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PURIFICAÇÃO, CARACTERIZAÇÃO E ATIVIDADES BIOLÓGICAS DE PROTEÍNA DE FOLHAS DE *Indigofera suffruticosa*.

Dewson Rocha Pereira

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Tese apresentada para o cumprimento parcial das exigências para obtenção do título de Doutor em Bioquímica e Fisiologia pela Universidade Federal de Pernambuco.

Orientadora: Profa. Dra. Vera Lúcia de Menezes Lima

Co-Orientadora: Profa. Dra. Patrícia Maria Guedes Paiva

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Aprovado por:

_____

Profa. Dra. Vera Lúcia de Menezes Lima -Presidente

_____

Profa. Dra. Maria Tereza dos Santos Correia – UFPE

_____

Profa. Dra. Márcia Vanuza da Silva – UFPE

_____

Prof. Dr. Antônio Fernando de Melo Vaz – UFCG

_____

Prof. Dr. Roberto Araújo Sá – UFPE

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“O futuro tem muitos nomes.
Para os fracos é o inalcansável.
para os temerosos, o desconhecido.
Para os valentes é a oportunidade”.
Victor Hugo

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LISTA DE ABREVIATURAS

Bis-ANS – 4,4'-dianilino-1,1'-binaftil-5,5'-sulfonato

BSA – Albumina bovina sérica

CFU – Unidade formadora de colônia

CL_{50} – Concentração letal (50%)

Con A – Concanavalina A

DPPH – 2,2-difenil-1-picrilhidrazil

DTA – Analise térmica diferencial

DTG – Termogravimetria derivada

FICI – Índice de concentração inibitória fracional

FPLC – Cromatografia líquida de desempenho rápido

FTIR – Infravermelho com Transformada de Fourrier

HA – Atividade hemaglutinante

HPLC-RP – Cromatografia líquida de alta resolução em fase reversa

IR – Infravermelho

IsuLL – Lectina de folhas de *Indigofera suffruticosa*

MBC - Concentração mínima bactericida

MIC – Concentração mínima inibitória

OMS – Organização Mundial de Saúde

SD – Desvio padrão

SDS-PAGE – Eletroforese em gel de poliacrilamida contendo sulfato sódico de dodecila

SDS – Sulfato sódico de dodecila

SHA - Atividade hemaglutinante específica

TG – Termogravimetria

TLC – Cromatografia de camada delgada

UV – Ultravioleta

RESUMO

Lectinas são proteínas de origem não imune que se ligam de forma reversível e não covalente a carboidratos e glicoconjugados. A espécie *Indigofera suffruticosa* Mill., encontrada na região do Semi-Árido Pernambucano, é utilizada popularmente como antiespasmódico, sedativo, diurético e purgativo. O objetivo deste estudo foi isolar, caracterizar e avaliar o potencial biológico de uma proteína de *I. suffruticosa*. Uma proteína de folhas de *I. suffruticosa* foi obtida por cromatografia de afinidade em coluna de quitina e demonstrou ser uma glicoproteína ácida com massa molecular de 35 kDa e 3,6 % de carboidratos neutros. Esta proteína apresentou maior atividade hemaglutinante específica para eritrócitos humanos do tipo A e foi inibida por glicoproteínas e monossacarídeos, demonstrando ser uma lectina que foi denominada IsuLL. Sua atividade hemaglutinante foi estável na faixa de pH entre 6,5 a 7,0, termoresistente e não foi alterada por cations divalentes. A espectroscopia de infravermelho revelou que IsuLL possui um maior percentual de estruturas do tipo folhas β. Espectroscopia de fluorescência intrínseca e extrínseca da estrutura molecular de IsuLL demonstrou a presença de resíduos triptófano e tirosina expostos na superfície da molécula e regiões hidrofóbicas parcialmente internalizadas. A estrutura de IsuLL revelou alta estabilidade térmica (T=200 ºC), avaliada pela curva de Termogravimetria. Esta lectina demonstrou atividade antioxidante (52 %) pela redução do radical DPPH**.**. IsuLL foi tóxica para larvas de *Artemia salina* com CL_{50} de 207.87±2 μg/mL, indicando toxicidade ambiental moderada e não reduziu, significativamente, a proliferação celular das linhagens humanas de nasofaringe (HeLa) e leucêmicas (KG1). IsuLL inibiu o crescimento de *Candida albicans* (MIC=64 μg mL^{-1}), *C. tropicalis* (MIC=16 μg.ml^{-1}) e *C. krusei* (MIC=8 μg mL^{-1}) e *Staphylococcus aureus* (MIC=15 μg mL^{-1}). O estudo do efeito de IsuLL sobre *S. aureus* revelou modificação na parede celular, utilizando técnicas de Microscopia Eletrônica de Transmissão, captação do cristal violeta e liberação de material genético. A associação de IsuLL com antibióticos (ampicilina e cifoxitina) foi sinérgica contra *S. aureus*. Os resultados indicam que IsuLL, isolada de folhas de *I. suffruticosa*, é uma nova lectina de estrutura estável com atividade antimicrobiana e antioxidante, de potencial aplicação biotecnológica.

Palavras-chaves: *Indigofera suffruticosa*, Fluorimetria, *Staphylococcus aureus*, DPPH, Candida, Sinergismo.

ABSTRACT

Lectins are proteins of non-immune origin that binding reversible and noncovalent with carbohydrate and glicoconjugated. The specie *Indigofera suffruticosa* Mill. found in Semi-arid region of Pernambuco is used in the folkmedicine as antispasmodic, sedative, diuretic and purgative. The aim of this study was isolate, characterize and evaluate the biologic potential of an *Indigofera suffruticosa* protein. An *I. suffruticosa* leaves protein was obtained by chitin affinity column chromatography, demonstrating be an acid glycoprotein with a molecular weight of 35 kDa and 3.6% neutral carbohydrate. This protein showed large hemagglutinating specific activity to type A human erythrocytes which was inhibited by glycoproteins and monosaccharides, demonstrating be a lectin called IsuLL. Its hemagglutinating activity was stable in 6.5 to 7.0 pH grade, termostable and was not dependent on the divalent cations. Infrared spectroscopy revealed a higher percent of β-sheet structures in the IsuLL molecule. Intrinsic and extrinsic fluorescence spectroscopy of IsuLL molecular structure, showed the presence of tryptophan and tyrosine residues exposed on molecule surface and hydrophobic regions partly internalized. IsuLL structure presented high thermostable (T=200ºC), evaluated by thermogravimetry curve. This lectin showed antioxidant activity (52%) by reduction of DPPH• radical. IsuLL was toxic for *Artemia salina* larvae with LC_{50} of 207.87±2 μg mL^{-1}, indicating moderate environment toxicity and did not impaired the cellular proliferation of HeLA (nasopharyngeal) and KG1 (leukemia) human cell lines. IsuLL inhibited the growing of *Candida albicans* (MIC=64 μg mL^{-1}), *C. tropicalis* (MIC=16 μg mL^{-1}), *C. krusei* (MIC=8 μg mL^{-1}), and *Staphylococcus aureus* (MIC=15 μg mL^{-1}). The study of IsuLL over *S. aureus* revealed modification on cell wall, using Transmission Electronic Microscopy techniques, evaluated by crystal violet uptake and release of genetic material. The association between IsuLL with antibiotics (ampicillin and cifoxitin) was synergic against *S. aureus*. The results indicate that IsuLL, isolated from *I. suffruticosa* leaves, is a new lectin with stable structure with antimicrobial and antioxidant activity, with a potential biotechnology application.

Keywords: *Indigofera suffruticosa*, Fluorimetry, *Staphylococcus aureus*, DPPH, *Candida*, Sinergism

INTRODUÇÃO

1. PLANTAS MEDICINAIS

A preservação da flora é um tema de destaque em reuniões internacionais por se tratar de uma importante fonte de recursos naturais fundamentais para a manutenção da biodiversidade. Além da função de reduzir as consequências do "efeito estufa" e de servirem como fonte de alimento, permitindo uma alimentação natural e saudável, as plantas são utilizadas para fins medicinais de grande importância, atuando no tratamento de diversas enfermidades. O homem faz uso das riquezas da flora para cura de doenças há milhares de anos, existindo relatos do uso de plantas com finalidades terapêuticas desde 3.000 A. C. (KO, 1999; TYLER, 1996).

As plantas medicinais são um tema de alta relevância na ciência brasileira e muitos estudos estão sendo realizados com intuito de explorar suas propriedades bioativas. No Brasil o uso de plantas para a melhoria da saúde aumentou devido à ampla biodiversidade vegetal existente em nosso país, acompanhada por uma aceitação do uso de plantas medicinais e pelo conhecimento tradicional associado. Os estudos aumentaram e as tecnologias evoluíram no decorrer dos tempos e no início do século 19, o desenvolvimento das ciências naturais e dos métodos científicos na medicina, proporcionaram mais conhecimentos sobre os fitomedicamentos. O isolamento da morfina da *Papaver sommiferum* (1803-1806), pelo farmacêutico Friedrich Sertürner, marcou o início do processo de extração de princípios ativos de vegetais através de métodos químicos e analíticos (SCHULZ; HÄNSEL; TYLER, 2001).

A fitoterapia foi reconhecida pela Organização Mundial de Saúde (OMS), na Conferência de Alma Ata em 1978, ressaltando as plantas medicinais como parte do Programa Saúde para Todos (WHO, 1978). Isso proporcionou a realização de mais estudos e a propagação do uso das plantas medicinais regionais como uma maneira de diminuir custos dos programas de saúde pública (YAMADA, 1998). A utilização de fitoterápicos como modalidade de terapia complementar ou alternativa para tratamento de doenças cresceu amplamente nas últimas décadas (CARVALHO *et al*., 2008; VEIGA-JUNIOR, 2008; CANTER; EMST, 2006). Na África, por exemplo, 80% da população dependem do uso de medicamentos naturais, já que suas condições financeiras não suportam os altos valores dos medicamentos sintéticos (ASCHWANDEN, 2001).

O Brasil é um país privilegiado no mercado de produtos naturais, devido a sua imensa e diversificada flora. Um terço da flora mundial se encontra no Brasil, representado pela Amazônia a maior reserva de produtos naturais com ação fitoterápica do planeta. Este fato estimulou o desenvolvimento de pesquisas para a produção de medicamentos fitoterápicos no cenário científico (YUNES *et al*., 2001; FRANÇA *et al*., 2008).

O crescimento na utilização de fitomedicamentos está relacionado ao desenvolvimento de medicamentos naturais reconhecidamente seguros e eficazes, mostrando ao mercado a credibilidade do produto. Além da necessidade da população por terapias de baixo custo e menos agressiva (YUNES *et al*., 2001). Aproximadamente 48% dos medicamentos empregados na terapêutica advêm, direta ou indiretamente, de produtos naturais, especialmente de plantas medicinais que permanecem em uma importante fonte para obtenção de medicamentos (BALUNAS; KINGHORN, 2005).

2. FAMÍLIA LEGUMINOSAE

A família Leguminosae Juss. ou Fabaceae Lindl., ordem Fabales, subclasse Rosidae, possui cerca de 727 gêneros e 19.325 espécies, incluindo árvores, arbustos, lianas e ervas. As leguminosas compreendem a terceira maior família de Angiospermas depois da Orchidaceae e Asteraceae, e a segunda em importância econômica depois da Poaceae (LEWIS *et al*., 2005). A sua importância advém do elevado número de espécies vegetais e pelo seu destaque como fonte de produtos alimentares, medicinais, ornamentais e madeireiros.

Um novo arranjo foi elaborado por Polhill (1994) em que são adotadas as seguintes divisões em subfamílias: Caesalpinioideae, Mimosoideae e Papilionoideae ou Faboideae. A subfamília Papilionoideae é a maior com, aproximadamente, 482 gêneros e cerca de 12.000 espécies de ampla distribuição mundial, além de ser um táxon bem representado no Semi-Árido nordestino (RODAL *et al.,* 1999; ARAÚJO; MARTINS, 1999). A Mimosoideae possui 77 gêneros e aproximadamente 3.000 espécies (DOYLE; LUCKOW, 2003) e a Caesalpinioideae é formada por 170 gêneros e aproximadamente 3.000 espécies (DOYLE, 1995).

Na subfamília Papilionoideae está incluído o gênero *Indigofera* que é formado por mais de 700 espécies, sendo na maior parte representadas por plantas herbáceas e arbustivas (LEWIS *et al*., 2005) com ocorrências em regiões tropicais e subtropicais das

Américas, desde o sul dos Estados Unidos até a Argentina, sendo também aclimatada na Ásia, África e Austrália (HASSEN *et al*., 2007). As espécies *Indigofera asperifolia, I. bongardiana* var. *bongardiana* e *I.guaranitica* estão distribuídas principalmente em direção à região sul do Brasil, atingindo o Paraguai e a Argentina e ocorre em campos e cerrados. Já a *I. truxillensis*, *I. suffruticosa*, *I. hirsuta* L., *I. guaranitica*, *I. lespedezioides*, *I. spicata*, *I. trita*, *I. sabulicola*, *I. pascuorum*, *I. lespedezioides*, *I. spicata* e *I. trita* estão mais amplamente dispersas ao norte e nordeste do Brasil, com a maioria de suas espécies distribuídas até o México (MOREIRA; AZEVEDO-TOZZI, 1997).

2.1 Indigofera suffruticosa

É uma espécie largamente encontrada na região do Semi-Árido Pernambucano e está incluída na lista de Leguminosas forrageiras da bacia leiteira do Estado de Alagoas (RIBEIRO *et al*., 1991). A *I. suffruticosa* é um arbusto que mede de 1 a 2 m de altura, possui ramos pubescentes, folhas pínadas contendo de 7 a 15 folíolos oblongos ou ovais, glabros na face e no verso, e apresenta flores miúdas, numerosas, albo-róseas ou amareladas, em racemos axilares (BRAGA, 1976). Ela também apresenta pequena vagem falciforme com 6-10 sementes, não comprimidas. As inflorescências são menores que as folhas e os frutos (Figura 1) são numerosos desde a base do eixo da inflorescência (BORTOLUZZI *et al*., 2003).

Figura 1 – *Indigofera suffruticosa*: ramos com folhas e inflorescência.

Fonte: http://flickr.com/photos/dinesh_valke/2681740889/

Esta planta é utilizada, popularmente, contra dores articulares, nefralgias, distúrbios circulatórios e afecções respiratórias. Como também, suas folhas são usadas na etnomedicina como um composto antiespasmódico, sedativo, diurético, purgativo ou estomáquico (HASTING, 1990; AGRA *et al*., 1996). A raiz é utilizada como febrífuga, diurética, purgativa, odontálgica e útil na cura da icterícia (VIEIRA, 1992).

Devido a sua elevada funcionalidade como fitoterápico, alguns estudos foram realizados a fim de tentar entender o potencial desta planta e identificar mais alguma atividade biológica. Estudos mostraram que extratos de *I. suffruticosa* apresentaram atividade anticonvulsivante (ALEJO *et al*., 1996), antigenotóxica (BADELL *et al*., 1998) e antiepiléptica (ROIG; MESA, 1974).

Mesmo sendo utilizada como forragem animal, tem sido reportado que a *I. suffruticosa* pode promover dano ao organismo animal, existem registros no estado do Rio de Janeiro, por exemplo, de anemia hemolítica e intoxicação no fígado e rim de bovinos, pelo consumo da *I. suffruticosa* (NETO *et al*., 2001), causando a morte destes animais sendo prejudicial à pecuária da região, o que enfatiza a necessidade de estudar a ação de novas drogas.

Trabalhos preliminares realizados pelo nosso laboratório (Laboratório de Química e Metabolismo de Lipídeos e Lipoproteínas) demonstraram que extrato aquoso bruto de folhas *I. suffruticosa* possui atividade citotóxica em células embrionárias em crescimento (LEITE *et al.,* 2004), atividade antimicrobiana contra *Staphylococcus aureus* e contra os fungos dermatófitos *Microsporium canis* e *Trichophyton rubrum* (LEITE *et al*., 2006). Contudo, nehuma dessas atividades biológica foi associada a metabólitos secundários ou primários que tenham sido purificados de *I. suffruticosa*.

3. PLANTAS COMO FONTE DE BIOMOLÉCULAS

As plantas sintetizam produtos químicos essenciais ao seu desenvolvimento, que são os compostos do metabolismo primário (açúcares, proteínas, purinas e pirimidinas – ácidos nucléicos e a clorofila). Os metabólitos secundários (alcalóides, terpenóides, substâncias fenólicas) são produzidos a partir das rotas do metabolismo primário, eles são característicos de uma única espécie ou grupo delas, relacionadas evolutivamente (MARTIN; DEMAIN, 1980; LIMA *et al*., 2008).

Os metabólitos primários possuem função estrutural, plástica e de armazenamento de energia. Os metabólitos secundários aparentemente não possuem

relação com crescimento e desenvolvimento da planta, contudo atuam protegendo as plantas contra herbívoros e patógenos, servem como atrativos para polinizadores e funcionam como agentes de competição entre plantas e de simbiose entre plantas e microrganismos (TAIZ; ZEIGER, 2006).

A maioria dos compostos presentes nas plantas faz parte do metabolismo primário. Dentre estes, as proteínas estão entre as macromoléculas biológicas mais abundantes sendo extremamente versáteis em suas funções. Elas participam da atividade celular como enzimas, inibidores de enzimas, hormônios, proteínas de transporte, proteínas de reserva, proteínas estruturais, proteínas regulatórias entre outros (VERPOORTE, 2000). Todas são formadas pelo mesmo conjunto de vinte aminoácidos, o que permite desempenhar funções biológicas variadas. A atividade biológica das proteínas é o resultado da sequência específica dos aminoácidos nas cadeias polipeptídicas, assim como da estrutura tridimensional resultante do estabelecimento de ligações entre os grupos de alguns dos seus aminoácidos (NELSON; COX, 2005). As características destas moléculas despertaram para o interesse em pesquisar suas funções e entender seus mecanismos de ação.

3.1 Lectinas

3.1.1 Generalidades

As lectinas, uma classe de proteínas com capacidade de se ligar especificamente a eritrócitos, foram relatadas inicialmente em 1888 por Stillmark estudando a toxicidade de *Ricinus communis* (mamona), o qual observou que no extrato desta planta havia a presença de uma proteína, denominada ricina, que aglutinava eritrócitos. Posteriormente, foi observado a presença de uma hemaglutinina em extratos de semente de jeriquiti por Hellin (SHARON, 1998). E em 1919, Sumner isolou de *Canavalia ensiformis* (feijão de porco) uma proteína cristalina, nomeada de Concanavalina A (Con A), obtendo uma hemaglutinina pura pela primeira vez (SHARON; LIS, 2004).

As lectinas foram denominadas inicialmente de aglutininas, hemaglutininas, fitoaglutininas ou fitohemaglutininas (SHARON; LIS, 1988). O termo Lectina (do latim “legere”, que significa selecionado) foi introduzido por Boyd e Shapleigh, em 1954, devido à capacidade destas moléculas de distinguir eritrócitos de diferentes tipos sanguíneos. A definição mais adequada para as lectinas foi proposta por Peumans e Van

Damme (1995), em que todas as proteínas ou glicoproteínas de origem não imune com pelo menos um domínio não catalítico que se liga reversivelmente a um mono ou oligossacarídeo específico sem alterar as propriedades dos carboidratos, são consideradas como lectinas.

Estudos iniciais com aplicação biológicas de lectinas foram importantes para verificar a importância destas proteínas como ferramenta biotecnológica. Um importante passo para imunologia ocorreu em 1960 em que a influência de lectinas de sementes de *Phaseolus vulgaris* (PHA) foi estudada, por Nowel, sobre a divisão celular de linfócitos (TEXEIRA *et al*., 2012). Como também, em 1963 quando foi observado que lectinas do germe do trigo (WGA) aglutinavam especificamente células malignas (NASCIMENTO *et al*., 2012)

Lectinas podem ser encontradas em todos os reinos desde vírus, microrganismos, plantas e animais (KUMAR *et al*., 2012). No reino vegetal, lectinas de leguminosas representam a maior e mais estudada família de proteínas dessa classe (SHARON; LIS, 1990). Em torno de 100 membros já foram bem caracterizados estando presentes principalmente em sementes maduras, onde podem representar até 10 % do peso seco das mesmas (KONOZY *et al.*, 2003) e em menor proporção em outros tecidos, como, folhas, cascas, frutos, raízes (NASCIMENTO et al., 2012).

Os fungos têm sido os microrganismos mais estudados para o isolamento e detecção de lectinas (GOLDSTEIN *et al*., 2007), contudo as bactérias também são utilizadas, mesmo que em menor frequência (LAKHTIN *et al*., 2006 ; LAMEIGNERE *et al*., 2008). Em animais invertebrados as lectinas podem ser isoladas de insetos (YU *et al*., 2000), esponjas (MOURA *et al*., 2006), crustáceos (ALPUCHE *et al*., 2005) ou de animais vertebrados como peixes (SILVA *et al*., 2012), bovinos (YE; NG, 2000), entre outros organismos.

3.1.2 Caracterização e Classificação

As lectinas são ricas em aminoácidos ácidos e hidroxilados, associadas por interações hidrofóbicas, pontes de hidrogênio e, em alguns casos, pontes dissulfetos (SHARON *et al*., 2004). Algumas lectinas apresentam um complexo glicídico, ligado covalentemente à sua estrutura, que corresponde a 4-10% da massa total da molécula (HELENIUS; AEBI, 2004).

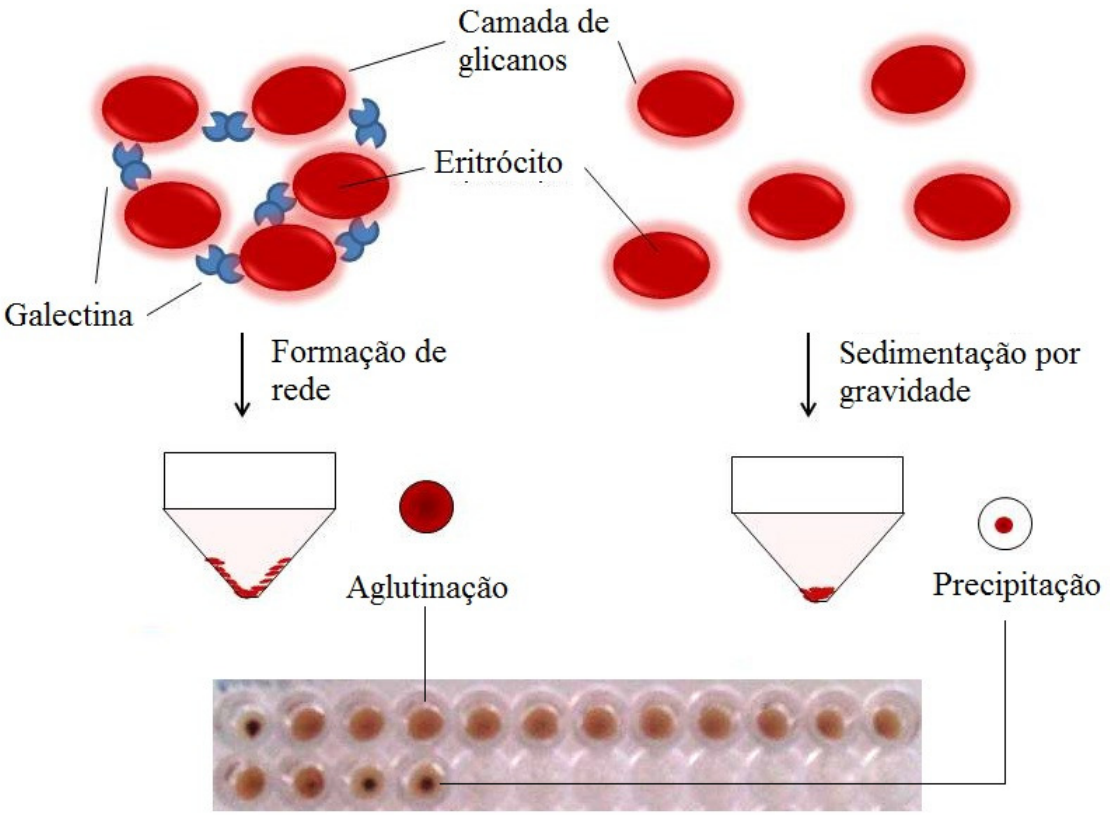
Estas moléculas possuem uma região conservada rica em aminoácidos hidrofóbicos, que forma uma cavidade na estrutura nativa. Esse sítio hidrofóbico possibilita interação entre as lectinas e grande número de compostos químicos distintos dos carboidratos como, por exemplo, a interação de compostos fenólicos, ligantes naturais dessa superfície hidrofóbica (BARRE *et al*., 2002).

Quando as lectinas são formadas por uma subunidade proteica podem ser denominadas monoméricas como a P-selectina (USHIYAMA *et al*., 1993) e a lectina de orquídea (*Gastrodia elata*) (LIU *et al.*, 2005). Quando apresentam duas subunidades polipeptídicas são denominadas diméricas, podendo ser semelhantes entre si (homodiméricas) ou diferentes (heterodiméricas). A lectina isolada a partir de feijão vermelho (*Phaseolus vulgaris*) é homodimérica com 67 kDa (YE *et al*., 2001), já a lectina presente no alho (*Allium sativum*) é heterodimérica, com uma subunidade de 11,5 kDa e outra de 12,5 kDa (VAN DAME *et al*., 1992). Existem outros tipos, como as triméricas, tetraméricas como a ConA, a lectina de soja (SBA) e de amendoim (PNA) (PRABU *et al*., 1999), pentaméricas, hexaméricas como a de sementes de *Hibiscus mutabilis* (LAM; NG, 2009) ou poliméricas.

As lectinas possuem uma característica específica de se ligar a um carboidrato específico ou grupo de carboidratos (oligossacarídeos ou glicoproteínas), através de seu sítio de ligação, denominado "domínio de reconhecimento de carboidrato" (CORREIA *et al*, 2008). Essa característica possibilita o reconhecimento específico de um tipo de eritrócito, e permite a identificação da presença de lectinas em uma amostra pelo ensaio de hemaglutinação (SANTOS *et al*., 2005). Os eritrócitos utilizados, de origem humana ou de outros animais, são tratados enzimaticamente (JUNG *et al.*, 2007) ou quimicamente (COELHO; SILVA, 2000).

Este ensaio consiste na diluição seriada da proteína e posterior incubação com eritrócitos humanos ou de outro animal permitindo a interação das lectinas com carboidratos da superfície celular dos eritrócitos (KENNEDY *et al.*, 1995). A hemaglutinação (Figura 2) ocorre quando há a formação de uma rede ou malha pela interação entre as proteínas e os carboidratos presentes na membrana dos eritrócitos (XIONG *et al*., 2006).

Figura 2 – Esquema ilustrativo de ensaio de hemaglutinação.



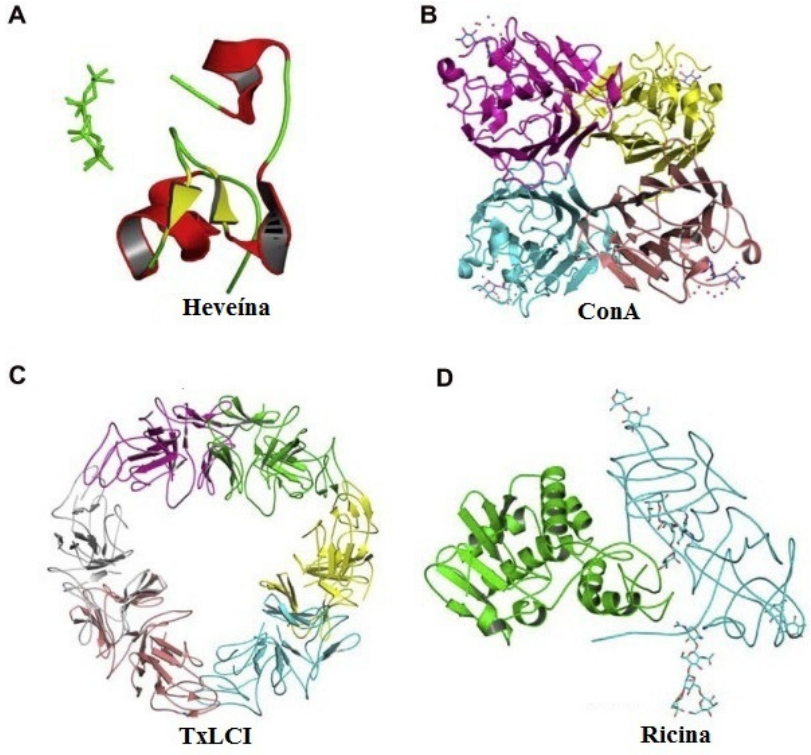
Fonte: Modificado de http://jcggdb.jp/GlycoPOD/protocolShow.action?nodeId=t123

A especificidade de uma lectina a determinado(s) carboidrato(s) é definida pelo ensaio de inibição da atividade hemaglutinante utilizando soluções de distintos carboidratos (CORREIA *et al*., 2008). Dependendo da afinidade a determinado(s) carboidrato(s) as lectinas foram divididas em grupos: glicose/manose; galactose; galactose/N-aceltilgalactosamina, fucose, N-acetilglicosamina, ácido siálico e grupos glicanos complexos (PEUMANS; VAN DAMME, 1998).

Outra forma de classificar as lectinas de plantas é de acordo com sua estrutura global (Figura 3). As merolectinas possuem um único sitio de ligação a carboidrato sendo incapazes de aglutinar uma célula ou precipitar glicoconjugados. A Heveína é um exemplo de merolectina isolada do látex de *Hevea brasiliensis*. As Hololectinas são constituídas por dois domínios de ligação a carboidrato, similares ou iguais, por isso estas lectinas (di ou multivalentes) podem aglutinar células e/ou precipitar glicoconjugados. Segundo Van Damme e colaboradores (1998), a maioria das lectinas de plantas pertence a este grupo, por exemplo, a Con A. Superlectinas possuem dois tipos de sítios de ligação a carboidratos diferente, a TxLCI, é uma lectina de tulipas que reconhece manose e N-acetil-galactosamina. Quimerolectinas são proteínas com duas

cadeias, com um ou mais sítios de ligação a carboidrato e outro sítio que pode ter atividade enzimática bem definida, ou outra atividade biológica, mas agem independentemente do domínio ligante a carboidrato, como exemplo a Ricina.

Figura 3 – Representação ilustrativa da estrutura de lectinas dependendo do domínio de ligação a carboidrato. (A) Merolectinas; (B) Hololectinas; (C) Superlectinas; (D) Quimerolectinas.



Fonte: Adaptado de Liu *et al.* (2010).

Esta interação das lectinas a carboidratos ocorre através de ligações do tipo pontes de hidrogênio em combinação a forças de van der Waals e interações hidrofóbicas com resíduos de aminoácidos aromáticos que estão próximos às porções hidrofóbicas de monossacarídeos (SHARON; LIS, 2002), sendo interações dominantes para a estabilidade da ligação proteína-carboidrato.

Íons metálicos divalentes como o Ca^{2+} Mg^{2+} e o Mn^{2+} podem influenciar na capacidade de interação com carboidratos de algumas lectinas, o que torna estas moléculas dependentes de íons. Os aminoácidos que coordenam estes íons metálicos são a asparagina e o ácido aspárticos (AUDETTE *et al*., 2000; SHARON e LIS, 2002).

3.1.3 Purificação

O isolamento das lectinas é realizado através de diferentes técnicas de purificação, devendo ser escolhida aquela que de maneira mais simples possibilite a obtenção de uma grande quantidade de lectinas e com suas propriedades mantidas (LAM; NG, 2011). Inicialmente, o material vegetal é submetido a uma extração através solução aquosa salina ou solução tampão (HUGHES; STOCK, 2001). Uma purificação parcial é realizada, na maioria dos casos, utilizando o fracionamento salino para precipitação das proteínas (HEU *et al*., 1995). Após, será escolhido um dos métodos convencionais de cromatografia que se baseiam nos aspectos gerais das proteínas, como carga elétrica (Cromatografia de Troca Iônica), tamanho (Cromatografia de Exclusão Molecular), especificidade (Cromatografia de Afinidade) (NASCIMENTO *et al*., 2012), alguns protocolos de purificação utilizam um ou mais métodos cromatográficos.

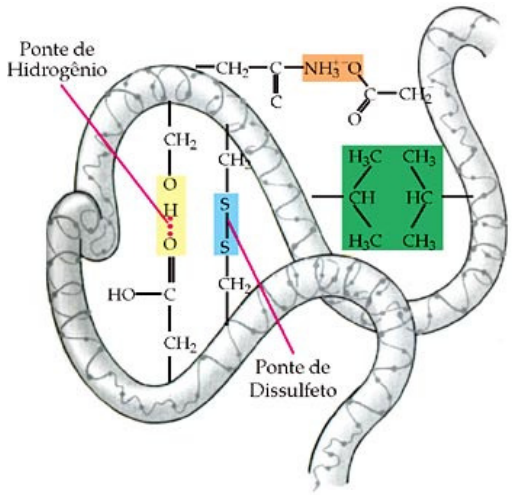
A técnica de eletroforese pode ser utilizada, adicionalmente, utilizando gel de poliacrilamida (PAGE) contendo o dodecilsulfato de sódio (SDS) ou β-mercaptoetanol, para a identificação do grau de pureza e do peso molecular, além de pontes dissulfeto, já que neste ensaio as proteínas são separadas de acordo com sua migração em um gel através de campo elétrico (REYNOSO-CAMACHO *et al*., 2003). Quando a eletroforese é realizada utilizando apenas PAGE, características em relação a sua carga líquida podem ser avaliadas sendo identificadas como proteínas ácidas (COELHO; SILVA, 2000) ou básicas (CORREIA; COELHO, 1995).

Outra forma de verificação de grau de pureza e para a obtenção de lectinas mais puras, após utilização de métodos de cromatografia convencionais, pode ser utilizado a cromatografia líquida de desempenho rápido (FPLC – do inglês: “Fast Protein Liquid Chromatography”) e a de alto desempenho (HPLC-RP – do inglês: High Performance/Pressure Liquide Chromatography-Reverse Phase) como um passo final no processo de purificação (WONG; NG, 2003). FPLC pode ser utilizado como um método de caracterização de massa molecular de proteínas, para estabelecer homogeneidade das lectinas puras, determinando se elas são monoméricas, diméricas ou até tetraméricas, havendo possibilidade de separação (WANG *et al*., 2001).

3.2 Estabilidade Proteica

A estabilidade estrutural das proteínas é determinada por uma variedade de forças físicas, que resultam de interações, que ocorrem dependendo da disposição e orientação dos grupos funcionais da molécula. Dentre as interações químicas que tem o efeito de estabilizar a conformação nativa proteica se incluem as pontes dissulfeto, interações hidrofóbicas e as interações não covalentes (fracas), como as pontes de hidrogênio, ligações hidrofóbicas e iônicas (Figura 4). O papel destas interações fracas é especialmente importante para o entendimento de como cadeias polipeptídicas formam estruturas específicas secundárias e terciárias, e como eles se combinam com outros polipeptídios de modo a formar estruturas quaternárias (NELSON; COX, 2005).

Figura 4 – Esquema ilustrativo de tipos de ligações químicas que podem estabilizar proteínas.



Fonte: NELSON; COX, 2005.

No contexto de estrutura proteica, o termo estabilidade pode ser definido como a tendência para manter a conformação nativa desta molécula. Proteínas nativas são apenas parcialmente estáveis, a energia que separa o seu estado enovelamento e desenovelamento é muito baixa. Interações hidrofóbicas são importantes na estabilidade da conformação proteica, pois seu interior é geralmente um núcleo denso com cadeias de aminoácidos hidrofóbicos (NELSON; COX, 2005).

Existem alguns fatores, como pH e temperatura, que podem afetar a estabilidade da proteína, causando mudanças na estrutura globular ou tridimensional (estrutura secundária e terciária) e consequentemente a desnaturação. Neste processo de

desenovelamento a proteína modifica seu estado físico, porém sua composição química permanece a mesma (MANNING *et al*., 2010).

A desnaturação ocasionada de elevadas temperaturas é provavelmente o estresse mais comum causado à estrutura globular das proteínas. A estabilidade termodinâmica não é uniformemente distribuída pela estrutura da proteína, a molécula pode ter dois domínios estáveis ligados por um segmento com menor estabilidade estrutural, ou uma pequena parte de um domínio pode ter uma menor estabilidade do que o restante. As regiões de baixa estabilidade permitem que a proteína altere sua conformação entre dois ou mais estados (NELSON; COX, 2005).

Para um entendimento mais aprofundado das características estruturais das lectinas algumas técnicas podem ser utilizadas para estudar o tipo de estrutura secundária, características da estrutura terciária, presença de região hidrofóbica, termoestabilidade. As metodologias adotadas, pela maioria dos estudos utilizam conceitos variáveis como técnicas espectroscópicas e análises térmicas.

3.2.1 Espectroscopia de Fluorescência

Luminescência é a emissão de luz a partir de alguma substância, e ocorre a partir de estados eletronicamente excitados, esta pode ser dividida em duas categorias: fluorescência e fosforescência (LAKOWICZ, 2009). Espectroscopia de absorbância é um método simples, não destrutivo e eficiente para a investigação da conformação e estabilidade proteica, através da fluorescência, ou seja, emissão de luz ultravioleta (UV) por moléculas aromáticas em estados eletronicamente excitados. O espectro de absorção das proteínas tem a informação contida dentro do espectro de 250 a 300 nm. Alguma interferência física ou química sobre a estabilidade da proteína vai ocasionar uma modificação das bandas de absorção dos espectros de fluorescência (JISKOOT; CROMMELIN, 2005).

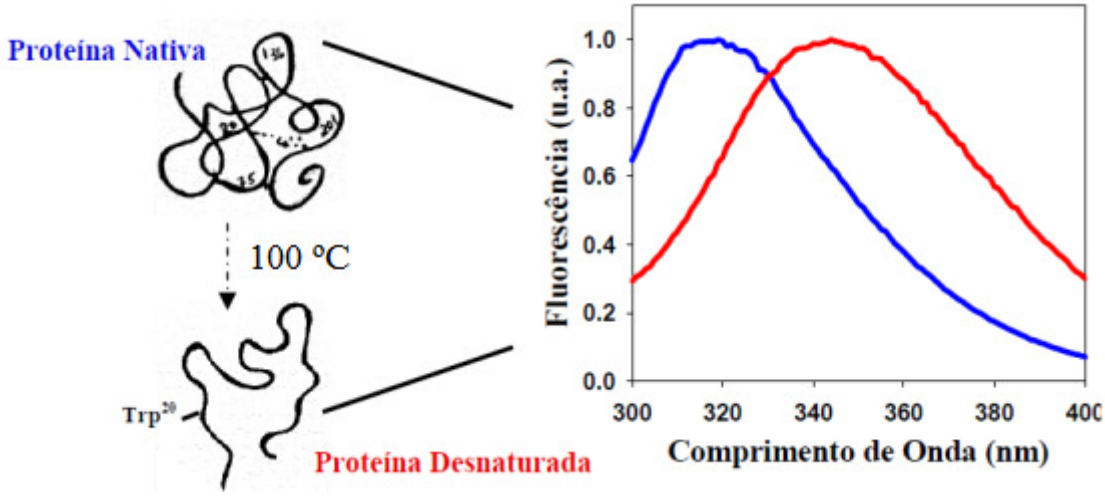
3.2.1.1 Fluorescência proteica intrínseca

Os aminoácidos aromáticos (triptófano, tirosina e fenilalanina) presentes nas proteínas, conhecidos também como fluoróforos naturais, são fontes de absorbância e emissão de luz UV. Estes aminoácidos são excitados por radiação ultravioleta e os

elétrons transferidos a níveis mais energéticos retornam ao estado fundamental por meio de emissão de fluorescência (LAKOWICZ, 2009).

O espectro de emissão máxima do triptófano é, aproximadamente, 340 nm e para as proteínas isso reflete uma exposição média dos seus resíduos aromáticos à fase aquosa (LAKOWICZ, 2009). Quando este espectro por influência química ou física diminui para menores comprimentos de onda pode ser afirmado que houve um "blue shifted", ou seja, o fluoróforo esta em uma região mais interna da proteína. Quando a sua emissão for modificada a comprimentos de onda maiores, "red shift", significa que o fluoróforo está e uma região mais externa da proteína (desenovelamento, Figura 5) (LAKOWICZ, 2009).

Figura 5 – Espectro de emissão de fluorescência do triptófano quando submetido a temperaturas elevadas (100 ºC).



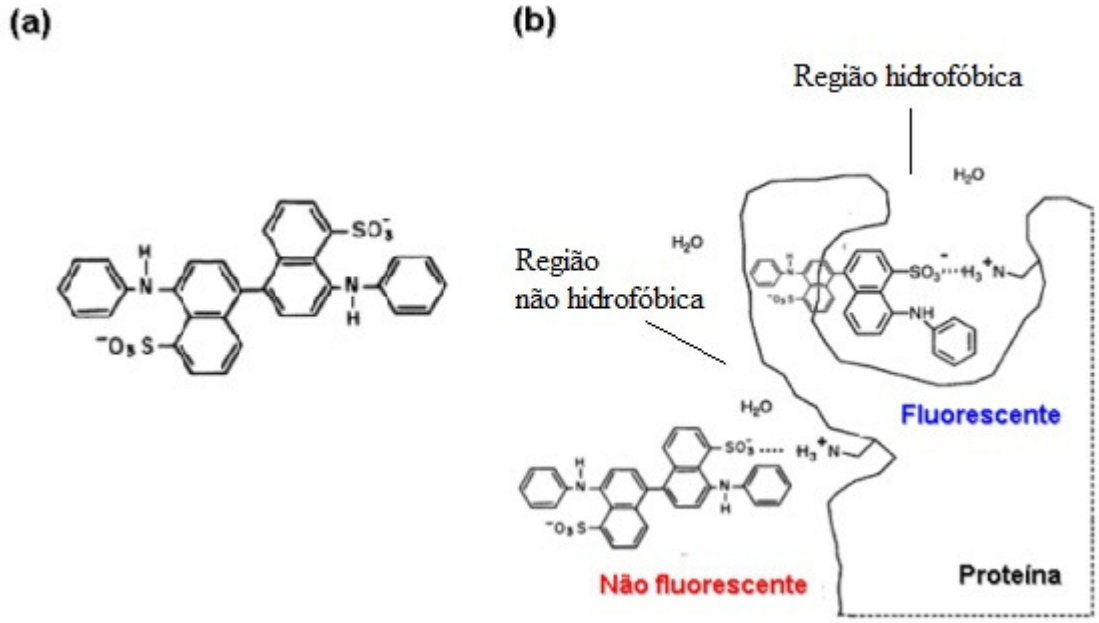
Fonte: Adaptado pelo autor.

3.2.1.2 Fluorescência proteica extrínseca

Os fluoróforos extrínsecos oferecem uma possibilidade adicional para o estudo de proteínas por espectroscopia de fluorescência, devido ao seu elevado coeficiente de absorção em relação aos resíduos aromáticos (JISKOOT; CROMMELIN, 2005).

O Bis-ANS (4,4'-dianilino-1,1'-binafitil-5,5'-sulfonato) é um componente derivado de naftaleno-anilina sulfato que é bem descrito por sua habilidade de se ligar não covalentemente a regiões hidrofóbicas de proteínas (Figura 6), resultando em um aumento em seu espectro de fluorescência (emissão máxima 510 nm) (FERRÃO-GONZALES *et al*., 2000).

Figura 6 – Fluorescência da molécula bis-ANS. (a) Fórmula estrutural de bis-ANS (4,4'dianilino-1,1'-naphtaleno 8-sulfonato). (b) Simulação de ligação do bis-ANS com regiões hidrofóbicas e não hidrofóbicas de uma proteína.



Fonte: Adaptado de TAKASHI, TONOMURA e MORALES (1977); MATULIS *et al*. (1999).

3.2.2 Termogravimetria

Para estudar a influência da temperatura sobre as lectinas é utilizado o ensaio de Termogravimetria (TG), o qual mede a massa da amostra que esta sujeita a uma variação de temperatura programada. Esta medida é realizada em uma atmosfera definida, geralmente, em condições inertes (nitrogênio) ou e um ambiente oxidativo, a massa é averiguada em uma balança eletrônica altamente sensível (WENDLANDT, 1986).

Análises termogravimétricas fornecem informações sobre o conteúdo volátil, tais como, solventes e água, sobre o comportamento de decomposição e sobre o conteúdo de cinzas e carga. Adicionalmente, a Termogravimetria Derivada (do inglês – Derivative Thermogravimetry - DTG) é o procedimento matemático que fornece a primeira derivada de uma curva TG, cada pico representa as áreas proporcionais às variações de massa (FORD; TIMMINS, 1989).

As informações obtidas como temperatura de maior perda de massa e etapas de degradação estão relacionadas à composição e estabilidade térmica da amostra, dos produtos intermediários e do resíduo formado. Assim, dependendo do peso molecular e das ligações químicas, por exemplo, uma proteína pode revelar diferença na

termoestabilidade. É possível conhecer, também, as regiões de temperatura em que o sistema apresenta processos endotérmicos e/ou exotérmicos, ou seja, com absorção ou liberação de energia através da curva de Analise térmica diferencial (do inglês – Differential Thermal Analysis – DTA) (VELASQUES *et al*., 2004).

Diferentes estudos foram realizados com produtos de plantas para avaliar o comportamento térmico dos seus componentes, incluindo as proteínas, através da curva de termogravimetria (KUMAR *et al*., 2008; FONTANARI *et al*., 2006, 2012).

3.2.3 Espectroscopia de Infravermelho

A estrutura secundária das proteínas tem um papel funcional importante para as atividades desta molécula, de acordo com a geometria espacial de uma cadeia, são reconhecidas algumas estruturas denominadas: α-hélice, folha β, β-turn (volta, giro) ou randômicas.

O método de espectroscopia de infravermelho (IR) é utilizado para estimar os componentes da estrutura secundária de proteínas sobre diferentes condições. O desenvolvimento do método de IR com Transformada de Fourier (FTIR) ampliou a aquisição de dados na espectroscopia de IR para analisar a estrutura secundária de polipeptídeos e proteínas em termos da vibração de unidades estruturais repetidas (KONG; YU, 2007).

O espectro de uma amostra é produzido pela diferença de intensidade da radiação infravermelho antes e depois de sua passagem pela amostra. O resultado são bandas de absorção originárias da interação entre discretos quanta de luz e os movimentos de vibração das moléculas excitadas pela absorção da radiação infravermelha (BEEKES, *et al*., 2007). A frequência precisa para a obtenção dos picos destas bandas depende de efeitos inter e intramoleculares, incluindo ângulo da ligação peptídica e padrões de pontes de hidrogênio (posição do hidrogênio) (BARTH, 2007).

A banda de espectro de absorção infravermelho Amida I (1700-1600 cm^{-1}) é uma das mais proeminentes do espectro e a mais sensível para proteínas. A amida I é praticamente toda representada por ligações do tipo C=O (± 80%), o que possibilita a identificação de pequenas variações na geometria molecular da proteína. Com isso, na banda Amida I estão localizados picos individualizados na região do espectro de infravermelho relacionados à estrutura secundária (Tabela 1) (CARBONARO; NUCARA, 2010).

Tabela 1 – Relação tipo de estrutura secundária e região do espectro de absorção infravermelho.

Estrutura secundária	Região (cm^{-1})
Turns	1682-1662
α-hélice	1662-1645
Estrutura randômica	1645-1637
Folhas β	1637-1613
	1682-1690

3.3 Papel Fisiológico das Lectinas

As funções das lectinas de plantas ainda não estão bem definidas, algumas funções são propostas dependendo de onde e como ela é expressa (KUMAR *et al*., 2012). Uma das vantagens é que são proteínas caracterizadas como secretoras, pois podem entrar no sistema secretório vegetal e se acumular em vacúolos. Durante o desenvolvimento da semente as lectinas e o estoque proteico são degradados para o fornecimento de amino ácidos para o crescimento dos embriões vegetais e encontrado também sobre as partes vegetativas da planta (WINDHOLZ *et al*, 1983).

A função de defesa contra diferentes organismos também é evidenciada para a maioria das lectinas (PEUMANS *et al*., 1995). Estas moléculas têm a capacidade de causar severa toxicidade a insetos ou outros animais atuando como proteínas de defesa específica para as plantas (BHUTIA *et al*., 2012). Além disso, atuam como mediadores na simbiose entre plantas e bactérias fixadoras de nitrogênio (SHARON; LIS, 2004).

Nos fungos, estas biomoléculas estão envolvidas na biossíntese da parede celular e diferenciação do micélio, a adesão de esporos de espécies patogênicas a insetos (TRIGUEROS *et al*., 2003). Já em animais algumas lectinas podem estar relacionadas com mecanismo de endocitose e translocação intracelular de glicoproteínas (SMETANA; ANDRE, 2008).

3.4 Aplicações das Lectinas

A ligação específica a carboidratos é a característica das lectinas chave para o crescimento de inúmeros estudos nas áreas médica, química e biológica, o que as torna proteínas versáteis. Algumas aplicações biológicas de lectinas estão relacionadas à comunicação celular, defesa, desenvolvimento celular, infecção parasitária, metástase,

inflamação, etc., o que pode ser atribuído a sua capacidade de reconhecer carboidratos distintos sobre a superfície celular (CAVADA *et al*., 2001; SHARON, 2007).

A capacidade de se ligar a carboidratos confere às lectinas uma valorização como reagentes para a investigação de carboidratos de superfície celular, para a avaliação do seu papel sobre o crescimento e a diferenciação celular ou em processos patológicos (SHARON *et al*., 2007). A primeira demonstração de que carboidratos de superfície celular poderiam servir como carreador de informações biológicas, através do reconhecimento das lectinas, foi realizada nos anos 50 utilizando hemaglutininas para demonstrar que o antígenos do tipo sanguíneo A é o carboidrato N-acetilgalactosamina α-ligado e que o determinante H (tipo sanguíneo O) é α-L-fucose (SHARON, 2007). Em 1960, uma importante etapa para a imunologia foi descrita para determinar o papel destas proteínas sobre a divisão celular de linfócitos, foi demonstrado que lectinas de feijão possuíam capacidade de estimular linfócitos a sofrer mitose (OLIVEIRA *et al*., 2011).

A ação de lectinas contra células neoplásicas, por exemplo, está relacionada à interação destas moléculas com carboidratos de superfície de membrana. Estudo verificou que as lectinas tiveram capacidade de aglutinar estas células e distinguir entre as normais e as malignas (SHARON, 2004). Yan *et al*. (2009) purificou uma lectina de *Astragalus mongholicus*, e observou atividade antineoplásica, *in vitro*, contra células cancerígenas humanas.

As lectinas podem ser utilizadas para o isolamento de outras moléculas, devido a sua específica afinidade a carboidratos, através de cromatografia de afinidade utilizando estas proteínas acopladas a suportes insolúveis (OHBA *et al.,* 2002). Um exemplo é o isolamento da enzima lecitina colesterol aciltransferase através da imobilização em Sepharose CL 4B da lectina de *Cratylia mollis* (LIMA *et al*., 1997). Como também, podem ser aplicadas na identificação sorológica do tipo sanguíneo, por exemplo, a lectina de *Dolichus biflorus* como anti-A e lectina de *Griffonia simplicifolia* como anti-B (KHAN *et al*., 2002).

Lectinas vegetais apresentaram, também, diferentes ações biológicas, como larvicida (SÁ *et al*, 2008; NAPOLEÃO *et al*., 2012), inseticida (SILVA *et al*., 2009; OHIZUMI *et al*., 2009; ARAÚJO *et al*., 2012), anti-inflamatória (BENJAMIM *et al.,* 1997; ARAÚJO *et al*., 2011) e relacionadas a coagulação (SANTOS *et al.*, 2009; LA BONTE *et al*., 2012). Estas moléculas são relatadas, ainda, como potentes agentes

contra o vírus da imunodeficiência (HIV) (SWANSON *et al*, 2010; WONG *et al*, 2003; LI *et al*, 2008).

3.4.1 Atividade Antioxidante

Antioxidantes são compostos que podem retardar ou inibir a oxidação de lipídios ou outras moléculas, evitando o início ou propagação das reações em cadeia de oxidação causadas pelos radicais livres.

Descobertos na ultima década, os radicais livres são substância perigosas formadas durante o processo metabólico normal, através da oxidação de carboidratos, lipídios e proteínas, para a geração de energia (SEN *et al*., 2010). Neste processo normal o O_2 sofre redução tetravalente, resultando na formação de H_2O, durante este processo são formados intermediários reativos como: radicais superóxido (O_2^-) e hidroxila (OH^-), e peróxido de hidrogênio (H_2O_2) (FERREIRA; MATSUBARA, 1997; NORDBERG e ARNÉR, 2001). Quando em excesso no organismo podem causar danos irreversíveis ao organismo, como: doenças crônicas degenerativas (doença cardiovascular, arteriosclerose, diabetes e câncer), bem como envelhecimento precoce (BOKOV *et al*., 2004).

A maioria dos radicais livres são instáveis e permanecem estáveis apenas por um curto tempo, contudo o radical 2,2-diphenil-1-picrilhidrazil (DPPH) é um dos que pode permanecer mais tempo estável mesmo em temperatura ambiente (CHEN *et al*., 2008). O modelo de captura do radical DPPH (DPPH**•**)é um método utilizado para avaliar atividades antioxidantes em um tempo relativamente curto comparado com outros métodos (MILARDOVIĆ *et al*., 2006). O princípio deste ensaio é baseado na redução do DPPH**•** na presença de um antioxidante doador de hidrogênio (próton), levando a formação de DPPH-H, não radical (LI *et al*., 2007).

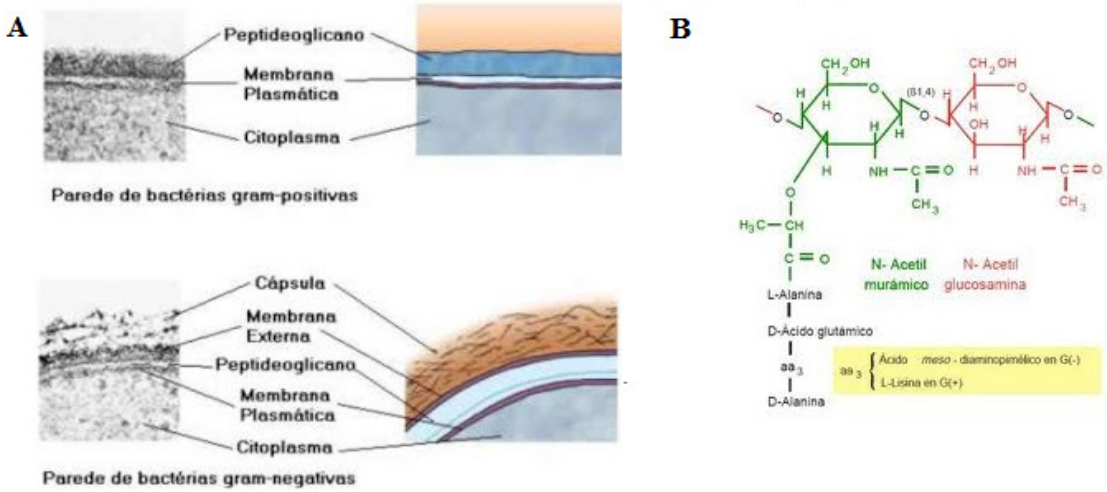
Alguns estudos reportam que aminoácidos como triptófano e tirosina são capazes de capturar quimicamente agentes oxidantes (SAITO *et al*., 2003). A presença destes resíduos aromáticos em uma lectina possibilitaria, então, que esta molécula participasse como uma doadora de prótons em ensaios de atividades antioxidantes com o DPPH, como demonstra a lectina de *Pleurotus ostreatus* (XIA *et al.,* 2011).

3.4.2 Atividade Antibacterinana

As bactérias são organismos procariontes uni ou pluricelulares, com organização celular relativamente simples. São organismos visíveis somente ao microscópio, em média se apresentam de 1 a 2 µm por 1 a 4 µm, podem ter as formas de cocos, bacilos ou espiraladas (TRABULSI *et al*., 2008). A microscopia, desde o século 17, é uma ferramenta fundamental para os microbiologistas, permitindo a contagem e a identificação de células bem como a determinação da sua morfologia. Atualmente, para uma visualização cada vez mais precisa, diferentes técnicas tem sido desenvolvidas utilizando a Microscopia Eletrônica de Transmissão (SCHEURING; DUFRÊNE, 2010).

A parede celular das bactérias é responsável pela manutenção da forma bacteriana e tem participação na divisão celular. Para as bactérias Gram positivas a parede celular (Figura 7) é composta de, aproximadamente, 90 % de peptidoglicano diferindo das Gram negativas que é mais complexa (TRABULSI *et al*., 2008). Sobre a sua superfície estão contidos polímeros aniônicos formados por carboidratos, que tem importante função em interações extracelulares e como suportes para enzimas exigidas no metabolismo da parede celular (SCHLAG *et al*. 2010). As duas classes principais de polímeros aniônicos são os ácidos lipoteicóicos associados à membrana plasmática, essenciais para uma adequada divisão celular e morfologia, e os ácidos teicóicos covalentemente ancorados ao peptidoglicano (WEIDENMAIER; PESCHEL, 2008).

Figura 7 – Parede celular bacteriana. (A) Estrutura da parede celular de bactéria gram negativa e gram positiva.(B) Estrutura química do monómero de peptidoglicano.



Fonte: (A) http://bionoensinomedio.blogspot.com.br/2012/03/1-ano-aula-2-celulas.html. (B) http://bioudescucuta.blogspot.com.br/2011/02/otras-estructuras-de-procariotas.html

Staphylococcus aureus é um coco Gram-positivo grande que cresce em cachos irregulares em forma de uvas devido às células se dividirem em pontos aleatórios e é uma das mais resistentes bactérias não formadoras de esporos (TORTORA *et al*., 2005). Este microrganismo é um patógeno que pode provocar endocardite, osteomielite hematogênica aguda, meningite ou infecção pulmonar, entre outras (TRABULSI *et al*., 2008). Possuem parede celular composta por peptidoglicano formando uma rede em que as fitas de glicanos estão entrelaçadas por peptídeos. As fitas de glicanos são compostas de unidades repetidas do dissacarídeo β-1-4-N-acetilglicosamina e do ácido N-acetilmuramico (PAIVA *et al*. 2010).

A utilização indiscriminada de fármacos contra infecções bacterianas têm aumentado a resistência dos microrganismos aos antibióticos, o que é uma pratica pouco segura devido à toxicidade que este medicamento pode provocar. A necessidade por novos agentes que combatam estes microrganismos multirresistentes culminou no crescimento de pesquisas para combater infecções bacterianas (GARVEY *et al*., 2011).

Recentes estudos têm investigado a influência das lectinas no campo da microbiologia, pela sua capacidade de interagir com patógenos e sua influência no desenvolvimento do microrganismo (TEIXEIRA *et al*., 2012). A parede celular microbiana não apenas impede alguma interação de glicoconjugados ou proteínas ligantes de carboidratos sobre sua membrana, contudo, também previne que os mesmos penetrem no citoplasma. Lectinas de plantas não alteram a estrutura ou prejudicam a ação de invasão da bactéria, elas têm o papel de defesa através de um mecanismo indireto que se baseia em interações com carboidratos da parede celular ou com carboidratos extracelulares (BHUTIA *et al*., 2012). Estudo com lectinas de *Eugenia uniflora*, utilizada para tratamento de hipercolesterolemia, gota e hipertensão, mostrou ação antimicrobiana contra *Staphylococcus aureus* (OLIVEIRA *et al*., 2008). Outros estudos verificaram a atividade de lectinas contra bactérias gram positivas e gram negativas (SANTI-GADELHA *et al.*, 2006; COSTA *et al.*, 2010; GOMES *et al*., 2012; NAIR *et al*., 2013).

Apesar do fato dos derivados vegetais possuírem menor reatividade do que os antibióticos, eles podem tratar as infecções com bastante sucesso, sendo evidente que a planta adota um paradigma diferente –"sinergia". A compreensão dos mecanismos moleculares de sinergia permite, assim, abertura de uma nova estratégia para o tratamento de doenças infecciosas, superando os patógenos resistentes e diminuindo o

uso de antibióticos, consequentemente, os efeitos secundários criados por eles (HEMAISWARYA; KRUTHIVENTI; DOBLE, 2008).

3.4.3 Atividade Antifúngica

Os fungos são organismos eucariontes que podem existir em uma forma unicelular, com um só núcleo como as leveduras ou podem ser multinucleados como os fungos filamentosos ou bolores e os cogumelos (TRABULSI *et al*., 2008). São, ainda, um grupo diversificado de organismos que ocupam muitos nichos no ambiente e são utilizados, por exemplo, na produção de antibióticos, ácidos orgânicos e esteróis (RAVEN *et al*., 2001)

Lectinas ligantes de quitina, extraídas de plantas, tem sido estudadas em relação ao seu potencial antifúngico, uma vez que a quitina é o componente-chave da parede celular de fungos (KUMAR *et al*., 2012). Muitos trabalhos relatam a ação de lectinas vegetais contra fungos patogênicos (TRINDADE *et al*., 2006; SITOHY *et al*., 2007; KLAFKE *et al.,*2013;). O mecanismo de ação pode ser a formação de canais iônicos nas membranas dos microrganismos ou a inibição competitiva da adesão das proteínas microbianas nos receptores polisacarídicos do hospedeiro. Esta interação pode afetar a viabilidade da parede celular do fungo devido a alterações na síntese ou por deposição de quitina na parede celular (SELITRENNIKOFF, 2001).

Dentre os fungos considerados como patogénos humanos, os membros do gênero *Candida* são frequentemente relatados, devido à alta frequência com que colonizam e infectam o hospedeiro humano (COLOMBO; GUIMARÃES, 2003). *Candida albicans* é a espécie mais frequentemente relacionada com infecções invasivas desse gênero (ZARDO *et al*., 2004). Os fungos do gênero *Candida* podem ser isolados das superfícies mucosas sadias, da cavidade oral, da vagina, do trato gastrointestinal em cerca de 80% da população na ausência de doença. No entanto, raramente é isolado da superfície da pele de seres humanos saudáveis, exceto esporadicamente de certas áreas intertriginosas, como na virilha (MURRAY *et al*., 2002).

As doenças infecciosas provocadas por *C. albicans* se apresentam primariamente através de três formas, muco-cutânea, cutânea e sistêmica, podendo atingir a mucosa oral, vaginal, traqueal, brônquios e o canal alimentar (KONEMAN *et al*, 2001). O tipo cutâneo da candidíase inclui áreas intertriginosas da pele das mãos, virilhas e axilas, já a

forma sistêmica pode invadir vários órgãos provocando candidíase pulmonar, fungemia e nefrite (LACAZ *et al*., 2002).

Candidíase, também denominada candidose, é uma micose oportunista primária ou secundária, podendo causar lesões superficiais ou profundas, envolvendo diversos órgãos tais como boca (Figura 5), língua, pele, couro cabeludo, genitálias, dedos, unhas (BARBEDO *et al*., 2010). Os fungos do gênero Candida sob certas circunstâncias têm acesso hematogênico a partir da orofaringe ou do trato gastroinstetinal, quando a barreira mucosa é rompida ou quando seringas e cateteres endovenosos estão contaminados (MURRAY *et al*., 2002). Os quadros clínicos mais rotineiramente reportados relacionados à candidíase são a do tipo cutâneo-mucosa, sistêmica/visceral e alérgica (LACAZ *et al*., 1991; ANAISSE, 1992; SIDRIM; MOREIRA, 1999).

Três espécies deste gênero são encontradas com menos frequência. *Candida krusei*, por exemplo, é uma levedura resistente a uma ampla variedade de antifúngicos, principalmente devido a sua resistência intrínseca ao fluconazol sendo uma importante espécie a ser monitorada (SHEMER *et al*., 2001; PFALLER *et al*., 2005). *Candida parapsilosis* é um micro-organismo comumente isolado de ambientes diferentes: do solo, da água e das plantas (LUPETTI, 2002). Esta espécie é a mais frequente em infecções na corrente sanguínea, em neonatos, pacientes transplantado, associadas a cateteres (COLOMBO *et al*., 2003; KOCSUBÉ *et al*., 2007). *Candida tropicalis* é uma levedura diplóide de reprodução assexuada e estudos recentes mostraram que é a terceira causa de candidemias em adultos, especialmente em pacientes com linfoma, leucemia, complicações hematológicas malignas, diabetes mellitus e câncer. Porém é raro encontrar esta espécie atuando sobre neonatos ou colonização mucocutânea (ZAUGG *et al*., 2001; VANDEPUTTE *et al*., 2005).

Entre as espécies de *Candida*, a resistência aos antifúngicos tem sido um problema crescente, pois muitas das espécies não-*albicans* mais comumente isoladas são menos susceptíveis aos derivados azólicos, o que dificulta o tratamento da candidíase e de outras infecções causadas por leveduras (SOJAKOVA, 2004; TORTORANO, 2006).

A candidíase permanece como a mais importante causa de infecção oportunistas no mundo e a principal agente de infecção em hospitais. Estão mais susceptíveis a desenvolver estas infecções ocorrem em pacientes que não possuem a capacidade de reagir, adequadamente, a um estímulo antigênico (imunodeprimidos) incluindo transplantados de órgãos ou tecido hematopoiético, pacientes com câncer, AIDS, bebês

prematuros, idosos e em pós-operatório. Com isso, a incidência de micoses oportunistas invasivas teve elevado crescimento na população de pacientes imunodeprimidos (NUCCI *et al*., 2010).

OBJETIVOS

1. GERAL

Purificar, caracterizar e identificar atividades biológicas de proteína de folhas de *I. suffruticosa*.

2. ESPECÍFICOS

- Preparar extratos aquosos de folhas de *I. suffruticosa*;
- Isolar e purificar componentes proteicos de extrato aquoso de folhas de *I. suffruticosa* por cromatografia e eletroforese;
- Avaliar capacidade hemaglutinante da proteína purificada e sua inibição frente a diferentes carboidratos e glicoproteínas
- Caracterizar a proteína purificada quanto a estabilidade térmica, variação do pH e dependência a íons;
- Caracterizar a proteína purificada quanto estrutura molecular**;**
- Investigar o potencial antioxidante, antimicrobiano e ação contra células cancerígenas da proteína purificada de folhas de *I. suffruticosa*
- Avaliar por Microscopia Eletrônica a ação da proteína purificada sobre microrganismos e identificar seu efeito sinérgico associado a antibióticos.

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CAPÍTULO I

ARTIGO I

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ISOLATION OF A CHITIN BINDING LECTIN FROM *Indigofera suffruticosa* LEAVES (IsuLL) WITH ANTI-STAPHYLOCCAL AND ANTIOXIDANT ACTIVITIES

Dewson Rocha Pereira[1]; Weber Melo Nascimento[1]; Eulália Camelo Pessoa de Azevedo Ximenes[2]; Maria Tereza dos Santos Correia[3]; Patrícia Maria Guedes Paiva[3]; Luana Cassandra Breitenbach Barroso Coelho[3]; Vera Lúcia de Menezes Lima*[,1]

[1]Laboratório de Química e Metabolismo de Lipídeos e Lipoproteínas, Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego s/n, Cidade Universitária. CEP 50670-420. Recife, PE, Brazil.
[2]Laboratório de Fisiologia e Bioquímica de Microrganismos, Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPE), Rua Artur de Sá s/n, Cidade Universitária. CEP 50670-901. Recife, PE, Brazil.
[3]Laboratório de Glipoproteínas, Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego s/n, Cidade Universitária. CEP 50670-420. Recife, PE, Brazil.

*Corresponding author

Vera Lúcia de Menezes Lima

Av. Professor Moraes Rego, S/N.

Cidade Universitária, Recife, Pernambuco, Brazil.

Cep: 50670-420. Phone fax: (+55 81) 2126-8540

E-mail address: lima.vera.ufpe@gmail.com

ABSTRACT

Aqueous extracts of *I. suffruticosa* have demonstrated activity against *Staphylococcus aureus*. Some plants lectins, carbohydrate recognition proteins, exhibit important antimicrobial and antioxidant activities. This study aimed to isolate proteins from aqueous extracts of *I. suffruticosa* leaves and investigate the antibacterial and antioxidant activities. *I. suffruticosa* Chitin Binding Lectin (IsuLL) obtained by chitin column chromatography is a glycoprotein with apparent molecular weight of 35 kDa SDS-PAGE. IsuLL had specific hemagglutinating activity for type A human erythrocytes which was inhibited by glycoproteins and monosaccharides. The highest hemagglutinating activity was stable on neutral pH and temperature up to 80^{o}C. IsuLL is an acidic glycoprotein with 3.6 % sugar content with effective bacteriostatic activity only against the Gram-positive bacteria *S. aureus*. The lectin exhibited 52% of antioxidant activity as indicated by DPPH radical scavenging. This study is the first report on isolation and preliminary characterization of IsuLL, a novel lectin from leaves of *I. suffruticosa* with biological activity. IsuLL revealed pharmacological interest in the search for new products of natural origin.

Keywords: Bacteriostatic effect; DPPH; Chitin-binding lectin; Fluorescence spectroscopy; *Staphylococcus aureus*.

Introduction

Indigofera suffruticosa Mill (Leguminosae - Papilionoideae) is a legume predominant from tropical and sub-tropical regions of the America, Asia, Africa and Australia [1]. This forage plant known as "anil" in Brazil is broadly found in the range of semi-arid region of northeastern Brazil [2]. The leaves are used in folk medicine as antispasmodic, sedative, diuretic and purgative [3]. Previous studies from our laboratory demonstrated that aqueous extract of *I. suffruticosa* leaves have antimicrobial activity against *Staphylococcus aureus*, *Microsporium canis* and *Trichophyton rubrum* [4], and also by in vitro assay, the extract present embryotoxicity activity on the early stage of mouse embryos development [5].

Lectins, protein of non-immune origin, bind specifically and reversibly to carbohydrate or glicoconjugate groups without promoting chemical modification in its covalent structure [6]. This group of proteins, found in plants, animal and microorganisms, can be directly isolated from a crude aqueous extract by affinity chromatography or following other steps of partial purification [7].

Various studies have shown that chitin-binding lectins, isolated by chitin-column chromatography, have great potential as antimicrobial and insecticidal agents [8-14] Therefore, plant lectins exhibit biological properties which are important to protect plant tissue against pests and pathogens [15].

Lectins have the selective ability to agglutinate erythrocyte of a particular human sanguine group [6] and antimicrobial activity [16,17] or to be utilized as affinity matrices [18]. This natural protein also showed ability of scavengers of DPPH radicals [19].

The present study aimed to isolate, partially characterize and to investigate antibacterial and antioxidant activities of lectin from aqueous extract of *I. suffruticosa* leaves of a new therapeutic product from a plant of the Brazilian semi-arid region.

Materials and methods

Chemicals

Sugars (N-acetil-D-glucosamine **-** GlcNac, D-arabinose, D-mannose, L-raffinose, D-galactose, D-fructose, D-glucose, sucrose, lactose, D-xylose, methyl-α-D-mannopyranoside), glycoproteins (azocasein, albumin from chicken egg white and

bovine serum albumin - BSA), chitin matrix and protein molecular mass markers were purchased from Sigma Chemical Company (USA). Solvents and other chemicals used were of analytical grade from Sigma or Merck (Germany). All solutions were prepared with water purified by a Milli-Q® system (Millipore). Mueller–Hinton Broth (MHB, Oxoid) and Mueller–Hinton agar (MHA, Oxoid) were also used.

Indigofera suffruticosa

Leaves of *Indigofera suffruticosa* were collected in the city of São Caetano-PE, localized in the semi-arid region of the Northeastern Brazil. The plant was identified by Dr. Marlene Carvalho de Alencar Barbosa of the Departamento de Botânica, Universidade Federal de Pernambuco, Brazil. This species is deposited in this institution in the Herbarium Geraldo Mariz UFP (nº 45.217).

Extract preparation

Leaves of *I. suffruticosa* were briefly washed with distilled water and left to dry at room temperature. The leaves powder was homogenized in water (10%) under constant agitation for 16 h. The water-based suspension was then paper-filtered and lyophilized.

Purification of *I. suffruticosa* leaf lectin

The lyophilized extract was solubilized (10%) in 0.15 M NaCl and applied to a chitin column (Sigma, USA, 20×7cm) previously equilibrated at 20 mL h^{-1} flow rate with 0.15 M NaCl. Protein elution was monitored by absorbance at 280 nm. Unbound proteins were removed with equilibrating solution until absorbance at 280nm was negligible. Bound proteins were eluted from the column with 2.0 M acetic acid, pH 4.0, pooled together, dialyzed against water, lyophilized and suspended in 0.15 M NaCl. Subsequently, the latter preparation was submitted to reverse- phase chromatography in a C-18 column (Restek Ultrapure 250x46 mm, 5 µm) performed in a HPLC system (Shimadzu LC-20AD, Kyto, Japan), with elution monitored at 215 nm. The column was equilibrated with solvent A (0.1% trifluoroaceti acid in H_2O) and eluted using solvent B (90% acetonitrile: 10% H2O: 0.1% TFA) in a 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.

FPLC-Gel filtration chromatography was carried out in an AKTA-purifier FPLC system equipped with a Superdex 75 10/300 GL column (GE Healthcare) equilibrated and eluted with 20 mM phosphate buffer (pH = 7.0 in 0.15 M NaCl) at a flow rate of 0.5 ml min^{-1} and monitored by absorbance at 215 nm.

Protein assay and neutral carbohydrate analysis

The protein content (mg mL^{-1}) was determined according to Lowry *et al*., (1951) [20] using a BSA calibration curve as the standard (0–500 μg mL^{-1}) and the total protein (mg) by ratio between a protein concentration and the volume value of the sample. Neutral carbohydrate contents of purified lectin were determined by the phenol–sulfuric acid method according to Masuko *et al*., (2005) [21] using mannose as a standard (0–100 μg mL^{-1}).

Hemagglutinating activity and hemagglutinating activity inhibition

Hemagglutinating activity (HA) assay was carried out in microtiter plates in pH 7.0 at 25 ºC (Kartell S.P.A., Italy) according to Paiva and Coelho (1992) [22] using a suspension (2.5% v/v; 50 μL) of rabbit and human A, B, AB and O-type erythrocytes treated with glutaraldehyde [23]. HA was defined as the highest title of the sample dilution that was able of agglutinate erythrocytes. Specific hemagglutinating activity (SHA) was determined by ratio between the HA and the total protein value of the sample. HA inhibition assay was performed in the presence of several carbohydrates and glycoproteins solutions before erythrocyte suspension addition.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Native polyacrylamide gel electrophoresis (Native PAGE)

Purified protein preparation was subjected to SDS-PAGE using a Mini-PROTEAN® 3 cell (BioRad). Gels consisted of a 12.5 % polyacrylamide resolving gel (pH 8.3) and a 5% stacking gel (pH 6.8) for subunit molecular mass determination [24]. Samples were dissolved in sample buffer (0.1 M Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 0.02% bromophenol blue) and loaded onto the gels (10 μl, 100 μg protein/well).

Following samples were heated in the presence or absence of β-mercaptoethanol for 5 min in a boiling water bath. The gel was stained with 0.125% (w/v) Coomassie brilliant blue R-250 in 7% acetic acid and 40% methanol (v/v) solution and destained with acetic acid 10% (v/v). Electrophoretic analysis under acidic and basic conditions was performed to evaluate the homogeneity of purified lectin preparation. PAGE of non-denatured purified lectin at pH 8.3 was performed by the method of Davis (1964) [25] and Bryan (1977) [26], and at pH 4.5 according to Reisfeld (1962) [27]. Glycoprotein staining was performed using the periodic acid-Schiff method [28].

Metal ion requirement, pH and temperature Stability

The samples were demetallized against EDTA by the method of [29] and assay utilized the divalent metal ions (Ca^{2+}, Mg^{2+} and Mn^{2+}). The activity in normal and demetallized samples was compared by the HA. The pH stability was performed with different pH values and buffers (10 mM citrate phosphate buffer, pH 4–7 and 10 mM tris–hydrochloric acid buffer, pH 8–11) for 1 h at room temperature; the result was evaluated by HA. Thermal stability of HA was determined by incubation of sample at different temperatures (30–100 ºC for 30 min) remained throughout the experiment (4 h) under temperature variations applied while different aliquots of sample remained (30 min) in each temperature range. After each step of incubation, HA was evaluated to study the effect of temperature.

Phytochemical analysis

The isolated material (1 mg ml^{-1} of protein) was submitted to phytochemical evaluation using silica gel Thin-Layer Chromatography (TLC) sheets (Merck, Germany). Different systems of development and adequate visualization techniques were used [30-32]: vanillin–hydrochloric acid for condensed tannins and indican (indigo component); Dragendorff's reagent for alkaloids; Lieberman–Burchard reagent for terpenes and steroids; anisaldehyde for saponins; Neu's reagent for gallic acid and flavonoids and UV light for coumarins.

Fluorescence emission spectroscopy

Fluorescence measurements of IsuLL were performed at 25ºC using a spectro fluorimeter (JASCO FP-6300, Tokyo, Japan) in a cuvette (1-cm pathlength rectangular quartz) and the sample to 0.2 mg ml^{-1} in 10 mM phosphate buffer, pH 7.0. The intrinsic fluorescence emission of sample protein to the excitation wavelength (λ_{exc}) was 280 and 295 nm; emission spectra (λ_{emiss}) were recorded at a range of 305–450 nm and band passes were 5 nm.

DPPH radical-scavenging activity

The DPPH free radical scavenging was based on a modified method of Brand-Williams [33]. In the assay, antioxidants present in the sample reduce the DPPH radicals, which have an absorption maximum at 517 nm. The samples (0.04 ml) in 0.15 M NaCl solution were added to microtiter plates (96 wells) with 0.25 ml of DPPH (2.22 mg mL^{-1}). The mixture was shaken and allowed to stand at room temperature in the dark for 30 min and measured at 517 nm. Extinction of the disposable cuvette with 250 µl of the methanolic DPPH solution and 0.04 ml of the 0.15 M NaCl solution was measured as blank. The quecertin (Sigma-Aldrich®) was used as positive control in the presence of the DPPH solution. All samples and standards were analyzed in concentrations of 0.25 to 1 mg mL^{-1} and in triplicate. DPPH free radical scavenging (FRS) activity was calculated according to the equation: $\mathbf{FRS\ (\%) = (\mathit{Ac} - \mathit{As})\ x\ 100)/\ \mathit{Ac}}$ where, *Ac* is the absorbance of the control and *As* is the absorbance of the sample.

Microbial strains

Antibacterial activity was assayed against clinical isolate strains of *Staphylococcus aureus* (UFPEDA15) and *Pseudomonas aeruginosa* (UFPEDAC1), in addition to standard strains of *Klebsiella pneumoniae* (ATCC-700603), *Staphylococcuss epidermidis* (ATCC-12228) and *Echerichia coli* (UFPEDA214) that were provided by the Departamento de Antibióticos, Universidade Federal de Pernambuco, Brasil. Stationary cultures were maintained in nutrient agar and stored at 4 °C. The microrganisms were cultured in nutrient broth (MHB) and incubated at 37 °C. The

inoculum was prepared using a 16 h culture adjusted by reference to the McFarland standard and further diluted with 0.15 M NaCl to achieve approximately 10^6 $CFUml^{-1}$.

Antibacterial activity assay

The antibacterial activity of purified lectin was investigated by broth method following the recommendations established by [34], with some modifications. Serial dilutions of the lectin (1 mg mL^{-1}) were prepared in sterile 96-well microplates containing Mueller Hinton broth (MHB) and the bacterial suspensions were inoculated in each well. The growth inhibition was demonstrated by absorbance using a microplate reader (Biorad XMark^{TM}) considering the total growth (100%) in the control well (MHB + bacteria), the percentage of growth reduction was attributed to the remaining well. The bacteriostatic concentration (Minimal inhibition concentration - MIC) was determined as the lowest concentration able to inhibit through microbial growth the well microdilutions after 24 h of incubation at 37°C. The bactericidal concentration (Minimal bactericide concentration - MBC) corresponded to the minimum concentration of the lectin that inhibited 100% growth. These definitions have been established by others workers. All assays were performed in triplicate.

Data analysis

All data were presented as mean ± standard deviation and an unpaired t-test was performed to compare the groups ($p < 0.05$ and $n = 3$). Graphpad Prism program was used to perform statistical analysis (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego CA).

Results and discussion

Aqueous extract of *I. suffruticosa* leaves showed HA for human and rabbit erythrocytes; the preparation was more specific for human erythrocytes type A than other sanguineous groups. The inhibition of the HA of extract by the monosaccharide GlcNac indicated that the use of affinity chromatography with chitin would be a choice for protein purification.

The unbound material from chitin column chromatography was devoid of HA. The bound fractions eluted with acetic acid revealed one active peak (SHA, 14,629) with human blood type A erythrocyte (Figure 1A). Results indicate that most protein content was adsorbed to chitin matrix which retained 80% of chromatographed HA (Table 1). Some plant proteins are able of reversibly bind to chitin matrix, a β-l,4-linked biopolymer of GlcNac [7]. This matrix has been used with efficacy to lectin isolation of different plant tissues including leaves [9].

The Superdex 75 column chromatography showed one peak with HA (Figure 1B) and C-18 reversed-phase column revealed one a single peak (Figure 1C). One gram of leaf powder yielded 1.2 mg of purified *I. suffruticosa* Leaf Lectin (IsuLL).

The electrophoresis analysis resolved IsuLL as a main band of 35 kDa by 12.5% SDS-PAGE under reducing or non-reducing conditions (Figure 1D) indicating the absence of disulfides bridges. PAGE for native acidic protein showed a polypeptide band (Figure 1D) and no bands were revealed in PAGE for native basic proteins. IsuLL was weakly stained by periodic acid-Schiff's reagent however has 3.6 % sugar content demonstrated by the phenol–sulfuric acid method.

IsuLL was purified by a single chromatographic step based on protein-carbohydrate interaction, with high HA recovery (80%). A lectin from the tubers of *Arisaema tortuosum* with affinity to GlcNac was isolated using one chromatographic step and recovered about 65% HA [35]. IsuLL yield per gram of leaf powder was very significant.

Inhibition assays of HA showed that IsuLL recognized glycoproteins (BSA, ovoalbumin, azocasein) and carbohydrates (xylose, galactose, GlcNac and sucrose) (Table 2). The results could explain the high lectin interaction to human erythrocyte type A, which has GlcNac on its plasmatic membrane surface [36].

The HA of IsuLL aliquoted samples remained stable up to 80°C whereas the samples that remained throughout experiment (4 h) on heating still showed HA up to 100°C (Figure 3A), demonstrating that IsuLL did not lose its interaction ability during heating. IsuLL activity was less susceptible to heating than lectins isolated of leguminoseae [37, 38], of fruit [39] and marine sponge [40]. The highest lectin activity occurred in neutral pH ranged from 6.5 to 7.0 (Figure 3B). In the basic and acid pH range HA was maintained; IsuLL lost activity between pH range of 3.0 to 5.0 (Figure 3B). The divalent ions (Mg^{2+}, Mn^{2+} e Ca^{2+}) did not alter lectin HA. Similar results also

demonstrated that lectin activity of *Erythrina lysistemon* [37] and *Eugenia uniflora* [41] are weakly ion dependence.

IsuLL phytochemical evaluation using TLC sheets on the different systems of development revealed total absence of secondary metabolites or non-proteins (alkaloids, condensed tanins, coumarins, flavonoids, hydrolysable tannins, indican (indigo component), polyphenols, saponins, steroids and terpenes).

IsuLL intrinsic fluorescence spectra showed a single major peak with maximum emission around 348 nm (Figure 3) suggesting that tryptophan and tyrosine residues in lectin native state are exposed to solvent.

In the figure 4 IsuLL antioxidant activity by DPPH free radical scavenging activity (52 %) was higher ($p < 0.0001$) than aqueous extract of *I. suffruticosa* (33 %), and less than standard sample quercetin (82%). However IsuLL (1 mg mL^{-1}) demonstrated best DPPH scavenging in comparison to protein binding polysaccharide purified from *Pleurotus ostreatus* in the same concentration [42].

Others proteins also demonstrated potential antioxidant with proteins isolated from lupin seeds [43]; a mannose-specific banana and garlic lectins were also reported with antioxidant activity [44]. This can be explained by the presence of aromatic amino acids in proteins which have been reported possess some degree about the reduction of free radicals [45]. The presence of exposed tryptophan and tyrosine residues on IsuLL (Figure 3) may contribute to the scavengers of free radicals, which is associated with many chronic degenerative diseases such as cardiovascular disease, arteriosclerosis, diabetes, cancer, as well as the aging process [46].

The antimicrobial assay showed that IsuLL exhibited antibacterial activity against the pathogenic strain of *Staphylococcus aureus* from clinical isolate. MIC values (Table 3) for *S. aureus* indicated that even in lowest concentrations IsuLL (31.25 µg mL^{-1}) was able to inhibit the microorganism growth. The lectin did not show activity against pathogenic bacteria *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. epidermidis* indicating a specific antimicrobial activity to a gram-positive bacterium.

A lectin purified from *Schinus terebinthifolius* also related antibacterial activity more expressive to Gram-positive than to Gram-negative bacteria [47]. Also, a chitin binding lectin isolated from *Myracrodruon urundeuva* with GlcNac affinity also showed more effective activity against *S. aureus* than to other tested bacteria [48].

Lectins demonstrated strongly interaction with complex carbohydrates (GlcNac, muramic acid, N-acetylmuramic acid) present in the peptidoglycan sheet of - microorganism cell wall [49].

IsuLL, a chitin binding lectin with affinity to GlcNac showed antibacterial activity against *S. aureus*, a gram-positive bacterium with cell wall containing GlcNac [50]. Lectin activity depends on protein-carbohydrate interactions, a relevant characteristic of these proteins and an essential role for IsuLL activity against *S. aureus* considered the third most important cause of disease in the world among the reported foodborne illnesses [51].

In conclusion, this study revealed that the new chitin-bind IsuLL, an acidic glycoprotein with carbohydrate and glycoprotein affinity, withstands heat, with hydrophobic surface and aromatic amino acids exposed, besides of showed antioxidant and antibacterial activity may be isolated from *I. suffruticosa* leaves by one step purification protocol with high yield of active protein.

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Figure 1. Isolation and partial characterization of *I. suffruticosa* leaf lectin. **(A)** Chitin **a**ffinity chromatography of IsuLL from aqueous extract of *I. suffruticosa*. Unbound fraction washed with 0.15 M NaCl. Single peak the bound fraction eluted with 2.0 M acetic acid. **(B)** Reverse phase chromatography in a C-18 column of a HPLC system. **(C)** Superdex 75 column coupled to an ÄKTA purifier system. **(D)** Electrophoresis 12.5% SDS-PAGE in absence (lane 1) and under reductions conditions (lane 2) of the Is-CBP (100µg) stained with Coomassie Brilliant Blue. (lane 3). Electrophoresis under native conditions for acid protein. Molecular Weight (MW) proteins: Albumin, bovine (66 kDa), Albumin, egg (45 kDa), Glyceraldehyde-3-phosphate Dehydrogenase, rabbit muscle (36 kDa), Carbonic anhydrase, bovine (29 kDa), Trypsinogen, bovine pancreas (24 kDa), Trypsin Inhibitor, soybean (20 kDa), a-Lactalbumin, bovine milk 14.2 kDa).

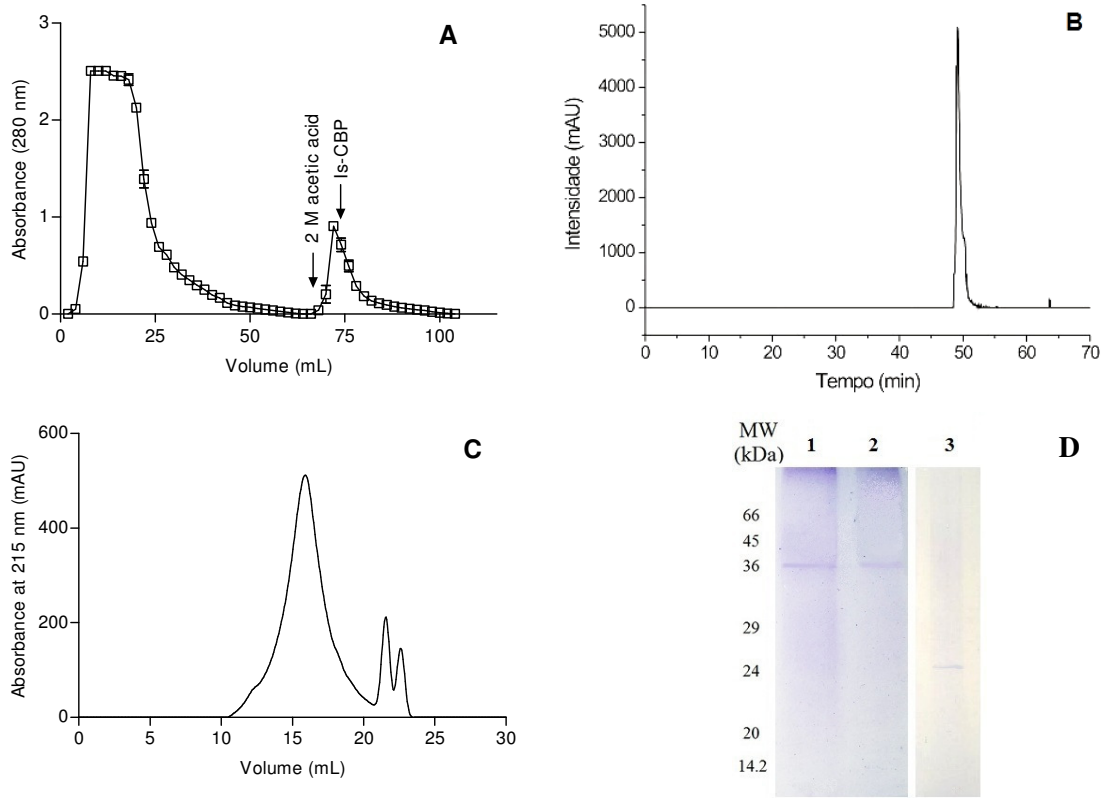

Table 1. Stages of purification process from aqueous extract of *I. suffruticosa*

Preparation	Volume (ml)	Total protein (mg)	Specific HA (HA/mg)	Total HA	Purifications[a] (folds)	Recovery[b] (%)
Crude extract	0.25	5.75	712	1024	1	100
IsuLL	0.2	0.28	14629	819.2	20.4	80

IsuLL - *Indigofera suffruticosa* Leaf Lectin.
HA - Hemagglutinating activity.
[a] Purification folds of IsuLL obtained by the ratio SHA of crude extract/SHA of lectin.
[b] Recovery of HA, obtained by ratio between total activity of crude extract and lectin.

Table 2. Inhibition test of IsuLL with carbohydrate and glycoproteins.

Inhibitor (20 mM)	SHA
Carbohydrate	
D-arabinose	14629
D-fructose	14629
D-galactose	3657
D-Glucose	14629
D-mannose	14629
D-xylose	3657
Lactose	14629
L-raffinose	14629
Methyl-α-D-mannopyranoside	14629
N-acetyl-D-glucosamine	7314
Sucrose	7314
Glycoprotein	
Azocasein	29
BSA	3657
Ovoalbumin	114

SHA- Specific Hemagglutinant activity.

Figure 2. Effects of temperature and pH on HA of IsuLL. (A) Effects of temperature on HA of (○) aliquoted and (■) remained throughout the experiment IsuLL samples. (B) Effects of pH on IsuLL (200 μgml^{-1}) HA.

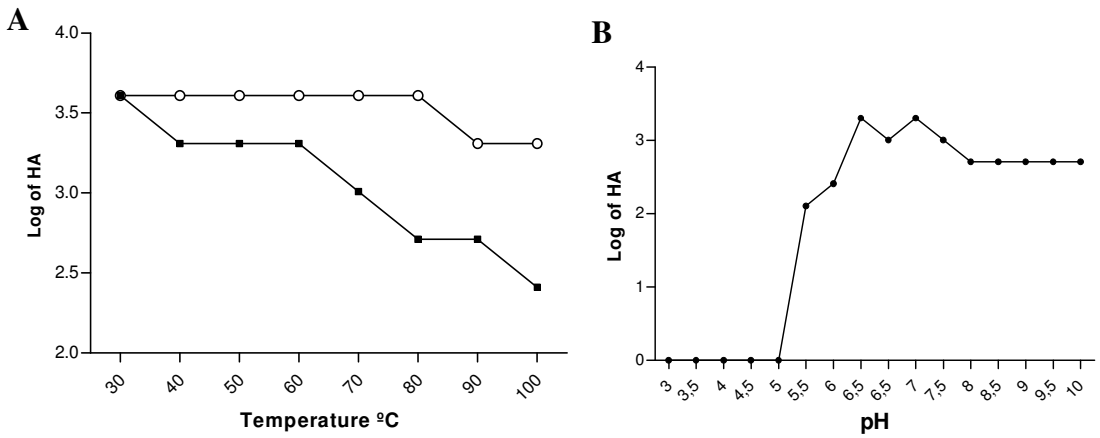

Figure 3. Fluorescence emission spectra of IsuLL. Intrinsic fluorescence at 280 nm (solid line) and 295 nm (dashed line) with wavelength light excitation.

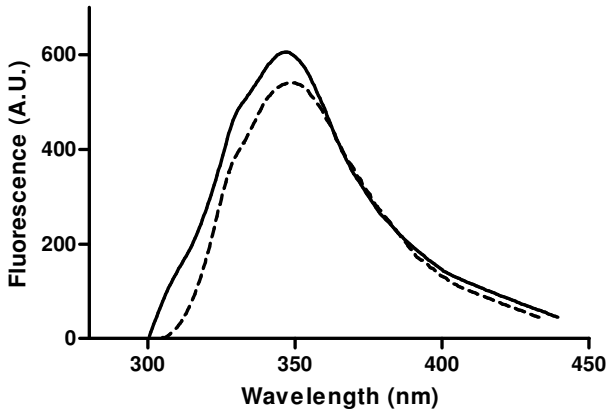

Figure 4. Comparison DPPH radical-scavenging activity of crude aqueous extract of *I. suffruticosa* and of IsuLL, utilizing the quercetin as standard. Each value represents a mean ± SD (n = 3). * (p<0,05) in coparison to control group.

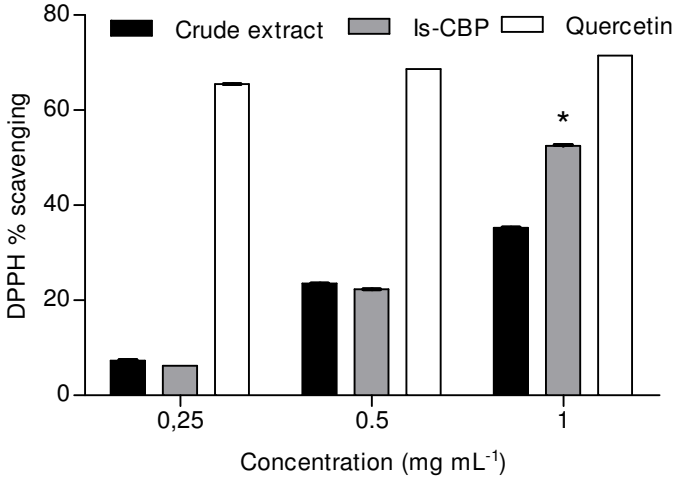

Table 3. Antibacterial activity of IsuLL. Minimal inhibition concentration - MIC, of IsuLL against assayed bacteria

Microrganism	Strain	MIC (µgml^{-1})
E. coli	UFPEDA214	ND
K. pneumoniae	ATCC-700603	ND
P. aeruginosa	UFPEDA14	ND
S. aureus	UFPEDA15	31.25
S. epidermidis	ATCC-12228	ND

MIC, minimal inhibitory concentration.
ND, not detected.

CAPÍTULO II

ARTIGO II

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CHARACTERIZATION OF AN CHITIN BINDING LECTIN FROM *Indigofera suffruticosa* LEAVES (IsuLL) WITH ANTIFUNGAL ACTIVITY AGAINST *Candida*

Pereira, D.R.[1]; Nascimento, W. M.[1]; Neves R. P.[2], Santos, N. P. S.[3]; Correia, M.T.S.[1]; Paiva, P. M. G.[1]; Coelho, L. C. B. B.[1]; Lima, V. L. M[1].

[1]Departamento de Bioquímica, [2] Departamento de Micologia, [3] Centro Acadêmico de Vitória, Universidade Federal de Pernambuco, Recife, PE, Brazil.

*Corresponding author
Vera Lúcia de Menezes Lima
Av. Professor Moraes Rego, S/N.
Cidade Universitária, Recife, Pernambuco, Brazil.
Cep: 50670-420. Phone fax: (+55 81) 2126-8540
E-mail adress: lima.vera.ufpe@gmail.com

ABSTRACT

An *Indigofera suffruticosa* Leaf Lectin (IsuLL) obtained by chitin column chromatography with apparent molecular weight of 35 kDa is composed predominantly by β-sheet structures. The Bis-ANS interaction with IsuLL indicated the presence of hydrophobic residues on the surface of the lectin. The high temperatures not changed the intrinsic fluorescence emission of tyrosine and tryptophan residues, but, the Bis-ANS fluorescence emission was slightly modified up to 100 ºC accompanied by a 7 nm blue shift. The TG curve revealed high temperature of thermal stability (T_{peak}=200°C). IsuLL demonstrated percentage deaths of *A. salina* with LC_{50} of 207.87 ± 2 μg mL^{-1}, however it didn't impaired the proliferation of human cancer cells. IsuLL inhibited the growth of *C. albicans*, *C. krusei*, *C. parapsilosis and C. tropicalis* with MIC of 64, 32, 8 and 16 μg mL^{-1}, respectively Thus IsuLL a lectin thermostable considered moderately toxic to the environment have antifungal activity against different species of candida, an opportunistic invasive mycoses with high incidence on the immunosuppressed.

Keywords: Lectins, leaves, extrinsic protein fluorescence, protein structure, antifungal activity

Introduction

Lectins are (glyco) proteins of non-imune origin that bind reversibly and noncovalent to carbohydrate or glicoconjugate groups whether free in solution or on cell surface (Correia *et al*., 2008). The lectins bound carbohydrate interaction is specific and essential for many biological processes, such as viral, bacterial, targeting of cells and soluble components, which increase the amount of studies in the medicinal and biotechnologies area (Sharon *et al*., 2007). The lectins have been studied because your toxic potential against insects, microorganisms and malignant cells (Napoleão *et al*., 2011; Costa *et al*., 2010; Singh *et al*., 2005; Yan *et al*., 2009).

The lectins ability to identify a target is determined by their broad selectivity of carbohydrate-binding site and the geometrical arrangement (Wang *et al*., 2011). The conformational change by different reason in the structure protein can be prejudicial to molecular stability and interactions with ligands. Therefore, the knowledge on the lectins characteristics and the importance of the structure for investigation of protein function is becoming increasingly commonplace (Varejão *et al*., 2011; Celej *et al*., 2003).

The fungi of Candida genus are pathogen responsible by infections in the oral and vaginal cavity, tracheal mucosa, bronchi and gastrointestinal tract that include a specter ample of surface and invasive opportunistic diseases (Alonso-Echanove *et al*., 2003). Epidemiologic studies have identified Candida species as the fourth most common cause of nosocomial bloodstream infection (BAR *et al*., 2006) and opportunistic infection (Nucci *et al.*, 2010).

Indigofera suffruticosa is a Leguminoseae distributed wideworld by tropical and subtropical regions and in Northeastern of Brazil is found in semi-arid region (Ribeiro *et al*., 1991). This plant is used as a source of indigo dye and in folk medicine as an antispasmodic, sedative, diuretic. Studies demonstrated inhibition the early development of mouse embryos in vitro (Leite et al., 2004).

Our aim is analyzed the structure of IsuLL and verified the capacity of inhibit the proliferation of human cells line and the growth of pathogens fungi.

Materials and methods

Chemicals

Sugars (N-acetyl-D-glucosamine, D-arabinose, D-mannose, L-raffinose, D-galactose, D-fructose, D-glucose, sucrose, lactose, D-xylose, methyl-α-D-mannopyranoside), glycoproteins (azocasein, albumin from chicken egg white and bovine serum albumin - BSA), the chitin matrix and the range protein molecular mass markers were purchased from Sigma Chemical Company (USA). The solvents and others chemicals used were of analytical grade from Sigma or Merck (Germany). All solutions were prepared with water purified by the Milli-Q® system (Millipore).

Purification of *I. suffruticosa* leaf lectin

The leaves of the *Indigofera suffruticosa* were collected in the city of São Caetano-PE, localized in the semi-arid region of the Northeastern Brazil. The plant was taxonomically identified by Dra. Marlene Carvalho de Alencar Barbosa of the Departamento de Botânica, Universidade Federal de Pernambuco, Brasil. This specie is deposited in this institution in the Herbarium Geraldo Mariz UFP (number 45.217). The leaves were briefly washed with distilled water and left to dry at environmental temperature. The powder of leaves of *I. suffruticosa* was homogenized in water (10%) about constant agitation for 16 hours. The water-based suspensions were then paper-filtered and lyophilized. The lyophilized extract was solubilized (10%) in 0.15 M NaCl and applied to chitin column (Sigma, USA, 20×7cm) previously equilibrated at 20 mL/h flow rate with 0.15 M NaCl. Protein elution was monitored by absorbance at 280 nm. The unbound proteins were removed with equilibrating solution until the absorbance at 280nm was negligible. The bound proteins were eluted from the column with 2.0 M acetic acid, pH 4.0 and after were pooled, dialyzed against water, lyophilized and suspended in 0.15 M NaCl.

Protein assay

The protein content was determined according to Lowry et al. (1951) using a BSA calibration curve as the standard (0–500 μg mL^{-1}) and the total protein (mg) by ratio between a protein concentration and the volume value of the sample.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified lectin preparation was subjected to SDS-PAGE, pH 8.3, using 12.5% (w/v) acrylamide slab gel for subunit molecular mass determination as described by Laemmli, (1970). The sample was heated for 5 minutes in boiling water bath and the gel was stained with Coomassie brilliant blue and destained with acetic acid 10% (v/v).

Infrared spectroscopy

Samples were carefully pressed into thin pieces. Then their Fourier transform infrared (FTIR) spectra were measured with a FTIR spectrometer model Perkin-Elmer System 2000 at room temperature. The wavenumber region scanned was from 4000 to 400 cm^{-1}. The resolution was 4 cm^{-1}. Each spectrum was scanned 100 times at a speed of 0.5 cm^{-1}/s.

Thermogravimetry

The analyses of thermogravimetry were realized by thermobalance Shimadzu®, model TGA Q60, in nitrogen atmosphere and flux 50 mL min^{-1}. The mass sample of protein was of about 0.5 mg, placed in a platinum crucible and an empty platinum crucible was used as a reference. The range of temperature was of 25 to 600 ºC at a temperature gradient of 10 ºC min^{-1}. The instruments calibration were verified utilizing a aluminium and zinc samples and the appropriate software (TA60 Shimadzu®)

Fluorescence emission spectroscopy

Fluorescence measurements of IsuLL were performed at 25ºC using a spectro fluorimeter (JASCO FP-6300, Tokyo, Japan) in a cuvette (1-cm pathlength rectangular quartz) and the sample to 0.2 mg mL^{-1} in 10 mM phosphate buffer, pH 7.0. To the intrinsic fluorescence emission of IsuLL the excitation wavelength (λ_{exc}) was 295 and 280 nm; emission spectra (λ_{emiss}) were recorded at a range of 305–450 nm and band passes were 5 nm. To the hydrophobic surface evaluation was used the same conditions as above however adding in the sample 5 μM Bis-ANS, the λ_{exc} was 360 nm and λ_{emiss} was 400–600 nm. The tubes were incubated, overnight, with stirring. The thermal

stability of IsuLL fluorescence intensity was performed by incubating lectin samples (0.2 mg mL^{-1} in 10 mM phosphate buffer, pH 7.0) at different temperatures (50–100°C) for 40 min and after cooling at room temperature (25ºC).

Environmental toxicity

A. salina (Brine Shrimp) encysted eggs (25 mg) were hatched in a beeker filled with seawater under artificial light at 30 ºC, pH 8–9 in constant aeration. After 28 h the nauplii were collected with a Pasteur pipette macroscopically and counted in the stem of a pipette against a lighted background. The nauplii were transferred to test tubes containing the samples. The lectin concentration ranged from 10 to 1000 µg mL^{-1} in vials containing 5 ml of seawater. Fifteen shrimp nauplii were added to each vial (45 shrimps per concentration). The plates were maintained under illumination. Survivors were counted after 24 h of incubation and the percentage of deaths at each dose and control was determined. The bioassay was performed as described previously by Meyer et al. (1982) and developed with minor modifications. When the LC_{50} < 80µg mL^{-1} was considered highly toxic; LC_{50} between 80 and 250µg mL^{-1} was considered moderately toxic; and LC_{50} > 250µg mL^{-1} was considered mildly toxic or non-toxic.

MTT Cytotoxicity Assay

Cell viability was assessed using the MTT colorimetric assay against human cells line KG1 (leukemia) and HeLa (nasofaringeal). MTT solution (1 mg ml^{-1}) was added to each 96-well culture plate and incubated for 4 h at 37 °C, and then the medium containing MTT was removed. The incorporated formazan crystals in the viables cells were solublized with 100 µl of dimethyl sulfoxide (DMSO) and the absorbance of each well was read at 540 nm using a microplate reader.

Antifungal Activity

Different strains of yeast species (*Candida albicans* 4990, *Candida albicans* 3719, *Candida tropicalis* 4790, *Candida tropicalis* 1150, *Candida krusei* 4802*, Candida krusei* 1059, *Candida parapsilosis* 4608) and of the clinical strains *Trichophyton mentagrophytes, Trichophyton rubrum, Trichophyton tonsurans, Epidermophyton*

flocossum e Fusarium solani were used for trial of antifungal susceptibly using microtitulation plates. The test was made with RPMI 1640 (Sigma), tamponed within Morpholin Propane Sulfônic (MOPS), pH 7,0 and for positive control was used Cetoconazol drug. Inocule of Candida species was prepared by equivalent turbidity of a control-solution from McFarland 0,5 scale. Firstly 100µL from RPMI 1640 (Sigma) ambient was put in every wells of the plate, and then was inserted in different wells of the plate 100µL of Cetoconazol, for control, and 100µL of protein (1024 µg/mL), performing serial dilutions. The distribution of fungal suspension was made in every wells, with exception of the designated well for ambient sterility control (line 12). The plate was incubated in stove at 37º C, after 24 and 48 hours of incubation was executed reading to determinate Minimal Inhibitory Concentration (MIC) and Minimal Fungicide Concentration (MFC). Fungi used were obtained on Fungal Culture Collection of the Mycology Department, in University Federal of Pernambuco.

Results and discussion

IsuLL (*Indigofera suffruticosa* Leaf Lectin) was isolated according to previously established protocols. The chitin affinity chromatography of aqueous extract of *I. suffruticosa* showed a bound protein isolated as a single peak and the electrophoresis SDS-PAGE resolved as a main band of 35 kDa.

The analysis of secondary conformational structure was analyzed by FTIR for the structural information like fractions of α-helix, β-sheet, β-turn and random coil structures of protein can be derived from the peak positions and intensity. IsuLL FTIR analysis are demonstrated by second derivative amide-I spectra (Figure 1). The individual peaks of great intensity were located between 1680 - 1688 cm^{-1} and with higher frequency between the peaks 1635 - 1637 cm^{-1} can be assigned to β-sheet structures. The bands appearing also at 1652 - 1659 cm^{-1} with less intensity can be attributed to α-helix (Carbonaro and Nucara, 2010). The results suggest that IsuLL is predominantly a β-sheet protein with a relative small α-helical content according with the conformational component (β-sheet) of most plant lectins (Konozy et al., 2003; Loris et al., 1998; Oliveira et al., 2002; Sinha et al., 2007).

The IsuLL intrinsic fluorescence emission on heating conditions (Figure 2A) revealed that the IsuLL fluorescence emission was initially increased at 60 °C and after only decreased at 100 °C, sharply accompanied with ~2 nm red shift (Figure 2B).

Kueltzo et al. (2003) have observed a new high-energy peaks appearing in the derivative spectral model amino acids at greater than 60°C. Studies of tyrosine derivatives show a curve-linear increase in peak position with increasing temperatures, with changes as great as 2 nm observed over 80°C temperature range (Jiskoot et al., 2005). The results indicated that during the heating the protein left their equilibrium state (25 °C) for a stable intermediate (60 °C) and after (100 °C) would unfolded the protein state. The thermostability is revealed by low variation of fluorescence intensity of the lectin structure conformation even on temperatures elevated. Similar results were related for lectins isolated of *Phthirusa pyrifolia* leaf (Costa et al., 2010).

The Bis-ANS interaction with the IsuLL indicated the presence of hydrophobic residues on the surface of protein. Extrinsic fluorescent dyes can provide information about folding and unfolding processes, and can be particularly valuable to evidence the presence or absence of molten globule intermediates (Hawe, Sutter and Jiskoot, 2008). The extrinsic fluorescence intensity was increased at 100 °C accompanied by a 7 nm blue shift of the fluorescence emission (Figure 3) The IsuLL thermostable capacity mentioned above can be confirmed since that even on temperatures elevated the lectin structure conformation remained stable. Same results were observed in the binding Bis-ANS−BSA which there is a blue shift in the spectra with a temperature midpoint of 75°C (Celej, Montich and Fidelio, 2003). Bis-ANS binding studies support the idea that organized hydrophobic surfaces persist, or can be formed, at high pressures (Chapeaurouge, Johansson and Ferreira, 2001).

TG and DTG curves of IsuLL showed four steps in the thermal decomposition process (Figure 4) and by Table 1 is possible indentified the stages of weight loss and the temperature range. The first stage revealed that the dehydration at the temperature range 30 – 90 ºC with a loss ~11% of the mass. In the second stage, the initial temperature (200 – 230 ºC), suggests high temperature of the thermal stability (T=200 ºC). DTA curve (Figure 4) demonstrated that in the second stage occurs a discrete endothermic peak (T_{peak}= 320 ºC), possibly associated to the elimination of the water and the consequent denature of the protein (Mohamed, 2002). The third and fourth step occurs with an intense heat release (exothermic) revealed by DTA curve (T_{peak}= 495 ºC), as expected to protein species, furthermore was ascribed by carbonization, with weight loss of ~30 %, to each stage. The endothermic transition process, after the removal of water, typically presents a large peak in the region of protein denaturation,

offering so a new characterization method for vegetable proteins (Rouilly *et al*., 2003), further indicate the absence of impurities (Pan *et al.,* 1989).

Sirtori *et al*. (2010) observed for the *Lupinus angustifolius* protein the presence of an endothermic peak around 71.49 ºC, attributed to the denaturation fraction Vicilin (β-conglutin) and the second peak, which occurred around 90.92 ºC, was attributed to the denaturation of legumin fraction (α-conglutin). The denaturation temperature was less than IsuLL suggesting better thermal stability.

Artemia salina Leach is a microcrustacean experimentally utilized as a toxic indicator due their sensibility to chemicals substances (Almeida, Silva, & Echevarria, 2002). IsuLL demonstrated percentage deaths of *A. salina* with LC_{50} of 207.87 ± 2 μg mL^{-1}, which is considerable as moderately toxic.

The anti-proliferative property of IsuLL against human cell line KG1 (leukemia) indicated a proliferation inhibition ratio of 30% for growth of malignant cells. The low antiproliferative activity might be attributed to toxic bioassay with *A. salina* which is recognized as a good predicator of anti-tumor activity (Meyer *et al*, 1982)

The antifungal activity of IsuLL against different species of *Candida* was showed in the Table 2, however was resistant to the others fungi utilized. IsuLL inhibited the growth of *C. albicans*, *C. krusei*, *C. parapsilosis and C. tropicalis* with MIC of 64, 32, 8 and 16 μg mL^{-1}, respectively. Similar results were viewed utilizing a lectin from Asiatic Fabaceae which showed a MIC of 56 μg ml^{-1} against *C. albicans* (Charungchitrak *et al*., 2011). Some lectins also demonstrated sensibility against *C. krusei*, *C. parapsilosis and C. tropicalis* but others were resistant (Gomes *et al*., 2012; Pan *et al*., 2010).

This activity can be related with the lectin carbohydrate binding property, which might occur by certain carbohydrate components in the fungal cell wall affecting its activity and viability (Damico *et al*., 2003). Since the major component of the fungal wall is chitin (N-acetylglucosamine polymer) (Ye *et al*., 2001), the chitin-binding proteins have shown to affect fungal growth and development (Sá *et al*., 2009; Trindade *et al*., 2006), due to disturbing the synthesis and/or deposition of chitin in cell wall (Selitrennikoff, 2001).

Oral candidosis is an opportunistic infection most commonly caused by *Candida albicans*, but *C. tropicalis* is the second most pathogenic of the *Candida* species, which has emerged as a clinically important pathogen, especially in deep fungal infecitions (Dar-Odeh *et al*., 2001; Zaug *et al*., 2001). Infections by C. krusei are rare, however is

recognized as a species of fungus resistant to a wide range of antifungal drugs (Barbedo and Scarbi, 2010).

The conventional treatment for this systemic mycoses have demonstrated limitations due to the cost of the essential medicaments to the major part of the population, the poor efficiency and high toxicity these drugs (Onishi *et al*., 2000).

The results demonstrated that molecular structure of IsuLL suggests the presence of β-sheets, aromatic residues exposed and hydrophobic region buried. The IsuLL structure conformation remained stable at 100 ºC. The TG curve revealed high temperature of thermal stability (200°C) and of endothermic transition (T_{peak}= 495 ºC). Water content and thermal stability parameters permit the control of the deterioration process during the storage period. A chintin-binding lectin of moderate effect on the enviroment revealed fungistatic activity against different species of candida being important for try to reduce the opportunistic invasive mycoses as well for avoid the use of synthetic drugs that can prejudice the health.

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Figure 1. IsuLL FTIR (Fourier-transform infrared) analysis. β-sheet (1,612 - 1,641 and 1670-1690 cm^{-1}), random coils RC (1, 640 - 1,650 cm^{-1}) , α-helix (1,648 - 1,660 cm^{-1}), turn (1,662 - 1,684 cm^{-1}).

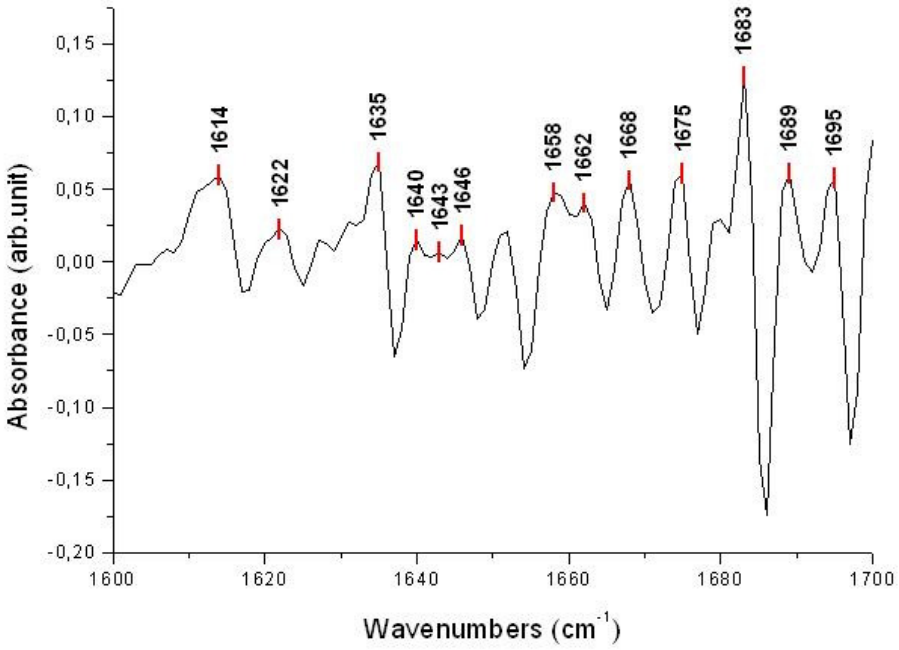

Figure 2. Intrinsic fluorescence emission spectra of IsuLL at different temperatures. (A) Fluorescence emission spectra at 280 nm wavelength light excitation at different temperatures. (B) Modified center of spectral mass plots of IsuLL at different temperatures. Each point on the lines represents the average of three replicates and data were expressed as the mean ± standard deviation (SD).

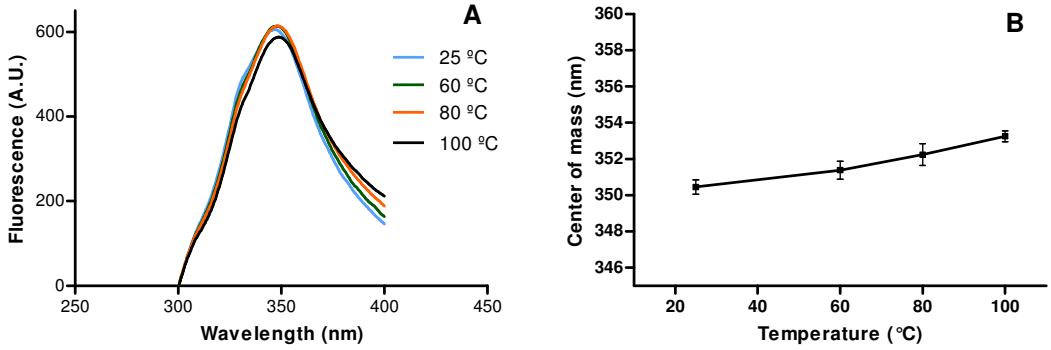

Figure 3. Extrinsic fluorescence emission spectra of IsuLL at different temperatures. (A) Fluorescence emission spectra of IsuLL at 360 nm wavelength light excitation by different temperatures (−− 25 °C, — 60 °C, --- 80 °C, ··· 100 °C). (B) Modified center of spectral mass plots of IsuLL at different temperatures. Each point on the lines represents the average of three replicates and data were expressed as the mean ± standard deviation (SD).

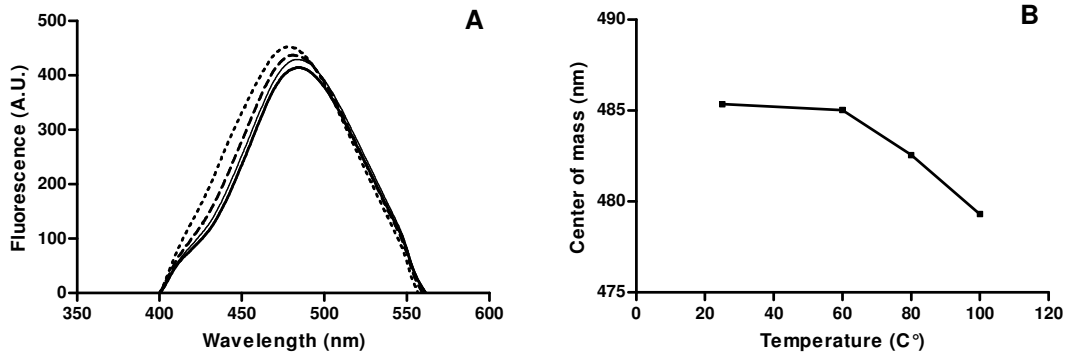

Figure 4. Typical Thermogravimetric Analysis (TGA) diagram of IsuLL. The direct (TG), Derivative (DTG) and Differential Thermal Analysis (DTA) curves were perfomed.

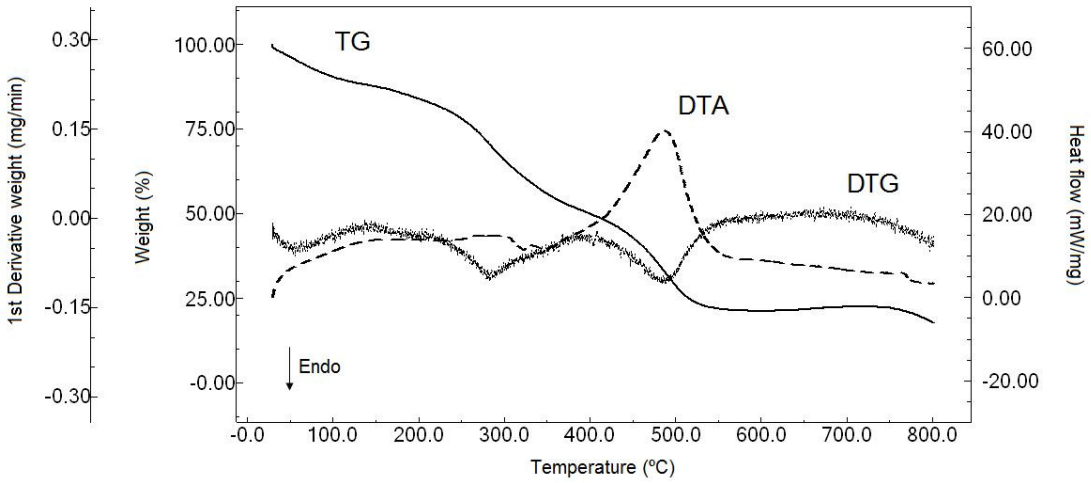

Table 1. Temperature range and weight loss under nitrogen atmosphere.

	Temperature (ºC)	**Weight (%)**
Stage I	43 90	10.9
Stage II	200 230	7.43
Stage III	257 325	30.20
Stage IV	451 517	29.65

Table 2. Antifungal activity of IsuLL. Minimal inhibition concentration - MIC, of IsuLL against tested fungi

Microorganisms	**Strains**	**MIC (μg mL^{-1})**
C. albicans	4990	128
C. albicans	3719	64
C. tropicalis	4790	64
C. tropicalis	1150	16
C. krusei	4802	8
C. krusei	1059	8
C. parapsilosis	4608	32

CAPÍTULO III

ARTIGO III

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EVALUATION OF THE EFFECT OF *Indigofera suffruticosa* LEAVES LECTIN (IsuLL) ON THE *Staphylococcus aureus*

Pereira, D.R.[1]; Nascimento, W. M.[1]; Ximenes, E. C. P. A[2]; Correia, M.T.S.[1]; Paiva, P. M. G.[1]; Coelho, L. C. B. B.[1]; Lima, V. L. M[1].

[1]Laboratório de Química e Metabolismo de Lipídeos e Lipoproteínas, Departamento de Bioquímica, Universidade Federal de Pernambuco (UFPE), Av. Prof. Moraes Rego s/n, Cidade Universitária. CEP 50670-420. Recife, PE, Brazil.
[2]Laboratório de Fisiologia e Bioquímica de Microrganismos, Departamento de Antibióticos, Universidade Federal de Pernambuco (UFPE), Rua Artur de Sá s/n, Cidade Universitária. CEP 50670-901. Recife, PE, Brazil.

*Corresponding author

Vera Lúcia de Menezes Lima

Av. Professor Moraes Rego, S/N.

Cidade Universitária, Recife, Pernambuco, Brazil.

Cep: 50670-420. Phone fax: (+55 81) 2126-8540

E-mail adress: lima.vera.ufpe@gmail.com

ABSTRACT

Plants isolated proteins interaction with *Staphylococcus aureus* is a predictor factor of cell death by membrane lysis or damage. *Indigofera suffruticosa* a plant (leguminosae) of the semi-arid clime demonstrated activity against microorganisms but without clearly. This paper aimed to investigate the effect of *I. suffruticosa* leaves lectin (IsuLL) on the *S. aureus*. IsuLL was isolated by chitin affinity columns chromatography with molecular weights of 35 kDa. This lectin inhibited the growth of different clinical isolates *S. aureus* strains. There was increased in uptake of crystal violet, but no release the 260-absorbing materials in *S. aureus* treated with IsuLL. Electron microscopy of IsuLL-treated microorganism cells showed cell wall deformity and change in eletron-density in comparison to control group, and not revealed lysis. The results indicated that the antimicrobial effect of the antibiotics (Cifoxitin and Ampicilin) can be potentiated when combined to IsuLL. Thus, IsuLL improves the antibacterial activity against the serious Gram-positive pathogen S. aureus strains and the action mechanism on this microorganism is probably by cell wall interaction without the lysis occurrence. Furthermore IsuLL represents a promising antibiotic compound from natural product

Keywords: *Indigofera suffruticosa*, lectins, Electron microscopy, Synergism, violet crystal, cell wall.

Introduction

Staphylococcus aureus a nonspore forming Gram-positive cocci can produce an enterotoxin with able for cause severe post-surgery (Okdakowska-Jedynak *et al*., 2003). This microorganism is the major cause of community and hospital-acquired infections as skin, urinary tract, gastrointestinal and lower respiratory tract infections, as well peritonitis, osteomyelitis, tropical myositis, endocarditis, and bacteremia (Fung *et al*., 2001). The indiscriminate antibiotic use increased the *S. aureus* strains resistant to penicillin by worldwide (Casal et al., 2005) and in most of the Asian countries 70–80% of the same strains are methicillin resistant (Chambers, 2001). The antibiotic resistance can occur naturally (intrinsic) or be acquired due the genetic ability of bacterial species (Silva *et al*., 2010). The necessity of development of new drugs including changing in molecular antibiotics, or on the other hand, the chose by natural products, may be employed to overcome these resistance mechanisms isolate or in combination of drugs (Hemaiswarya *et al*., 2008).

Natural components are still major sources for new therapeutic agents, since Brazil possess such great plant biodiversity (Silva *et al*., 2010). Some molecules can be utilized within the context to play a role in plant defense being known as antimicrobial proteins (De Candido Souza *et al*., 2011).

Lectins are proteins that possess at least one noncatalytic domain that binds reversibly to carbohydrate and recognize different cells through binding with cell surface glicoconjugates (Texeira *et al*., 2012).

Indigofera suffruticosa Mill. (Fabaceae) is an arbustive plant found in the semi-arid regions in the Northeast of Brazil (Ribeiro *et al*., 1991). The leaves are used by folk medicine due the purgative, sedative and diuretic actions (Hasting, 1990). Preliminary results demonstrated that the aqueous extract of *I. suffruticosa* have antimicrobial activity against *S. aureus* (Leite *et al*., 2006).

There a few studies that try understand the mode as the drugs acting on the microorganisms. In this study were evaluated the anti-staphylococcal activity, the membrane interaction and was tested the antibiotic adjuvant activity of proteics compounds from I. suffruticosa against S. aureus.

Materials and methods

Chemicals

Bovine serum albumin (BSA), the chitin matrix and the range protein molecular mass markers were purchased from Sigma Chemical Company (USA). The solvents and others chemicals used were of analytical grade from Sigma or Merck (Germany). All solutions were prepared with water purified by the Milli-Q® system (Millipore). Inocula were prepared in Mueller–Hinton Broth (MHB; Oxoid) and Mueller–Hinton agar (MHA; Oxoid).

Indigofera suffruticosa

The leaves of the *Indigofera suffruticosa* were collected in the city of São Caetano-PE, localized in the semi-arid region of the Northeastern Brazil. The plant was taxonomically identified by Dra. Marlene Carvalho de Alencar Barbosa of the Departamento de Botânica, Universidade Federal de Pernambuco, Brasil. This specie is deposited in this institution in the Herbarium Geraldo Mariz UFP (number 45.217).

Crude Extract preparation

The leaves were briefly washed with distilled water and left to dry at environmental temperature. The powder of leaves of *I. suffruticosa* was homogenized in water (10%) about constant agitation for 16 hours. The water-based suspensions were filtered in paper-filtered and lyophilized.

Purification of *I. suffruticosa* leaf lectin

The lyophilized extract was solubilized (10%) in 0.15 M NaCl and applied to chitin column (Sigma, USA, 20×7cm) previously equilibrated at 20 mL/h flow rate with 0.15 M NaCl. Protein elution was monitored by absorbance at 280 nm. The unbound proteins were removed with equilibrating solution until the absorbance at 280nm was negligible. The bound proteins were eluted from the column with 2.0 M acetic acid, pH 4.0 and after were pooled, dialyzed against water, lyophilized and suspended in 0.15 M NaCl.

Protein assay

The protein content (mg/ml) was determined according to Lowry et al. (1951) using a BSA calibration curve as the standard (0–500 μg mL^{-1}) and the total protein (mg) by ratio between a protein concentration and the volume value of the sample.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Purified protein preparation was subjected to SDS-PAGE using a Minin-PROTEAN® 3 cell (BioRad). Gels consisted of a 12.5 % polyacrylamide resolving gel (pH 8.3) and a 5% stacking gel (pH 6.8) for subunit molecular mass determination as described by Laemmli, (1970). Samples were dissolved in sample buffer (0.1 M Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 0.02% bromophenol blue) and loaded onto the gels (10 μl, 100 μg protein/well). After was heated for 5 minutes in boiling water bath. The gel was stained with 0.125% (w/v) Coomassie brilliant blue R-250 in 7% acetic acid and 40% methanol (v/v) solution and destained with acetic acid 10% (v/v). Electrophoretic analysis under acidic and basic conditions was performed to test the homogeneity of purified lectin preparation.

Microbial strains

Antibacterial activity was assayed against eight strains *Staphylococcus aureus* ATCC (6538) and Clinical isolates (13, 14, 15, 17, 27, 138 and 311) that were provided by the Departamento de Antibióticos, Universidade Federal de Pernambuco, Brazil. Stationary cultures were maintained in nutrient agar and stored at 4 °C. The microrganisms were cultured in nutrient broth (MHB) and incubated at 37 °C. The inoculum was prepared using a 16 h culture adjusted by reference to the McFarland standard and further diluted with 0.15 M NaCl to achieve approximately 10^8 $CFUml^{-1}$.

Antimicrobial activity assay

The antibacterial activity of purified lectin was investigated by broth method following the recommendations established by Clinical Laboratory Standards Institute (2010), with some modifications. Serial dilutions of the lectin (1 $mg.ml^{-1}$) were prepared in

sterile 96-well microplates containing Mueller Hinton broth (MHB) and the bacterial suspensions were inoculated in each well. The growth inhibition was demonstrated by absorbance using a microplate reader (Biorad XMark^{TM}). Considering the total growth (100%) in the control well (MHB + bacteria), the percentage of growth reduction was attributed to the remaining well. The bacteriostatic concentration (Minimal inhibition concentration - MIC) was determined as the lowest concentration able to inhibit through microbial growth the well microdilutions after 24 h of incubation at 37◦C. The bactericidal concentration (Minimal bactericide concentration - MBC) corresponded to the minimum concentration of the lectin that inhibited 100% growth. These definitions have been established by other workers. All assays were performed in triplicate.

Electron microscopy

The *S. aureus* inoculum (strain 27) cultured as describe above was incubated with the sample (MIC and 2xMIC) during 16 h at 37 ^{o}C. The suspension was centrifuged at 2,000 x*g* for 5 min, the pellets were fixed overnight in 2.5% glutaraldehyde at room temperature. After it was done a post-fixation with osmium 1% and washed with phosphate 0.1 M and distilled water. The sample was placed in uranyl 5% block for 1h and washed with distilled water. The preparation was dehydrated in acetone 30%, 50%, 70%, 90% and three times in 100%. The material was embedding in epon resin with acetone overnight for four days in proportion different. In the fifth day it was in block with epon pure at 60°C for 48h. Ultrathin sections were cut in a Reichert Jung microtome and stained with uranyl acetate and examined with a FEI Morgagni 268(D) transmission electron microscope at an accelerating voltage since 40 at 100 kV.

Violet Crystal assay

The alteration in membrane permeability was detected by crystal violet assay (Vaara and Vaara, 1981). Suspensions of *Staphylococcus aureus* were prepared in LB broth. Cells were harvested at 4500×g for 5 min at 4 ºC. The cells were washed twice and resuspended in PBS (pH-7.4). IsuLL (MIC and 2xMIC) and ciprofloxacin (2 µg/ml) were added to the cell suspension and incubated at 37 ºC for 30 min. Control samples were prepared similarly without treatment. The cells were harvested at 9300×g for 5min. After that the cells were resuspended in PBS containing 10µg mL^{-1} of crystal

violet. The cell suspension was then incubated for 10 min at 37 ºC. The suspension was then centrifuged at 13,400×g for 15 min and the OD_{590} of the supernatant was measured in spectrophotometer (Biorad XMark^{TM}). The OD value of the crystal violet solution, which was originally used in the assay, was taken and it was considered as 100%. The percentage of crystal violet uptake of all the samples was calculated using the following formula: OD_{value} of the sample/ OD_{value} of crystal violet solution × 100

Release of cellular material

The measure of the release of 260-nm-absorbing material from *S. aureus* cells (strain 27) was carried out in aliquots of the bacterial inoculum in MHB added of purified protein (MIC and 2xMIC) at 37 ºC. Additional samples were removed after 60, 120 min and 16h of treatment and cells were centrifuged at 4000 x *g*. The nucleic acid content of the supernatants was directly measured at 260 nm using a UV microplate spectrophotometer (Biorad XMark^{TM}) (Carson, Mee, & Riley, 2002). Control flasks without protein purified were tested similarly. Results were expressed by mean ± SD (n = 3) of 260-nm-absorbing material in each interval with respect to the ultimate time.

Synergy assay

Synergy testing was performed using the chequerboard assay, allowing multiple test concentrations of ampicillin, cefoxitin and ciprofloxacin to be assayed in combination of various concentrations of plant purified compound in a 96-well microtiter tray. Each well was inoculated with bacterial suspension (at approximately 10^6 $CFUml^{-1}$) and the plates were incubated at 37 ºC for 24 h The fractional inhibitory concentration index (FIC) was calculated using the following formula: $FIC_A + FIC_B = FIC$, where FIC_A = MIC of antibiotic in combination/MIC of antibiotic alone and FIC_B = MIC of drug in combination/MIC of drug alone. Synergy was defined whereby the FIC was ≤ 0.5, additive when it was 0.5 to ≤ 1, and antagonistic when ≥ 2 (Eliopoulus and Moellering, 1996).

Results and discussion

IsuLL (*Indigofera suffruticosa* Leaf Lectin) was isolated according to previously established protocols. The chitin affinity chromatography of aqueous extract of *I. suffruticosa* showed a bound protein isolated as a single peak and the electrophoresis SDS-PAGE resolved as a main band of 35 kDa.

The antimicrobial assay of the chitin-bound material on *Staphylococcus aureus* is reported in the Table 1. The results showed an antibacterial activity against different pathogenic *S. aureus* strains, with lower MIC for the clinical isolate strains 15 (31.25 µg mL^{-1}) and 27 (15.62 µg mL^{-1}), but not demonstrated bactericidal activity. IsuLL showed elevated inhibition of the growth to different strains indicating a specific capacity of lectin-carbohydrate recognition through the cell surfaces which may be used as specific target against certain microorganisms (Paiva *et al*, 2010). Recent studies utilizing lectin also demonstrated antimicrobial activity against *S. aureus* (Gomes *et al*., 2012; Costa *et al*., 2010; Oliveira *et al*., 2007)

The cell membrane damage of *S. aureus* treated with IsuLL was evaluated by crystal violet assay and quantification of release of UV-absorbing materials. The uptake of crystal violet by *S. aureus* cells was of 21 % in the absence of IsuLL, but increased, significantly ($p<0,05$), to 54 % and 64 % after 15.62 µg mL^{-1} and 31.25 µg mL^{-1} treatment, respectively (Figure 1) in comparison to control group. Cifoxitin (2 µg ml^{-1}) demonstrated 53 % of uptake of crystal violet by *S. aureus*. The crystal violet assay available changes on outer membrane permeability due to penetration of this molecule in cell, but the crystal violet easily enter when the membrane is defective. A significant enhancement in the uptake of crystal violet was observed in *S. aureus* treated with IsuLL when compared to control cells. In the release of 260-absorbing materials assay the IsuLL treatment not produced significant differences in comparison to control group (Figure 2). This assay is an index of cell lysis (Zhou *et al*., 2008) suggesting that the effect of IsuLL on *S. aureus* not is by damages cytoplasmic membrane or subsequent leakage of intracellular constituents. However, cifoxitin cause lysis on *S. aureus* demonstrated by release of 260-absorbing materials.

Transmission Electron Microscopy (TEM) revealed that treatment with IsuLL (MIC and 2xMIC) promoted morphology and structural alterations on *S. aureus* cells. There was a decrease in the volume cell volume and the cytoplasm electron density (Figure 3B - black arrow). Furthermore the cell wall presented corrugated and

detachment in comparison to control group (Figure 3C - black arrow). IsuLL interfered in the development of the *S. aureus* in the cell wall since which its principal component (peptidoglycan) is involved in the multiplication of the gram positive microorganisms (Vollmer and Seligman, 2010).

The plants lectins acting on microrganisms through the interaction with N-acetylglucosamine, N-acetylmuramic acid (MurNAc) and tetrapeptides linked to MurNAc present in the cell wall of Gram-positive bacteria or to lipopolysaccharide present in the cell walls of Gram-negative bacteria (Dziarski *et al*., 2000). An indirect role as antimicrobial agent, due to interesting mechanism of action is the blocking of bacterial movements, causing loss of motility and preventing invasion of seedling roots by the pathogens. A lectin from thorn apple (*Datura stramonium*) also showed the same mechanism against pathogenic microorganism (Candido *et al*., 2011).

There varying degrees of antimicrobial toxicity by perforation, membrane destabilization, metabolic inhibitors, and triggering of bacteriolysis (Brouwer, Wulferink and Welling, 2007). The antimicrobial effect of IsuLL on *S. aureus* without cell lysis confirmed by TEM may have beneficial clinical consequences due the reduction of release of pro-inflammatory bacterial components (English *et al*., 2005).

S. aureus is an immotile Gram-positive coccus responsible for the majority of superficial and invasive skin infections, resulting in more than 11,000,000 outpatient/emergency room visits and 464,000 hospital admissions annually in the United States (Daum, 2007).

The increasing resistance at the antibiotics has created a need for the knowledge new antimicrobial agents. Therefore more drugs were research as well as plants component have been performed for available the action on microorganism humans pathogens (Santos et al., 2008, Agizzio et al., 2003). The use of natural products in combination with current antibacterial drugs can lead to the development of new therapies to combat otherwise resistant infections.

The synergistic interaction has the objective of available the antimicrobial effect adjuvants of IsuLL with antibiotic on *S. aureus*. The results demonstrated that combination of Cifoxitin and Ampicilin with IsuLL the antibiotics reduced the bacterial growth of the *S. aureus* strain 27 (6:4) and 15 (7:3), respectively, in proportions different (Table 2). Furthermore, the Fractional inhibitory concentration index (FICI) showed in table 3 confirm a synergistic interaction between Cifoxitin and Ampicilin with IsuLL on *S. aureus* strain 27 (6:4) and 15 (7:3 and 8:2), respectively, with

reference to the FIC scale, synergistic effect (<0.5). Hence, the combination therapy can reduce use of antibiotics required to achieve the same level of inhibition. Consequently, can be avoided the increase in the number and variety of bacterial strains resistant to these drugs, well as this antibiotics are often toxics (Sibanda and Okoh, 2007).

Taylor et al., (2002) suggested that the use of agents that do not kill pathogenic bacteria but modify them to produce a phenotype that is susceptible to the antibiotic could be an alternative approach to the treatment of infectious disease.

In conclusion, IsuLL improves the antibacterial activity against the serious Gram-positive pathogen *S. aureus* strains. The action mechanism on this microorganism is probably through their cell wall without produce lysis. Furthermore IsuLL represents a promising antibiotic adjuvant lead compound.

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Table 1. Antistaphylococcal activity of IsuLL. Minimal inhibition concentration - MIC, of IsuLL against tested bacteria

Microrganism	*Strains*	*MIC* (µg ml^{-1})
S. aureus	ATCC 6538	1000
	13	250
	14	500
	15	31.25
	17	1000
	27	15.62
	138	1000
	311	1000

Figure 1. Crystal violet uptake of IsuLL treated *Staphylococcus aureus* (strain 27). The mean±SD for three replicates are illustrated. * ($p<0,05$) in coparison to control group.

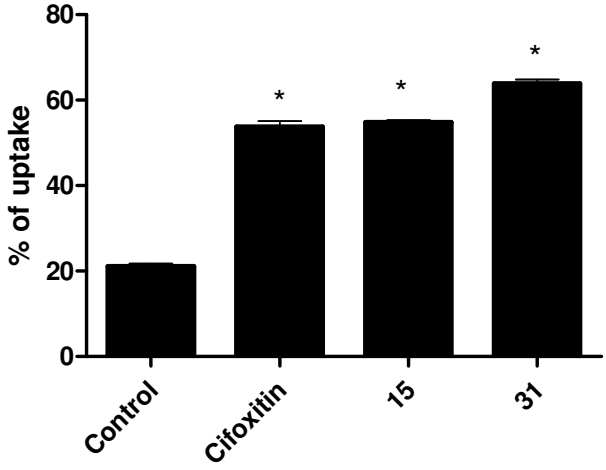

Figure 2. Measuring absorbance of the cell materials contents at 260nm releasing. The *S. aureus* cells were treated with 15.62 µg, with 31.25 µg of IsuLL and Cifoxitin (2 µg) at 1, 2 and 16 hours, compared to *S. aureus* control suspension. The data are expressed as means ± standard deviations (n = 3).

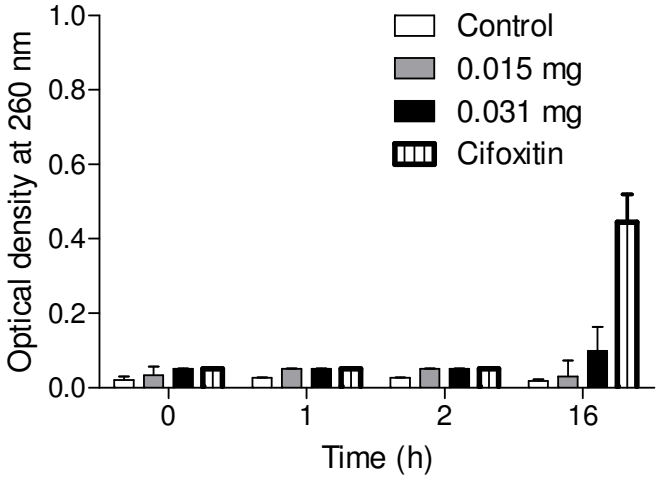

Figure 3. Electron micrographs of *S. aureus* (strain 27) cells after treatment with IsuLL. The microrganisms were stained with uranyl acetate after no treatment (A) and after treatment with MIC (B) and 2xMIC (C) of IsuLL.

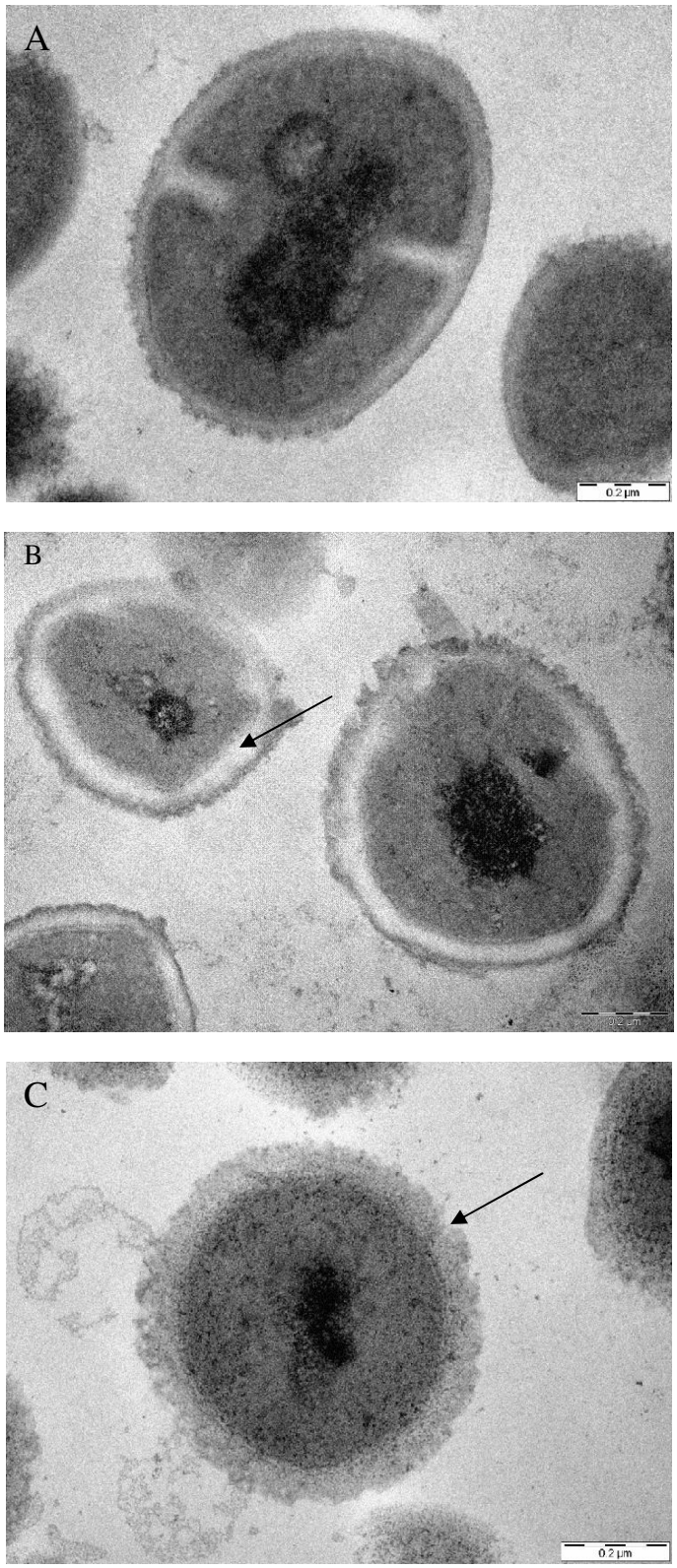

Table 2. Effect of IsuLL on the Minimum Inhibitory Concentrations (MICs) of ampicillin (AMP), cifoxitin (CF) and ciprofloxacin (CP) against *S. aureu*. IsuLL was utilized in different proportions.

Strains	MIC (µgml^{-1})						
	IsuLL	Cf	Cf + IsuLL	Amp	Amp + IsuLL	Cp	Cp + IsuLL
S. aureus 15	31.25	2	2 (6:4) 5.6 (7:3)	16	14.4 (6:4) 0.7* (7:3) 7.2 (8:2)	0.25 0.125	0.5 (6:4) 0.25 (7:3)
S. aureus 27	15.62	2	2 (5:5) 0.6*(6:4) 1.4 (7:3) 1.6 (8:2)	0,062 5	0.08 (5:5) 0.05 (6:4) 0.11 (7:3) 0.03 (8:2)		

Table 3. Fractional inhibitory concentration index (FICI) values of cifoxitin (CF), ampicilin (AMP) and ciprofloxaxin (CP) with IsuLL against *S. aureus*. IsuLL was utilized in different proportions. Synergy was defined as a FICI <0.5, no interaction was defined as a FICI of >0.5–4 and antagonism was defined as a FICI >4.

Strains	FICI		
	Cf + IsuLL	Amp + IsuLL	Cp + IsuLL
S. aureus 15	1.5 (6:4) 2.6 (7:3)	0.75 (6:4) 0.06* (7:3) 0.5* (8:2)	5.6 (6:4) 2.6 (7:3)
S. aureus 27	3 (5:5) 0.5* (6:4) 1.3 (7:3) 1.2 (8:2)	2.28 (5:5) 1.27 (6:4) 2.39 (7:3) 0.6 (8:2)	

CONCLUSÕES

- Folhas de *Indigofera suffruticosa* possuem uma lectina (IsuLL) que pode ser isolada por eficiente protocolo de cromatografia de afinidade com coluna de quitina;
- IsuLL é uma glicoproteína ácida termoestável, de 35 kDa, constituída por estruturas em folhas beta, com atividade hemaglutinante a eritrócitos humanos do tipo A, com afinidade a N-acetil-D-glicosamina e galactose, estável em pH neutro e independente de íons divalentes;
- IsuLL tem potencial efeito antioxidante, com moderado toxicidade ambiental pelo ensaio com *A.salina*, não demonstrando significante ação contra células humanas cancerígenas;
- IsuLL demonstrou específica ação fungistática contra espécies de Candida e bacteriostática contra *Staphylococcus aureus*, pela formação de poros na parede celular de *S. aureus* e potencialização do efeito dos antibióticos ampicilina e cifoxitina.

ANEXO

NORMAS DAS REVISTAS

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

Footnotes to the text are numbered consecutively; those to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data). Footnotes to the title or the authors of the article are not given reference symbols.

Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section before the reference list. The names of funding organizations should be written in full.

Manuscript Preparation

- Preferred length of Original Papers is 6-8 printed pages. The indicated length includes figures, tables and references: one printed page corresponds to approximately 6,500 characters (9,000 characters in the sections "Materials and methods" and "References").
- Manuscripts must be written in English and should be typed (12 pt throughout) in double-line spacing and wide margins.
- Mini-Reviews on biotechnological products and processes or an apparatus and reactors used for biotechnological processes are welcome. They should provide a short historical outline of the development, extensively review the current state and conclude with an outline of trends and prospects for the future. For biotechnological products the competitiveness of the biotechnological process as compared to chemical processes or to processes, which rely on the isolation of the product from natural sources, should be provided. Applications and cost figures should be provided. Mini-reviews should contain a structural formula or a photograph of the biotechnological product, an outline of the biosynthetic pathway, a scheme of the apparatus, plant or reactor or a flow scheme of the process. Authors are asked to contact the Editor-in-Chief before submitting a mini-review.

Please arrange your manuscript for Original Papers as follows:

- Abstract

  Each paper must be preceded by an abstract presenting the most important results and conclusions in no more than 250 words.

- Footnotes

  Essential footnotes to the text should be numbered consecutively and placed at the bottom of the page to which they refer.

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- Introduction

  The Introduction should state the purpose of the investigation and give a short review of the pertinent literature (max. one printed page).

- Materials and methods

  The Materials and methods section should follow the Introduction and should provide enough information to permit repetition of the experimental work. The microorganisms used in the study and in particular new isolates must be deposited in a publicly accessible culture collection (e.g. DSM, ATCC, NCIMB etc.), and the

authors should refer to the collection and the strain number in the text to ensure that the strains are available to other scientists. If nucleic acid or amino acid sequences are presented, a GenBank/EMBL accession number for primary nucleotide and/or amino acid sequence data should be included in a separate paragraph at the end of the Materials and methods section.

- Results

The Results section should describe the outcome of the study. Data should be presented as concisely as possible, if appropriate in the form of tables or figures, although very large tables should be avoided.

- Discussion

The Discussion should be an interpretation of the results and their significance with reference to work by other authors.

Scientific style
References
Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat 1995; Kelso and Smith 1998; Medvec 1993).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work.

- Journal article

Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. doi: 10.1007/s00421-008-0955-8

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

- Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see
ISSN Website

Please note:

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- Please do not use an EndNote Style abbreviating long author lists with "et al."!

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- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

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- For vector graphics, the preferred format is EPS; for halftones, please use TIFF format. MS Office files are also acceptable.
- Vector graphics containing fonts must have the fonts embedded in the files.
- Name your figure files with "Fig" and the figure number, e.g., Fig1.eps.

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- Definition: Black and white graphic with no shading.
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