaOH, pH 10-12). The effect of temperature on HA was evaluated by incubating 1 mL of purified lectin at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C for 30 min [6].

2.8. Fluorescence spectrum by heating

Lectin fluorescence emission spectra were obtained at 25°C (± 1°C) using a JASCO FP-6300 (Tokyo, Japan) spectrofluorimeter. A slit width of 5 nm was used on the excitation and emission monochromators. The evaluation of the thermal stability of lectin fluorescence intensity was performed by incubating lectin samples (200 µg mL^{-1} in 10 mmol L^{-1} phosphate buffer, pH 7.0) at different temperatures (50ºC to 100ºC) for 40 min and after cooling at room temperature (25°C). Measurements were performed by lectin excitation at 280 nm to selectively excite tyrosine and tryptophan residues of the protein, and the emission spectra were recorded over the range of 305–450 nm. The contribution of the buffer was always subtracted. The aromatic amino acids tyrosine and tryptophan are moderately polar, and their contribution to the emission spectra were calculated by subtracting the emission spectrum measured at λ_{exc} 280 nm multiplied by a factor from that measured at λ_{exc} 275 nm. The factor was obtained from the ratio

between the fluorescence intensities measured with λ_{exc} 275 and λ_{exc} 280 nm at wavelengths above 380 nm. All measurements were performed in duplicate at 25°C.

2.9. Antibacterial activity

Bacteria were provided by the Department of Antibiotics (DA), Universidade Federal de Pernambuco (UFPE), Brazil in Difco™ Nutrient Agar (NA) and stored at 4°C. Gram-positive strains were *Staphylococcus aureus* (ATCC 6538), *Staphylococcus epidermidis* (UFPEDA9), *Streptococcus faecalis* (ATCC 6057), and *Bacillus subtilis* (UFPEDA16), and Gram-negative strains were *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (ATCC 29665). Bacteria were grown in shaker flasks (250 mL) containing Difco™ Nutrient Broth (NB) and incubated overnight in a orbital shaker at 100 rpm and 37°C. The biomass concentration was determined by measuring the suspension turbidity at 600 nm and then converted to colony forming units (10^5–10^6 CFU mL^{-1}) using appropriate calibration curves (turbidity equivalent to 0.5 in the McFarland scale). One CFU represents a viable cell capable of promoting bacterial growth. Lectin antibacterial activity was investigated by the disc diffusion method [19]. One-hundred millilitres of warm NA (43°C) and 0.5 mL of bacteria suspensions (10^5–10^6 CFU mL^{-1}) were mixed, and 10 mL volumes were distributed in sterile Petri plates (90x15 mm) and allowed to solidify. Sterile blank paper discs (6 mm diameter) impregnated with 15 μL of sterile lectin solution (5.3 mg mL^{-1} in 0.15 mol L^{-1} NaCl) was added on the center agar plates. A positive control was carried out with discs of the antibiotics chloramphenicol (100 μg) for Gram-negative bacteria and vancomycin (30 μg) for Gram-positive bacteria, and a negative control was discs with 0.15 mol L^{-1} NaCl (15 μL). Plates were incubated at 37°C for 24 h. A transparent ring around the

paper disc revealed antimicrobial activity. Zones of growth inhibition around discs were measured in millimeters.

The minimum inhibitory concentration (MIC) corresponded to the minimum lectin concentration that inhibited visible bacterial growth. MIC was determined by the dilution tube test [20]. Briefly, serial dilutions of purified lectin in 0.15 mol L^{-1} NaCl were prepared and added to the bacteria cultures (in NB) containing 10^5–10^6 CFU mL^{-1} in the exponential growth phase. The samples were incubated for 24 h at room temperature (25°C). Afterwards, cultures were seeded onto NA plates and incubated for 24 h at 37°C. The minimum bactericidal concentration (MBC) corresponded to the minimum concentration of the lectin that inhibited 100% growth [21].

Inhibitory assays on bacterial growth were also performed along the time in buckets of sterile plastic according to the modified method of Gaidamashvili and Staden [22] using a Smart 3000 spectrophotometer at 600 nm wavelength light. To each bucket was added 800 µL of bacteria cultures (in NB) in the exponential growth phase plus 200 µl of lectin solution (0.5 µg mL^{-1} in 0.15 mol L^{-1} NaCl), and the absorbance at 600 nm was determined every hour for 6 hours. The buckets were kept at 37°C, and the content was homogenized manually before each reading. The assays were carried out with four replicates. A positive control was carried out using bacterial suspensions in NB, and a negative control was carried out using lectin solution in NB.

2.10. Antifungal activity

Aspergilus niger (URM2813), *A. flavus* (URM2814), *A. fumigatus* (URM2815), *Rhizopus arrhizus* (URM2816), *Paecilomyces variotti* (URM2818), *Fusarium moniliforme* (URM2463), *F. lateritium* (URM2665), *Candida albicans* (UFPE-DA1007), *C. burnenses* (UFPEDA4674), *C. tropicalis* (URM1150), *C. parapsilosis*

(URM3624), *Saccharomyces cerevisae* (UFPEDA5107), and *Rhizoctonia solani* (URM 2820) were obtained from the Cultures Collection "Micoteca" (URM) of the Department of Mycology and from the Department of Antibiotic (DA), Universidade Federal of Pernambuco (UFPE), Brazil.

Fungi were grown at 28°C on potato dextrose agar (PDA) plates for an 8-15 day period until the surfaces of the plates were completely covered by them. Afterwards, fungal mycelium discs with a 6-mm diameter were removed from the peripheral part of the colonies, placed in the center of the PDA plate and incubated for 48 h. After the mycelia colony had developed, sterile blank paper discs (6 mm in diameter) were impregnated with 15 μL of lectin solution (6.5 mg μL^{-1}) and deposited at a distance of 5 mm away from the mycelial colony. Cercobin (10 μg) was used as a positive control, and 0.15 mol L^{-1} NaCl was used as a negative control. The plates were incubated at 28°C for 72 h, and the antifungal activity was observed as an inhibition line forming around the disc (23). To determine the concentration required to produce 50% inhibition of mycelial growth (IC_{50}), PDA plates (40x10mm) were prepared with different concentrations of lectin (0 - 1 mg mL^{-1}), and a small amount of mycelia was placed in the center of each plate and incubated at 28°C for 48 h. The area of the mycelial colony was measured (in millimeters), and the IC_{50} was calculated.

3. Results and Discussion

3.1. Seasonal effects on lectin extraction

Seasonality is a factor that acts directly on photosynthesis in plants. Plants use a variety of ways to regulate foliar light absorption, thereby preventing light energy absorption in excess, which may cause modifications to the synthesis of biomolecules.

Considering that plant lectins have been purified and broadly used in biotechnological applications, our investigation of seasonal dynamics in the *P. pyrifolia* leave extracts under monthly seasonal evaluation by SHA analysis revealed a large variation between heavy rain and drought periods. During the experiment, it was observed that in May and June, the rainy period, no lectin activity was detected (Fig. 1). We suggested that the rainy time may have limited the use of light energy by the plant. According to Newell & Mulkey [11], seasonal variations in water, light and nutrient availability may limit the productivity of the plants, thereby altering the production of biomolecules. SHA was higher in March, so this extract (CE) was chosen for the experiments in this work. A different response was obtained in the seasonal protein variation of European mistletoe; expression was enhanced during the winter season [24]. Leaves of *Hymenaea courbaril* have also been shown to change the contents of glucose and fructose under different climatic periods, revealing another association between seasonality and the production of compounds by plants [25].

3.2 Purification and partial characterization of Phthirusa pyrifolia leaf lectin (PpyLL)

Among the fractions saturated with ammonium sulfate from CE, F20-40% showed the most SHA and was, therefore, chosen for the lectin purification protocol using Sephadex G-100 (affinity support) followed by CM-cellulose (ion-exchange matrix).

The Sephadex G-100 chromatographic step revealed a single active peak (SAP) eluted with glucose, suggesting the homogeneity of the sample (Fig. 2A). The SAP applied on the CM-cellulose column (second purification step) eluted with Tris-HCl (Fig. 2B) gave an active peak (PpyLL) with a yield of 69.37% and a 36.4-fold purification factor (Table 1), yielding a total of 28.58 mg of PpyLL from 10 g of *P. pyrifolia* leaves. This result showed that PpyLL was obtained in a higher quantity than

reported for other lectins. *Dolichos lablab* lectin yielded 15 mg of purified lectin from 100 g of *D. Lablab* vegetative tissues [26]. The purification process of four isoforms of Himalayan mistletoe from *Viscum album* gave a yield of 1.5 mg of pure lectin MBLI from 100 g of fresh weight of plant tissue [27]. Besides these two chromatographies, the PpyLL was submitted to Superdex-G75 followed by C-18 chromatography to evaluate its purity. The chromatograms indicated the really efficiency of the PpyLL purification protocol, where we observed one peak representing HA (Fig. 2C) from the Superdex-G75 chromatography and one peak from the C-18 column (Fig. 2D).

SDS-PAGE of the SAP (Fig. 3A) showed three polypeptide bands between (40-14 kDa), while PpyLL revealed a single 15.6 kDa polypeptide band (Fig. 3B). The result suggested that the Sephadex chromatography step was not enough to purify the lectin. However, this chromatography was necessary because all the pigment contained in F20-40% was present in the unabsorbed fractions. Upon reduction with 2-mercaptoethanol, this lectin consistently dissociated into two bands whose molecular weights were 15 kDa and 7 KDa (Fig. 3C). This might be due to lectin structural disulfide bridges breaking down into subunits under β-mercaptoethanol action. Similar results were reported by Lin [28] for *Bungarus multicinctus* lectin and by Mishra *et al.* [27] for the purification of four isoforms of Himalayan mistletoe when they observed that, in the absence of reducing agent only, a single band of protein was revealed in their samples. However, in the presence of a reducing agent, two polypeptides bands were visualized. PpyLL submitted to glycoprotein Schiff staining (a stain that may identify picograms of sugar in the electrophoretic procedures) after SDS-PAGE showed a single strongly stained band. According to Won-Kyo [29], *Katsuwonus pelamis* lectin is a glycoprotein result confirmed by a similar method using Schiff reagent. PpyLL has an acidic character and is seen as a single band when submitted to native PAGE. In contrast,

Himalayan mistletoe (HmRip) lectin was seen as two bands under PAGE for acidic native proteins, suggesting the presence of lectin isoforms [27].

PpyLL was not specific to human erythrocytes (types A, B, and O), and no HA was detected to human AB erythrocytes. Furthermore, PpyLL showed high affinity to rabbit erythrocytes and did not agglutinate chicken or rat erythrocytes (Table 2). According to Oliveira et al. [30], agglutination differences may be due to the nature of glycoproteins protruding from cell surfaces, which are distinctly recognized by the lectin. PpyLL showed a unique affinity for the phosphate derivative of fructose, fructose-1-6-biphosphate (12.25 mM), which has not been reported to be a lectin inhibitor but lacked affinity for glucose-6-phosphate and glucose-1-phosphate. To the best of our knowledge, the affinity for fructose-1-6-biphosphate has not been documented for other mistletoe lectins. Recognition of the sugar chains on immunologically active cells is the first step in lectin binding to induce biological activity. Like the lectins from Himalayan mistletoe, which represented a new class of plant lectins having shared affinity for L-rhamnose and galactose that are not yet reported, interestingly PpyLL does not fit into any structural class of lectins currently described. Therefore, the novel sugar affinity of PpyLL may be significant to determining its biological potential. The glycoprotein casein inhibited PpyLL activity at a lower concentration of 15.62 µg ml^{-1} (Table 3). Roman and Sgarbieri [31] reported that casein is a protein that shows in its structure a small carbohydrate fraction and an important compound with phosphorus as a constituent of the biomolecule. Thus, this property suggests the high recognition of PpyLL for this glycoprotein. PpyLL HA was neither affected by demetallization nor was it dependent on mono or divalent cations (K^+, Na^+, Ca^{2+}, Mg^{2+} and $Mn2^+$). According to Santos et al. [32], the lectin HA from *Moringa oleifera* increased in the presence of ions Mg^{2+}, Ca^{2+} and K^+. Furthermore, Sun et al. [7] revealed that the

activity of *Fenneropenaeus chinensis* shrimp plasma lectin was completely recovered by Ca^{2+} addition.

PpyLL HA was stable at different pH values, and the highest HA (512) was obtained at pH 7.5 (Fig. 4A). Nevertheless, PpyLL was able to retain activity over a large pH range (4.5 at 12) in contrast to all of the lectin isoforms from Himalayan mistletoe that failed to retain their biological activity under basic pH. PpyLL is a glycoprotein composed of 19% of covalently linked carbohydrates, revealed by the phenol–sulfuric acid assay. The percentage of carbohydrates in the plant lectin structure has shown variations. The carbohydrate content of *S. tuberosum*, *L. esculentum* and *D. stramonium* lectins was reported to be about 40–50% in weight and for *Cyphomandra betacea* lectins was 24% for CBL1 and CBL2 and 13.6% for CBL3 [33]. In some fungal lectins [34] and isoforms of Himalayan mistletoe lectin [27] variable quantities of carbohydrates, which have been related to thermostability, have been observed.

PpyLL was heat-stable up to 70°C with a total loss of HA at 80ºC (Fig. 4B). Chemical or physical stress, such as pH and temperature, promoted abrupt changes in structural protein, disturbing the amplitude and time scales of intramolecular motions [35], which can be evaluated through fluorescence spectroscopy. The fluorescence intensity of native PpyLL by heating showed an increase and maximum emission fluorescence at 342 nm (Fig. 4C), strongly suggesting that both of these tyrosine and tryptophan residues are in a slightly hydrophobic environment of the protein. In active lectin (up to 70°C), the changes of the fluorescence signals suggested the existence of quenching fluorescence related to protonated acidic groups and neighboring tryptophan residues. Furthermore, the spectra revealed that the quenching fluorescence is lower in heating than at room temperature (25°C), resulting in a significant decrease in the accessibility of the quencher to the aromatics residues. Additionally, above 80°C the

PpyLL center of mass of emission spectra shifts the emission maximum to 344 nm (Fig. 4D), clearly indicating protein unfolding.

Unlike in the studies reported here, in general, high temperatures induce denaturation for the majority of proteins, with a shift of fluorescence, as in maximum emission to 350 nm, and a decrease of intensity [36]. Although, thermal unfolding of PpyLL involves different behaviors reported in the literature, the content of carbohydrates in its structure could be responsible for significant thermal stabilization without affecting the protein folding pathways or their conformations. A few studies showed the higher tendency of the deglycosylated protein to aggregate during thermal inactivation, suggesting that glycosylation could also prevent partially folded or unfolded proteins from aggregating [37]. So, further experiments are required to draw firm conclusions on this.

3.3 Antibacterial activity

Several studies have been performed to evaluate interactions involving plant lectins with bacteria [6, 21, 22, 38]. Bacterial infections are increasingly frequent in hospitals. While nosocomial infections increase in a logarithmic progression, the number of antibiotics synthesized grows in a linear progression, and this fact has provided incentive for scientific research to obtain new antibiotics derived from plants.

In vitro antibacterial assays demonstrated that the PpyL exhibited antibacterial activity against the most tested pathogenic bacteria (*Staphylococcus epidermidis, Streptococcus faecalis, Bacillus subtilis, Klebsiella pneumonia*), being the diameter of the corresponding inhibition halos shown in Table 4. The lectin was shown to be active against most of the bacteria, and was more effective for Gram-positive than for Gram-negative species of bacteria. This greater interaction observed with Gram-positive

bacteria may be explained by the high levels of peptidoglycan on the wrapper. Lectin from *Araucaria angustifolia* caused the presence of pores and severe disruption of the Gram-positive bacterial membrane and substantial bubbling in the Gram-negative bacterial cell wall, besides some destroyed bacteria, demonstrating stronger antimicrobial activity against Gram-positive than Gram-negative bacteria [39].

The obtained values for MIC and MBC are presented in Table 4, and among the tested microorganisms, the *B. subtilis* demonstrated lower MIC (0.125 mg mL^{-1}). Furthermore, it was the only one that showed MBC (0.5 mg mL^{-1}). Although the lectin was able to inhibit the growth of *Klebsiella pneumoniae* in the agar diffusion test, the MIC was undetectable, even at a concentration of 2 mg mL^{-1}. Superior concentrations of lectin were not performed in this assay due to the non-viability of using high concentrations of proteins in the antimicrobial activity assays because high concentrations may disturb microorganism growth, not by its direct action but by increased osmolarity causing microorganism death and thus giving a false-positive result.

Bacteria (*S. epidermidis, S. faecalis, B. subtilis* and *K. pneumoniae*) that showed some kind of interaction with lectin were submitted to a new assay to evaluate a possible growth inhibition. PpyLL was able to abolish the growth of *B. subtilis* (Fig. 5A) within a short time of 6 h but not of other bacteria (data not shown), corroborating the results described above.

Interestingly, an important finding in this study was that PpyLL, although not bactericidal for most of the tested bacteria, caused a clustering of cells at the bottom of the assay tube that could be observed without the aid of a microscope (Figure 5B) after 6 hours. This show that PpyLL, like other lectins, has the ability to recognize carbohydrates present on the surface of cells, such as bacterial cells, is able to

agglutinate them, promotes their immobilization, and inhibits their growth or even destroys the bacteria. This kind of interaction (lectin-bacteria cells) may exist by covalent/or non-covalent aggregation, depending on the molecular weight of the oligomers and its subunits [40]. A similar agglutination phenomenon was also reported for the lectin from *Fenneropenaeus chinensis* that agglutinated Gram-positive cells and was examined through fluorescence microscopy [7]. The ability of PpyL to agglutinate the bacteria is important because promoting agglutination when associated with a topical antibiotic could be used to increase the effectiveness of treatment; therefore, the concentrations of this antibiotic could be lower because the cells will be grouped.

3.4 Antifungal activity

PpyLL was devoid of antifungal activity against most of the fungi tested but markedly inhibited the growth of *F. lateritium* and *R. solani,* and these results agree with the results obtained for other plant lectins [41]. These species are correlated and responsible for a portion of the losses involved in the production of *Phaseolus vulgaris* beans and carrots. Figure 6 shows that the PpyLL disc formed an inhibition line on *F. Lateritium* growth. PpyLL concentrations were able to inhibit 50% (IC_{50}) of the growth of *R. solani* and *F. Lateritium* at 0.33 mg mL^{-1} and 0.25 mg mL^{-1}, respectively. According to Li et al. [42], only a few lectins have been reported to possess remarkable antifungal activity. In addition, Chen et al. [43] in their study reported that the lectin from *Phaseolus coccineus* exhibited a potent antifungal activity towards four commonly agronomically harmful fungi (*Helminthosporium maydis, Sclerotinia sclerotiorum, Gibberalla sanbinetti and Rhizoctonia solani)*, suggesting that the lectin might play an important role against fungal infections in plants.

Nonetheless, further efforts should be directed towards investigating the characterization and molecular configuration that may affect the binding specificity of PpyLL to better understand the role and interaction of this lectin in plant defense mechanisms. In addition, this research shows that the biomolecules recognized by PpyLL in these microorganisms reveal different lectin binding sites among the mistletoe species.

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Conclusion

This first report of PpyLL, a lectin from the South American hemi-parasitic mistletoe obtained from *P. pyrifolia* leaves, demonstrates its sensitive to seasonality, requiring sunny weather to promote the highest protein expression. A purification protocol using Sephadex-G100 followed by CM-cellulose allowed us to obtain a high purity lectin in milligram quantities, and this acidic glycoprotein was resolved as one polypeptide band by SDS-PAGE and as two bands under reduced conditions. The lectin was thermo-stable and active against bacterial and fungal pathogens, properties that are favorable for therapeutic and biotechnological applications.

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Figure Captions

Figure 1. Effect of seasonality in the lectin *P. pyrifolia* leaf production. SHA was determined in different extracts [10% (w/v) in 0.15 mol L^{-1} NaCl] from leaves collected monthly.

Figure 2. Elution profile of chromatography columns. (A) The extensively dialyzed fraction 20–40% (ammonium sulfate saturated) was loaded onto the affinity column packed with Sephadex G-100 and eluted with glucose. (B) The active peak from Sephadex (SAP) was applied to ion exchange chromatography on CM-cellulose, and the bound fraction containing active protein showed a sharp single peak when eluted with Tris-HCl (PpyLL). (C) Superdex 75 HR 10/30 column coupled to an ÄKTA purifier system equilibrated and eluted with 0.3 M NaCl. (D) Reverse phase chromatography in C-18 column HPLC system. Chromatogram: Abs_{280} of samples (**-●-**); Log HA of samples (**-Δ-**).

Figure 3. Molecular characterization. (A) 10% SDS-PAGE of SAP obtained by Sephadex-G100 purification stained with Coomassie Brilliant Blue. (B) 12% SDS-PAGE of PpyLL obtained by CM-cellulose purification, silver stained. (C) 12% SDS-PAGE of PpyLL obtained by CM-cellulose purification under reducing conditions with 2-mercaptoethanol and stained with Coomassie Brilliant Blue. Standard marker proteins were used as follows: (1) bovine serum albumin (66 kDa); (2) albumin from chicken egg white (45 kDa); (3) carbonic anhydrase (30 kDa); and (4) lysozyme (14.3 kDa).

Figure 4. (A) The effect of pH on PpyLL hemagglutinating activity (HA) by incubating equal amounts of sample with universal buffers at a pH range of 3.5 - 12. (B) Effect of temperature on the PpyLL HA. PpyLL initial HA corresponded to 512 towards rabbit erythrocytes suspension, and each point on the lines represents the average of three replicates. (C) Intrinsic fluorescence emission spectra of PpyLL at 280 nm wavelength light excitation. Temperature values: (—) 50 to 70°C; (---) 80 to 90°C; (▬) 100°C. (D) Modified center of spectral mass plots of PpyLL at different temperatures. Each point on the lines represents the average of three replicates.

Figure 5. Growth inhibition assay. (A) *B. subtilis* growth under lectin effect. Bacteria cultures (in NB) in the exponential growth phase were added to buckets of lectin solution (0.5 mg mL^{-1} in 0.15 mol L^{-1} NaCl), and the absorbance (600 nm) was determined every hour for 6 h. (B) A clustering of *B. Subtilis* cells by PpyLL observed without the aid of a microscope.

Figure 6. Agar diffusion assay showing the antifungal activity of PpyLL (100 μg) against Fusarium lateritium. The antifungal activity was observed as an inhibition line formed around the discs.

Figure 1.

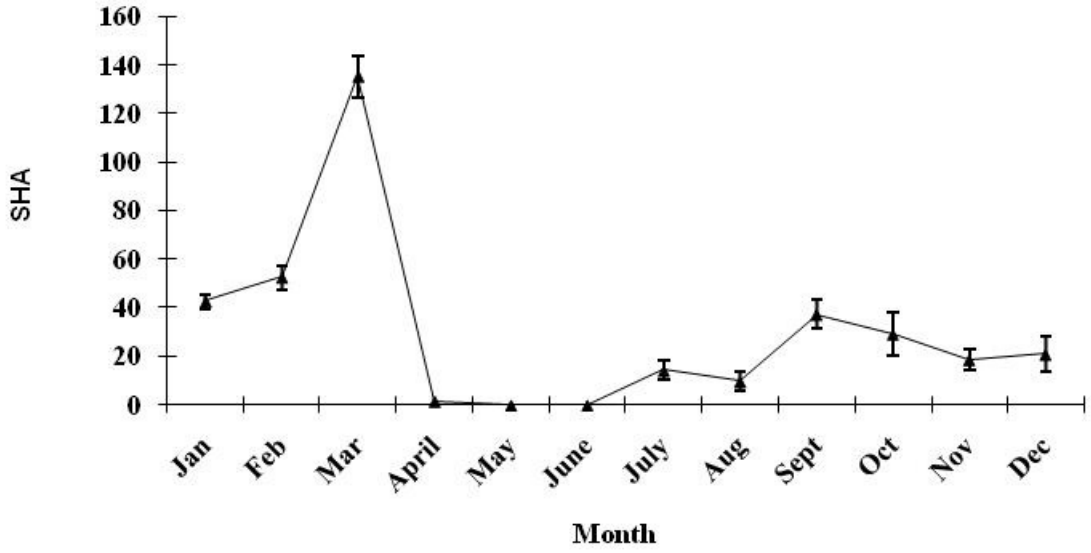
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Figure 2.

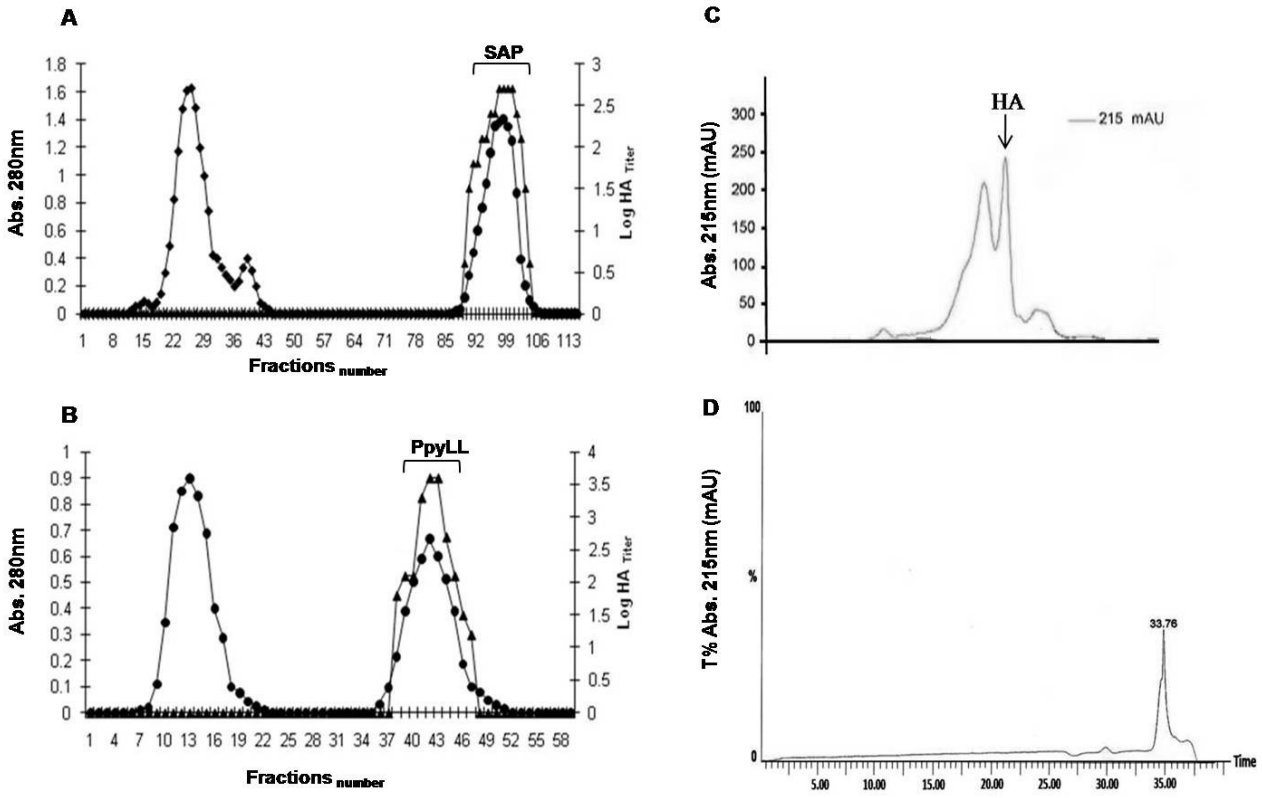
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Figure 3.

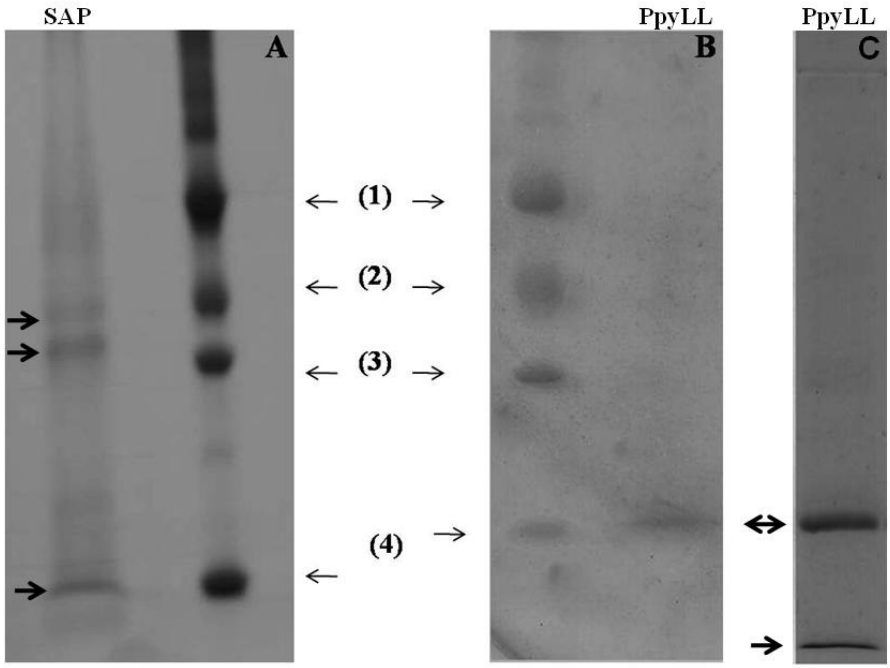
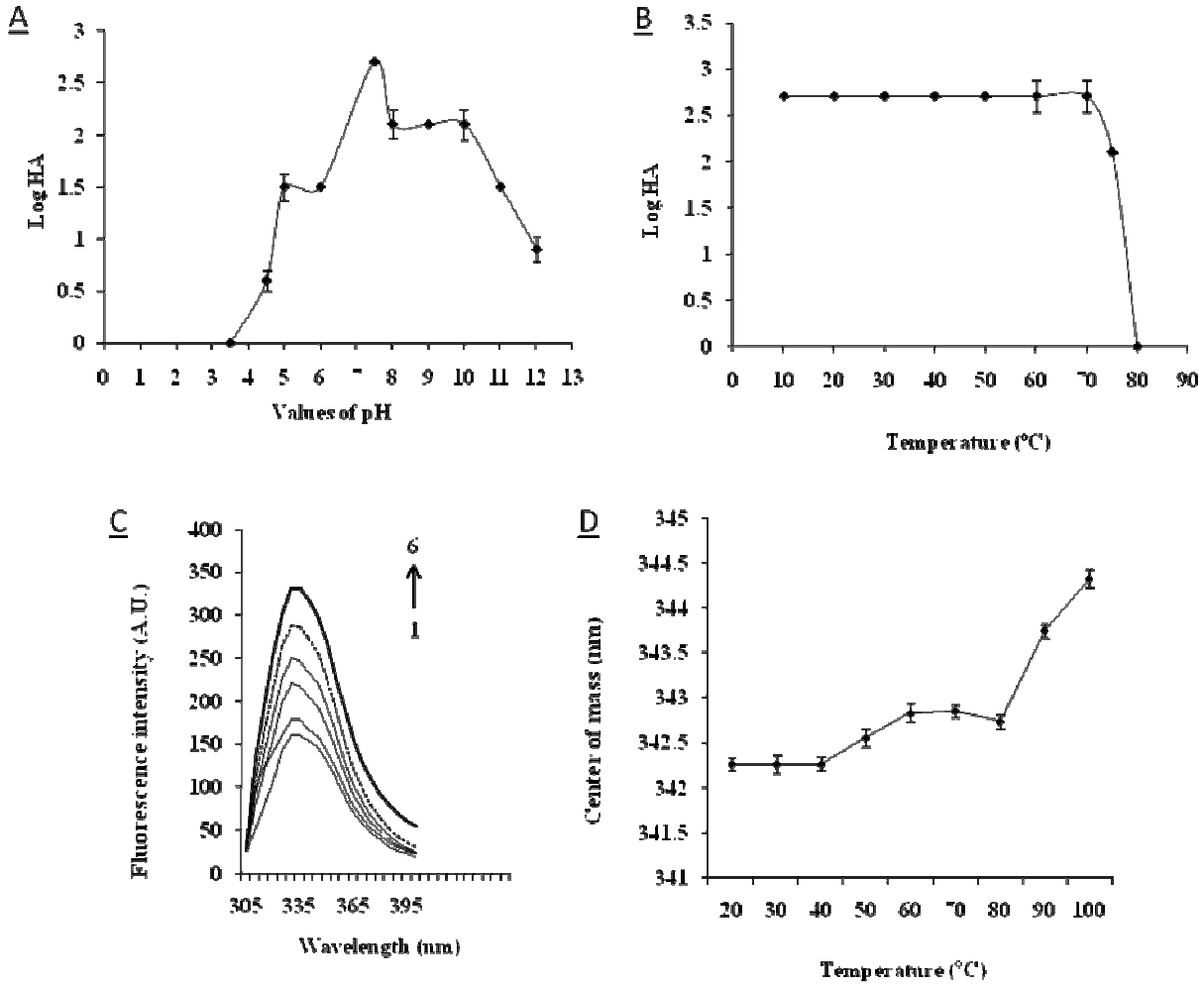


Figure 4.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Temperature (°C)

Figure 5.

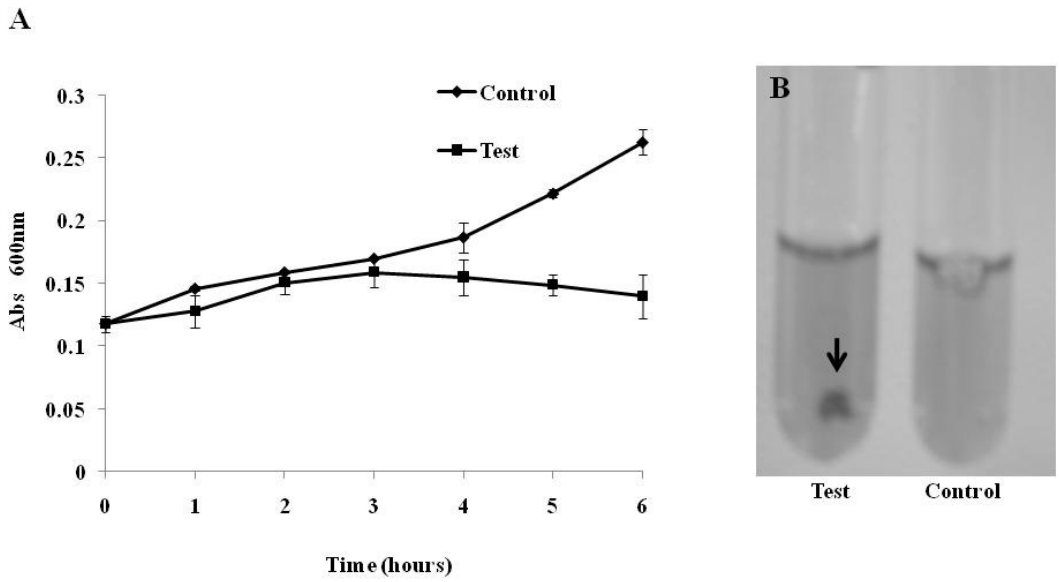
A
Control
Test
Abs 600nm
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
6
Time (hours)
B
Test
Control

Figure 6.

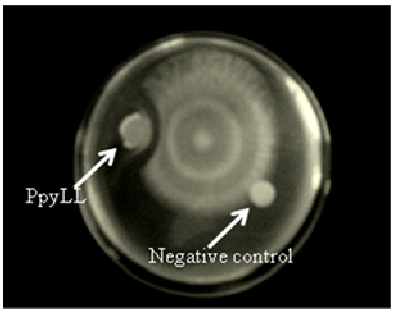

Table 1. Summary of *P. pyrifolia* leaf lectin purification.

Lectin Fraction	*Volume* *(ml)*	*Protein* *(mg)*	*Total activity* *HA*	[a]*SHA* *HA/mg*	[b]*Purification* *Fold*	[c]*Recovery* *(%)*
Crude Extract	100	1500	204800	136.5	1.0	100.0
F 20-40%	45	333	92160	276.8	2.0	45.0
SAP	444	81.58	56832	696.6	5.1	27.8
PpyLL	277.5	28.58	142080	4971.3	36.4	69.4

[a] Specific hemagglutinating activity; agglutinating against rabbit erythrocytes.

[b] Purification fold: relation between SHA of crude extract and SHA of lectin.

[c] Recovery: relation between total activity of crude extract and lectin.

SAP: Sephadex G-100 chromatographic active peak.

PpyLL: *P. pyrifolia* leaf lectin obtained by CM-cellulose chromatography.

Table 2. Details of specific hemagglutinating activity of PpyLL with seven erythrocyte types belonging to human and three animals species.

Species	*Specific Hemagglutinating Activity (HA/mg)**
Rabbit	640
Rat	*NA*
Chicken	*NA*
Human blood erythrocytes	
A^+	80
B^+	80
O^+	1280
AB^+	*NA*

*Specific hemagglutinating activity was expressed as HA/mg.

NA, no agglutination even at 250 μg mL^{-1} lectin concentration.

Table 3. Inhibition of PpyLL agglutination activity using rabbit red blood erythrocytes.

Glycoprotein ($\mu g\ mL^{-1}$)	500	250	125	62.50	31.25	15.62
Asialofetuin	-	-	-	-	-	-
*ACEW	-	-	-	-	-	-
Fetuin	-	-	-	-	-	-
*BSA	+	+	+	-	-	-
Casein	+	+	+	+	+	+
Azocasein	+	+	+	+	-	-

Lectin concentration, 250 $\mu g\ mL^{-1}$.

Initial HA_{titer} corresponded 512.

*ACEW: Albumin from chicken egg white.

*BSA: Bovine serum albumin.

Assay achieved at room temperature (25°C) using a 2.5% suspension of erythrocytes.

Table 4. Antibacterial activity, MIC and MBC of PpyLL against bacteria.

Microorganisms	**Inhibition zone** (mm)	**MIC** $\mu g\ mL^{-1}$	**MBC** $\mu g\ mL^{-1}$
Staphylococcus epidermidis (+)	10.25 ± 0.5	250	NT
Streptococcus faecalis (+)	12.45 ± 0.5	500	NT
Staphylococcus aureus (+)	NT	NT	NT
Bacillus subtilis (+)	10.75 ± 0.5	125	500
Pseudomonas aeruginosa (-)	NT	NT	NT
Klebsiella pneumonia (-)	11.50 ± 1.3	>2000	NT

PpyLL, *Phthirusa pyrifolia* leaf lectin.

Lectin concentration: 80 μg per disc. Each point on the line represents the average of five replicates.

NT, not determined.

Gram-positive (+) and Gram-negative (-) bacteria.

MIC, Minimum inhibitory concentration corresponded to the minimum lectin concentration that inhibited visible bacterial growth. MIC was determined by the dilution test tube.

MBC, Minimum bactericidal concentration was determined as the highest dilution (lowest concentration) at which no growth occurred on agar plates.

6. CONCLUSÃO

Através de um protocolo envolvendo dois processos cromatográficos, cromatografia de afinidade e troca iônica, foi possível isolar e purificar uma lectina de folhas de *Phthirusa pyrifolia* denominada PpyLL. Os resultados da avaliação mensal da AHE do Extrato Bruto demonstraram que o clima é um fator importante no processo de extração, interferindo diretamente na Atividade Hemaglutinante da lectina. Quando submetida à eletroforese sob condições nativas, a lectina apresentou caráter ácido (carga líquida negativa) e a coloração com Schiff caracterizou a lectina PpyLL como uma glicoproteína. PpyLL aglutinou diferentes eritrócitos, obtendo melhor AHE para o Tipo O^+. Nenhum carboidrato simples inibiu a AH da lectina, excetuando-se a frutose-1,6-bifosfato, como carboidrato fosfatado. A fosfo-glicoproteína Caseína conseguiu abolir a AH em uma concentração mínima de 62,5 mg/ml, apresentando alta afinidade por essa biomolécula. A PpyLL mostrou-se termoresistente até 70ºC e quando submetida a variações de pH, a mesma apresentou maior AH em solução Tris-HCl 10 mM, pH 7,5. No ensaio para avaliação da atividade antimicrobiana, PpyLL apresentou maior atividade de inibição diante de bactérias Gram-positivas e inibiu o crescimento de fungos fitopatogênicos.. Assim, a nova lectina obtida por um simples protocolo de purificação apresenta potencial para aplicações biotecnológicas.

Perspectivas futuras para a caracterização estrutural da lectina das folhas da *Phthirusa pyrifolia*

A espectrometria de massa é uma técnica que permite a determinação da massa molecular de compostos com altíssima precisão e também pode ser usada para seqüenciar pequenas regiões de peptídios. O uso das pequenas sequências obtidas em conjunto com as informações de massas moleculares constitui uma poderosa técnica de identificação de proteínas conhecida como sequence tag. Um dos mais modernos métodos empregados na Proteômica para análise de massas está relacionado com o uso do acoplamento líquido cromatográfico bidimensional (LC/LC/MS) ao espectrômetro de massa (MALDI, MALDI-TOF, MALDI- TOF TOF).

Macromoléculas biológicas, como proteínas, carboidratos e ácidos nucléicos, interagem com a luz polarizada e a alteram. A técnica de Dicroísmo Circular (DC)

detecta a atividade óptica de moléculas quirais originada pela interação de centros assimétricos com a luz circularmente polarizada. Este Fenômeno é expresso pela diferença da absorção da luz circularmente polarizada à direita e à esquerda após esta passar através de uma amostra. Em proteínas, os cromóforos responsáveis pelo espectro de DC são a ligação amida, os resíduos aromáticos (triptófano e tirosina) e as pontes dissulfeto. A forma do espectro de DC de proteínas depende do seu conteúdo de estrutura secundária. Isso permite que as proporções de hélices, estruturas □, alças (turns) e estrutura secundária sejam determinasdas.

A função de uma proteína está diretamente relacionada com sua estrutura. A capacidade de um cientista em entender esta relação nos seus maiores detalhes é o fator limitante para compreender os processos biológicos e a base molecular da vida. Existem duas maneiras de obter informações detalhadas (resolução anatômica) sobre estruturas protéicas: Cristalografia e Ressonância Magnética Nuclear (RMN). As duas técnicas são complementares. Enquanto a cristalografia, em geral permite uma maior resolução e determinação de estruturas maiores, RMN fornece, além de informação estrutural, detalhes sobre processos dinâmicos relacionados ao intercâmbio entre conformações em solução, algumas das quais podem ser transitórias e resistentes a cristalização.

ANEXO

Guide for Authors

Process Biochemistry is an application-orientated research journal devoted to reporting advances with originality and novelty, in the science and technology of the processes involving bioactive molecules or elements, and living organisms ("Cell factory" concept). These processes concern the production of useful metabolites or materials, or the removal of toxic compounds. Within the segment "from the raw material(s) to the product(s)", it integrates tools and methods of current biology and engineering. Its main areas of interest are the food, drink, healthcare, energy and environmental industries and their underlying biological and engineering principles. Main topics covered include, with most of possible aspects and domains of application: fermentation, biochemical and bioreactor engineering; biotechnology processes and their life science aspects; biocatalysis, enzyme engineering and biotransformation; downstream processing; modeling,optimization and control techniques.

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Moreover the authors must give a list of all related manuscripts/papers, whether in submission or in press.

Format and type of manuscripts

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