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TESE DE DOUTORADO

PURIFICAÇÃO E CARACTERIZAÇÃO DE LECTINAS E INIBIDOR DE TRIPSINA PRESENTES EM TECIDOS DE *Myracrodruon urundeuva* E *Schinus terebinthifolius*: AÇÃO ANTIMICROBIANA DE PREPARAÇÕES

FRANCIS SOARES GOMES

ORIENTADORA: Profª. Dra. PATRÍCIA MARIA GUEDES PAIVA

CO-ORIENTADOR: Prof. Dr. JESÚS L. ROMALDE

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"Purificação e caracterização de lectinas e inibidor de tripsina presentes em tecidos de *Myracrodruon urundeuva* e *Schinus terebinthifolius*; ação antimicrobiana de preparações"

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Profa. Dra. Luana Cassandra Breitenbach Barroso Coelho

Prof. Dr. Thiago Henrique Napoleão

Dr. Emmanuel Viana Pontual

Profa. Dra. Liline Maria Soares Martins

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“Foi o tempo que dedicaste a tua rosa
que fez tua rosa tão importante”

Antoine de Saint-Exupéry

Dedico

Aos meus pais, Francisco Gomes e
Ileana Martins, pelo eterno amor,
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RESUMO

Schinus terebinthifolius (aroeira-da-praia) e *Myracrodruon urundeuva* (aroeira-do-sertão) são plantas utilizadas na medicina popular para tratamento de doenças humanas causadas por microorganismos. Este trabalho relata 1) o isolamento e caracterização estrutural das lectinas isoladas de folha (SteLL) e entrecasca (SteBL) de *S. terebinthifolius* e do inibidor de tripsina isolado de entrecasca de *S. terebinthifolius* (SteBI) e 2) a investigação da atividade antimicrobiana de infusão e extrato metanólico de entrecasca de *S. terebinthifolius*, de SteLL, SteBL, SteBI e de lectinas de entrecasca (MuBL), folha (MuLL) e cerne (MuHL) de *M. urundeuva* isoladas de acordo com procedimentos previamente estabelecidos. Bactérias e fungo que causam doenças em humanos e em peixes foram utilizados nos ensaios e os valores de concentrações mínima inibitória (CMI), bactericida (CMB) e fungicida (CMF) foram determinados. Adicionalmente foi avaliado o efeito das lectinas de *M. urundeuva* na aderência e capacidade invasiva de bactérias em células de peixe (SAF-1) e humanas (HeLa) bem como na produção de produtos extracelulares por bactérias. SteLL, isolada após eluição da coluna de quitina com ácido acético 1 M, é um glicopeptídeo de 14 kDa com atividade hemaglutinante (AH) inibida por N-acetilglicosamina, não afetada por Ca^{2+} e Mg^{2+}, estável entre 30 e 100 °C e pH 5,0-8,0. SteBL, isolada após eluição da coluna de quitina com ácido acético 1 M, é um peptídeo não-glicosilado de 20 kDa, com maior AH em pH 7,5 e inibida por N-acetilglicosamina e fetuína. SteBI, isolado durante o mesmo procedimento cromatográfico que isolou SteBL, representando a fração protéica ao suporte cromatográfico, é um peptídeo de 16 kDa que inibiu em 89,5% a atividade de tripsina bovina. SteLL apresentou atividade antibacteriana contra *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* e *Staphylococcus aureus*. Maiores efeitos bacteriostáticos e bactericidas foram detectadas para *S. enteritidis* (CMI: 0,45 µg/ml) e *S. aureus* (CMB: 7,18 µg/ml), respectivamente. SteLL inibiu o crescimento (CMI: 6,5 µg/ml) e matou (CMF: 26 µg/ml) *Candida albicans*. SteBL mostrou atividade bacteriostática contra *E. coli*, *S. aureus*, *Enterococcus faecalis* (CMI: 200 µg/ml), atividade bactericida contra *S. aureus* (CMB: 400 µg/ml) e não apresentou atividade antifúngica contra *C. albicans*. SteBI não apresentou atividade antibacteriana e foi fungicida (CMF: 7,5 µg/ml) contra *C. albicans*. MuBL, MuHL e MuLL apresentaram atividade bacteriostática e bactericida contra *S. aureus*, *E. coli* e *Photobacterium damselae* subsp. *piscicida* (CMI e CMB de 1 a 122 e 2 a 225 µg/ml, respectivamente) e bacteriostática contra linhagens de *Yersinia ruckeri* (CMI entre 34 a 489,8 µg/ml). A infusão da entrecasca de *S. terebinthifolius* foi bactericida contra *S. aureus*, *E. faecalis* e *E. coli* (CMB de 14,85 a 29,7 mg/mL de proteínas e 0,026 a 0,052 mg/mL de fenóis) e fungicida contra *C. albicans* (CMF de 3,71 mg/mL de proteínas e 0,007 mg/mL de fenóis). Extrato metanólico mostrou atividade bactericida contra *S. aureus* e *E. faecalis*, com CMB de 13,19 e 3,29 mg/mL de proteínas e 4,1 e 1,02 mg/mL de fenóis, respectivamente e não contem atividade antifúngica. As lectinas de *M. urundeuva* promoveram redução na adesão e capacidade invasiva de *S. aureus*, *E. coli*, *Y. ruckeri* e *P. damselae* subsp. *piscicida* em SAF-1 e HeLa e alteração no padrão de produtos extracelulares liberados por *S. aureus*, *E. coli*, *Y. ruckeri* e *P. damselae* subsp. *piscicida*. O estudo demonstrou a purificação, caracterização e propriedades biológicas de lectinas ligadoras de quitina e inibidor de tripsina presentes em tecidos de *S. terebinthifolius* e *M. urundeuva*. Adicionalmente o estudo confirmou a ação antimicrobiana da infusão da entrecasca de *S. terebinthifolius* usada pela população para tratar infecções.

Palavras-chave: Aderência, atividade antimicrobiana, capacidade invasiva, inibidor de tripsina, lectina, *Myracrodruon urundeuva*, *Schinus terebinthifolius*.

ABSTRACT

Schinus terebinthifolius (aroeira-da-praia) and *Myracrodruon urundeuva* (aroeira-do-sertão) are plants commonly used in folf medicine for treating human diseases caused by micro-organisms. This work reports 1) the isolation and structural characterization of lectins isolated from leaf (SteLL) and bark (SteBL) from *S. terebinthifolius* and of trypsin inhibitor isolated from bark of *S. terebinthifolius* (SteBI), and 2) the antibacterial activity of infusion and methanolic extract from *S. terebinthifolius* bark, SteLL, SteBL, SteBI and *M. urundeuva* bark (MuBL), leaf (MuLL) and heartwood (MuHL) lectins isolated according to procedures previously established. Bacteria and fungi pathogenic to human and fishes were used and Minimal inhibitory (MIC), bactericide (MBC) and fungicide (MFC) concentrations were determinated. Additionaly, it was evaluated the effect of *M. urundeuva* lectins on adherence and invasive capacities of bacteria to fish (SAF-1) and human (HeLa) cell lines and on production of extracellular products bacteria. SteLL, isolated after elution in chitin column with 1M acetic acid, is a 14 kDa glycopeptide with haemagglutinating activity (HA) inhibited by N-acetylglucosamine, not affected by ions (Ca^{2+} and Mg^{2+}) and stable between 30 and 100°C and at pH 5.0–8.0. SteBL, isolated after elution in chitin column with 1M acetic acid, is a non-glycosylated peptide of 20 kDa with higher HA at pH 7.5 and inhibited by N-acetylglucosamine and fetuin. SteBI, isolated during the same chromatographic procedure of SteBL isolation representing the protein fraction to the chromatographic support, is a peptide of 16 kDa that inhibited 89.5% of bovine trypsin activity. SteLL showed antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Staphylococcus aureus*. Highest bacteriostatic and bactericide effects were detected for *S. enteritidis* (MIC: 0.45 µg ml^{-1}) and *S. aureus* (MBC: 7.18 µg ml^{-1}), respectively. SteLL inhibited the *Candida albicans* growth (MIC: 6.5 µg ml^{-1}) and survival (MFC: 26 µg ml^{-1}). SteBL showed bacteriostatic activity against *E. coli*, *S. aureus* and *Enterococcus faecalis* (MIC of 0.2 µg ml^{-1}) and bactericide activity against *S. aureus* (MBC: 0,4 mg ml^{-1}) and did not show antifungal activity against *C. albicans*. SteBI did not show antibacterial activity and was fungicide (MFC: 7,5 µg/ml) againt *C. albicans*. MuBL, MuHL and MuLL showed bacteriostatic and bactericidal activity against *S. aureus*, *E. coli* and *Photobacterium damselae subsp. piscicida* (MIC and MBC from 1 to 122 and from 2 to 225 µg ml^{-1}, respectively) and bacteriostatic activity against stains of *Y. ruckeri* (MIC from 34 to 489,8 µg ml^{-1}). Bark infusion was bactericide against *S. aureus*, *E. faecalis* and *E. coli* (MBC ranging from 14.85 to 29.7 mg/mL of protein and 0.026 to 0.052 mg/mL of phenol) and fungicide against *C. albicans* (MFC of 3.71 mg/mL of protein and 0.007 mg/mL of phenol). Methanolic extract showed bactericide activity against *S. aureus* and *E. faecalis* with MBC of 13.19 and 3.29 mg/mL of protein and 4.1 and 1.02 mg/mL of phenol, respectively and did not contain antifungal activity. The *M. urundeuva* lectins promoted reduction in adhesion and invasive capacities of *S. aureus*, *E. coli*,*Y. ruckeri* and *P. damselae subsp. piscicida* in fish (SAF-1) and human cells (HeLa), and changes in the extracellular products pattern released by *S. aureus*, *E. coli*, *Y. ruckeri* e *P. damselae subsp. piscicida*. The study demonstrated the purification, characterization and biological properties of chitin-bind lectins and trypsin inhibitors present in tissues of *S. terebinthifolius* and *M. urundeuva*. Additionally, the study confirmed the antimicrobial action of the infusion of bark from *S. terebinthifolius* that is used by people to treat infections.

Keywords: adherence, antimicrobial activity, invasive capacity, lectin, *Myracrodruon urundeuva*, *Schinus terebinthifolius*, trypsin inhibitor.

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LISTA DE ABREVIATURAS

AH: atividade hemaglutinante;
ANVISA: Agência Nacional de Vigilância Sanitária
CFU: unidade formadora de colônia, do inglês "colony forming units"
CMI: concentração mínima inibitória;
CMB: concentração mínima bactericida;
CMA : concentração mínima aglutinante;
D-Gal: galactose**;**
Fru-1,6-P2: frutose-1,6-bifosfato;
Glc: glicose;
GlcNAc: *N*-acetilglicosamina;
IBAMA: Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis;
IPA: Instituto Agronômico de Pernambuco
IPT : Instituto de Pesquisa Tecnológica;
Man: manose.
MuBL: lectina de entrecasca de *Myracrodruon urundeuva*, do inglês "*M. urundeuva* bark lectin".
MuHL: lectina do cerne de *M. urundeuva*, do inglês "*M. urundeuva* heartwood lectin".
MuLL: lectina de folha de *M. urundeuva*, do inglês "*M. urundeuva* leaf lectin".
NA: Ágar nutrient, do inglês "nutrient agar";
NB: Caldo nutriente, do inglês "nutriente broth"
PAGE: eletroforese em gel de poliacrilamida, do inglês "poliacrilamide gel electrophorese"
SF40: fração sobrenadante 40%
SteBI: inibidor de tripsina de entrecasca de *Schinus terebinthifolius*, do inglês "*S. terebinthifolius* bark inhibitor"
SteBL: lectina de entrecasca de *S. terebinthifolius*, do inglês "*S. terebinthifolius* bark lectin;
SteLL: lectina da folha de Schinus terebinthifolius, do inglês "S. terebinthifolius leaf lectin"

SUMÁRIO

1. INTRODUÇÃO

A indiscutível riqueza da flora brasileira, que desde muitos séculos tem despertado o interesse de pesquisadores em todo o mundo, é ressaltada principalmente pelos estudos etnofarmacológicos, abrangendo conhecimentos de origem ameríndia, enriquecidos historicamente pelas presenças africanas e européias a partir do século XVI (SANDES & DI BLASI, 2000).

O Brasil é o país com maior diversidade genética vegetal do mundo, contando com 42.898 espécies catalogadas, cerca de 15% de um total de 250 a 500 mil espécies de plantas existentes na flora mundial (Forzza *et al.*, 2012). As plantas constituem ainda uma fonte importante para a descoberta de novas substâncias biologicamente ativas. Em meados do século XIX, o uso terapêutico de plantas medicinais era o único meio de tratamento, porém após o início do século XX, o uso de plantas medicinais foi marginalizado por não conter um embasamento científico e os fitoterápicos foram substituídos por alopáticos. Contudo a partir dos anos 80 e 90 daquele mesmo século, a fitoterapia começou a ser resgatada para atuar de forma complementar às práticas de saúde vigentes (ALVIM, 2006).

Graças à valorização do saber popular e à tentativa de suprir a falta de recursos financeiros para os setores de saúde, o uso de fitoterápicos tem crescido substancialmente. O comércio de medicamentos fitoterápicos vem crescendo a uma taxa anual média de 15% e no Brasil e, entre 2011 e 2012, o crescimento das vendas de fitoterápicos superou a casa dos 25% (ABFISA, 2012).

Plantas com valor medicinal, segundo a Organização Mundial de Saúde, são aquelas que possuem, em um ou em vários de seus órgãos, substâncias usadas com finalidade terapêutica ou substâncias que sejam ponto de partida para a síntese de produtos químicos e farmacêuticos. A essas substâncias é dado o nome de princípios ativos. As funções fisiológicas de muitos desses princípios ativos ainda não estão completamente esclarecidas, mas associa-se a sua produção à defesa da própria planta contra agentes externos, como doenças, pragas e radiação solar, entre outros. Assim, essas substâncias possuem funções ecológicas importantes para a sobrevivência da espécie (BOCHNER *et al.*, 2012) e podem ser utilizadas, com a devida responsabilidade, em benefício do ser humano.

A co-evolução existente entre plantas e microorganismos tem sido explorada, então, na busca por fontes naturais de substâncias antimicrobianas, já que as mesmas são produzidas pelo vegetal em resposta a um ataque. Usualmente os compostos naturais são separados em compostos do metabolismo primário e do metabolismo secundário (Braz-Filho, 2010). Os

metabólitos primários são amplamente distribuídos nos seres vivos, enquanto os metabólitos secundários são de ocorrência restrita, embora essenciais para os organismos que os produzem (WINK *et al.*, 2003).

O metabolismo primário leva à produção de substâncias com a introdução de nutrientes tirados do solo (nitrogênio, fósforo e sais minerais). Essas substâncias têm a função de promover os processos básicos da planta, como os ácidos carboxílicos do ciclo do ácido cítrico (ciclo de Krebs), os cerca de vinte aminoácidos que constituem a maioria das proteínas, os ácidos graxos e os açúcares comuns e seus derivados. São substâncias que existem em todas as plantas e constituem a matéria-prima de reações posteriores, catalisadas por enzimas e controladas geneticamente. São essas reações posteriores que levam à produção dos compostos do metabolismo secundário das plantas (flavonóides, alcalóides, terpenos, entre outros) (DIAS & DIAS, 2007).

Os metabólitos secundários são conhecidos por suas ações na proteção contra estresse biótico e abiótico e na manutenção da integridade estrutural. Essa vasta gama de compostos naturais biologicamente ativos pode apresentar também ação farmacológica, agindo como tranqüilizante, analgésico, antiviral, fungicida e bactericida, cujas diversas aplicações estendem-se tanto à terapêutica médica quanto à indústria de cosméticos e de alimentos (WINK et al., 2011).

Entre os diversos exemplos de substâncias de uso terapêutico, oriundas do metabolismo secundário de plantas, podemos citar a artemisina (DHINGRA *et al.*, 1999), presente em artemísia, *Artemisia annua*, que possui atividade antimalárica; a morfina (KALSO, 2007), obtida do látex da papoula, *Papaver somniferum*, que ainda é o analgésico mais potente para uso clínico, e o diterpeno taxol (CHENG *et al.*, 2008), isolado de *Taxus brevifolia*, teixo-do-pacífico, usado para o tratamento de câncer dos ovários e pulmões.

Além dos metabólitos secundários, alguns componentes do metabolismo primário das plantas têm sido relacionados a mecanismos de defesa. As lectinas de plantas, por exemplo, são proteínas que podem assumir diferentes papéis biológicos. Todavia, não existe uma função universal para todas elas. De maneira abrangente, as lectinas podem assumir papéis exógenos como, por exemplo, agentes antimicrobianos contra fitopatógenos (SÁ *et al.*, 2009a) ou inseticida (SÁ *et al.*, 2008; Sá *et al.*, 2009b; SILVA *et al.*, 2009) ou terem papéis endógenos ao interagirem com ligantes do próprio organismo para, por exemplo, auxiliar a deposição de proteínas de reservas nos corpos protéicos (LIMPENS & BISSELING, 2003).

A atividade antibacteriana das lectinas resulta da interação com ácidos teicóicos e teicurônicos, peptidioglicanos e lipopolissacarídeos presentes na parede celular bacteriana e

tem sido sugerido que lectinas formam um canal na parede celular e a morte bacteriana ocorre pelo extravasamento do conteúdo celular (RATANAPO *et al.* 2001; CORREIA *et al.*, 2008). A atividade antifúngica de lectinas resulta da interação com a parede celular de hifas resultando em redução na absorção de nutrientes assim como na interferência no processo de germinação de esporos (SÁ *et al.*, 2009a).

Os inibidores de proteases podem pertencer a diferentes classes, tais como metabólitos secundários e proteínas, mas têm em comum a capacidade de bloquear a atividade catalítica de enzimas proteolíticas (PAIVA *et al.*, 2012). Nas plantas, eles são expressos com o objetivo de controlar a atividade proteolítica endógena e também podem atuar em mecanismos de defesa. A tripsina é uma das enzimas sobre as quais inibidores de proteases podem atuar, podendo ser encontrada no sistema digestivo de vertebrados, sendo também expressa por microorganismos, como fungos (BARATA *et al.*, 2002). A importância desta enzima no arsenal bioquímico de diferentes grupos taxonômicos tem estimulado a busca por inibidores como agentes terapêuticos (FEAR *et al.* 2007; KANSAL *et al.*, 2008; MACEDO *et al.*, 2010; PRASAD *et al.*, 2010; MACEDO *et al.*, 2011).

A entrecasca e folhas da aroeira da praia (*Schinus terebinthifolius* Raddi.) são utilizadas pela medicina popular para cicatrização e reparo tecidual de feridas cutâneas, no tratamento de cervicites, corrimento genital e de diversas infecções no aparelhos respiratório, digestório e ginecológico (AMORIM & SANTOS, 2003; RIBAS *et al.*, 2006).

Myracrodruon urundeuva (aroeira-do-Sertão) é uma espécie considerada madeira de lei devido a sua elevada resistência aos microorganismos fitopatógenos. A entrecasca dessa planta é bastante utilizada na medicina popular no tratamento de infecções no trato gênito-urinário, pele, tecido subcutâneo e sistema digestivo. Macerada, na forma de infusão em água ou como xarope são algumas das maneiras de uso popular (MONTEIRO *et al.*, 2006). Lectinas já foram isoladas de cerne, entrecasca e folhas de *M. urundeuva* e os protocolos definidos produzem as lectinas em quantidades miligrama (SÁ *et al.*, 2009b; NAPOLEÃO *et al.*, 2011). As lectinas de entrecasca e cerne de *M. urundeuva* possuem atividade inseticida contra *Aedes aegypti* (Diptera) e *Nasutitermes corniger* (Isoptera) e a lectina do cerne de *M. urundeuva* também possue atividade antifúngica contra espécies de fungos fitopatógenos do gênero *Fusarium* e atividade antibacteriana sobre *B. subtilis, Corynebacterium callunae, S. aureus, Enterococcus faecalis, E. coli, Klebsiella pneumoniae* e *Pseudomonas aeruginosa* (SÁ *et al.*, 2008; SÁ *et al.*, 2009a,b). A lectina de folhas de *M. urundeuva* é inseticida contra o cupim da espécie *Nasutitermes corniger* e o gorgulho-do milho, *Sitophilus zeamais*, e larvicida contra *Aedes aegypti*, o mosquito vetor da dengue (Napoleão et al., 2011, 2012,

2013). Metabólitos secundários também já foram detectados em extrato metanólico do cerne de *M. urundeuva* com ação antioxidante, antifúngica contra *F. lateritium*, *F. oxysporum* e *F. moniliforme* e repelente contra cupins da espécie *Nasutitermes corninger* (Sá *et al.*, 2009c).

As propriedades encontradas para *S. terebinthifolius* e *M. urundeuva* estimulam a investigação das atividades biológicas das lectinas de espécies de aroeira sobre microorganismos e o estudo do mecanismo de ação antimicrobiano, visando determinar o seu potencial uso como agentes antimicrobianos naturais.

1.1. CONSIDERAÇÕES SOBRE A FAMÍLIA ANACARDIACEAE E AS ESPÉCIES *Schinus terebinthifolius* RADDI E *Myracrodruon urundeuva* Fr. All.

1.1.1. A Família Anacardiaceae

Anacardiaceae é uma família botânica representada por 70 gêneros e cerca de 600 espécies, conhecidas por suas espécies frutíferas, entre elas a mangueira *(Mangifera indica*), originária da Ásia, e o cajueiro *(Anacardium occidentale*), nativo do Brasil. A família distingue-se de outras pela combinação de um disco intra-estaminal, com a presença de fruto drupáceo e vasos resiníferos que, quando expostos por injúrias, têm um cheiro característico (RAVEN *et al.*, 2004).

Há dois centros vegetativos desta família: um na Malásia e outro na América Andina, estendendo-se da zona tropical até ambas as zonas temperadas. No Brasil conhecem-se aproximadamente 40 espécies. A família possui representantes de hábito arbóreo ou arbustivo, e menos freqüentemente há rasteiras, pequenos arbustos e lianas. (SMITH *et al.*, 2004). As folhas desta família são alternantes, em sua maioria, simples ou imparipenadas, às vezes ternadas, sempre sem estípulas. Suas flores são diclamídeas e podem ser terminais ou axilares, com inflorescência racemosa, em sua maioria. A polinização ocorre mediante a presença de insetos. Sua madeira é de boa qualidade e muitas substâncias são extraídas para uso na indústria e na medicina. Os canais resiníferos são ricos em taninos (CRONQUIST, 1981; SOUZA & LORENZI, 2008).

O termo aroeira tem sido usado para designar plantas classificadas em três gêneros da família Anacardiaceae*: Lithraea*, *Schinus* e *Myracrodruon*. *S. terebinthifolius* Raddi pertence ao gênero *Schinus* e é conhecida por diferentes nomes como aroeira da praia, aroeira marrom, aroeira vermelha, aroeira pimenteira, fruto da raposa, dentre outras denominações. A aroeira-do-sertão, ou aroeira-preta, é a *Myracrodruon urundeuva* Fr. All., nome dado por Francisco

Allemão e Cysneiros em 1862. Alguns sinônimos também podem ser encontrados, tais como *Astronium juglandifolium* Griseb. e *Astronium urundeuva* Engl (GARRIDO & POGGIANI, 1979).

1.1.2 A espécie *Schinus terebinthifolius* Raddi

No Brasil, a aroeira da praia é encontrada desde Pernambuco até Mato Grosso do Sul e Rio Grande do Sul, nas mais variadas formações vegetais. É uma planta dióica, terrestre e de hábito arbóreo (Figura 3A) podendo atingir uma altura entre 5 a 10 m, com tronco de 30-60 cm de diâmetro revestido com casca grossa (LORENZI, 2008).

Figura 1: *Schinus terebinthifolius*. Parte aérea (A); inflorescência (B); infrutescência não madura (C). Fotos: Thamara Figueiredo Procópio.

Suas flores são pentâmeras, actinomorfas, pequenas, dialissépalas e dialipétalas, estão dispostas em inflorescência composta do tipo panícula racemosa que saem da axila das folhas (Figura 3B), com sépalas pequenas e verdes de formato triangular, pétalas pequenas, brancas e ovais. As flores estaminadas têm dez estames heterodínamos, dispostos em duas fileiras

concêntricas, com anteras basifixas que apresentam deiscência longitudinal, pólen amarelo, gineceu bastante reduzido (resquício de ovário) e possuem um disco nectário. As flores pistiladas possuem estigma trilobado, ovário súpero e unilocular e estames reduzidos. A placentação é apical lateral (FAGUNDES, 2007).

O fruto (Figura 3C) é tipo drupa, sendo numerosos e pequenos, inicialmente de coloração verde tornando-se vermelho brilhante quando maduros. As folhas, fortemente aromáticas, são alternas espiraladas, compostas e imparipenadas, normalmente com cinco ou sete folículos de consistência membranácea sempre sem estípulas e a venação é do tipo peninérvea (FAGUNDES, 2007; LORENZI, 2008). Altas concentrações de monoterpenos e alguns hidrocarbonetos sesquiterpenos foram relatadas nas folhas e frutos de *S. terebinthifolius* (STAHL *et al.*, 1983; MALIK *et al.*, 1994; SINGH *et al.*, 1998).

A espécie é amplamente usada na recuperação de áreas degradadas, em programas de reflorestamento e arborização devido ao longo período em que as flores e frutos persistem nas plantas, sendo de grande importância ornamental (KAGEYAMA & GANDARA, 2000; SOUZA *et al.*, 2001). Sua casca, rica em taninos, pode ser utilizada na produção de tinta para tecidos ou para curtimento de couro e fortalecimento de redes de pesca. A madeira resistente é utilizada para cercados devido a sua durabilidade, e como lenha e carvão devido às suas características energéticas comparáveis a eucaliptos usados para esse fim. É uma espécie muito procurada por aves, possivelmente dispersoras de seus frutos (pimenta rosa), os quais são apreciados como condimento alimentar na cozinha nacional e internacional. Suas flores são melíferas tendo um alto valor apícola para a produção de mel de qualidade (SOUZA *et al.*, 2001; GUIMARÃES, 2003).

Atualmente, a aroeira da praia está incluída na Relação Nacional de Plantas Medicinais de Interesse ao SUS (MINISTÉRIO DA SAÚDE, 2010). A sua casca, folhas e frutos são utilizados na medicina popular como antiinflamatório, antitérmico, analgésico e cicatrizante no tratamento de cervicites, corrimento genital, em diversas infecções no aparelho respiratório, digestivo e ginecológico e como reparador tecidual de feridas cutâneas (CORSI *et al*, 1994; AMORIM & SANTOS, 2003; RIBAS *et al.*, 2006). Aos frutos atribuem-lhes propriedades diuréticas e segundo Degáspari (2005) o seu extrato alcoólico possui efeito inibitório contra *Staphylococcus aureus* e *Bacillus cereus*.

A casca é recomendada como chá para curar diarréias, hemoptises e como banhos contra ciática, gota, reumatismo e bactérias que se manifestam sob a forma de edemas do tipo erisipela. Índios do Paraná e Santa Catarina utilizavam brotos novos e casca do caule contra odontalgia (dor de dente) (BALBACHAS, 1959; LINDENMAIER, 2008). Melo Júnior *et al.*

(2002) mostraram que o tratamento com o extrato etanólico da casca de *S. terebinthifolius* foi de eficiência semelhante ou superior a antibióticos utilizados atualmente contra *Enterococcus*, *Bacillus corineforme* e *Streptococcus viridans*. Estudo também mostrou que um extrato hidroalcoólico da casca apresentou um efeito anti-inflamatório, aliado com a ação anti-histamínica, além de ação antimicrobiana contra *S. aureus* (MATOS, 1988). Santos (2007) constatou a atividade antibacteriana do extrato hidroalcoólico contra *S. aureus, Pseudomonas aeruginosa, Escherichia coli e Bacilus subtilis.*

As folhas são antirreumáticas e um valioso remédio na cura de úlceras e feridas, sua infusão é utilizada pelos índios Guarani para combater o popular sapinho, causado pelo fungo *Candida albicans*, na boca das crianças (BALBACHAS, 1959; LINDENMAIER, 2008). Extrato etanólico de folhas de *S. terebinthifolius* promoveu inibição no crescimento de *S. aureus, Pseudomonas aeruginosa* e *C. albicans* (MARTINEZ *et al.*, 1996; GUERRA *et al.* 2000). O óleo essencial ds folhas apresentou potencial inibitório frente a alguns agentes etiológicos da infecção otológica em cães como *Staphylococcus* spp., *Malassezia, Streptococcus sp.* e *Corynebacterium sp.* (CAMPOS *et al.*, 2010). COUTINHO *et al.* (2006) comprovaram por meio da análise histológica o efeito positivo do extrato hidroalcoólico de folhas no processo de cicatrização de anastomoses do cólon em ratos. Segundo Cavalher-Machado *et al.* (2008), a fração de acetato de folhas de *S. terebinthifolius* possui uma importante propriedade antialérgica, que é marcada pela inibição da formação de edema, degranulação dos mastócitos e influxo de eosinófilos. O extrato etanólico de folhas de *S. terebinthifolius,* apresentou fenóis, flavonas, flavonoides, xantonas e leucoantocianidinas, flavononas e esteróides livres (LIMA *et al.*, 2006).

É recomendado, no entanto, precaução no uso da planta, principalmente das folhas e cascas, devido às suas propriedades tóxicas, apesar de não haver dúvidas quanto às suas qualidades adstringentes, tônica e estimulante (LORENZI, 2008; BAGGIO, 1988; LINDENMAIER, 2008). A ingestão de frutos verdes ou maduros por crianças pode causar reações como vômitos, erupções cutâneas e inchaço das mãos, braços e rosto(CARVALHO *et al.*, 2013), mas Lima *et al.* (2009) mostraram que a administração oral de extratos secos de cascas de *S. terebinthifolius*, durante 45 dias, em ratos não induziu nenhum efeito tóxico.

1.1.3. A espécie *Myracrodruon urundeuva* Fr. All.

No Brasil, essa planta é conhecida como *aroeira, aroeira-do-sertão, aroeira-preta, aroeira-do-campo, aroeira-verdadeira* ou *urundeúva,* entre outras denominações. O nome

urundeuva vem de um conceito guarani para "incorruptível na água", uma referência à grande resistência que a madeira dessa planta possui (LORENZI, 2008).

Considerada madeira de lei, a aroeira-do-sertão é muito densa (densidade = 1,00 a 1,21 g/cm^3), dura, elástica e resistente a fungos fitopatógenos. Recebe excelente polimento e, quando seca, é de difícil trabalhabilidade. A madeira é muito pesada, tem textura média e uniforme, grã irregular, possuindo o alburno bem diferenciado do cerne e facilmente decomposto (MORAIS *et al.*, 1999). Estudos realizados pelo Instituto de Pesquisas Tecnológicas (IPT) indicam que um pedaço do cerne desta planta do tamanho de uma caixa de fósforos suporta 6 toneladas de carga, sem se deformar. A madeira da aroeira-do-sertão suporta cerca de três vezes mais peso do que o concreto, sendo excelente para obras externas (postes, moirões, esteios, estacas, vigas, armações de pontes e moendas de engenho) e para a construção civil (caibros, vigas, tacos, assoalhos, ripas e peças torneadas) (MAINIERI & CHIMELO, 1989).

A madeira é um material que possui propriedades energéticas, medicinais, químicas e alimentícias (LEPAGE *et al.*, 1986), apresentando uma vasta gama de utilização nos meios rural e urbano. Entretanto, em virtude da sua estrutura e constituição química, boa parte das madeiras é passível de sofrer ataque de vários microorganismos que utilizam os polímeros naturais da parede celular como fonte de nutrição, entre os quais os fungos são responsáveis pelos maiores danos (CAVALCANTE, 1982).

A durabilidade natural da madeira é interpretada pela capacidade que a mesma possui de resistir à ação dos agentes deteriorantes, tanto biológicos quanto físico-químicos. Para sobreviver a essas adversidades, as plantas, ao longo de sua evolução, desenvolveram mecanismos de resposta relacionados à sua defesa e proteção (SOARES & MACHADO, 2007). O conhecimento da resistência natural da madeira é de suma importância na recomendação de sua utilização, para que sejam evitados gastos desnecessários com a reposição de peças deterioradas e seja reduzido o impacto sobre as florestas remanescentes (PAES *et al.*, 2007).

As defesas anatômicas são encontradas em diferentes partes da aroeira-do-sertão, tendo como um dos principais objetivos formarem uma barreira mecânica ao acesso de organismos às estruturas vegetais. Observando-se um corte transversal de um tronco, podem-se notar dois tecidos bem distintos em termos de cor (Figura 1): a parte central, mais escura, é denominada cerne, enquanto a parte mais clara é denominada alburno e envolvendo-os, temos a casca interna (entrecasca) e externa.

O cerne é considerado um tecido morto, sem atividade vegetativa. A transformação do alburno em cerne é iniciada internamente, e não por condições externas. A morte da maioria das células após o espessamento celular é marcada pelo desaparecimento do núcleo e do protoplasma, pela mudança química do citoplasma, redução em amido, açúcares e materiais nitrogenados. Entretanto, algumas células retêm seu protoplasto, como as células do parênquima, que ocorrem como células longitudinais e radiais (RAVEN *et al.*, 2004).

O alburno é responsável por processos metabólicos como a respiração e digestão (SILVA, 2002), além de fornecer suporte ao tronco, conduzir a seiva bruta até as folhas e armazenar alimentos. O cerne, por outro lado, não armazena alimento nem faz condução de seiva, funcionando, segundo Hunt & Garratt (1967), somente como suporte. Já a casca funciona como primeiro impedimento à entrada de agentes deteriorantes.

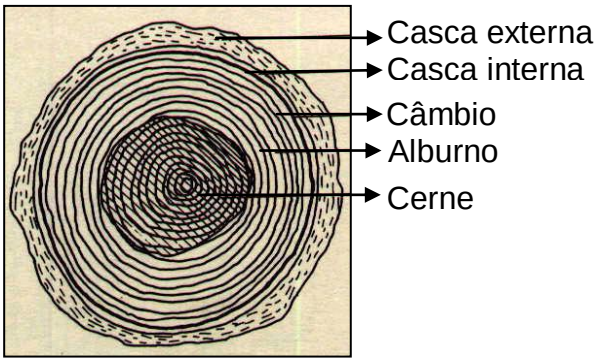

Figura 2. Anatomia da Madeira. Fonte: Silva, 2002

A característica de durabilidade é encontrada em apenas 1 a 5 % das madeiras e apenas menos de 1 % delas são muito duráveis. Além das propriedades mecânicas dessas madeiras, que formam uma barreira física de proteção, existe também uma barreira química formada pelos extrativos e outras substâncias, que possuem efeito fungicida. Essas substâncias se formam principalmente no processo de transformação do alburno em cerne (MAINIERI & CHIMELO, 1989).

A aroeira-do sertão tem tronco de 50 a 80 cm de diâmetro, cerca de 6 a 14 m de altura no Cerrado e na Caatinga, e até 20 a 25 m em solos mais férteis da floresta latifoliada semidecídua. Apesar de ser considerada típica de regiões da Caatinga e do Cerrado, formando agrupamentos densos, é também encontrada em formações muito úmidas e fechadas, incluindo florestas pluviais. Ocorre desde o México a Argentina (BARKLAY, 1968; GARRIDO & POGGIANI, 1979); no Brasil, essa espécie possui ampla distribuição do

Maranhão até o Paraná e Mato Grosso do Sul, sendo mais freqüente nos estados da Bahia, Minas Gerais, São Paulo, Mato Grosso e Goiás. É uma planta característica de terrenos secos e rochosos, ocorrendo em grupamentos densos (LORENZI, 2008).

A flor de *M. urundeuva* é do tipo inflorescência em racemo. As folhas são compostas, imparipinadas, ovaladas com 10 a 30 cm de comprimento (Figura 3). O fruto é do tipo drupa e de estrutura carnosa e as sementes são pequenas medindo cerca de 0,2 cm (FAO, 1986; LEITE, 2002).

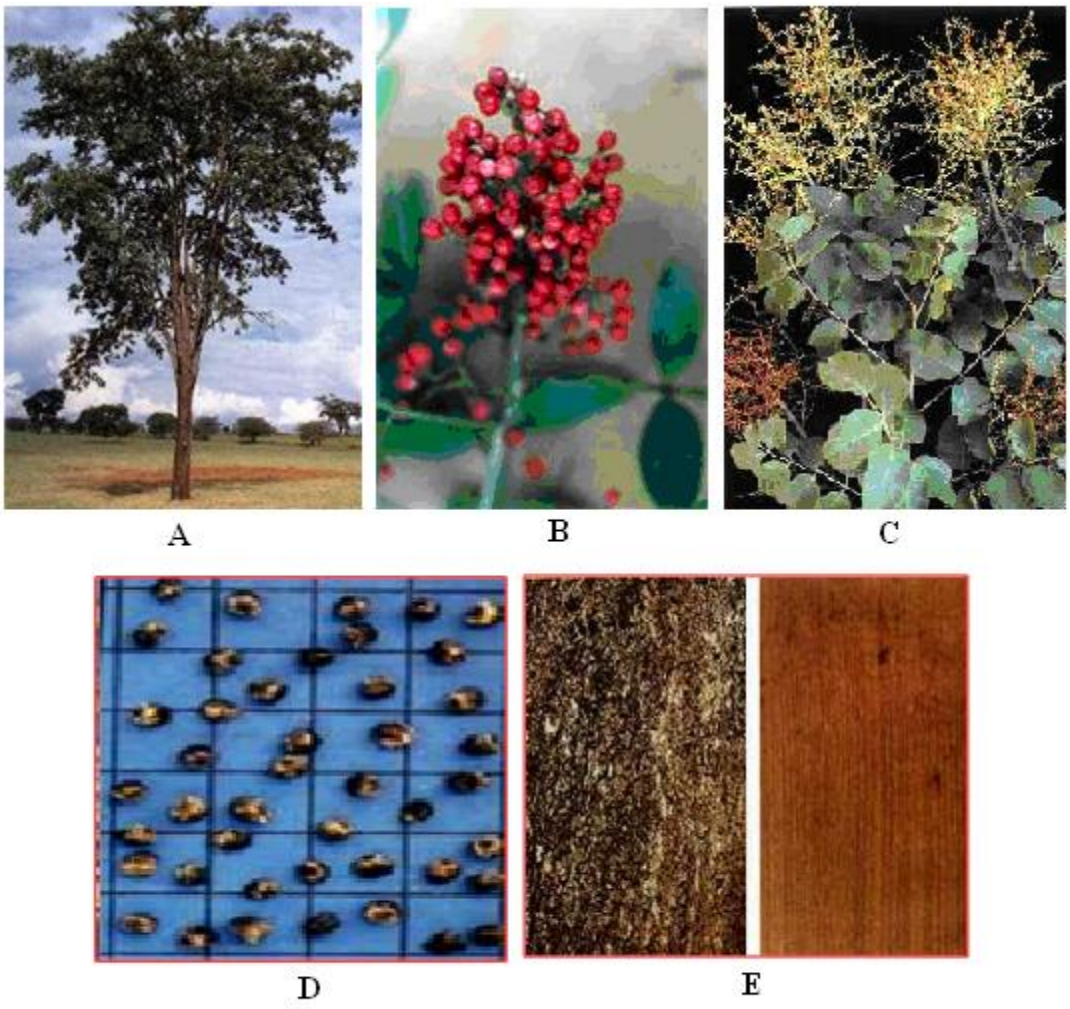

Figura 3. *Myracrodruon urundeuva:* Parte aérea (A), inflorescência (B), folhas (C), frutos (D) e madeira (E). Fonte: VIANA, 1995.

A casca da aroeira é empregada na farmacologia popular, como planta medicinal, sendo recomendada para o tratamento de inflamação de garganta, gastrite e prisão de ventre. Sua resina amarelo-clara, proveniente das lesões da casca, possui aplicação em larga escala como tônico entre os sertanejos (BRAGA, 1976).

Por causa de suas qualidades, a aroeira sofreu superexploração, tornando-se escassa em todas as áreas de ocorrência, fato que a colocou na categoria "vulnerável", ou seja, em perigo de extinção, conforme a Lista Oficial de Espécies da Flora Brasileira Ameaçadas de Extinção (IBAMA, 2008).

1.2. LECTINAS

O primeiro relato a respeito de lectinas se deu em 1888, quando Stillmark, ao estudar a toxicidade de extratos de *Ricinus communis* (mamona), observou sua capacidade para aglutinar eritrócitos, devido à presença de uma proteína extraída, a ricina, descoberta que marcou o início das pesquisas envolvendo lectinas (KENNEDY *et al.*, 1995). Pouco tempo depois, outra hemaglutinina, chamada abrina, foi encontrada em sementes de *Abrus precatorius* (jequiriti). Entretanto, o estudo sobre estas proteínas só começou a ganhar ímpeto em 1960, abrindo uma vasta área de aplicação para as lectinas (GABOR *et al.*, 2004).

O termo *lectina* (originado do latim "lectus", que significa selecionado) refere-se à habilidade dessas proteínas ligarem-se seletivamente e reversivelmente a carboidratos (SHARON & LIS, 2002). Ao contrário dos anticorpos, não são produtos de uma resposta imune. A ênfase que é dada quanto à origem não-imunológica das lectinas serve para distingui-las de anticorpos anticarboidratos que aglutinam células. Os anticorpos são estruturalmente similares, enquanto as lectinas diferem entre si quanto à composição aminoacídica, requerimentos de metais, peso molecular e estrutura tridimensional (VAN DAMME *et al.*, 1998).

1.2.1. Fontes de lectinas

Lectinas estão largamente distribuídas na natureza, sendo encontradas em microrganismos (BHOWAL *et al.*, 2005; KHAN *et al.*, 2007), invertebrados (BATTISON & SUMMERFIELD, 2009) e vertebrados (LOPES-FERREIRA *et al.*, 2011; NUNES *et al.*, 2011). Nos vegetais, as lectinas têm sido encontradas em cerne (SÁ *et al.*, 2008), folhas (NAPOLEÃO *et al.*, 2012), flores (SANTOS *et al.*, 2009), sementes (SILVA *et al.*, 2012), cascas (VAZ *et al.*, 2010), raízes (AGRAWAL *et al.*, 2011) e rizomas (YANG *et al.*, 2011; SANTANA *et al.*, 2012).

Lectinas já foram isoladas de cerne (MuHL), entrecasca (MuBL) e folhas (MuLL) de *M. urundeuva* e os protocolos definidos produzem as lectinas em quantidades miligrama (Sá

et al., 2009b; Napoleão *et al.*, 2011). MuBL e MuHL possuem atividade inseticida contra *Aedes aegypti* (Diptera) e *Nasutitermes corniger* (Isoptera) e MuHL possui atividade antifúngica contra espécies de fungos fitopatógenos do gênero *Fusarium* e atividade antibacteriana sobre *B. subtilis*, *Corynebacterium callunae*, *S. aureus*, *Streptococcus faecalis*, *E. coli*, *Klebsiella pneumoniae* e *Pseudomonas aeruginosa* (Sá *et al.*, 2008; Sá *et al.*, 2009a,b). A lectina de folhas de *M. urundeuva* (MuLL) possui ação inseticida contra o cupim da espécie *Nasutitermes corniger* e o gorgulho-do milho, *Sitophilus zeamais*, e é larvicida contra *Aedes aegypti* (Napoleão et al., 2011, 2012, 2013).

1.2.2. Detecção e Especificidade de lectinas

As lectinas são, em sua maioria, di ou polivalentes e são capazes de formar pontes entre carboidratos (Figura 4) ou glicoproteínas, que se apresentam em solução ou ligadas à membrana celular (CORREIA *et al.*, 2008).

A presença de lectinas em uma amostra pode ser facilmente detectada a partir de ensaios de aglutinação, nos quais elas interagem com carboidratos da superfície celular através de seus sítios, formando diversas ligações reversíveis entre células (Figura 5). As lectinas podem aglutinar diversos tipos de células. O ensaio mais comumente utilizado é o de hemaglutinação, o qual é realizado através de uma diluição seriada da amostra contendo lectina e de posterior incubação com eritrócitos; a rede formada entre os eritrócitos constitui o fenômeno de hemaglutinação. Os eritrócitos utilizados podem ser de humanos ou de animais, os quais podem ser tratados enzimaticamente (com tripsina, papaína, entre outras) ou quimicamente (com glutaraldeído ou formaldeído), aumentando ou não a sensibilidade das células à lectina (COELHO & SILVA, 2000; SANTOS *et al.*, 2005). O inverso da maior diluição em que se observa a hemaglutinação (título) corresponde à atividade hemaglutinante (AH) (NAPOLEÃO *et al.*, 2012).

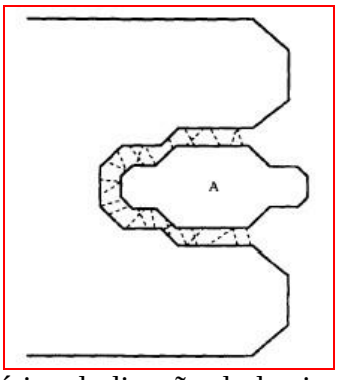

Figura 4. Representação esquemática da ligação da lectina a um carboidrato (A). As linhas pontilhadas representam pontes de hidrogênio. Fonte: Kennedy *et al.* (1995)

Para assegurar que o agente aglutinante é uma lectina, uma vez que alguns compostos, tais como taninos, lipídios ou íons bivalentes podem dispersar eritrócitos dando um falso resultado, são necessários ensaios subseqüentes de inibição da AH, utilizando-se o carboidrato livre em solução (WU *et al.*, 2006).

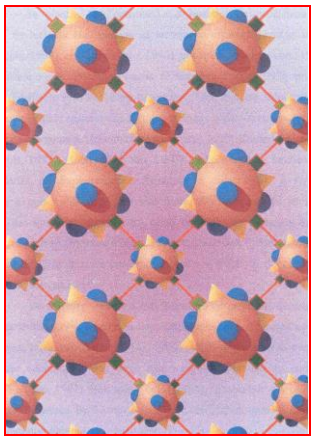

Figura 5. Representação esquemática de aglutinação por lectinas, baseada em Kennedy *et al.*(1995). Lectina  , e seus ligantes de superfície da célula  , carboidratos ou não-carboidratos, ligantes ou não.

A grande maioria de lectinas de plantas apresenta especificidade por carboidratos simples (monossacarídeos) ou complexos (oligossacarídeos e glicanas), tais como manose, N-acetilglicosamina e ácidos N-glucurônico, galacturônico, xilurônico, L-idurônico, siálico e N-acetilmurâmico (VAN DAMME *et al.*, 1998).

As lectinas podem apresentar especificidade para eritrócitos, como a lectina da polpa do fruto de *Aegle marmelos*, específica para eritrócitos do tipo A (RAJA *et al.*, 2011), e do cogumelo *Marasmius oreades* (WINTER *et al.*, 2002), específica para eritrócitos do tipo B. Outras lectinas, no entanto, são caracterizadas como não específicas para grupos sanguíneos como a lectina da esponja *Cinachyrella apion* (MEDEIROS *et al.*, 2010), que aglutina todos os tipos de eritrócitos humanos.

1.2.3. Purificação de Lectinas

Métodos comuns utilizados na purificação de proteínas são aplicados para purificar as lectinas. O primeiro passo para a purificação consiste na extração de proteínas (YAO *et al.*, 2010). Extratos podem ser feitos utilizando solução salina, como no caso do isolamento da lectina de rizoma de *M. vaccinifolia* (SANTANA *et al*., 2012) ou usando tampões, como na obtenção das lectinas de cotilédones de pau-serrote, *Luetzelburgia auriculata,* (OLIVEIRA *et al.*, 2002), dos tubérculos de tupinambo, *Helianthus tuberosus,* (SUSEELAN *et al.*, 2002), e da semente de *Salvia bogotensis* (VEGA & PÉREZ, 2006).

Para a preparação do extrato, o material é submetido à extração sob período de tempo e condições de temperatura estabelecidas. A partir do extrato bruto, as proteínas podem ser isoladas por alguns métodos, tais como o fracionamento de proteínas com sais. O sulfato de amônio, altamente hidrofílico, remove a camada de solvatação das proteínas fazendo com que as mesmas se precipitem (DELATORRE *et al.*, 2006).

As lectinas parcialmente purificadas pelo tratamento salino são geralmente submetidas ao processo de diálise em membranas semipermeáveis, método baseado na separação de moléculas por diferenças de peso molecular; as proteínas ficam retidas dentro da membrana enquanto moléculas menores (como carboidratos ou sais), presentes na amostra, passam para a solução solvente (THAKUR *et al.*, 2007).

As lectinas podem ser purificadas à homogeneidade através de métodos cromatográficos que utilizam matrizes cuja escolha dependerá da especificidade a carboidratos (cromatografia de afinidade), carga líquida (cromatografia de troca iônica) ou tamanho molecular da proteína (cromatografia de gel filtração) (MOURA *et al.*, 2006; SANTI-GADELHA *et al.*, 2006; SUN et al, 2007).

A cromatografia de afinidade, técnica mais amplamente utilizada, tem como princípio de separação a habilidade das lectinas se ligarem especificamente a suportes polissacarídicos, através de ligações não-covalentes. A proteína desejada é obtida com alto grau de pureza, alterando-se as condições de pH, força iônica ou pela eluição com uma solução contendo um competidor (PEUMANS & VAN DAMME, 1998).

O isolamento de lectinas é estimulado pela sua potencial utilização em diversas áreas da medicina clínica, bem como para o estudo estrutural e funcional dessa classe de proteínas (DURHAM & REGNIER, 2006; BIES *et al.*, 2004).

1.2.4. Características estruturais das lectinas

Com base na estrutura geral das proteínas, as lectinas de plantas têm sido subdivididas em merolectinas, hololectinas, quimerolectinas e superlectinas (PEUMANS & VAN DAMME *et al.*, 1998). Merolectinas são aquelas que possuem apenas um domínio para ligação a carboidratos. São monovalentes e por isso não podem precipitar glicoconjugados ou aglutinar células. Hololectinas também possuem domínio específico para ligação a carboidratos, mas contêm, pelo menos, dois domínios idênticos ou mais domínios homólogos ligantes a açúcares; sendo di ou multivalentes, aglutinam células e/ou precipitam glicoconjugados. A maioria das lectinas de plantas pertence a esse grupo.

Quimerolectinas são proteínas com um ou mais domínios de ligação a carboidratos e um domínio não-relacionado. Esse domínio diferente pode ter uma atividade enzimática bem definida ou outra atividade biológica, mas age independentemente dos domínios de ligação a carboidratos. Superlectinas consistem exclusivamente de pelo menos dois domínios de ligação a açúcares diferentes. Esse pode ser considerado um grupo especial de quimerolectinas, consistindo de dois domínios estruturalmente e funcionalmente diferentes de ligação a carboidratos (VAN DAMME *et al.*, 1996). Portanto, as lectinas apresentam uma grande variedade estrutural, mas uma característica comum a todas é a presença de ao menos um sítio específico de ligação a carboidrato em cada cadeia polipeptídica, que corresponde ao chamado domínio de reconhecimento de carboidrato (GABIUS, 1994; ZANETTI, 2007).

Algumas lectinas requerem a presença de íons bivalentes para que, por intermédio da ligação dos íons aos seus sítios metálicos, a proteína possa se tornar ativa e pronta para exercer sua função biológica. Lectinas que não requerem íons metálicos já possuem a conformação estrutural necessária para o reconhecimento aos carboidratos (SHARON & LIS, 1990). As lectinas podem ainda apresentar uma porção glicídica, sendo denominadas de glicoproteínas. Essa porção aumenta a estabilidade da lectina diminuindo a degradação proteolítica e a desnaturação por temperatura e pH, bem como influencia na associação com outras moléculas, na solubilidade e viscosidade em solução aquosa (KILPATRICK, 1986).

1.2.5. Propriedades biológicas e potencial biotecnológico de lectinas

As lectinas, por terem a habilidade de se ligar a mono e oligossacarídeos, apresentam uma variedade de efeitos biológicos, alguns dos quais servindo como base para a aplicação de lectinas na investigação de atividades biológicas, tais como ação contra fungos (SITOHY *et al.*, 2007), bactérias (SANTI-GADELHA *et al.*, 2006) e na identificação de espécies

bacterianas por diferença na aglutinação em placas de microtitulação (ATHAMNA *et al*., 2006).

Lectinas têm sido utilizadas na detecção e separação de glicoconjugados (PAIVA *et al.*, 2006). A lectina de *Cratylia mollis* (feijão camaratu) foi capaz de isolar a enzima lecitina colesterol aciltransferase, importante no metabolismo do colesterol (LIMA *et al.*, 1997). O complexo pôde ser, então, utilizado para o estudo de glicoproteínas de soro humano.

Lectinas também podem ser usadas na determinação de tipos sangüíneos (KHANG *et al.*, 1990) e diagnóstico de processos de desenvolvimento, diferenciação e transformação neoplásica (LI *et al.*, 2008) e no tratamento de condições pré-cancerosas como a colite ulcerativa através de conjugação com drogas (WROBLEWSKI *et al.*, 2001).

A lectina do rizoma de *Setcreasea purpureae* (família Commelinaceae) apresentou atividades antiviral e indutora de apoptose (YAO *et al.*, 2010). Devido ao fato de algumas lectinas possuírem habilidade para mediar mucoadesão, citoadesão e citoinvasão de drogas (GABOR *et al.*, 2004), essas moléculas têm sido exploradas em sistemas de liberação de drogas. Lectina de folhas de *Bauhinia monandra* (pata-de-vaca) e a lectina de *Lens culinaris* (lentilha) foram incorporadas e também adsorvidas na superfície de nanopartículas, mostrando ser ferramentas potenciais em medicamentos de administração oral, com liberação controlada (RODRIGUES *et al.*, 2003).

Algumas lectinas são capazes de atuar sobre linfócitos, fazendo com que tais células passem de um estado quiescente para um estado de crescimento e proliferação. A lectina da babosa *Aloe arborescens* (KOIKE et al, 1995) e a lectina de semente de *Cratylia mollis* (MACIEL *et al.*, 2004) são alguns exemplos de lectinas com atividade mitogênica que podem ser utilizadas em ensaios *in vitro*.

1.2.5.1. Atividade antimicrobiana de lectinas

O uso contínuo de antimicrobianos comerciais leva a seleção de organismos cada vez mais resistentes, tornando necessária a busca por novas substâncias com propriedades antimicrobianas (BASTOS, 2008). As proteínas antimicrobianas, em animais, constituem parte do sistema imune inato. Em plantas, elas também estão envolvidas no mecanismo de defesa (YE & NG, 2001). As plantas são fonte de diversos compostos que já tiveram a sua eficácia comprovada contra microrganismos (CIMANGA *et al.*, 2011; WANG & BUNKERS, 2000). Neste sentido, muitas substâncias, inclusive lectinas, estão sendo avaliadas quanto ao seu efeito antimicrobiano.

A quitina é um polissacarídeo de ocorrência natural composto por monômeros de N-acetilglicosamina (GlcNAc) os quais formam uma estrutura molecular complexa. Lectinas ligadoras de quitina têm sido isoladas de diversas fontes, incluindo bactérias, insetos, plantas e mamíferos. Muitas delas apresentam atividade antifúngica, uma vez que a quitina é o componente-chave da parede celular de fungos (TRINDADE *et al.*, 2006; SITOHY *et al.*, 2007). Lectinas de plantas, com seu potencial antifúngico, podem ser exploradas através da introdução de material genético que codifique a expressão destas lectinas (VAN DAMME et al, 1996; FIELDS & KORUNIC, 2000).

As lectinas ligadoras de quitina também têm sido estudadas do ponto de vista estrutural. Lectinas que se ligam a quitina são proteínas muito estáveis e acredita-se que essa estabilidade está relacionada ao alto teor de pontes dissulfeto intracadeia dando-lhes rigidez e estabilidade em uma ampla faixa de pH e temperatura (TRINDADE *et al.*, 2006). As mais estudadas são aquelas pertencentes à família das heveínas, assim chamadas por possuírem em comum o domínio heveínico como motivo estrutural de reconhecimento da quitina. A heveína é uma lectina constituída por 43 aminoácidos (cerca de 4,5 kDa), encontrada na seringueira (*Hevea brasiliensis*). É especialmente rica em resíduos de glicina e cisteína e sua estrutura é mantida por 4 pontes dissulfeto, o que lhe confere uma estabilidade notável, característica que se estende às demais lectinas da família das heveínas. Mesmo depois de aquecida a 90 ºC por 10 minutos, a heveína ainda inibe o crescimento de fungos (NEUMANN *et al.*, 2004).

Atividade antifúngica foi observada para uma lectina isolada de sementes de *Castanea mollissima* (castanha-da-China) frente aos fungos *Botrytis cinerea, Mycosphaerella arachidicola* e *Physalospora piricola* (WANG & NG, 2003), bem como para a lectina de sementes de *Talisia esculenta* (pitombeira), a qual inibiu o crescimento dos fungos *Fusarium oxysporum, Colletotrichum lindemuthianum* e *Saccharomyces cerevisiae* através da interação da lectina com as estruturas dos fungos (FREIRE *et al.*, 2002). Xu *et al.* (1998) purificaram e caracterizaram uma lectina da *Gastrodia elata*, que inibiu o crescimento dos fungos fitopatógenos *Valsa ambiens, Rhizoctonia solani, Gibberella zeae, Ganoderma lucidum* e *B. cinerea*.

Apesar do pouco conhecimento a cerca do mecanismo de ação antifúngico, já foi observado que as lectinas possuem a capacidade de se ligarem especificamente a hifas fúngicas e atuarem impedindo o consumo de nutrientes e a incorporação de precursores necessários para o crescimento do fungo. Atuam ainda sobre a germinação de esporos fúngicos, provavelmente num estágio muito inicial do processo, inibindo-a, de modo que há um prolongamento do período latente que precede a germinação (LIS & SHARON, 1981).

Lectinas têm sido usadas com grande sucesso na identificação de fungos, uma vez que esses compostos são altamente específicos aos carboidratos presentes na parede celular dos mesmos (ZABEL & MORRELL, 1992). O conhecimento do perfil sacarídico na superfície fúngica habilita o uso de lectinas como promissoras sondas celulares, que podem servir como carreadores de agentes antifúngicos que utilizam, como alvos específicos, os carboidratos existentes na superfície da célula do microorganismo (LEAL *et al.*, 2007).

As bactérias possuem em suas paredes celulares ácidos teicóicos e teicurônicos, peptidioglicanos e lipopolissacarídeos que podem interagir com lectinas. A habilidade de lectinas em reconhecer especificamente carboidratos permite o emprego dessas biomoléculas como sondas-diagnóstico para identificação de bactérias patógenas, que estão baseadas na reação de aglutinação seletiva entre lectina e bactéria (DOYLE & SLIFKIN, 1994). Athamna *et al.* (2006) analizaram os diferentes padrões de aglutinação de bactérias promovidas por 23 lectinas e mostraram que a interação lectina-bactéria é uma boa ferramenta para identificar rapidamente espécies de *Mycobacterium.* Ratanapo *et al.* (2001) mostraram a interação de duas lectinas com especificidade para ácido N-glicosilneuramínico contra bactérias fitopatogênicas, propondo uma possível função na defesa de plantas.

Lectinas com atividade antibacteriana tem potencial uso como antibióticos naturais. Lectinas antibacterianas já foram isoladas de animais como *Bothrops leucurus e Crassostrea hongkongensis* e plantas tais como *Phthirusa pyrifolia* e *Myracrodruon urundeuva* (Costa *et al.*, 2010; He *et al.*, 2011; Nunes *et al.*, 2011; Sá *et al.*, 2009a) e tem sido sugerido que lectinas podem formar poros na parede celular, induzindo a morte bacteriana pelo extravasamento do conteúdo celular (Correia *et al.*, 2008).

1.2.5.1.1 Bactérias

As bactérias são organismos unicelulares, procariontes e pertencentes ao reino Monera. Sua classificação é feita de acordo com a constituição da parede celular em dois grupos: Gram-positivas (+) e Gram-negativas (+). As bactérias Gram-positivas apresentam em sua parede celular polissacarídeos, ácidos teicóicos e peptidoglicanos, enquanto as Gram-negativas apresentam na sua parede celular peptideoglicanos, lipídeos, proteínas e lipopolissacarídeos (TRABULSI, 2000).

Dentre as bactérias Gram (+), destaca-se *Staphylococcus aureus*, de forma esférica, geralmente formando cachos irregulares semelhantes a cachos de uvas. É um patógeno responsável por muitas infecções graves nos seres humanos. Pode provocar endocardite,

osteomielite hematogênica aguda, meningite ou infecção pulmonar, entre outras. Outras bactérias Gram (+) são as do gênero *Streptococcus,* de forma esférica, tipicamente formando pares ou cadeias durante o seu crescimento. Apresentam-se amplamente distribuídas na natureza. Podem estar presentes na microbiota humana normal, como também estar associadas a importantes doenças humanas, que podem ser atribuídas, em parte, à infecção por *Streptococcus* (TRABULSI, 2000; CANTÓN *et al.*, 2007).

Entre as Gram (-), destacam-se: 1) *Escherichia coli*, que se apresenta na forma de bastonete, além de formar colônias lisas, convexas e circulares; faz parte da microbiota normal, mas pode causar doenças (infecção do trato urinário, diarréia, meningite e septicemia). 2) *Klebsiella,* também em forma de bastonete, forma colônias grandes e mucóides. Encontrada no trato respiratório e nas fezes, é responsável por uma pequena fração de pneumonias bacterianas, provocando extensa consolidação necrozante hemorrágica nos pulmões. 3) *Pseudomonas aeruginosa*, um bacilo aeróbico móvel amplamente distribuído na natureza, sendo comum seu achado em ambientes úmidos de hospitais. Consegue colonizar seres humanos normais, nos quais é saprófita, sendo encontrada em pequenos grupos na flora intestinal normal e na pele de humanos (JAWETZ *et al.*, 1991). 4) *Yersinia ruckeri*, bactéria causadora da "doença da boca vermelha" ou yersiniose por provocar hemorragia subcutânea da boca, barbatanas, e olhos de peixes como os salmonídeos, acarretando elevadas perdas econômicas em todo o mundo. Embora geralmente bem controlada por meio de vacinação e tratamento com antibióticos, surtos da doença ainda ocorrem, especialmente em áreas endêmicas (Fernández *et al.*, 2007). 5) *Photobacterium damselae* subsp. *piscicida*, bactéria em forma de bastonete causadora de "pasteurelose" em peixes, provocando nódulos em vísceras interna, em particular do rim e no baço, acompanhada por necrose interna generalizada (Romalde, 2002)

1.2.5.2. Fungos

Os fungos são organismos não-fotossintéticos que crescem como uma massa de filamentos (fungos filamentosos) entrelaçados e ramificados, conhecida como micélio ou na forma de leveduras (fungos leveduriformes). Os fungos, em sua maioria, têm sua parede celular constituída por celulose ou quitina (JAWETZ *et al.*, 1991). Esses microrganismos são ubíquos, encontrados no solo, água, vegetais, homem e detritos em geral (TRABULSI, 2000). Interagem com a natureza das mais variadas maneiras, podendo agir tanto de forma benéfica como deletéria.

A espécie *Candida albicans* pertencente a família Saccharomycetaceae, é um fungo polimorfo, podendo ser oportunista ou patogênico (NOBRE *et al.*, 2002). De grande importância médica para o homem, *C. albicans* pode causar infecções superficiais como doenças de pele e mucosa ou em tecidos mais profundos, sendo responsável por mais de 85% dos casos de candidíase (VAL & ALMEIDA FILHO, 2001; CROCCO *et al.*, 2004). A forma sistêmica pode alcançar diversos órgãos, causando candidíase pulmonar, endocardite dentre outros, podendo levar os pacientes a óbito (MENEZES *et al.*, 2004)

A virulência e patogenicidade de *C. albicans* são atribuidas a diversos fatores como a capacidade de se transformar em uma célula adaptada para invadir os tecidos dos hospedeiros (KUNAMOTO *et al.*, 2005). A capacidade que esse fungo possui em desenvolver formas filamentosas (hifas e pseudo-hifas) está intimamente associada a sua capacidade de causar doenças, pois sua forma filamentosa facilita a invasão dos tecidos e roforça a colonização. Além disso sugere-se que temperaturas acima de 37 ºC e pH maior que 5,5 podem aumetar a formação de hifas (BASTOS, 2008). A elevada versatilidade de *C. albicans* faz com que essa espécie fúngica seja considerada o patógeno mais comum ao humano (Hoehamer *et al*., 2010).

1.3 INIBIDORES DE PROTEASES

Os inibidores de proteases são compostos pertencentes a diferentes classes, tais como metabólitos secundários e proteínas, que interagem com enzimas proteolíticas bloqueando sua atividade catalítica (Paiva *et al.*, 2012). Inibidores podem atuar competindo com substratos pelo sítio ativo das proteases, com o qual estabelecem interações estáveis através de pontes de hidrogênio e interações eletrostáticas ou hidrofóbicas. No caso de inibidores não competitivos, modificações conformacionais na molécula da protease impossibilitam o reconhecimento do substrato (BODE e HUBER, 2000; LIAO *et al.*, 2007).

Inibidores de proteases são promissores agentes antimicrobianos, atuando no bloqueio da atividade de enzimas que desempenham papéis-chave no ciclo de vida de microorganismos Além disso, a interação entre o inibidor e proteínas de membrana pode resultar em mudanças na permeabilidade celular e indução da morte do microrganismo (SUPURAN *et al.*, 2002; KIM *et al.*, 2005; FEAR *et al.*, 2007; LI *et al.*, 2007). Inibidores de tripsina ainda possuem a vantagem de não sofrerem hidrólise por enzimas tripsina-símile, fator fortemente limitante para a aplicação de peptídeos antimicrobianos (LI *et al.*, 2007). Inibidores de proteases de origem vegetal têm sido descritos como agentes antifúngicos (CHEN *et al.*, 1999; GIUDICI *et*

al., 2000; YANG *et al.*, 2006; LOPES *et al.*, 2009) e antibacterianos (NGAI & Ng, 2004; KIM *et al.*, 2006).

A tripsina é uma endopeptidase do grupo das serinoproteases amplamente encontrada no sistema digestivo de vertebrados, sendo também expressa por alguns microorganismos, como fungos, e invertebrados, como moluscos e insetos (BAHGAT *et al.*, 2002; BARATA *et al.*, 2002; BHATTACHARYYA *et al.*, 2007). Atividade inibidora de tripsina pode ser detectada pela incubação da preparação teste com a tripsina em presença do substrato cromogênico N-benzoil-DL-arginil-ρ-nitroanilida (BApNA). A hidrólise de ligações peptídicas pela tripsina ocorre preferencialmente em resíduos de arginina e, dessa forma, a enzima é capaz de hidrolisar a molécula do BApNA liberando ρ-nitroanilina no meio reacional. Essa espécie química absorve a luz no comprimento de onda correspondente à região do amarelo e pode ser detectada pela medida da absorbância a 405 nm. A presença do inibidor é indicada quando a liberação de ρ-nitroanilina no meio é reduzida ou abolida em relação a um tratamento onde a preparação teste foi omitida (PAIVA *et al*., 2013).

2. OBJETIVOS

2.1. Objetivo Geral

- Definir procedimentos experimentais para isolamento de lectinas de casca e folha e de inibidor de tripsina de casca de *S. terebinthifolius.* Determinar a atividade antimicrobiana das lectinas e inibidor de tripsina de *S. terebinthifolius* e de lectinas isoladas de entrecasca, cerne e folhas de *Myracrodruon urundeuva*.

2.2. Objetivos específicos

- Purificar e caracterizar as lectinas de folhas (SteLL) e entrecasca (SteBL) de *S. terebinthifolius*.
- Purificar o inibidor de tripsina da casca de *S. terebinthifolius* (SteBI).
- Preparar infusão aquosa e extrato metanólico de entrecasca de *S. terebinthifolius*
- Isolar as lectinas de entrecasca (MuBL), cerne (MuHL) e folhas (MuLL) de *M. urundeuva* de acordo com procedimentos previamente estabelecidos.
- Investigar a atividade antibacteriana de infusão e extrato metanólico de entrecasca de *S. terebinthifolius*, bem como de SteLL, SteBL, SteBI, MuBL, MuHL e MuLL, contra bactérias patogênicas a humanos.
- Investigar a atividade antifúngica da infusão e extrato metanólico de entrecasca de *S. terebinthifolius*, bem como de SteBI, SteBL e SteLL sobre *Candida albicans*.
- Investigar a atividade antibacteriana de MuBL, MuHL e MuLL contra bactérias patogênicas a peixes.
- Determinar o efeito de MuBL, MuHL e MuLL sobre a aderência e capacidade invasiva das bactérias *E. coli, S. aureus, Photobacterium damselae* subsp. *piscicida* e *Yersinia ruckeri* em células de peixes (SAF-1) e humanos (HeLa) .
- Determinar a composição de produtos extracelulares produzidos por bactérias patogênicas através de dosagem protéica, atividade enzimática.

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3. CAPÍTULO 1

Antimicrobial lectin from *Schinus terebinthifolius* leaf

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Antimicrobial lectin from *Schinus terebinthifolius* leaf

F.S. Gomes, T.F. Procópio, T.H. Napoleão, L.C.B.B. Coelho and P.M.G. Paiva

Departamento de Bioquímica, CCB, Universidade Federal de Pernambuco, Recife, Brazil

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Correspondence
Patrícia M.G. Paiva, Departamento de Bioquímica, CCB, Universidade Federal de Pernambuco, Avenida. Prof. Moraes Rego S/N, Cidade Universitária, 50670-420, Recife-PE, Brazil. E-mail: ppaivaufpe@yahoo.com.br

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Abstract

Aims: *Schinus terebinthifolius* leaves are used for treating human diseases caused by micro-organisms. This work reports the isolation, characterization and antimicrobial activity of *S. terebinthifolius* leaf lectin (SteLL).

Methods and Results: The isolation procedure involved protein extraction with 0·15 mol l^{-1} NaCl, filtration through activated charcoal and chromatography of the filtrate on a chitin column. SteLL is a 14-kDa glycopeptide with haemagglutinating activity that is inhibited by *N*-acetyl-glucosamine, not affected by ions (Ca^{2+} and Mg^{2+}) and stable upon heating (30–100°C) as well as over the pH 5·0–8·0. The antimicrobial effect of SteLL was evaluated by determining the minimal inhibitory (MIC), bactericide (MBC) and fungicide (MFC) concentrations. Lectin was active against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Staphylococcus aureus*. Highest bacteriostatic and bactericide effects were detected for *Salm. enteritidis* (MIC: 0·45 μg ml^{-1}) and *Staph. aureus* (MBC: 7·18 μg ml^{-1}), respectively. SteLL impaired the growth (MIC: 6·5 μg ml^{-1}) and survival (MFC: 26 μg ml^{-1}) of *Candida albicans*.

Conclusions: SteLL, a chitin-binding lectin, purified in milligram quantities, showed antimicrobial activity against medically important bacteria and fungi.

Significance and Impact of the Study: SteLL can be considered as a new biomaterial for potential antimicrobial applications.

Introduction

Antimicrobials have been used to treat diseases caused by bacteria and fungi and therefore have significantly contributed to a reduced mortality rate in humans and animals. The emergence of micro-organisms resistant to frequently used commercial antimicrobial drugs, such as methicillin, oxacillin and penicillin, has stimulated the evaluation of medicinal plants as sources of compounds for inhibiting the growth or survival of micro-organisms. Plant lectins with antibacterial and antifungal activities against micro-organisms that cause human diseases have been isolated from seeds, heartwood, cladodes and leaves (Santi-Gadelha *et al.* 2006; Oliveira *et al.* 2008; Sá *et al.* 2009; Santana *et al.* 2009; Costa *et al.* 2010; Charungchitrak *et al.* 2011).

The antibacterial activity of lectins occurs 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). Previous studies have revealed that isolectin I from *Lathyrus ochrus* seeds binds to muramic acid and muramyl dipeptide – two components commonly found in bacterial cell walls – through hydrogen bonds between ring hydroxyl oxygen atoms of the sugar and the carbohydrate-binding site of lectin as well as through hydrophobic interactions with the side chains of residues Tyr^{100} and Trp^{128} of isolectin I (Bourne *et al.* 1994).

The antifungal activity of lectins occurs through an interaction with the fungal cell wall, which is composed of chitin, glucans and other polymers (Adams 2004). Chitin-binding lectins can impair the synthesis and/or deposition of chitin in the cell wall as well as prevent hyphal development and spore germination (Lis and Sharon

1981; Selitrennikoff 2001; Trindade *et al.* 2006). It has been also suggested that small antifungal lectins such as hevein (4·7 kDa) and poutein (14 kDa) can penetrate the fungal cell wall to reach the plasma membrane, where they can block the active sites of enzymes involved in cell wall morphogenesis (Van Parijs *et al.* 1991; Boleti *et al.* 2007).

Schinus terebinthifolius Raddi (Anacardiaceae family), known as Brazilian pepper, is a tree that is distributed worldwide. The leaves are used topically in Brazil for healing and tissue repair of skin wounds. Moreover, its leaves are commonly used as an infusion for treating infections in the respiratory, digestive and urinary tracts, as well as against rheumatism and oral candidiasis (Martinez *et al.* 1996; Ribas *et al.* 2006; Lindenmaier 2008). The essential oil of the *S. terebinthifolius* leaf is used to treat respiratory problems, mycosis and candidal infections (topical use). Its activity has been attributed to its high concentrations of monoterpenes (Lloyd *et al.* 1977). *S. terebinthifolius* leaf essential oil has shown antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigella dysenteriae*, *Staphylococcus albus*, *Staphylococcus aureus* and *Staphylococcus intermedius* as well as antifungal activity against *Aspergillus niger*, *Aspergillus parasiticus* and *Candida albicans* (El-Massry *et al.* 2009; Silva *et al.* 2010). Extracts from *S. terebinthifolius* leaves in ethanol and dichloromethane containing secondary metabolites such as phenols, flavones, flavonoids, xanthones, leucoanthocyanidins, flavanones and free steroids were active against *E. coli*, *Ps. aeruginosa*, *Staph. aureus* and *C. albicans* (Lima *et al.* 2006; El-Massry *et al.* 2009). Among 23 extracts from 12 Cuban plants, an aqueous extract from the leaves of *S. terebinthifolius* showed the highest activity against *Staph. aureus*, and it could inhibit the growth of *B. subtilis* (Martinez *et al.* 1996).

This work reports the isolation and characterization of a chitin-binding lectin from *S. terebinthifolius* leaf (SteLL) and the effect of this protein on the growth of bacteria and fungi that cause human diseases.

Materials and methods

Leaf extract

Leaves of *S. terebinthifolius* were collected in Recife City, State of Pernambuco, in north-eastern Brazil. A voucher specimen is archived (number 73 431) in the *Instituto Agronômico de Pernambuco* (IPA), Recife, Brazil.

Dried leaves (20 g) were crushed to powder (40 mesh) and suspended in 0·15 mol l^{-1} NaCl (200 ml). After agitation for 16 h at 4°C, the sample was filtered through gauze and centrifuged at 3000 ***g*** for 15 min. The supernatant (crude extract) was passed through activated charcoal (10%, w/v; Reagen, Paraná, Brazil), and the filtrate (leaf extract) was collected.

Isolation of *Schinus terebinthifolius* leaf lectin (SteLL)

Leaf extract (11·4 mg of protein) was loaded onto a chitin (Sigma-Aldrich, MO, USA) column (7·5 × 1·5 cm) equilibrated (20 ml h^{-1} flow rate) with 0·15 NaCl. After extensive washing with equilibrating solution (100 ml), SteLL was recovered by elution using 1·0 mol l^{-1} acetic acid (80 ml). The elution product was dialysed in a 10-kDa cut-off membrane (Sigma-Aldrich) against distilled water (4 h, 4°C) and in sequence against 0·15 mol l^{-1} NaCl (4 h, 4°C).

Total phenol content

Total phenol content of the crude extract, leaf extract and SteLL was determined using the Folin–Ciocalteu method based on the reduction of phosphomolybdic–phosphotungstic acid reagent in an alkaline medium (Morais *et al.* 1999). The Folin–Ciocalteu's reagent (1 : 10 solution in distilled water; 2·5 ml) and sodium carbonate (75 g l^{-1}; 2 ml) were added to the samples (0·5 ml), and the mixtures were incubated at 50°C for 5 min. After cooling for 30 min, absorbance was measured at 760 nm. Phenol content was determined based on a standard curve of tannic acid (9·6–48 mg ml^{-1}).

Haemagglutinating activity

In lectinology, the haemagglutinating assay is the classic tool to assess the carbohydrate-binding property of a lectin, making sure that it is active. A haemagglutinating assay was carried out in microtitre plates (TPP-Techno Plastic Products, Trasadingen, Switzerland) according to the method described by Paiva and Coelho (1992). A serial 2-fold dilution of the lectin preparation (50 μl) was prepared with 0·15 mol l^{-1} NaCl before incubation with a suspension (2·5% v/v) of glutaraldehyde-fixed rabbit erythrocytes (Bing *et al.* 1967). One haemagglutination unit (titre) was defined as the reciprocal of the highest dilution of the sample promoting full erythrocyte agglutination (Napoleão *et al.* 2011). Specific haemagglutinating activity was defined as the ratio between the titre and protein concentration (mg ml^{-1}) determined according Lowry *et al.* (1951). Increase in specific haemagglutinating activity reveals lectin concentration and purification.

Haemagglutinating activity of SteLL (0·5 mg ml^{-1}; 50 μl) was also evaluated in the presence of metal ions, at different pH values, or after heating to different temperatures. The effect of Ca^{2+} and Mg^{2+} was evaluated

according to Pajic *et al.* (2002) using the haemagglutinating activity assay described above but by replacing the 0·15 mol l^{-1} NaCl with 0·02 mol l^{-1} ion solution prepared with 0·15 mol l^{-1} NaCl. SteLL was incubated at 25°C for 45 min in the presence of metal ions before the addition of a rabbit erythrocyte suspension (50 μl). For pH assays, aliquots of SteLL (50 μl) were serially diluted 2-fold in 0·01 mol l^{-1} citrate phosphate (pH 5·0–6·0), 0·01 mol l^{-1} sodium phosphate (pH 7·0), 0·01 mol l^{-1} Tris-HCl (pH 8·0–9·0) or 0·01 mol l^{-1} glycine–NaOH (pH 10·0–11·0); all these buffers were prepared with 0·15 mol l^{-1} NaCl. The effect of temperature was evaluated by incubating (30 min) SteLL at 30, 40, 50, 60, 70, 80, 90 and 100°C before conducting the haemagglutinating assay.

Polyacrylamide gel electrophoresis (PAGE)

SteLL was evaluated using PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) and β-mercaptoethanol on a 15% (w/v) gel according to Laemmli (1970). Polypeptide bands and molecular mass standards (bovine serum albumin, 66 000 Da, ovalbumin, 45 000 Da, glyceraldehyde-3-phosphate dehydrogenase, 36 000 Da, carbonic anhydrase, 29 000 Da, trypsin inhibitor, 20 000 Da, α-lactalbumin, 14 400 Da, from Sigma-Aldrich) were stained with 0·02% (w/v) Coomassie Brilliant Blue prepared with 10% (v/v) acetic acid. Glycoprotein staining was performed using Schiff's reagent (Pharmacia Fine Chemicals 1980).

Antibacterial activity

Gram-positive (*Staph. aureus* WDCM 00032) and Gram-negative (*E. coli* WDCM 00013, *Klebsiella pneumoniae* ATCC 29665, *Ps. aeruginosa* WDCM 00025 and *Salmonella enteritidis* MM 6247) strains were provided by the *Departamento de Antibióticos*, *Universidade Federal de Pernambuco*, Brazil. The Gram-negative *Proteus mirabilis* WDCM 00023 was provided by the *Fundação Oswaldo Cruz*, Brazil. Stationary cultures were maintained in nutrient agar (NA) and stored at 4°C. To determine the antibacterial activity, bacteria were cultured in nutrient broth (NB) and incubated while shaking at 37°C overnight. Cultures were adjusted turbidimetrically to $1{\cdot}5 \times 10^8$ colony forming units (CFU) ml^{-1} at a wavelength of 600 nm.

Aliquots (100 μl) of leaf extract (1·9 mg ml^{-1} of protein) or SteLL (0·23 mg ml^{-1}) were diluted 1 : 2 in NB (100 μl) and submitted to a series of 10 double dilutions to a final ratio of 1 : 2048. A 180-μl aliquot of each dilution was dispensed into a microtitre plate well. All wells were inoculated with 20 μl of the bacterial culture and incubated at 37°C for 24 h. Assays were performed in triplicate for each concentration. Negative control wells contained NB medium and the micro-organisms. After incubation, optical density was measured at 490 nm (OD_{490}) using a microplate reader (Biotek Instruments Inc., VT, USA). Minimal inhibitory concentration (MIC) was determined as the lowest protein concentration at which there was $\geq$50% reduction in optical density relative to the control well OD_{490} (Amsterdam 1996).

To determine minimal bactericide concentration (MBC), inoculations (10 μl) from wells treated with leaf extract or SteLL that was found to inhibit bacterial growth were transferred to NA plates and incubated at 37°C for 24 h. The lowest protein concentration showing no bacterial growth was recorded as the MBC. The assay was performed in triplicate.

Antifungal activity

Candida albicans was obtained from the Culture Collections at University Recife Mycologia, *Departamento de Micologia*, *Universidade Federal de Pernambuco*, Brazil. Antifungal activity was evaluated using the same method used for antibacterial activity, changing the incubation temperature (28°C) and replacing the culture medium used. Sabouraud dextrose was used to determine the MIC, while Sabouraud agar was used to determine the minimal fungicide concentration (MFC) – the lowest protein concentration showing no fungal growth. Assays were performed in triplicate.

Results

From 20 g of *S. terebinthifolius* leaves, 3·7 g of protein was extracted using 0·15 mol l^{-1} NaCl. *S. terebinthifolius* crude extract (specific haemagglutinating activity: 80·3) was treated with activated charcoal to eliminate tannins and other phenol compounds. After a filtration step, the filtrate (leaf extract) showed no colour, but it showed higher specific haemagglutinating activity (2155) than the crude extract. To confirm that activated charcoal treatment was efficient, the total phenol content of the crude extract and leaf extract was determined. Values obtained for the crude extract and leaf extract (after activated charcoal treatment) were 0·019 mg ml^{-1} and 0·005 mg ml^{-1}, respectively.

Lectin was further purified by chromatography of the leaf extract over a chitin column. Haemagglutinating activity for the leaf extract adsorbed onto the chitin column and one active protein peak (SteLL; specific haemagglutinating activity of 29467) was eluted using 1·0 mol l^{-1} acetic acid (Fig. 1a). From 20 g of leaf powder, 125 mg of SteLL was isolated with a yield of 1224%

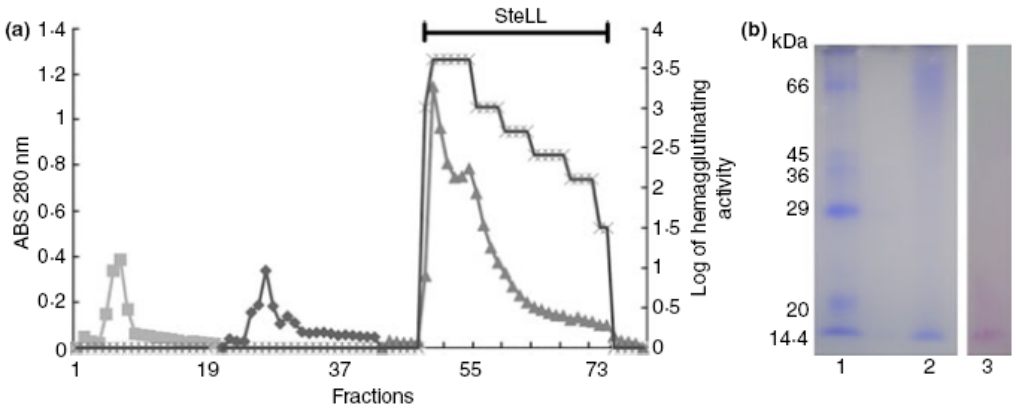

Figure 1 Purification of *Schinus terebinthifoilus* leaf lectin (SteLL). (a) Chromatography of leaf extract on chitin column. Washing step used 0·15 mol l^{-1} NaCl. Fractions of 2·0 ml were collected and evaluated for haemagglutinating activity and absorbance at 280 nm in spectrophotometer. (b) SDS-PAGE of SteLL under reducing conditions with β-mercaptoethanol; molecular weight markers (1) and SteLL (2) were stained with Coomassie Brilliant Blue. SDS-PAGE of SteLL stained using Schiff's reagent (3). (–■–) 0.15 mol l^{-1} NaCl; (–◆–) 1 mol l^{-1} NaCl; (–▲–) 1 mol l^{-1} - acetic acid and (–✕–) log of haemagglutinating activity.

and 367-fold purification relative to the crude extract (Table 1). SDS-PAGE revealed that SteLL is a glycosylated polypeptide of 14·0 kDa (Fig. 1b). The same electrophoretic profile was detected after treating lectin with the reducing agent β-mercaptoethanol. Phenolic compounds were not detected in SteLL.

The specific haemagglutinating activity of SteLL (29467) was not altered at pH 5·0, 6·0, 7·0 and 8·0, but was reduced at pH 9·0, 10·0 and 11·0 for 58, 16 and 8, respectively. Heating to 100°C and the addition of Ca^{2+} and Mg^{2+} did not alter the haemagglutinating activity.

Antibacterial assays with leaf extract revealed an inhibitory effect on *E. coli*, *Pr. mirabilis* and *Staph. aureus* growth and no effect on *Kl. pneumoniae*, *Ps. aeruginosa* and *Salm. enteritidis* (Table 2). Bactericidal activity was only detected against *Staph. aureus* (MBC of 950 μg ml^{-1} of protein). SteLL was active against all tested bacteria, MIC values ranged from 0·45 to 28·75 μg ml^{-1}, and MBC values ranged from 7·18 to 115 μg ml^{-1} (Table 2).

The antifungal assay revealed that *C. albicans* growth was inhibited by the leaf extract (MIC of 12·75 μg ml^{-1} of protein) and SteLL (MIC of 6·5 μg ml^{-1}). However, only SteLL showed fungicide activity, with an MFC value of 26 μg ml^{-1}.

Discussion

Extraction and isolation of pharmacologically active compounds from medicinal plants have received attention in the search for new economically viable alternatives for treating human infections. Plant lectins have been reported as active components in aqueous extracts that have antimicrobial activity (Oliveira *et al.* 2008; Sá *et al.* 2009; Costa *et al.* 2010).

The crude extract of *S. terebinthifolius* leaves showed strong pigmentation, indicating the presence of a high concentration of phenol compounds such as tannins. Leaf extract obtained after treatment with activated charcoal showed no colour, a phenol content of 73% lower than that in the crude extract and specific haemagglutinating activity higher than that in the crude extract. Studies have shown that activated charcoal is efficient for adsorbing

Table 1 Summary of SteLL isolation

Sample	Protein (mg ml^{-1})	Haemagglutinating activity (titre)	Total protein (mg)	Total haemagglutinating activity	Specific haemagglutinating activity	Purification (fold)*	Yield (%)†
Crude extract	25·5	2048	3700	296960	80·3	1	100
Leaf extract	1·9	4096	260	546406	2155	27	184
SteLL	0·278	8192	125	3634790	29467	367	1224

*Purification fold corresponds to the ratio between specific haemagglutinating activity of the sample and specific haemagglutinating activity of crude extract. Specific haemagglutinating activity was defined as the ratio between the titre and protein concentration (mg ml^{-1}).
†Yield corresponds to the percentage of total haemagglutinating activity from crude extract recovered.

Table 2 Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *Schinus terebinthifolius* leaf extract and lectin (SteLL)

	Leaf extract		SteLL	
Bacteria	MIC	MBC	MIC	MBC
Escherichia coli (−)	12750	ND	28·75	115
Klebsiella pneumoniae (−)	ND	ND	3·59	115
Pseudomonas aeruginosa (−)	ND	ND	1·79	14·37
Proteus mirabilis (−)	950	ND	3·59	14·37
Staphylococcus aureus (+)	118	950	1·79	7·18
Salmonella enteritidis (−)	ND	ND	0·45	115

MIC and MBC values are expressed in μg ml^{-1} of protein. ND: not detected. (+) Gram-positive and (−) Gram-negative bacteria.

tannins and other phenol compounds (Mohan and Karthikeyan 1997; Aerts *et al.* 1999; Mukherjee *et al.* 2007).

SteLL, a glycosylated protein, was isolated with yield >100% using chromatography on a chitin matrix. The molecular mass of SteLL was smaller than that of glycosylated lectin isolated from the *Myracrodruon urundeuva* leaf (14·2 kDa), and the purification fold as well as the yield achieved for SteLL was higher than that obtained for this lectin (Napoleão *et al.* 2011).

The high yield obtained in the SteLL isolation procedure likely reflects the elimination of tannins from the crude extract after activated charcoal treatment and chitin chromatography. Phenol compounds form soluble or insoluble complexes with proteins, thereby interfering with their biological activity (Hagerman 1992). Suzuki and Mori (1989), using a combination of affinity, anion exchange and gel filtration chromatographies, isolated a lectin from the haemolymph of *Pinctada fucata martensii* with a yield of 200%. The authors suggested that the high recovery ratio was likely due to the elimination of haemagglutinating activity inhibitors.

SteLL haemagglutinating activity was not affected in the presence of divalent cations, and it was stable at broad pH and temperature ranges. Haemagglutinating activity of lectins from *Setcreasea purpurea* rhizome and from *Artocarpus integrifolia* fruit was also shown to be stable at pH 6·0–9·0 and until 80°C, respectively (Trindade *et al.* 2006; Yao *et al.* 2010). The pH and heat stability of SteLL may be due to its glycosylation. In a previous study, the oligosaccharide moiety of *Erythrina corallodendron* lectin showed dynamically stable interactions, forming long-range contacts between amino acids, which were important for maintaining the structure of this glycoprotein (Kaushik *et al.* 2011).

The stability of SteLL at different pH and temperatures is a physicochemical characteristic desirable for its use as an antibiotic. An antimicrobial agent should act at wide pH and temperature ranges as pathogenic bacteria are able to grow at a temperature range between 4 and 60°C and at high or low pH values (Hill *et al.* 1995). Additionally, fungi can also grow at a wide range of temperatures, and most fungi, including *C. albicans*, tolerate wide variations in pH (Gostinčar *et al.* 2011; Vylkova *et al.* 2011). SteLL was active at temperatures and pH around those found in the human body (37°C; pH 6·5–7·5), indicating its potential use for treating human infectious diseases.

Leaf extracts showed weak inhibitory and bactericidal effects on only *E. coli*, *Pr. mirabilis* and *Staph. aureus*. Ethanolic extracts from *S. terebinthifolius* leaves were also not effective antibacterial agents against *Staph. aureus*, showing only a bacteriostatic effect with MIC values <100 mg ml^{-1} (Martinez *et al.* 1996; Guerra *et al.* 2000; Lima *et al.* 2006). An aqueous leaf extract from this plant was shown to be active against *Staph. aureus* and *B. subtilis* through a qualitative agar diffusion assay, with growth inhibition zones of 14 and 17 mm, respectively (Martinez *et al.* 1996).

SteLL showed higher antibacterial activity than leaf extract against *E. coli*, *Pr. mirabilis* and *Staph. aureus,* and it was active against *Kl. pneumoniae*, *Ps. aeruginosa* and *Salm. enteritidis*, which were not affected by the leaf extract. The increment of antibacterial activity was likely due to the concentration of SteLL, indicating that it is one of the main active components present in the leaf extract. A thermo-resistant lectin isolated from *Eugenia uniflora* seeds inhibited the growth of *Staph. aureus* and *Ps. Aeruginosa* with an MIC (1·5 μg ml^{-1}) similar to those determined for SteLL, but it was less effective in inhibiting (MIC of 16·5 μg ml^{-1}) the growth of *B. subtilis* and *E. coli* (Oliveira *et al.* 2008).

The MBC for bactericidal drugs is generally the same or not more than fourfold higher than the MIC value. In contrast, the MBC of bacteriostatic drugs is many fold higher than their MIC (Levison 2004). Based on the MBC/MIC ratio, SteLL was a bactericide drug against *E. coli*, *Staph. aureus* and *Pr. mirabilis*. Regarding the effects on *Kl. pneumoniae*, *Ps. aeruginosa* and *Salm. enteritidis*, SteLL can be classified as a bacteriostatic agent because MBC values were 32-, 8- and 255-fold greater than MIC values, respectively. Lectins isolated from *Bothrops leucurus* venom and *M. urundeuva* heartwood can also be considered as bacteriostatic agents against *Staph. aureus*, because MBC values of these lectins were 15·8- and 13·9-fold greater than their MIC values, respectively (Sá *et al.* 2009; Nunes *et al.* 2011).

SteLL was more efficient in killing the Gram-positive *Staph. aureus* than the Gram-negative bacteria such as *E. coli*, *Kl. pneumoniae*, *Ps. aeruginosa* and *Salm. enteritidis*. Similarly, the *N*-acetyl-D-glucosamine-binding lectin from *Araucaria angustifolia* seeds was also more active against Gram-positive (*Clavibacter michiganensis*) than

Gram-negative (*Xanthomonas axonopodis*) bacteria, promoting the formation of pores and severe disruption of the *C. michiganensis* membrane and bubbling on the *X. axonopodis* cell wall (Santi-Gadelha *et al.* 2006). The difference in the susceptibility of Gram-positive and Gram-negative bacteria may be linked to the difficulty of lectins to cross the Gram-negative bacteria outer cell wall to reach the periplasmic space (Nunes *et al.* 2011). Additionally, the high level of peptidoglycan (which contains GlcNac) in the cell wall of Gram-positive bacteria may provide more interaction sites for chitin-binding lectins.

SteLL was more efficient than leaf extract in inhibiting *C. albicans* growth than the lectins from *Archidendron jiringa* seeds (MIC of 56·7 μg ml^{-1}), rhizome of *Curcuma longa* (MIC of 46 μg ml^{-1}) and the lectin-rich fraction from *Hypnea musciformis*, which showed only weak fungistatic action (Cordeiro *et al.* 2006; Petnual *et al.* 2010; Charungchitrak *et al.* 2011).

Plant lectins are active against fungi by recognizing and immobilizing the micro-organisms via binding to carbohydrate components, thereby preventing their subsequent growth and multiplication (Cordeiro *et al.* 2006). The chitin-binding property of SteLL may be involved in its antifungal mechanism. Hevein, a chitin-binding lectin, showed inhibitory activity against *C. albicans* with a MIC value of 95 μg ml^{-1} (Kanokwiroon *et al.* 2008).

In conclusion, SteLL is a glycosylated and chitin-binding lectin that can be purified in milligram quantities in one chromatographic step. SteLL showed strong antimicrobial activity against species that cause human diseases. SteLL haemagglutinating activity was inhibited by *N*-acetylglucosamine, a component of chitin found in bacterial and fungal cell walls. The characteristics of SteLL, including its ability to interact with *N*-acetylglucosamine, its pH and heat stability and the insensitiveness of haemagglutinating activity to divalent cations, indicate that studies should be conducted focusing its application as a biomaterial with bactericide and fungicide properties for treating infections as well as for evaluating its toxicity in humans.

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4. CAPÍTULO 2

Medicinal *Schinus terebinthifolius* bark contains trypsin inhibitor, lectin, and phenols with antimicrobial activity

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Medicinal *Schinus terebinthifolius* bark contains trypsin inhibitor, lectin, and phenols with antimicrobial activity

Francis Soares Gomes[1], Ana Mary Viana Jorge[1], Pollyanna Michelle da Silva, Thiago Henrique Napoleão, Luana Cassandra Breitenbach Barroso Coelho, Patrícia Maria Guedes Paiva[*]

Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Avenida Prof. Moraes Rego, S/N, Cidade Universitária, Recife-PE, 50670-420, Brazil.

[*] Corresponding author: Tel.: +55 8121268540. Fax: +55 81 21268576.

E-mail: ppaivaufpe@yahoo.com.br (P.M.G. Paiva).

[1]These authors equally contributed to this study.

Abstract

Schinus terebinthifolius bark infusion is an herbal remedy used to treat female genital infections. Trypsin inhibitors, carbohydrate recognizing proteins (lectin) and secondary metabolites have been identified as antimicrobial compound from plants. This work reports: 1) the presence in *S. terebinthifolius* bark infusion of trypsin inhibitor (SteBI), lectin (SteBL) and phenols, 2) the isolation of SteBI and SteBL, and 3) determination of minimal bactericide (MBC), inhibitory (MIC) and fungicide (MFC) concentrations of bark infusion, isolated proteins and methanolic extract on growth and survival of microorganisms that causes human diseases. Bark infusion (59 mg/mL of protein) containing SteBI (0.28 Umg^{-1}), SteBL (specific hemagglutinating activity of 551) and phenol (0.11 mg/mL) was bactericide against *S. aureus*, *E. faecalis* and *E. coli* (MBC ranging from 14.85 to 29.7 mg/mL of protein and 0.026 to 0.052 mg/mL of phenol) and fungicide against *C. albicans* (MFC of 3.71 mg/mL of protein and 0.007 mg/mL of phenol). SteBI and SteBL were isolated without phenol contamination by the same chromatographic procedure using different eluent solutions. SteBI (0.67 Umg^{-1}) was a peptide of molecular mass 16 kDa with fungicide action on *Candida albicans* (MFC of 0.0075 mg/mL) and without antibacterial activity. SteBL (specific hemagglutinating activity of 1,067) with molecular mass 20 kDa recognized N-acetylglucosamine and fetuin, showed bactericide activity on *Staphylococcus aureus* (MBC of 0.4 mg/mL) and bacteriostatic action against *S. aureus*, *Enterococcus faecalis* and *Escherichia coli* (MIC of 0.2 mg/mL). The lectin did not show antifungal activity on *C. albicans*. Methanolic extract (26.3 mg/mL of protein and 8.3 mg/mL of phenol) containing SteBL (specific hemagglutinating activity of 19,874) showed bactericide activity against *S. aureus* and *E. faecalis* with MBC of 13.19 and 3.29 mg/mL of protein and 4.1 and 1.02 mg/mL of phenol, respectively. Methanolic extract did not contain SteBI and antifungal activity. This study confirms the antimicrobial action of infusion from *S. terebinthifolius* bark that has been used by people to treat infections and identifies SteBI, SteBL and phenols as constituents of infusion with antimicrobial activity.

Keywords: *Schinus terebinthifolius*; bark infusion; lectin; trypsin inhibitor; phenols; antimicrobial activity.

1. Introduction

The use of commercial antibiotics and fungicides in large scale can promote the development of multidrug-resistant strains of bacteria and fungi stimulating an intensive search for new ones (Cowen et al., 2000; Oliveira et al., 2002; Yim et al., 2013). Drug resistance of *C. albicans* to the fungicide fluconazole accompanied by increased resistance to ketoconazole and itraconazole has been reported and *S. aureus* isolates also shown resistance to penicillin, streptomycin, tetracycline, and erythromycin (Pereira and Siqueira-Júnior, 1995; Marchaim et al., 2012).

In the tropics**,** aqueous infusion from bark of *Schinus terebinthifolius* is widely used topically to treat female genital tract disorders (Lorenzi and Matos, 2008; Braga et al., 2007). Studies conducted aiming to find antimicrobial agents from plants have identified trypsin inhibitors, lectins and secondary metabolites which promote damage effects on growth and survival of bacteria and fungi (Paiva et al., 2010, 2013).

Protease inhibitors can be protein or peptides that form stable complexes with enzymes promoting inhibition of activity (Li et al., 2007), and are thought to be involved in plant defense against pathogens. Trypsin inhibitors are able to interfere on the trypsin-mediated activation of the chitin synthase zymogen, affecting development of fungi cell wall (Chilosi et al., 2000). Protease inhibitors also act against bacteria by inhibiting bacterial proteases as well as by interacting with the cell wall or proteins from plasma membrane leading to permeability changes (Supuran et al., 2002; Li et al. 2007; Kim et al., 2009).

Lectins are proteins that recognize carbohydrates and their binding to carbohydrates on erythrocyte surface promotes hemagglutination, phenomenon broadly used for lectin detection (Santana et al., 2012). Plant lectins showed antibacterial and antifungal activities against species that cause human diseases (Sá et al., 2009a; Gomes et al., 2013) and it was demonstrated that the antimicrobial activities of lectin from *Araucaria angustifolia* seeds were

linked to carbohydrate binding sites (Santi-Gadelha et al., 2006). Chitin, glucans and mannans present on fungal cell wall and glycoconjugates present on bacterial cell surfaces, such as peptidoglycans, lipopolysaccharides and teichoic acids, constitute potential lectin targets (Gomes et al., 2013).

Many classes of plant secondary metabolites (e.g. flavonoids, terpenes, essential oils, tannins and coumarins) have shown antifungal and antibacterial activities. There is a variety of mechanisms involved in the antimicrobial effect of these compounds such as damage of plasma membrane, inhibition of DNA and RNA metabolism, reduction in cell respiration, and inactivation of enzymes (Paiva et al., 2010).

This work reports 1) the presence of proteins with trypsin inhibitor and hemagglutinating activities as well as phenols in the bark infusion used in folk medicine, 2) the isolation of trypsin inhibitor (SteBI) and lectin (SteBL) from *S. terebinthifolius* bark by the same chromatographic procedure and 3) the determination of antimicrobial activity on *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Candida albicans* of bark infusion, SteBI, SteBL and methanolic extract containing phenols.

2. Materials and Methods

2.1. Plant material

Bark of *S. terebinthifolius*, popularly known as "aroeira-da-praia" or Brazilian pepper, was collected in Recife City, State of Pernambuco, Brazil. A representative specimen is archived under number 73,431 in the *Instituto Agronômico de Pernambuco* (IPA). The bark was powdered (40 mesh) and stored at -20 ºC. The authors have authorization from the *Instituto Chico Mendes de Conservação da Biodiversidade* from Brazilian Ministry of the Environment for plant collection (number 38690-1).

2.2. S. terebinthifolius bark infusion

Bark infusion was prepared according to the instructions of the Brazilian Sanitary Surveillance Agency (ANVISA, 2010) by adding the bark powder (1 g) to boiling water (150 ml). After 10 min, the liquid was passed through filter paper and the filtrate (bark infusion) was stored at -20 ºC.

2.3. Isolation of SteBI and SteBL

Bark powder (10 g) was suspended in 0.01 M citrate phosphate buffer pH 4.0 (100 ml) and after agitation (8 h at 28 °C) followed by centrifugation (5000 *g* for 15 min at 4 °C), the collected supernatant was passed through activated charcoal (10 %, w/v; Reagen, Brazil). The filtrate (buffer extract) was treated with ammonium sulphate to a final saturation of 40% according to Green and Hughes (1955) and after 4 h at 28 ºC, the mixture was centrifuged (5000 *g* for 15 min at 4 °C). The collected supernatant fraction (SF40) was dialyzed for 4 h against two changes of 0.15 M NaCl, using a volume of 2 L for dialysis fluid. The dialyzed SF40 (3.0 mg of protein) was loaded onto a chitin (Sigma, USA) column (7.5 x 1.5 cm) equilibrated with 0.15 M NaCl. After washing step with equilibrating solution (100 ml), the adsorbed material was eluted from column with 0.015 M sodium borate buffer pH 7.4 followed by 1.0 M NaCl. The fractions eluted with 0.015 M sodium borate buffer, containing trypsin inhibitor activity, were pooled and called *S. terenbithifolius* bark inhibitor (SteBI) and those eluted with 1.0 M NaCl, containing hemagglutinating activity, were pooled and called *S. terenbithifolius* bark lectin (SteBL).

2.4. Methanolic extract from S. terebinthifolius bark

Methanolic extract was obtained soaking three grams of the leaf powder in 10 ml of methanol at 25 ºC for 3 h. After this period, the mixture was filtered through filter paper and evaporated to dryness on a rotary evaporator. The resulting extract was then dissolved in distilled water.

2.5. Protein and total phenol contents

Protein concentration was determined according to Lowry et al. (1951) using bovine serum albumin as standard (31-500 μg ml^{-1}). Total phenol content was determined using the Folin–Ciocalteu method based on the reduction of phosphomolybdic–phosphotungstic acid reagent in an alkaline medium (Morais et al., 1999). Phenol content was determined based on a standard curve of tannic acid (9.6–48 mg ml^{-1}).

2.6. Trypsin inhibitory activity

The determination of trypsin inhibitor activity used 0.1 mg/ml bovine trypsin as described by Kakade et al.. (1969). One unit of trypsin activity was defined as the amount of enzyme that hydrolyzes 1 μmol of N-benzoyl-DL-arginyl-ρ-nitroanilide (BApNA) per minute. Bovine trypsin (5 μl) was incubated (5 min, 37 ºC) with sample (100 μl) in Tris-HCl buffer pH 8.0 (95 μl). Following, BApNA (8 mM) dissolved in dimethyl sulfoxide was added (5 μl) and the mixture was incubated (30 min, 37 ºC). The substrate hydrolysis was followed by measurement of absorbance at 405 nm and the inhibitory activity was determined by remaining hydrolytic activity towards BApNA. One unit of trypsin inhibitor activity was defined as the amount of inhibitor that decreases the absorbance in 0.01 per 30 min at 37 °C (Oliveira et al., 2012).

2.7. Hemagglutinating activity

Hemagglutinating activity was determined according to method described by Paiva and Coelho (1992). The sample (50 µl) was two-fold serially diluted in 0.15 M NaCl on microplate wells. Next, a suspension of 2.5% glutaraldehyde-treated rabbit erythrocytes (Bing et al., 1967) was added (50 µl) to each well. One hemagglutination unit ($titer^{-1}$) was defined as the reciprocal of the highest sample dilution which promoted full erythrocyte agglutination (Napoleão et al., 2011). Specific hemagglutinating activity was defined as the ratio between the titer and protein concentration ($mg.ml^{-1}$).

Hemagglutinating activity was also determined after previous incubation (15 min, 28 ºC) of sample with 0.1 M monosaccharide (D(-)-fructose, D(+)-glucose, D(+)-maltose, D(+)-mannose, N-acetyl-D-glucosamine, D(+)-trehalose, D(+)-xylose) or 0.5 $mg.ml^{-1}$ fetuin solutions.

2.8. Polyacrylamide gel electrophoresis

Electrophoresis gels containing sodium dodecyl sulphate (SDS) was carried out according to Laemmli (1970) on a 10% (w/v). Polypeptide bands of proteins from bark and standards (bovine serum albumin, 66 000; ovalbumin, 45 000; carbonic anhydrase, 29 000; trypsinogen, 24 000; trypsin inhibitor, 20 000 and α-lactalbumin, 14 200; from Sigma, USA) were stained with 0.02% (w/v) Coomassie Brilliant Blue in 10% (v/v) acetic acid.

2.9. Antibacterial activity

Stationary cultures of Gram-positive (*Staphylococcus aureus* WDCM 00032, *Enterococcus faecalis* WDCM 00117) and Gram-negative (*Escherichia coli* WDCM 00013) bacterial strains, maintained in nutrient agar (NA) at 4 °C, were provided by the *Departamento de Antibióticos, Universidade Federal de Pernambuco*, Brazil. Bacteria were cultured overnight in nutrient broth (NB) under permanent shaking at 37 °C. Then, the

cultures were turbidimetrically adjusted to 1.5 x 10^8 colony forming units (CFU) per ml measuring the optical density at 490 nm.

An aliquot (100 µL) of bark infusion (59.4 mg/mL of protein and 0.11 mg/mL of phenol), SteBI (0.03 mg/mL), SteBL (0.4 mg/mL) or methanolic extract (26.3 mg/mLof protein and 8.2 mg/mL of phenol) was diluted 1:1 in NB and then submitted to a series of ten double dilutions, until a final ratio of 1:2048. Next, a 180-µl aliquot of each dilution was dispensed into each well of a 96-well microplate. All wells were inoculated with 20 µl of the bacterial culture and incubated at 37 °C for 24 h. Assay for each concentration was performed in triplicate. Control wells contained only NB medium and microorganism. After incubation, the optical density at 490 nm (OD_{490}) was measured using a microplate reader. Minimal inhibitory concentration (MIC) was determined as the lowest lectin concentration at which there was ≥50% reduction in OD_{490} in comparison with the control (Amsterdam, 1996).

To determine the minimal bactericide concentration (MBC), inoculations (10 µl) from the sample wells at which inhibition of bacterial growth was detected were transferred NA plates and incubated at 37 °C for 24 h. The MBC corresponded to the lowest sample concentration at which no bacterial growth was observed. The assay was performed in triplicate.

2.10. Antifungal activity

Candida albicans was obtained from the Culture Collections at University Recife Mycologia, Departamento de Micologia, Universidade Federal de Pernambuco, Brazil. Antifungal activity was evaluated using the same method used for antibacterial activity, changing the incubation temperature (28°C) and replacing the culture medium used. Sabouraud dextrose was used to determine the MIC, while Sabouraud agar was used to

determine the minimal fungicide concentration (MFC) – the lowest protein concentration showing no fungal growth. Assays were performed in triplicate.

2.11. Statistical analysis

Data were expressed as mean ± standard deviation, and the Student's *t*-test were used to determine the significance of the differences between the control and experimental groups using Origin^{TM} 6.0 (Microcal, USA) computer package.

3. Results

S. terebinthifolius bark infusion showed high protein content (59.4 mg/mL), trypsin inhibitor (0.28 U/mg), lectin (specific hemagglutinating activity of 551, which was reduced to 2.15 by fetuin) and phenol content of 0.11 mg/mL. Table 1 shows that bark infusion inhibited the growth of *E. coli*, *E. faecalis* and *S. aureus* with MIC values of 7.42, 7.42 and 14.85 mg/mL of protein and 0.013, 0.013 and 0.026 mg/mL of phenol, respectively and was able to kill these bacteria (MBC values of 14.85, 14.85 and 29.7 mg/mL of protein and 0.026, 0.026 and 0.052 mg/mL of phenol for *E. coli*, *E. faecalis* and *S. aureus*, respectively). The infusion showed antifungal activity on *C. albicans* with MIC of 1.85 mg/mL of protein and 0.0035 mg/mL of phenol and MFC of 3.71 mg/mL of protein and 0.007 mg/mL of phenol (Table 2).

Buffer extract (preparation used for isolation of SteBI and SteBL) contained protein (23.7 mg/mL), trypsin inhibitor (0.47 Umg^{-1}), lectin (specific hemagglutinating activity of 238) and phenolic compounds (0.097 mg/mL). The inhibition of hemagglutinating activity from buffer extract by *N*-acetylglucosamine and fetuin (specific hemagglutinating activity of 1.8) revealed that the lectin bound to these glycan molecules.

Ammonium sulphate fractionation of buffer extract yielded a protein fraction (SF40). Chromatography of SF40 on chitin (*N*-acetylglucosamine polymer) column resulted in the

isolation of SteBI (0.67 Umg^{-1}) in the fractions eluted with 0.015 M sodium borate buffer pH 7.4 and SteBL (specific hemagglutinating activity of 1,067) eluted with 1.0 M NaCl (Figure 1). The isolated proteins did not show phenol contamination. The hemagglutinating activity of SteBL was inhibited for 1.08 by *N*-acetylglucosamine and fetuin and SDS-PAGE revealed SteBI and SteBL as polypeptides of molecular masses 16 kDa and 20 kDa, respectively (Figure 1). SteBI did not affect the growth and survival of all assayed bacteria but was active against *C. albicans* with MIC of 0.00375 mg/mL and MFC of 0.0075 mg/mL (Table 2). Table 1 show that SteBL inhibited the growth of all tested bacteria with similar MIC and was bactericide on *S. aureus* (MBC of 0.4 mg/mL). The data from antifungal assays revealed that SteBL did not show antifungal activity.

Methanolic extract, containing protein (26.38 mg/mL) and phenol (8.2 mg/mL) did not inhibit trypsin but showed specific hemagglutinating activity of 19,874, which was reduced to 0.15 by fetuin. The results from antibacterial assays (Table 1) showed that methanolic extract inhibited the growth and killed *E. faecalis* (MIC of 1.67 mg/mLof protein and 0.51 mg/mLof phenol; MBC of 3.29 mg/mLof protein and 1.025 mg/mL) and *S. aureus* (MIC of 6.59 mg/mL of protein and 2.05 mg/mL of phenol; MBC of 13.19 mg/mL of protein and 4.1 mg/mL of phenol). The methanolic extract did not show antifungal activity against *C. albicans*.

4. Discussion

Infusion of *S. terebinthifolius* bark is widely used topically to treat female genital tract disorders caused by bacteria and fungi and this fact stimulated us to investigate the antimicrobial activities of bark preparations containing trypsin inhibitor, lectin and phenols, that are know as antimicrobial agents. *E. coli, E. faecalis, S. aureus* and *C. albicans* were used in antimicrobial assays because they are species that cause urinary and genital tract infections

in humans (Pellati et al., 2008; Boucher et al., 2010; Kuo et al., 2010; Yasufuku et al., 2011). The data from antimicrobial assays performed in this study revealed that *S. terebinthifolius* bark infusion contains three antimicrobial agents.

Bark infusion containing trypsin inhibitor, lectin and phenols promoted deleterious effects on growth and survival of all tested bacteria and was also active against the fungi *C. albicans*, the major cause of genital tract disorders (Ge et al., 2012). The detected antimicrobial activity of bark infusion confirms the efficiency of this preparation as bactericide and fungicide agent and thus corroborates with the traditional use of *S. terebinthifolius* bark infusion to treat diseases caused by microrganisms. Infusion of *Hofmeisteria schaffneri* reported by Pérez-Vásquez et al. (2011) also exhibited an antibacterial activity against *Staphylococcus aureus* (MIC value of 64 µg/ml of dry weight) and antifungal activity against *C. albicans* (MIC of 128 µg/ml of dry weight).

Procedure used to isolate the trypsin inhibitor and lectin present in *S. terebinthifolius* protein fraction from bark was developed aiming to determine if these proteins are antimicrobial compounds. Buffer extract was selected as initial preparation for protein purifications due to the fact that temperature used in its preparation is lower than that used to bark infusion preparation as well as phenol concentration was lowest in comparison to that of bark infusion. High temperature could fragile to protein structures and phenol can cause interference in the following steps of purification (Doonan, 1996). Electrophoretic pattern of isolated proteins on SDS-PAGE showed the presence of single polypeptide band in SteBI and SteBL revealing the efficiency of isolation procedure. Molecular mass of SteBI is smaller than that determined by Liu et al. (2009) for trypsin inhibitor from *Sapindus mukorassi* bark (23 kDa). SteBL molecular mass is similar to that of *Sophora japonica* bark lectin, smaller than that determined to *Maackia amurensis* bark lectins and bigger than *Myracrodruon urundeuva* bark lectin (Ueno et al., 1991; Van Damme et al., 1997; Sá et al., 2009).

In response to attack by proteinases produced by pathogenic microorganisms, plants synthesize inhibitory polypeptides that can suppress enzymes involved in fungal development (Lorito et al., 1994; Liao et al., 2007). SteBI showed antifungal activity against *C. albicans* higher than the trypsin–chymotrypsin protease inhibitor, isolated from the tubers of *Solanum tuberosum* L cv. Gogu, with MIC and MFC values of 0.56 mg/mL (Kim et al., 1999), and than *Capsicum annuum* L. trypsin inhibitor, with MIC and MFC values of 0.016 and 0.064 mg/mL, respectively (Ribeiro et al., 2012). The antifungal activity of trypsin inhibitor against *C. albicans* can be through induction of cellular agglomeration, release of cytoplasmic content, generation of nitric oxide and exhibition of morphological alterations, such as the formation of pseudohyphae, cellular aggregates and elongated forms (Ribeiro et al., 2012).

The antibacterial assay with SteBL showed that the lectin has a bacteriostatic action against *E. coli* and *E. faecalis* and was bactericide against *S. aureus*, showing a MBC/MIC ratio of 2. The MBC for bactericidal drugs is generally the same or not more than four-fold higher than the MIC value (Levison, 2004). The chitin-binding property of SteBL can be involved in its antibacterial mechanism, since *N*-acetylglucosamine is component of the peptidoglycan found in bacterial cell walls. The mechanism of antimicrobial action of lectins are not fully understood but studies have shown that lectin can promote the formation of pores and severe membrane disruption of Gram positive bacteria as well as bubbling on the cell wall of Gram negative bacteria (Santi-Gadelha et al., 2006).

Phenols are compounds generally extracted from plant tissues by use of organic solvents (Garcia-Salas et al., 2010). Lima et al. (2006) extracted phenol compounds from *S. terebinthifolius* bark using ethanol. The determination of high phenol concentration of methanolic bark extract revealed that methanol used by us was also able to solubilize secondary metabolite present in *S. terebinthifolius* bark.

Antimicrobial activity of organic solvent extracts rich in phenols has been reported. The ethanolic *S. terebinthifolius* bark extract described by Lima et al. (2006) showed bacteriostatic activity against *S. aureus* (MIC of 100 μg ml^{-1} of dry weigh) and ethanolic and methanolic extracts from bark of *Jatropha curcas* showed both antibacterial and antifungal activities (Igbinosa et al., 2009). Phenol compounds are probably involved in the antibacterial activity of methanolic bark extract from *S. terebinthifolius*. The phenols after being adsorbed to bacteria can react with proteins in the cytoplasm and in the cell wall promoting inactivation microbial adhesins, enzymes and cell envelope transport proteins as well as inhibit bacterial adherence by interference in the availability of receptors on the cell surface (Cowan, 1999).

5. Conclusion

This study confirms the antimicrobial action of infusion from *S. terebinthifolius* bark that has been used by people to treat infections. Also, reveals that damage promoted by *S. terebinthifolius* bark infusion on growth and survival of bacteria and fungi is due to the presence of trypsin inhibitor, lectin and phenol compounds. The investigation of antimicrobial activities of isolated SteBI and SteBL as well as of methanolic extract rich in phenol allowed us to define the antimicrobial activity of each of these three constituents of bark infusion. SteBL and phenols were the antibacterial agents while SteBI was the fungicide. In conclusion, *S. terebinthifolius* bark is a source of distinct antimicrobial agents against microorganisms that cause female genital tract disorders.

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Figure captions

Fig. 1. Isolation of SteBI and SteBL by affinity chromatography on chitin column. **Sample of SF40 (3.0 mg of protein) was applied onto a chitin column (7.0 x 1.5 cm) equilibrated with 0.15 M NaCl. The retained proteins were eluted at a flow rate of 30 ml.h^{-1} with 0.015 M sodium borate buffer pH 7.4 and 1.0 M NaCl. Fractions of 1.5 ml were collected. A 280 nm (●). The dotted bar indicates fractions with trypsin inhibitor activity. The solid bar indicates fractions with hemagglutinating activity. The insets show the electrophorectic profiles of molecular weight standards, SteBI (pool of fractions 43-50) and SteBL (pool of fractions 57-65) on a 10 % gel containing SDS. The proteins were stained with Coomassie Brilliant Blue. Arrows indicate the presence of polypeptide bands.**

Table 1. Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of preparations from *Schinus terebinthifolius* bark.

Sample	Bacteria		
	Escherichia coli	***Enterococcus faecalis***	***Staphylococcus aureus***
Bark infusion			
MIC (protein)	7.42	7.42	14.85
MIC (phenol)	0.013	0.013	0.026
MBC (protein)	14.85	14.85	29.7
MBC (phenol)	0.026	0.026	0.052
Methanolic extract			
MIC (protein)	ND	1.64	6.59
MIC (phenol)	ND	0.51	2.05
MBC (protein)	ND	3.29	13.19
MBC (phenol)	ND	1.02	4.1
SteBL			
MIC	0.2	0.2	0.2
MBC	ND	ND	0.4
SteBI			
MIC	ND	ND	ND
MBC	ND	ND	ND

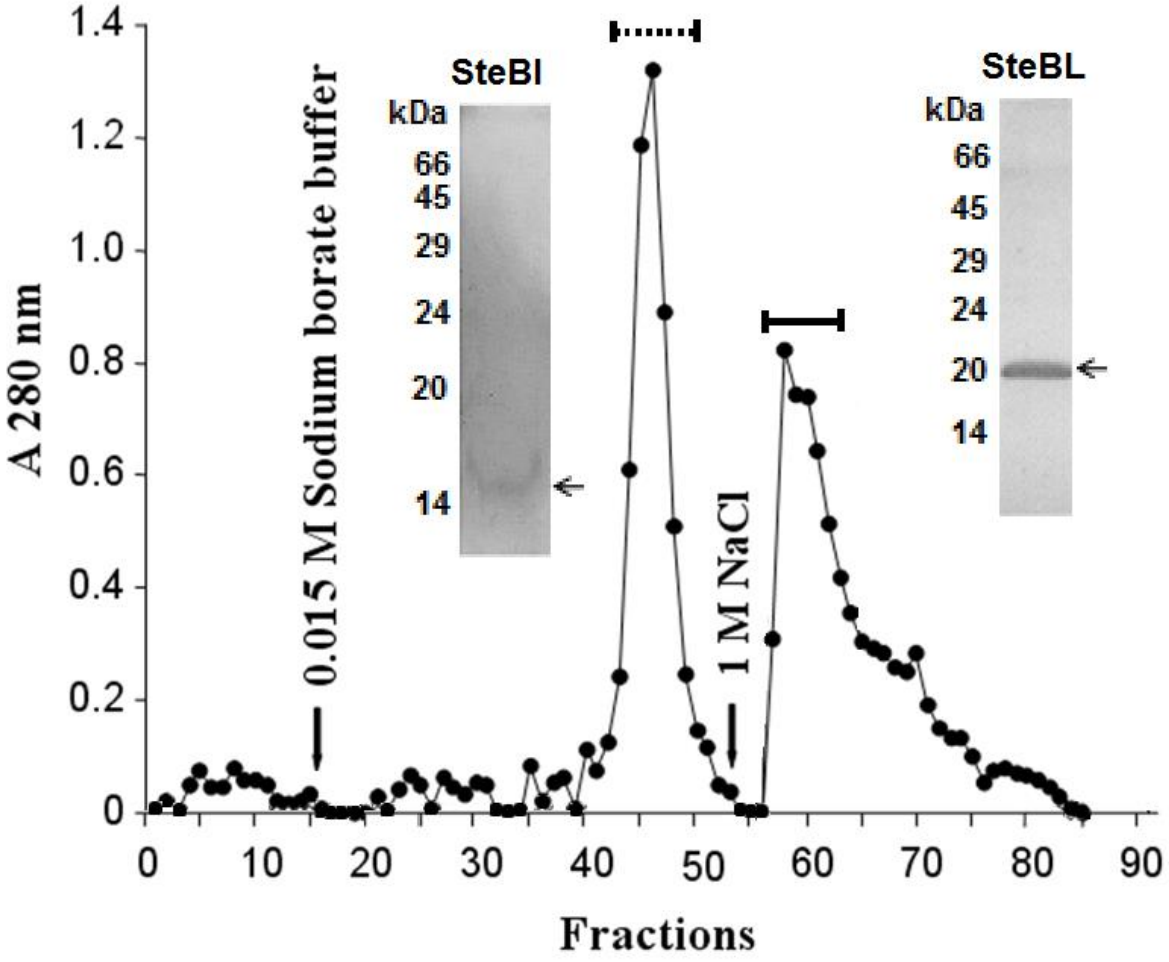
MIC and MBC expressed in mg/mL of protein and mg/mL of phenol. ND: not detected

Table 2. Minimum inhibitory (MIC) and minimum fungicidal concentrations (MFC) of preparations from *Schinus terebinthifolius* bark.

Sample	*Candida albicans*
Bark infusion	
MIC (protein)	1.85
MIC (phenol)	0.0035
MFC (protein)	3.71
MFC (phenol)	0.007
Methanolic extract	
MIC (protein)	ND
MIC (phenol)	ND
MFC (protein)	ND
MFC (phenol)	ND
SteBL	
MIC	ND
MFC	ND
SteBI	
MIC	0.00375
MFC	0.0075

MIC and MFC expressed in mg/mL of protein and mg/mL of phenol. ND: not detected

Figure 1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SteBI
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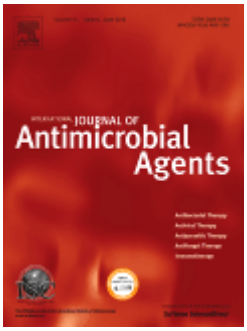
5. CAPÍTULO 3



Lectins from *Myracrodruon urundeuva* interfere on growth, survival, adherence, invasion and extracellular products releasing of bacteria pathogenic to human and fish

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Lectins from *Myracrodruon urundeuva* interfere on growth, survival, adherence, invasion and extracellular products releasing of bacteria pathogenic to human and fish

Francis Soares Gomes[a], Sabela Balboa[b], Thiago Henrique Napoleão[a], Roberto Araújo Sá[c], Jesús Lópes Romalde[b], Patrícia Maria Guedes Paiva[a,*]

[a]*Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Pernambuco, Avenida Prof. Moraes Rego, S/N, Cidade Universitária, Recife-PE, 50670-420, Brazil.*

[b]*Departamento de Microbiología y Parasitología, CIBUS-Facultad de Biología, Universidad de Santiago de Compostela, 15782, Santiago de Compostela, Spain.*

[c]*Centro Acadêmico do Agreste, Universidade Federal de Pernambuco, Nova Caruaru, 55002-970, Caruaru, Pernambuco, Brasil.*

[*] Corresponding author: Tel.: +55 8121268540. Fax: +55 81 21268576.

E-mail: ppaivaufpe@yahoo.com.br (P.M.G. Paiva).

Abstract

This work investigates the antibacterial activity of lectins from bark (MuBL), heartwood (MuHL) and leaf (MuLL) of *Myracrodruon urundeuva* against *Escherichia coli*, *Staphylococcus aureus*, *Photobacterium damselae* subsp. *piscicida* and *Yersinia ruckeri* by determining minimal inhibitory (MIC) and bactericide (MBC) concentrations. Also, bacteria treated with lectins were investigated for adherence and invasive capacity on fish fibroblastic-like (SAF-1) and human cancer (HeLa) cell lines as well as enzyme activity of extracellular products released (ECPs). The lectins were bacteriostatic against *S. aureus*, *E. coli*, *P. damselae* subsp. *piscicida* and *Y. ruckeri* (MIC from 1 to 489 µg ml^{-1}) and bactericidal to *S. aureus*, *E. coli*, and *P. damselae* subsp. *piscicida* (MBC from 2 to 489 µg ml^{-1}). All lectins inhibited adherence of all assayed bacteria to SAF-1 and to HeLa cells. MuBL, MuHL and MuLL inhibited invasion of SAF-1 cells by *S. aureus* (89, 87 and 76%, respectively) and *P. damselae* subsp. *piscicida* (50, 20 and 10%, respectively) as well as invasion of HeLa cells by *E. coli* (43, 42 and 44%, respectively) and *Y. ruckeri* (18, 49 and 8%, respectively). Invasion of SAF-1 cells by *Y. ruckeri* was inhibited by MuHL (95%) and MuLL (53%) and MuBL and MuHL inhibited HeLa invasion (31 and 16%, respectively) by *S. aureus*. Comparison between ECPs released by bacteria incubated or not with *M. urundeuva* lectins revealed that the treatment interfered on release of protein (all bacteria) and reduced the activities of DNAse (*S. aureus*, *P. damselae* subsp. *piscicida*), phospholipase (*S. aureus*, *Y. ruckeri*), amylase (*P. damselae* subsp. p*iscicida)* gelatinase (*Y. ruckeri*) and caseinase (*Y. ruckeri*). This study revealed that *M. urundeuva* lectins were antibacterial agents on *S. aureus*, *E. coli*, *Y. ruckeri* and *P. damselae subsp. piscicida* as well as inhibited adherence and invasion of these bacteria species on human and fish cells. Evaluation of ECPs suggests that interference of MuBL, MuHL and MuLL on physiologic processes of bacteria can be due to reduction in release of bacterial enzymes.

Keywords: lectin, *Myracrodruon urundeuva*, antibacterial activity, adhesion, invasion.

1. Introduction

There are several bacteria species that cause diseases in humans and animals. *Escherichia coli* and *Staphylococcus aureus* are human pathogens that can be transmitted from contaminated food and cause gastroenteritis, among other diseases. The *Photobacterium damselae* subsp. *piscicida* and *Yersinia ruckeri* are bacteria that cause diseases in many species of fishes resulting in major economic losses worldwide (Romalde et al., 1993; Magariños et al., 1996; Novotny et al., 2004). The prevention of bacterial diseases in fish is essential for the improvement of farming production, and increase in use of fish resources.

Lectins are hemagglutinating proteins that bind specifically and reversibly carbohydrates. Antibacterial lectins have been isolated from animals and plants (Costa et al., 2010; He et al., 2011; Nunes et al., 2011). The antibacterial activity results from the interaction of lectins with teichoic and teicuronic acids, peptidoglycans and lipopolysaccharides present in the bacterial cell wall and it has been suggested that lectins form a channel in the bacterial cell wall causing death by leakage of cellular contents (Ratanapo et al., 2001; Correia et al., 2008). Furthermore, contact with an antibacterial agent can change protein expression pattern altering bacteria virulence (Hecker et al., 2006).

Myracrodruon urundeuva is a plant with high resistance to phytopathogenic microorganisms, mainly in the heartwood tissue. The bark of this plant is widely used in folk medicine to treat infections in the genitourinary tract, skin, subcutaneous tissue and digestive system. Macerated, infusion and syrup are some of the ways of popular use (Monteiro et al., 2006). Lectins have been isolated from *M. urundeuva* heartwood (MuHL), bark (MuBL) and leaf (MuLL) and the defined protocols yield milligram quantities of these proteins (Sá et al., 2009b; Napoleão et al., 2011). MuBL, MuHL and MuLL have insecticidal activity against *Aedes aegypti* (Diptera) and *Nasutitermes corniger* (Isoptera), and MuHL showed antifungal activity against pathogenic *Fusarium* species and antibacterial activity against *Bacillus*

subtilis, *Corynebacterium callunae*, *S. aureus*, *Streptococcus faecalis*, *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Sá et al., 2008; Sá et al., 2009a,b; Napoleão et al., 2011, 2012).

This work reports the antibacterial activity of MuBL, MuLL and MuHL against *E. coli*, *S. aureus*, *P. damselae* subsp. *piscicida* and *Y. ruckeri* by determining the effect of lectins on growth and survival as well as on adherence and invasive capacity to fish (SAF-1) and human (HeLa) cell lines. Additionally, evaluation of enzyme activities in extracellular products (ECPs) released by bacteria treated with *M urundeuva* lectins was described.

2. Materials and Methods

2.1. Isolation of lectins

Lectins were isolated according to protocols established by Sá et al. (2009b) and Napoleão et al. (2011). The powder (10 g) of bark, heartwood or leaves was mixed with 0.15 M NaCl (100 mL). After stirring (16 h at 4 ° C), filtration and centrifugation (15 min at 8,000 g, 4 ° C), the supernatants were collected and corresponded to bark, heartwood or leaf extracts. Proteins precipitated from each extract using ammonium sulfate were applied on chitin (Sigma-Aldrich, USA) columns and MuBL, MuHL and MuLL were recovered as the fractions eluted with 1.0 M acetic acid.

2.2. Protein content and hemagglutinating activity

Protein concentration was determined according Lowry et al. (1951) using bovine serum albumin as standard (31-500 μg ml^{-1}). Hemagglutinating activity was determined according to method described by Paiva and Coelho (1992). The sample (50 μl) was two-fold serially diluted in 0.15 M NaCl on microplate wells. Next, a suspension (2.5%, v/v) of

glutaraldehyde-treated rabbit erythrocytes (Bing et al., 1967) was added (50 µl) to each well. One hemagglutination unit ($titer^{-1}$) was defined as the reciprocal of the highest sample dilution which promoted full erythrocyte agglutination (Napoleão et al., 2011). Specific hemagglutinating activity was defined as the ratio between the titer and protein concentration ($mg\ ml^{-1}$).

2.3. Antibacterial activity

Stationary cultures of *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Photobacterium damselae* subsp. *piscicida* (P3334) and *Yersinia ruckeri* (FP 13) were provided by the *Departamento de Microbiología y Parasitología* of *Universidad de Santiago de Compostela*. Bacteria were cultured overnight in trypticase soy broth supplemented with 1% NaCl (TSB-1) under permanent shaking at 37 °C (*S. aureus* and *E. coli*) or 25 °C (*P. damselae subsp. piscicida* and *Yersinia ruckeri*). Then, the cultures were turbidimetrically adjusted to 1.5×10^8 colony forming units (CFU) per ml by measuring the optical density at 490 nm.

An aliquot (100 µL) of MuBL (0.07 mg ml^{-1} of protein), MuLL (0.34 mg ml^{-1} of protein) or MuHL (0.98 mg ml^{-1} of protein) was diluted 1:1 in TSB-1 and then submitted to a series of ten double dilutions, until a final ratio of 1:2048. Next, an aliquot (180 µl) of each dilution was dispensed into each well of a 96-well microplate. All wells were inoculated with 20 µl of the bacterial culture and incubated at 37 °C or 25 °C for 24 h. Assays for each concentration were performed in duplicate. Control wells contained only TSB-1 medium and microorganism. After incubation, the optical density at 490 nm (OD_{490}) was measured using a microplate reader. Minimal inhibitory concentration (MIC) was determined as the lowest lectin concentration at which there was ≥50% reduction in OD_{490} in comparison with the control (Amsterdam, 1996).

To determine the minimal bactericide concentration (MBC), inoculations (10 µl) from the sample wells at which inhibition of bacterial growth was detected were transferred to trypticase soy agar (TSA-1) plates and incubated at 37 °C or 25 °C for 24 h. The MBC corresponded to the lowest sample concentration at which no bacterial growth was observed. The assay was performed in triplicate.

2.4. Evaluation of in vitro adherence and invasive capacities

MuBL, MuHL and MuLL were evaluated for effects on adherence and invasive capacities of the bacteria (*E. coli*, *S. aureus*, *P. damselae subsp. piscicida* and *Y. ruckeri*) to fibroblastic-like fish (SAF-1) and human cancer (HeLa) cell lines. The assays were performed as described by Ling et al. (2000) with modifications. Semiconfluent cells monolayers were prepared in 24-well culture plates (Costar) using Leibovitz L-15 medium for SAF-1 and Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum and 50 µg mL^{-1} of gentamicine for HeLa. Bacteria cultures were turbidimetrically adjusted to 1.5 x 10^8 CFU per ml and incubated with 500 µL of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2) or with 500 µL of the lectins at MIC concentrations in PBS during 2 h. The monolayer was then infected with bacterial cultures and incubated with PBS (negative control) or lectin during 3 h to reach an adequate infection level. Monolayer infected with mock buffer (sterile PBS) was taken as uninfected control.

To measure adherence, the monolayer was washed three times with PBS after 3-h infection and lysed with 1% (v/v) Triton X-100 in PBS. Next, appropriate dilutions of the material removed by washing were placed on TSA-1 to determine the number of CFU. To measure internalization, the monolayers were washed 3 times after 3-h infection and incubated for another 2 h in the cell culture medium containing gentamicine (200 µg mL^{-1}), in

order to kill all remaining extracellular bacteria. The monolayer was then washed three times with PBS and treated with 1% (v/v) Triton X-100, and counting of cells in plate was performed as described above. The adherence and invasive capacity rates were calculated from the mean of triplicates performed in three independent experiments.

2.5 Determination of enzyme activities of extracellular products (ECPs) released by bacteria

The ECP released by *E. coli*, *S. aureus*, *P. damselae* subsp. *piscicida* and *Y. ruckeri* strains were obtained by the classical cellophane plate method described by Magariños et al. (1992) with slight modifications. Overnight bacteria cultures were incubated with 3 mL of PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2) or with lectins at MIC concentration in PBS. The cultures were turbidimetrically adjusted to 1.5 x 10^8 CFU per ml and applied on cellophane plate containing TSA-1. After 24 h, bacterial suspensions (1.5 mL) were centrifuged and the supernatants were filtered (0.22 µm of diameter) to separate bacteria from ECP. The protein concentration of each ECP sample was determined by the method of Bradford (1976) using bovine serum albumin as standard (10-100 µg ml^{-1}.

The enzyme activities of the ECP from bacteria were assayed through radial diffusion method using plates with 1% (w/v) of the specific substrate of caseinase, DNAase, gelatinase, phospholipase or amylase following the methodology previously described by Magariños et al. (1992). Hemolytic activity was evaluated on TSA plates supplemented with a 5% of human erythrocytes. Briefly, plates were inoculated with 10 µL of each ECP and incubated until zones of hemolysis appeared in positive control (ECP from bacteria not incubated with lectins).

2.7. Statistical analysis

Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA), and data were expressed as the mean of replicates ± SD. Significant differences between treatment groups were analyzed using Student's t-test (significance level at $p < 0.05$) with Origin 6.0 software.

3. Results

Table 1 shows the values of MIC and MBC for *M. urundeuva* lectins determined through the antibacterial assay. MuBL, MuHL and MuLL showed bacteriostatic and bactericidal activities against *S. aureus* and *P. damselae* subsp. *piscicida*, being this last the most sensitive to the lectins. *E. coli* and *Y. ruckeri* were not killed by the three lectins, which acted as bacteriostatic agents on these bacteria.

MuBL, MuHL and MuLL promoted reduction in adherence capacities of *S. aureus*, *E. coli*, *Y. ruckeri* and *P. damselae* subsp. *piscicida* to SAF-1 cells. The lectins also reduced the invasion capacity of bacteria in SAF-1 cells (Figure 1). MuBL, MuHL and MuLL caused a reduction in invasion by *S. aureus* and *P. damselae* subsp. *piscicida* while only MuHL and MuLL were able to inhibit invasion by *Y. ruckeri*. Invasion capacity of *E. coli* was not detected on SAF-1 in treatments with or without previous incubation with lectins.

M. urundeuva lectins also reduced adherence capacities on HeLa of *S. aureus*, *E. coli*, *Y. ruckeri* and *P. damselae* subsp. *piscicida* in comparison with controls. Regarding the invasive capacity of tested bacteria on HeLa cells, MuBL, MuLL and MuHL caused a reduction of invasion by *E. coli* and *Y. ruckeri* but only MuBL and MuHL were inhibitory of cell invasion by *S. aureus* (Figure 2). The number of invading *P. damselae* subsp. *piscicida*

cells into HeLa cells was not significantly ($p>0.05$) different between control and treatments with the lectins.

Evaluation of ECP from *S. aureus* revealed that MuBL, MuLL and MuHL reduced the release of protein and that enzymatic activity of DNAse and phospholipase as well as hemolytic activity were detected only in ECP sample from control treatment. For *P. damselae* subsp. *piscicida*, MuBL, MuLL and MuHL reduced the release of protein, DNAse and amylase. MuBL and MuLL reduced the protein release by *E. coli* whereas MuHL enhanced protein release in 29%. The ECP sample from MuBL-treated *Y. ruckeri* showed a lower protein content than control; on the other hand MuLL and MuHL enhanced protein release in 28 and 24%, respectively. MuBL reduced gelatinase, caseinase and phospholipase activities in ECP sample from *Y. ruckeri* while MuLL and MuHL reduced caseinase and phospholipase activities (Figure 3).

4. Discussion

Lectins are able to bind carbohydrate moieties on the surface of cells leading to several and distinct responses and biological activities (Lam and Ng, 2011). The *M. urundeuva* lectins (MuBL, MuLL and MuHL) are N-acetyl-glucosamine-binding proteins with molecular masses ranging from 14.0 to 14.4 kDa (Sá et al., 2009a; Napoleão et al., 2011). The molecular masses of these lectins are especially interesting for biological applications because it has been suggested that small lectin molecules are less antigenic than larger plant lectins (Rogers and Hori, 1993). Lately, with the emerging problem of multiple drug resistance by bacteria, research on discovering new antibiotic agents has gaining momentum.

Sá et al. (2009a) described the antimicrobial activity of MuHL against several bacteria strains including *S. aureus* (ATCC 6538) with MIC and MBC of 0.58 and 8.1 μg mL^{-1}, respectively. In this work, we report that MuBL, MuHL and MuLL showed inhibitory and

bactericidal activities against another *S. aureus* strain (ATCC 25923). MuHL was bacteriostatic and bactericide for ATCC 25923 with MIC and MBC of 122 and 489.8 µg mL^{-1}, respectively. The distinct values determined for strains ATCC 25923 and ATCC 6538 of *S. aureus* is probable correlated with factors associated with resistance demonstrated by different strains. Day et al. (2001) identified through DNA sequencing of various gene alleles a wide variety of bacterial strains with differences in ecological fitness and virulence.

Initial adherence and invasion of epithelial cells are two important factors which lead to the pathogenesis. Infection by pathogenic strains usually involves interactions with multiple components of the cell wall, including carbohydrate-containing molecules that are directly linked to bacterial adherence and may act as virulence factors (Leoff et al., 2008). To avoid infections, humans possess physical barriers, such as the skin, assisted by bacteriostatic or bactericidal compounds.

To the best of our knowledge, the inhibitory effect of plant lectins on adherence of *S. aureus, E. coli, Y. ruckeri* and *P. damselae* subsp. *piscicida* to eukaryote cells has not been described in the literature. Teichoic acid, an important staphylococcal adhesin, has *N*-acetylglucosamine in its structure. Also the lipid derivatives of teichoic acid (lipoteichoic acids) mediate the binding of staphylococci to host cells (Carruthers and Kabat, 1983). In this sense, these molecules can be targets involved in adherence inhibition by MuBL, MuLL and MuHL, which are *N*-acetylglucosamine binding proteins (Sá et al., 2009b; Napoleão et al., 2011).

Lectins have been described to interfere in adherence of bacteria to host cells. Lectin-like proteins from uroepithelial cells (50 µg ml^{-1}) inhibited 50% adhesion of *Staphylococcus saprophvticus*, *Lactobacillus* sp. and *Bacteroides intermedius* to uroepithelial cells (Graham et al., 1992). It is also known that the adherence of *Shigella dysenteriae* to human colon tumor cells (HT29) was inhibited in presence of *Aegle marmelos* fruit lectin at 625 µg mL^{-1}; the

authors suggested that the specificity of lectin to *N*-acetylgalactosamine, mannose and sialic acid might have played a key role in inhibition of initial adherence (Raja et al., 2011). Plant lectins from *Datura stramonium*, *Robinia pseudoacacia* and *Dolichos biflorus* agglutinated streptococcal bacterial cells which prevented the adherence of them to human cell surfaces (Kellens et al., 1994).

Some microorganisms have the ability to invade cells and to replicate and persist in the intracellular compartment, where secreted enzymes and bacterial scavenger molecules can detrimentally interfere with the functioning of host cells and tissues (Finlay and Falkow, 1989; Strauss and Falkow, 1997)**.** This internalization is a clinically important feature, since it usually confers protection against killing by the most of antibiotics (Von Eiff et al., 2001). Therefore, the study on drugs that affect this invasion capacity is essential for combating these microorganisms. In presence of lectins from *M. urundeuva*, the number of bacteria that adhered and invaded fish cell line (SAF-1) was significantly decreased, similarly to observed for *Aegle marmelos* fruit lectin on invasion of HT29 cells by *S. dysenteriae* (Raja et al., 2011). *E. coli* is reported as a non-invasive strain to fish cells (López-Dóriga et al., 2000) and in the present work it was observed the absence of bacteria from this species invading SAF-1 cells. Strong invasion inhibition was observed against *S. aureus* by MuBL (89%), MuLL(76%) and MuHL (87%). MuHL also showed high inhibition value (95%) of invasion by *Y. ruckeri*.

In human cell line (HeLa), MuLL and MuHL inhibited adherence of all bacteria more than MuBL. The highest values for adherence inhibition were shown by MuBL, MuLL and MuHL against *S. aureus* and by MuLL and MuHL against *E. coli*. The effect of lectins on adherence of *S. aureus* and *E. coli* to human line cell was stronger than that observed for fish cell line. Similarly to SAF-1, MuHL showed better inhibitory effect than MuBL and MuLL on adherence and invasion of HeLa by *Y. ruckeri*. Despite the high adherence inhibition of *S. aureus* promoted by MuLL, this lectin did not avoid HeLa invasion by this bacterium. Unlike

the results from SAF-1 cells, the invasion of HeLa cells by *P. damselae* subsp. *piscicida* was not significantly different from negative control. These results suggest that the mechanism of internalization of *S. aureus*, *E. coli*, *Y. ruckeri* and *P. damselae* by human cells is different from the mechanism of internalization of these bacteria into fish cells. The invasive properties of a bacteria depend greatly on specific features of the bacterial strain and cell lines, as demonstrated for *Salmonella*, whose invasion efficiency varied between 6 and 38% depending on the cell lines and the strain tested (Mills & Finlay, 1994).

Bacteriostatic compounds may act by inhibiting enzyme activities associated with the growth and/or lysis systems of the bacterium (Matsumoto et al., 1999). Lectins from *M. urundeuva* changed the patterns of extracellular products released by *S. aureus*, *E. coli*, *Y. ruckeri* and *P. damselae* subsp. *piscicida*, showing reduction of enzymatic activity. Total inhibition of DNAse, fosfolipase and hemolitic activity of ECPs from *S. aureus* was detected after incubation with MuBL, MuLL and MuHL at MIC values and the protein content of *S. aureus* was also greatly reduced in contact with these lectins. *S. aureus* synthesizes a broad spectrum of compounds that are secreted in the growth environment and are frequently synthesized in a manner that seems to be coordinated with adhesion molecule synthesis. For instance, *S. aureus* secretes a polypeptide called ‘‘extracellular adherence protein’’ (eap), which binds to a variety of plasma proteins and to the *S. aureus* surface (Palma et al., 1999). Therefore, the reduction of protein content after incubation with *M. urundeuva* lectins can undermine some process linked to infection of host by *S. aureus*, including adherence.

The functions of the enzymes secreted by pathogens are diverse. Frequently, some of these enzymes (such as caseinase and amylase) are secreted in order to provide a source of free amino acids or monosaccharides, which are then taken up by the microorganism for energy use or as building material (Travis et al., 1995). MuBL and MuHL abolished the amylase activity in ECPs released by *P. damselae subsp. piscicida*. The strong antimicrobial

activity of these lectins can be related with reduction of energy metabolism of *P. damselae* subsp. *piscicida* due to unavailability of free monosaccharides as substrates. MuBL, the lectin with the lowest value of MIC (34 µg mL^{-1}) against *Y. ruckeri*, showed higher inhibition of caseinase and gelatinase activities than MuLL and MuHL, which may account for the differences in the effects of these lectins on *Y. ruckeri*.

In some cases enzymes secreted by pathogens may play a more direct role in pathogenesis including hydrolysis of proteins from host cell membranes to facilitate adhesion and tissue invasion, activation or inactivation of cytokines, and damage of cells and molecules of the host defense system such as antibodies and complement factors (Souza et al., 2008). Incubation with MuBL, MuHL and MuLL resulted in absence of phospholipase activity in ECPs released by *Y. ruckeri*. Phospholipases hydrolyze phospholipids, molecules common to all cell membranes (Souza et al., 2008), and thus the fact that MuHL showed the best effect on adherence and invasion of SAF-1 and HeLa cells can be related with the reduction in this enzyme activity promoted by this lectin.

ECPs released by *E. coli* contained lower protein content after contact with MuBL and MuLL. None of the assayed enzymatic activities were determined in ECPs from *E. coli* since this bacterium does not produce the kind of evaluated enzymes. The protein content detected in ECPs from *E. coli* previously incubated with MuHL was higher than in ECPs from bacteria without contact. Lectins can induce the formation of pores in the membrane leading to a outflowing of cellular contents (Oliveira et al., 2008; Terras et al., 1993), which together with our data pointing this as a possible mechanism for inhibition of *E. coli* growth by MuHL.

In conclusion, *M. urundeuva* lectins were active against human and fish pathogens with effects on growth and survival of bacteria as well as adherence and invasion of bacteria on fish and human cells. The alterations in bacterial physiologic processes promoted by

lectins can be related with the ability of them to bind to *N*-acetylglucosamine and to alter the extracellular products released by bacteria.

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Figure captions

Fig. 1. Inhibition of adherence (A) and invasive (B) capacities of *Staphylococcus aureus, Escherichia coli, Photobacterium damselae subsp. piscicida* and *Yersinia ruckeri* to SAF-1 cell line after incubation with MuBL, MuLL and MuHL.

Fig. 2. Inhibition of adherence (A) and invasive (B) capacities of *Staphylococcus aureus, Escherichia coli, Photobacterium damselae subsp. piscicida* and *Yersinia ruckeri* to HeLa cell line after incubation with MuBL, MuLL and MuHL.

Fig. 3. Composition of extracellular products (ECP) released by *Staphylococcus aureus, Escherichia coli, Photobacterium damselae subsp. piscicida* and *Yersinia ruckeri* without incubation and after incubation with MuBL, MuLL and MuHL.

Table 1: Antibacterial activity of *Myracrodruon urundeuva* lectins.

Microorganisms	MuBL		MuLL		MuHL	
	MIC	MBC	MIC	MBC	MIC	MBC
Bacteria						
E. coli	34	ND	168.5	ND	490	ND
S. aureus	34	68	42	168.5	122	490
P. piscicida	1	2	1	2.6	3.8	7.6
Y. ruckeri	34	ND	84	ND	245	ND

MIC and MBC or MFC expressed in mg ml^{-1} of protein. ND: not detected

Figure 1

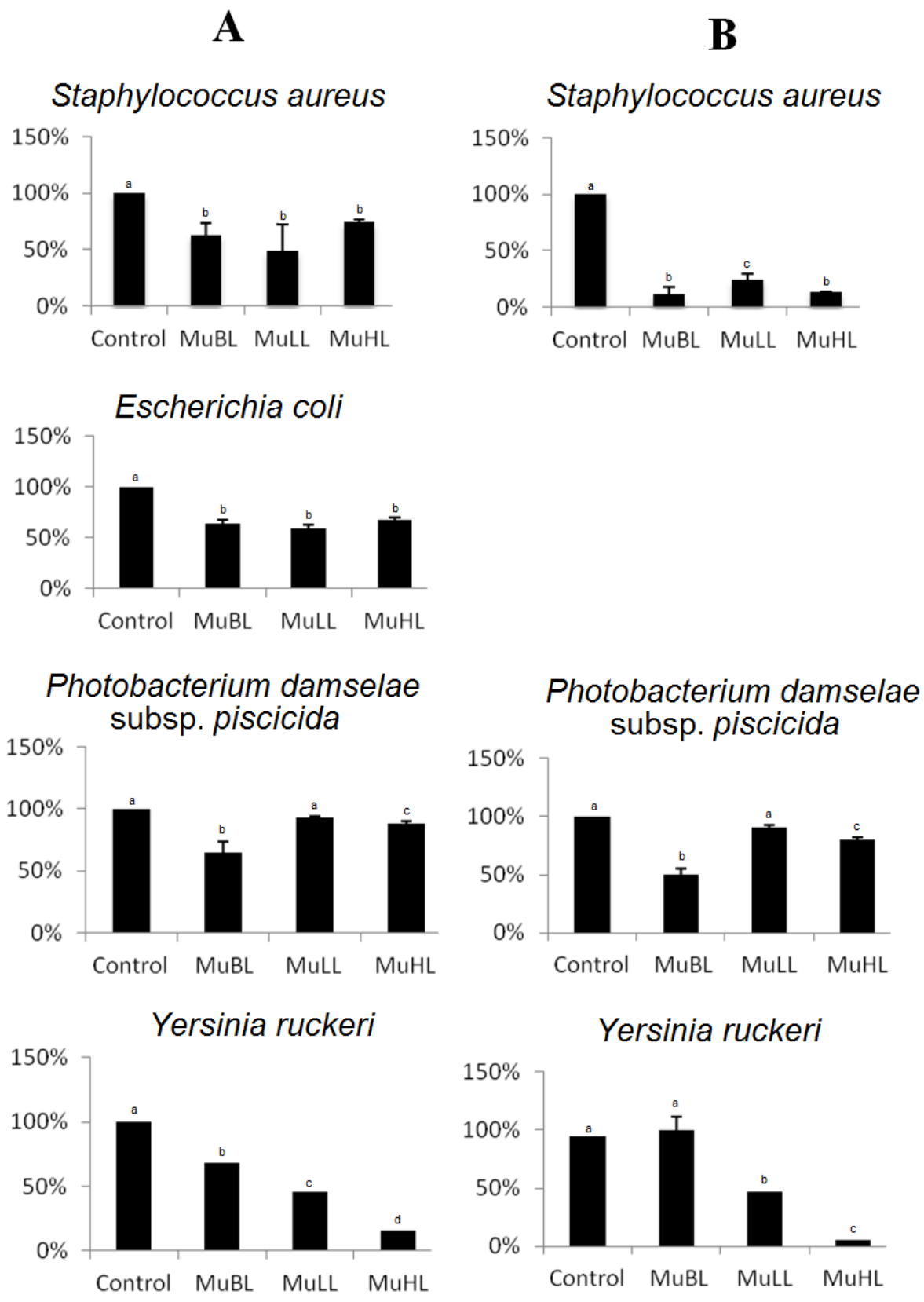

A
B
Staphylococcus aureus
Staphylococcus aureus
Escherichia coli
Photobacterium damselae subsp. piscicida
Photobacterium damselae subsp. piscicida
Yersinia ruckeri
Yersinia ruckeri
150%
100%
50%
0%
Control
MuBL
MuLL
MuHL

Figure 2

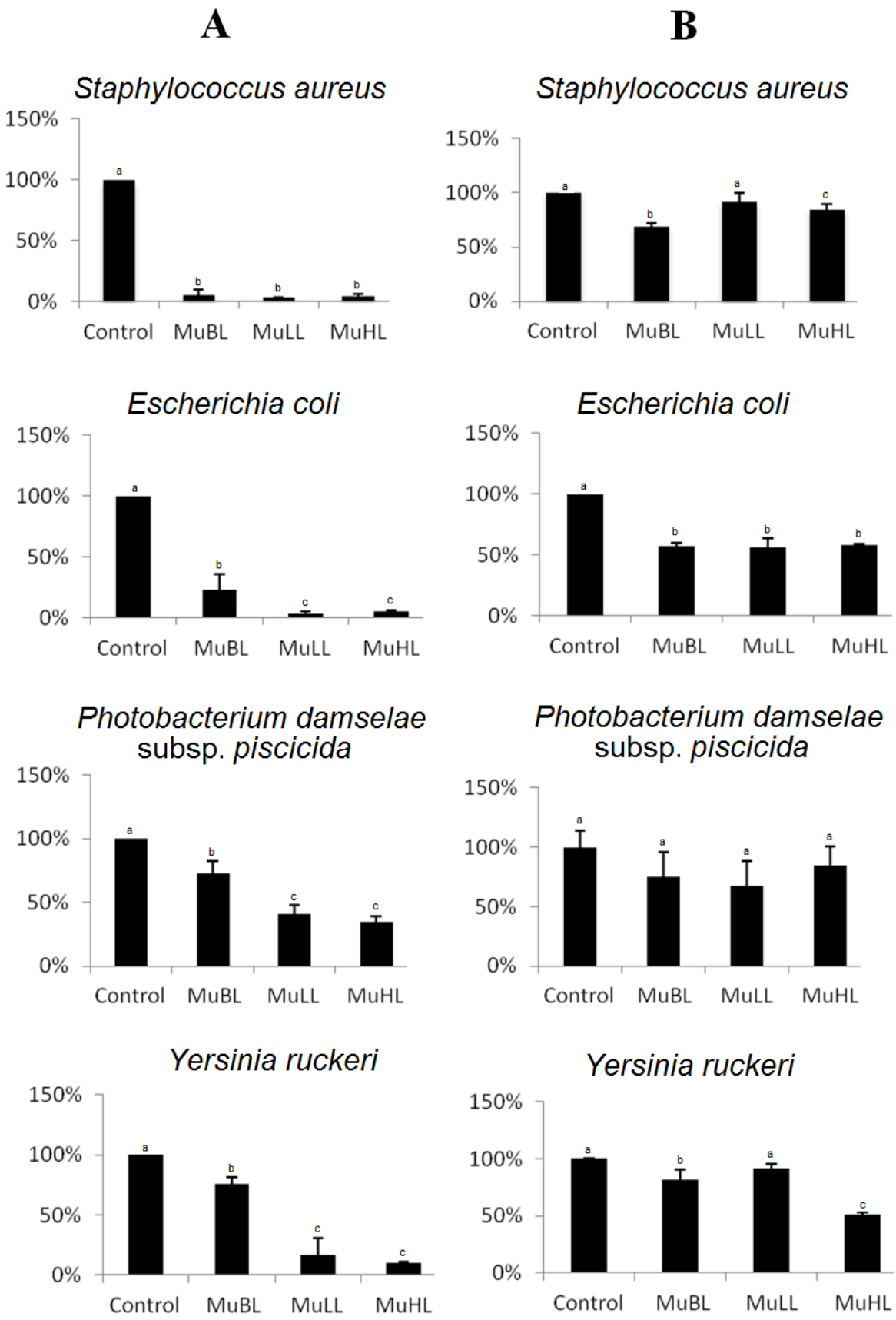
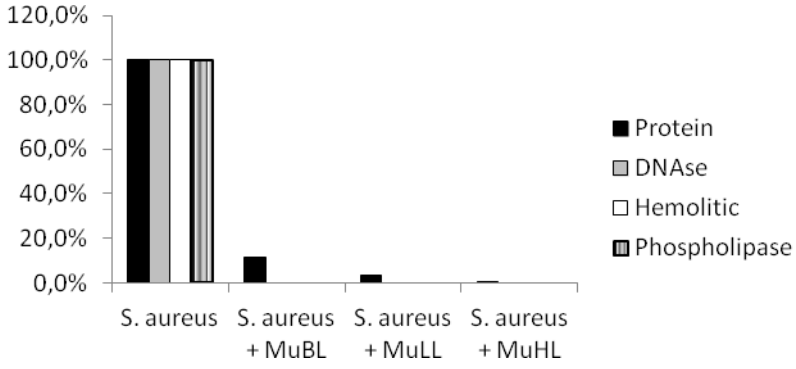
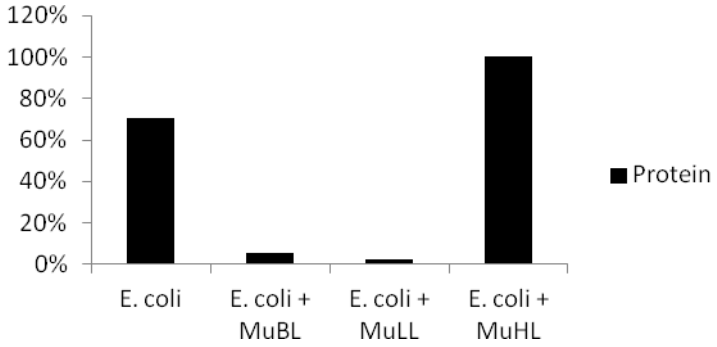

A
B
Staphylococcus aureus
Escherichia coli
Photobacterium damselae subsp. piscicida
Yersinia ruckeri
150%
100%
50%
0%
Control
MuBL
MuLL
MuHL

Figure 3:


120,0%
100,0%
80,0%
60,0%
40,0%
20,0%
0,0%
S. aureus
S. aureus + MuBL
S. aureus + MuLL
S. aureus + MuHL
Protein
DNAse
Hemolitic
Phospholipase


120%
100%
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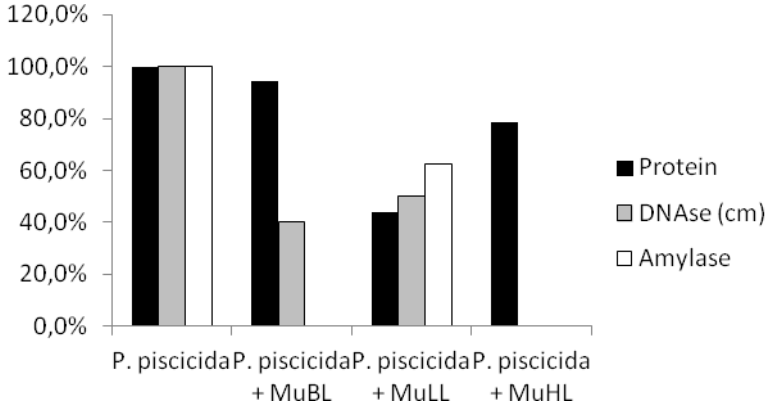
E. coli
E. coli + MuBL
E. coli + MuLL
E. coli + MuHL
Protein


120,0%
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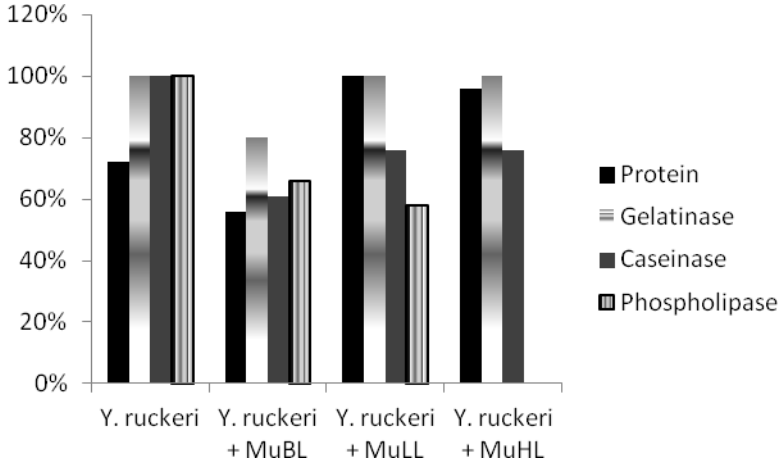
P. piscicida
P. piscicida + MuBL
P. piscicida + MuLL
P. piscicida + MuHL
Protein
DNAse (cm)
Amylase


120%
100%
80%
60%
40%
20%
0%
Y. ruckeri
Y. ruckeri + MuBL
Y. ruckeri + MuLL
Y. ruckeri + MuHL
Protein
Gelatinase
Caseinase
Phospholipase

6. CAPÍTULO 4

Antimicrobial activity of secondary metabolites and lectins from plants

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Antimicrobial activity of secondary metabolites and lectins from plants

P.M.G. Paiva[1], **F.S. Gomes**[1], **T.H. Napoleão**[1], **R.A. Sá**[2], **M.T.S. Correia**[1] and **L.C.B.B. Coelho**[1]

[1] Departamento de Bioquímica, Universidade Federal de Pernambuco, Av. Prof. Moraes Rêgo s/n, 50670-420 Recife, Brazil
[2] Centro Acadêmico do Agreste, Universidade Federal de Pernambuco, 55002-970 Caruaru, Brazil

This review outlines the antimicrobial activity of secondary metabolites and lectins, compounds usually associated to defense mechanisms of plants. Secondary metabolites are separated into nitrogen compounds (alkaloids, non-protein amino acids, amines, alcamides, cyanogenic glycosides and glucosinolates) and non-nitrogen compounds (monoterpenes, diterpenes, triterpenes, tetraterpenes, sesquiterpenes, saponins, flavonoids, steroids and coumarins). Lectins are carbohydrate-binding proteins and their biological properties include cell-cell interactions. This chapter reports solvent organic extracts (mixture of secondary metabolites), isolated secondary metabolites and lectins from plants with antimicrobial activity against Gram-negative and Gram-positive bacteria as well as antifungal activity towards human and plant pathogens. Mechanisms proposed for antimicrobial activity of secondary metabolites and lectins against bacteria and fungi are also discussed. The effects of plant secondary metabolites and lectins on deleterious human and plant microorganisms indicates their perspectives of antimicrobial uses.

Keywords antibacterial activity; antifungal activity; plant lectins; secondary metabolites.

1. Secondary metabolites with antimicrobial activity

Solvent organic extracts contain a mixture of secondary metabolites including alkaloids, flavonoids, terpenoids, and other phenolic compounds; these molecules are associated to defense mechanisms of plants by their repellent or attractive properties, protection against biotic and abiotic stresses, and maintenance of structural integrity of plants. Polar solvents (such as organic acids), solvents of intermediate polarity (such as methanol, ethanol, acetone, and dichloromethane) and solvents of low polarity (such as hexane and chloroform) are used to extract plant secondary metabolites that differ in structure and polarity. Then extracts from the same plant material obtained with solvents of different characteristics have distinct biological properties. Extracts from aerial parts of *Salvia tomentosa* were evaluated for antibacterial activity and it was reported that non-polar extracts showed moderate activity and polar extracts were inactive [1].

Solvent organic extracts from aerial parts, bark, flowers, fruits, heartwood, leaves, twigs and root from medicinal plants have been investigated aiming to validate their ethnopharmacological use. Extracts from plants used to treat diarrhea (*Indigofera daleoides*, *Punica granatum*, *Syzygium cordatum*, *Gymnosporia senegalensis*, *Ozoroa insignis*, *Elephantorrhiza elephantina*, *Elephantorrhiza burkei*, *Ximenia caffra*, *Schotia brachypetala* and *Spirostachys africana*) contained agents against bacteria that cause gastrointestinal infections (*Vibrio cholerae*, *Escherichia coli, Staphylococcus aureus*, *Shigella dysentery*, *Shigella sonnei*, *Shigella flexneri*, *Shigella boydii* and *Salmonella typhi*) and this strengths their usefulness in the treatment of diarrhea [2]. Extracts from *Calophyllum brasiliense* leaves (obtained with acetone), *Mammea americana* fruit peels (obtained with acetone and hexane) and dichloromethane extract of the resinous exudate from *Baccharis grisebachii* were also effective against methicilline-resistant and sensible *S. aureus* strains [3, 4]. Table 1 shows that solvent organic extracts may be antibacterial agent only on Gram-positive or both Gram-positive and Gram-negative bacteria. Differential sensitivity of Gram-positive and Gram negative bacteria to plant extracts may be explained by the morphological differences between these microorganisms. Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components; this makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da. The Gram-positive bacteria should be more susceptible since they have only an outer peptidoglycan layer which is not an effective permeability barrier [5].

Different types of secondary metabolites have been identified as the active principles of antimicrobial solvent organic extracts (Table 2). The tannins methyl gallate and gallic acid from *Galla rhois* inhibit cariogenic (*Actinomyces viscosus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus salivarus*, *Streptococcus mutans* and *Streptococcus sobrinus*) and periodontopathic (*Porphyromonas gingivalis*) bacteria and the *in vitro* formation of *S. mutans* biofilms; authors suggested the use of these compounds to prevent the formation of oral biofilms [6].

Solvent organic extracts with antifungal activity against species that cause diseases in humans and plants have been reported. Dichloromethane extract of the resinous exudate from *Baccharis grisebachii* containing diterpene (labda-7,13*E*-dien-2β,15-diol) and coumaric acids (3-prenyl-*p*-coumaric acid and 3,5-diprenyl-*p*-coumaric acid) was active against *Epidermophyton floccosum*, *Microsporum canis*, *Microsporum gypseum*, *Trichophyton mentagrophytes* and

Trichophyton rubrum [3]. Ethanolic extracts of *Caesalpinia mimosoides* showed potent activity against fungi (*M. gypseum* and *T. rubrum*); gallic acid (a tannin) was detected as the main principle of the extract [7]. A methanolic extract from *Myracrodruon urundeuva* containing cinamic derivatives, flavonoids, gallic acid, luteolin, and tannins showed antifungal activity on *Fusarium* [8]. The extract had important role in growth inhibition of *Fusarium lateritium* and *Fusarium oxysporum*, as evidenced by inhibition superior to commonly used antifungal Cercobin. *F. oxysporum* is a phytopathogen and opportunistic human pathogen.

Table 1 Organic solvent extracts from medicinal plants with antibacterial activity.

Medicinal use	Plant	Antibacterial activity	
		Gram + and -	Only Gram +
Respiratory disease	*Abuta grandifolia, Cordia alliodora*	X	
	Acacia nilotica, Caesalpinia pyramidalis, Cupania oblongifolia, Cupania platycarpa		X
Digestive disease	*A. grandifolia, Maytenus macrocarpa, Naucleopsis glabra, Annona cherimola, Calophyllum brasiliense, Ozoroa insignis*	X	
	Commiphora parvifolia, Ocotea glomerata, Simarouba amara, Talisia esculenta		X
Skin disease	*Guazuma ulmifolia, Solanum incanum*		X
	Lipia adoensis, Mammea americana, Eryngium creticum, Juglans regia, Lycium europeum, Micromeria nervosa	X	
Malaria	*Aegiphila lhotskiana, Hedychium coronarium, Simarouba amara*		X
Anti-inflammatory	*Annona salzmanni, C. pyramidalis, Pterodon polygalaeflorus*		X
	Schinus terebinthifolius	X	
Antirheumatic	*Annona muricata, Marsdenia altissima, P. polygalaeflorus, T. esculenta*		X
	C. alliodora, N. glabra	X	
Healing activity	*Pterocarpus rohrii, Plantago lanceolata, Pinus gerardiana*	X	
	A. nilotica, Syzygium jambolanum, Dipteryx micrantha, Andira inermis, Auxemma oncocalyx		X
Renal disease	*Sarcopoterium spinosum, Pistacia lentiscus, E. creticum, Retama aculeatus*	X	
	Indigofera spinosa, Cadaba glandulosa		X
Fever	*Anogeissus schimperi, Bauhinia thonningi, Cassia goratensis, Butyrospernum parkii, Boswellia dalzielli*	X	
Veneral disease	*Abutilon indicum, Vitex nigundo, Boswellia serrata, Commiphora mukul, Bixa orellana, Raphanus sativus*	X	
Eye disease	*Syzygeum guineense, Lippia adoensis, Zizyphus jujube, Capparis spinosa, Lycium europeum, Retama raetam, Zizyphus spina-christi, Albezzia lebbeck*	X	

Gram + and – means Gram-positive and Gram-negative bacteria and (X) means bacteriostatic or bactericide effects. References: [2, 4, 9-19].

Prenylated flavonoids purified from Asian medicinal plants *Broussnetia papyrifera*, *Echinosophora koreensis*, *Morus alba*, *Morus mongolica* and *Sophora flavescens* showed antifungal activity against *Candida albicans*; the authors highlighted the high potential use of them in Asian traditional medicine to treat infections [20]. A mixture of linear aliphatic primary alcohols isolated from cyclohexane extract of *Solanecio mannii* leaves was an antifungal agent on *C.*

albicans (minimal inhibitory concentration of 1.6 μg/ml) while fatty acid esters of diunsaturated linear 1,2-diols from cyclohexane extract of *Monodora myristica* fruits were active against on *C. albicans* and *Candida krusei* [21].

Table 2 Antimicrobial activity of secondary metabolites from medicinal plants.

Microorganism	Compound	Plant
Bacillus cereus, Bacillus subtilis, Micrococus kristinae, Staphylococcus aureus, Aspergillus flavus, Cladosporium sphaerospermum	3,5,7-Trihydroxyflavone (galangin)	*Helichrysum aureonitens*
B. cereus, S. aureus, Staphylococcus epidermis, Candida albicans, Cryptococcus neoformans	Benzoquinone and benzopyran	*Gunnera perpensa*
B. cereus, B. subtilis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, S. aureus, C. albicans, C. neoformans	Helihumulone	*Helichrysum cymosum*
B. cereus, S. aureus	Carnosol and 7-O-methyl-epirosmanol	*Salvia chamelaeagnea*
B. subtilis, E. coli, K. pneumoniae, S. aureus	Anolignan B	*Terminalia sericea*
B. subtilis, E. coli, K. pneumoniae, S. aureus	Sesquiterpenoid	*Warburgia salutaris*
B. subtilis, E. faecalis, E. coli, P. aeruginosa, S. aureus, Aspergillus niger	Flavonoids	*Combretum erythrophyllum*
E. coli, S. aureus, C. albicans		*Erythrina burttii*
E. coli, Salmonella typhimurium, Staphylococcus epidermis, S. aureus, C. albicans, Saccharomyces cerevisiae		*Broussnetia papyrifera, Echinosophora koreensis, Morus alba, Morus mongolica* and *Sophora flavescens*
E. coli, S. aureus	Terpenoids	*Spirostachys africana*
E. coli, K. pneumoniae, S. aureus	Vernolide and vernodalol	*Vernonia colorata*
Epidermophyton floccosum, Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum, S. aureus	Diterpene and coumaric acids	*Baccharis grisebachii*
Actinomyces viscosus, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus salivarus, Porphyromonas gingivalis, Streptococcus mutans, Streptococcus sobrinus	Methyl gallate and gallic acid	*Galla rhois*
C. albicans	Alkaloids	*Aniba panurensis*
S. aureus	Naphtoquinones	*Tabebuia avellanedae*
A. niger, Botrytis cinerea	Saponin	*Astragalus verrucosus*
S. aureus, B. cereus, Clostridium perfrigens, E. faecalis, Micrococcus luteus, Aeromonas hydrophila, Enterobacter sakazakii, K. pneumoniae, E. coli, Enterobacter cloacae, P. aeruginosa, Vibrio vulnificus, Pseudomonas luteola, Chryseobacterium indologenes, C. albicans, A. niger, Penicillium sp.	Glucosinolates	*Aurinia sinuata*
Mycobacterium tuberculosis	Quassinoids	*Ailanthus altissima*
M. tuberculosis	Xanthones	*Canscora decussata*
Mycobacterium smegmatis, Mycobacterium intracellulare, Mycobacterium chelonae, Mycobacterium xenopi	Ferruginol	*Juniperus excelsa*
Mycobacterium avium, M. tuberculosis	Gingerols	*Zingiber officinale*

References: [2, 3, 6, 20, 22-39]

Essential oils are a bioactive mixture of complex compounds synthesized as secondary metabolites by buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark. The lipophilic nature of essential oils makes them permeable to cellular membrane; cytotoxic effects include cell alterations in plasma membrane, cytoplasm and nucleus [40]. *Lippia rugosa* oil containing geraniol, nerol and geranial as main components was able to inhibit *Aspergillus flavus* growth as well as the production of aflatoxin, probably due to interference on fungal cellular metabolism [41]. Antifungal activity on dermatophytes *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum* and *Microsporum canis* was also found in the essential oil from *Moringa oleifera* leaves [42]. *Salvia pisidica* and *Achillea ligustica* oils showed antibacterial activity against Gram (+) bacteria and were suggested as food preservatives and anti-cariogenic agent [43, 44]. The essential oil from *Salvia tomentosa*, composed of β-pinene (39.7%), α-pinene (10.9%) and camphor (9.7%), was highly active and showed minimal inhibitory concentration ranging from 0.54 mg/mL (*Clostridium perfringens*) to 72.00 mg/mL (*Moraxella catarrhalis*, *Enterobacter aerogenes* and *Klebsiella pneumoniae*). The essential oil was not active on *Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa* [1].

Antimicrobial activity of phenolic compounds present in plants change according its structure; flavone, quercetin and naringenin were effective in inhibiting the growth of *Aspergillus niger*, *Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, *Staphylococcus aureus* and *Staphylococcus epidermidis* while gallic acid inhibited only *P. aeruginosa*; rutin as well as catechin did not show any effect on the tested microorganisms [45].

The mechanisms of antimicrobial action of plant secondary metabolites are not fully understood but several studies have been conducted in this direction. Flavonoids may act through inhibiting cytoplasmic membrane function as well as by inhibition of DNA gyrase and β-hydroxyacyl-acyl carrier protein dehydratase activities [46, 47]; the isoflavone genistein was able to change cell morphology (formation of filamentous cells) and inhibited the synthesis of DNA and RNA of *Vibrio harveyi* [48]. It has been suggested that terpenes promote membrane disruption, coumarins cause reduction in cell respiration and tannins act on microorganism membranes as well as bind to polysaccharides or enzymes promoting inactivation [49-51]. Although a number of publications have focused on the isolation and identification of bio-active compounds, it is important to keep in mind that a single compound may not be responsible for the observed activity but rather a combination of compounds interacting in an additive or synergistic manner.

2. Antimicrobial lectins

Lectins are carbohydrate-recognizing proteins that bind to cells promoting hemagglutination and antimicrobial effect. Plant lectins have been isolated from bark, cladodes, flowers, leaves, rhizomes, roots and seeds. Alternatively, plant recombinant lectins have been expressed in heterologous systems [52]. Plant lectins can be glycosylated molecules and staining on polyacrylamide gel specific for glycoprotein can easily reveal the presence of glycan in the lectin structure; carbohydrate moiety characterization can be performed after lectin tryptic digestion in gel followed by enzymatic deglycosylation and mass spectrometric analysis [53]. The compact globular structures, molecular aggregation and glycosylation in general result in high structural stability of lectins [54, 55]; high temperature is a powerful denaturing agent leading to protein unfolding through breaking of hydrogen bonds that maintain protein structure and heated lectins can or not lose their biological properties.

Lectins are distributed in fucose, mannose, sialic acid, *N*-acetylglucosamine, *N*-acetylgalactosamine and glycan-complex groups according to carbohydrate specificity [56]. The selectivity of binding is achieved through hydrogen bridges, van der Waals and hydrophobic interactions between sugar and lectin site. The presence of multiple molecular forms of protein is a frequent phenomenon in some plant species; they may have distinct carbohydrate specificity, charge, mobility on polyacrylamide gel and biological property [57]. Molecular forms with different electrophoretic mobility which belong to the same species are called isolectins [58] and the term isoform was proposed for lectins belonging to the same species when heterogeneity of genetic origin was not well defined [59].

Hemagglutinating activity is the most commonly used assay for the detection of lectin in a sample due to the simplicity of implementation and ease visualization of agglutination. Aliquot of sample is serially diluted in microtitration plate before addition of erythrocytes and hemagglutinating activity (titer) is defined as the reciprocal of the highest dilution of sample promoting full agglutination of erythrocytes. The hemagglutinating activity occurs when the lectin binds to carbohydrate from erythrocyte surface promoting a network among them (Figure 1A); sometimes lectin is not detected due to steric hindrance in the lectin-carbohydrate interaction and previous enzymatic treatment of erythrocytes is needed to occur hemagglutination [60].

The hemagglutination assay allows the assessment of lectin stability to pH and temperature values and thus can determine the conditions to be used in the biotechnological application of lectin. Additionally the assay may reveal lectin carbohydrate specificity defined by carbohydrate that more effectively inhibits the hemagglutinating activity (Figure 1B). Alternative strategies to detect the carbohydrate specificity of lectins are surface plasmon resonance method using carbohydrate immobilized on a gold-coated glass prism and enzyme-linked adsorbent assay using monosaccharide-polyacrylamide conjugates on the microplates [61, 62].

Lectins can be extracted from plant tissue with water, 0.15 M NaCl or buffer solutions when it is necessary to control pH for the maintenance of hemagglutinating activity. The temperature and extraction time depends on stability and

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solubility of the lectin and may vary from 4 to 27° C, from minutes to hours. Lectin can also be extracted using a reversed micelle system of the anionic surfactant sodium di(2-ethylhexyl)sulfosuccinate in isooctane; protein solubilization is strongly dependent on pH, concentration of surfactant and on the size of the micelle relative to that of the protein [63]. Lectin present in a mixture of proteins can be isolated by column chromatography that promotes separation due to differential migration of proteins adsorbed to the matrix. Disruption of interactions lead to the release of proteins in distinct fractions, dependent on the binding of each protein component of sample to the matrix. Presence of oil and pigments in vegetal tissues can interfere in lectin isolation by chromatography since non-specific adsorption of these contaminants on matrix constitutes an impediment to lectin-matrix interaction. Plant tissues with high oil and polysaccharide content can be previously treated before protein extraction aiming to eliminate contaminants; polyethylene glycol (PEG 8000) is effective in removing polyphenolic compounds [64].

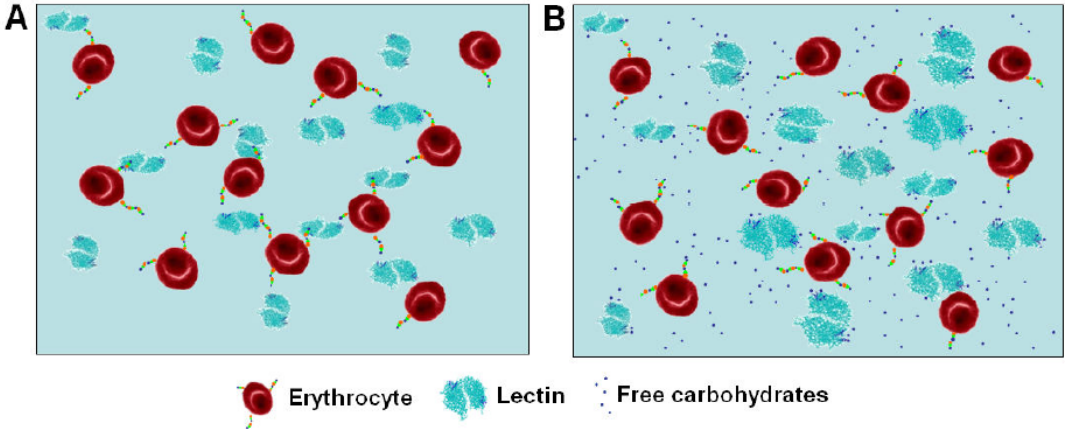

Fig. 1 Schematic representation of erythrocyte network promoted by lectin binding to surface carbohydrates (A) and inhibition of hemagglutinating activity by free carbohydrates (B).

The conditions used in the chromatographic steps (washing, lectin adsorption, and desorption) including volume and protein concentration of sample, pore size and matrix charge, column length, temperature and solution used for lectin desorption, flow velocity and volume of fraction collected are defined in order to increase yield and degree of purity. The choice of chromatographic method is performed according to lectin biochemical characteristics and isolation procedures can use one or sequential chromatographic processes.

Affinity chromatography is present in almost all purification procedures of lectin with defined specificity due to advantages such as high recovery and high specificity. The method provides a high degree of protein purification, in a single step, with maintenance of the biological activity. Polysaccharide matrices such as Sephadex, chitin and Sepharose consisting of glucose, N-acetylglucosamine and galactose units, respectively, are selected according to the specificity of the lectin to be isolated. Lectins that recognize glycoconjugates may be isolated by affinity chromatography on columns containing glycoproteins immobilized on Sepharose activated with cyanogen bromide [65]. A method was developed to immobilize egg proteins and the affinity matrix was efficient to purify lectins from extracts of *Phaseolus vulgaris* (complex saccharide binding), *Lens culinaris* (mannose and glucose binding), and wheat germ (sialic acid, acetyl-glucosamine, and its polymer binding) in terms of milligrams per gram of matrix [66]. Another alternative for lectin affinity isolation is the use of ferromagnetic levan particles, a composite of the carbohydrate levan from *Zimomonas mobilis* and magnetite. Lectins are eluted with 0.3 M monosaccharide solutions and recovered from particles by a magnetic field [67].

Cytotoxic effects of lectins may be revealed by antitumoral and antiviral activities and also by deleterious effect on microorganisms (Table 3); lectins of different carbohydrate specificities are able to promote growth inhibition or death of fungi and bacteria. Table 4 shows proposed applications of lectins for detection, typing, and control of bacteria and fungi that cause damage to plants and humans.

Antibacterial activity on Gram-positive and Gram-negative bacteria occurs through the interaction of lectin with components of the bacterial cell wall including teicoic and teicuronic acids, peptidoglycans and lipopolysaccharides; study revealed that the isolectin I from *Lathyrus ochrus* seeds bind to muramic acid and muramyl dipeptide through hydrogen bonds between ring hydroxyl oxygen atoms of sugar and carbohydrate binding site of lectin and hydrophobic interactions with the side chains of residues Tyr^{100} and Trp^{128} of isolectin I [68].

The inhibition of fungi growth can occur through lectin binding to hyphas resulting in poor absorption of nutrients as well as by interference on spore germination process [58]. The polysaccharide chitin is constituent of fungi cell wall and chitin-binding lectins showed antifungal activity; impairment of synthesis and/or deposition of chitin in cell wall may be the reasons of antifungal action [69]. Probably the carbohydrate-binding property of lectin is involved in the

antifungal mechanisms and lectins of different specificities can promote distinct effects. Plant agglutinins are believed to play a role in plant defense mechanism against microorganism phytopathogens [70].

Table 3 Plant lectins with antimicrobial activity.

Plant (tissue)	Lectin specificity	Antimicrobial activity
Araucaria angustifolia (seed)	GlcNAc	*Clavibacter michiganensis*, *Xanthomonas axonopodis* pv. *passiflorae*
Artocarpus incisa (seed)	GlcNAc	*Fusarium moniliforme*, *Saccharomyces cerevisiae*
Artocarpus integrifolia (seed)	GlcNAc	*F. moniliforme*, *S. cerevisiae*
Astragalus mongholicus (root)	Lactose/D-Gal	*Botrytis cincerea, Fusarium oxysporum, Colletorichum sp., Drechslera turcia*
Eugenia uniflora (seeds)	Carbohydrate complex	*Bacillus subtilis, Corynebacterium bovis, Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa, Streptococcus sp., Staphylococcus aureus*
Gastrodia data (corms)	α-Man/ GlcNAc	*B. cinerea, Ganoderma lucidum, Gibberella zeae, Rhizoctonia solani*, *Valsa ambiens*
Hevea brasiliensis (latex)	Chitotriose	*B. cinerea, Fusarium culmorum, F. oxysporum* f. sp. *pisi, Phycomyces blakesIeeanus, Pyrenophora triticirepentis, Pyricularia oryzae, Septoria nodorum, Trichoderma hamatum*
Myracrodruon urundeuva (heartwood)	GlcNAc	*B. subtilis, Corynebacterium callunae, E. coli, Klebsiella pneumoniae, P. aeruginosa, S. aureus, Streptococcus faecalis.* *Fusarium solani*, *F. oxysporum*, *F. moniliforme*, *Fusarium decemcellulare*, *Fusarium lateritium*, *Fusarium fusarioides*, *Fusarium verticiloides*
Ophiopogon japonicus (rhizome)	Man	*Gibberella saubinetii, R. solani*
Opuntia ficus indica (cladodes)	Glc/Man	*Colletrotrichum gloesporioides*, *Candida albicans, F. oxysporum, F. solani*
Phaseolus coccineus (seeds)	Sialic acid	*Helminthosporium maydis*, *Gibberalla sanbinetti*, *R. solani*, *Sclerotinia sclerotiorum*
Phthirusa pyrifolia (leaf)	Fru-1,6-P2	*B. subtilis*, *K. pneumoniae*, *Staphylococcus epidermidis*, *S. faecalis* *F. lateritium, R. solani*
Pisum sativum (seed)	Man	*Aspergillus flavus*, *F. oxysporum, Trichoderma viride*
Sebastiania jacobinensis (bark)	Carbohydrate complex	*F. moniliforme*, *F. oxysporum*
Talisia esculenta (seeds)	Man	*Colletotrichum lindemuthianum*, *F. oxysporum*, *S. cerevisiae*
Triticum vulgaris (seeds)	GlcNAc	*Fusarium graminearum, F. oxysporum*
Urtica dioica (rhizome)	GlcNAc	*B. cinerea*, *C. lindemuthianum*, *Phoma betae*, *Phycomyces blakesleeanus*, *Septoria nodorum*, *Trichoderma hamatum*, *T. viride*

D-Gal: galactose; Fru-1,6-P2: fructose-1,6-biphosphate; Glc: glucose; GlcNAc: *N*-acetylglucosamine; Man: mannose.
References: [70-85].

Myracrodruon urundeuva Fr. All is broadly distributed in Brazil. Considered a hardwood, it is very resistant to degradation by microorganisms; its heartwood contains antimicrobial lectin [70]. The heartwood lectin inhibited Gram-positive (*Bacillus subtilis, Corynebacterium callunae, Staphylococcus aureus* and *Streptococcus faecalis*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) bacteria but was more effective on Gram-positive than on Gram-negative bacteria. The lowest minimal inhibitory concentration (MIC) was determined for *S. aureus* (0.58 μg/mL) and minimum bactericidal concentration (MBC) for this bacterium was 8.1 μg/mL; *K. pneumoniae* was the least sensitive microorganism (MIC of 9.37 μg/mL). The lectin is a chitin-binding protein with antifungal activity against *Fusarium* strains; the highest percentage of growth inhibition was obtained for *F. oxysporum* (60.8% ± 2.9) and similar inhibition was detected against *Fusarium decemcellulare* (51.1% ± 3.8) and *Fusarium fusarioides* (51.1% ± 1.9).

Phthirusa pyrifolia leaf lectin with a unique affinity for fructose-1-6-biphosphate showed antimicrobial activity [71]. Antibacterial activity was detected against *Klebsiella pneumoniae*, *Staphylococcus epidermidis* and *Streptococcus faecalis* but bactericide effect was only detected on *Bacillus subtilis* (MBC of 0.5 mg/mL). The lectin was an antibacterial agent more effective for Gram-positive than for Gram-negative bacteria and it was suggested that the

bacteria sensitivity was related to levels of peptideoglycan on the wrapper. The *P. pyrifolia* lectin was an antifungal agent on *Fusarium lateritium* and *Rhizoctonia solani* but did not affect the growth of *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Rhizopus arrhizus*, *Paecilomyces variotti*, *Fusarium moniliforme*, *Candida albicans*, *Candida burnenses*, *Candida tropicalis*, *Candida parapsilosis* and *Saccharomyces cerevisiae*.

Table 4 Application of lectins in the study of microrganisms

Application	Lectin source	Year
Antifungal agent	*Artocarpus incisa*, *Artocarpus integrifolia*, *Ophiopogon japonicus*, *Opuntia ficus indica*, *Phaseolus coccineus*, *Pisum sativum*, *Sebastiania jacobinensis*, *Talisia esculenta*	2002, 2006, 2007, 2008, 2009, 2010
Antimicrobial agent on bacteria and fungi	*Myracrodruon urundeuva*	2009
Biotinylated lectins applicable to large scale typing of *Staphylococcus epidermidis*	*Triticum vulgaris*, *Glicine max*, *Lens culinaris*, *Canavalia ensiformis*	1992
Clinical microbiology and therapeutic applications	*Eugenia uniflora*, *Phthirusa pyrifolia*	2008, 2010
Colloidal gold-labeled lectin for the direct microscopic observations of bacterial exopolysaccharides in Cheddar cheese matrix using transmission electron microscopy	*Ricinus communis*	2005
Fluorescein-conjugated lectins for rapid visualization of *Candida albicans, Aspergillus fumigatus* and *Fusarium solani*	*Canavalia ensiformis*, *Lens culinaris*, *Triticum vulgaris*, *Ulex europeus*	1986
Identification of *Mycobacterium* species (*M. tuberculosis*, *M. avium*) by different agglutination in a microtiter plate	*Canavalia ensiformis*, *Cladrastis lutea*, *Galanthus nivalis*, *Narcissus pseudonarcissus*, *Vicia fava*, *Vicia sativa*	2006
Lectin-magnetic microspheres to distinguish between bacterial species from aqueous suspensions	*Helix pomatia*	1996
Quartz crystal microbalance lectin-based biosensor to identify the presence of bacteria	*Canavalia ensiformis*, *Lens culinaris*, *Maackia amurensis*, *Triticum vulgaris, Ulex europeus*	2008
Selectivity in targeting to skin-associated bacteria by Con A-bearing liposomes (*Streptococcus sanguis* and *Corynebacterium hofmanni*) and WGA-bearing liposomes (*Staphylococcus epidermidis*)	*Triticum vulgaris*, *Canavalia ensiformis*	1995
Tool for studying bacterial infections and inflammatory processes	*Araucaria angustifolia*	2006

References: [70-78; 80-92]

A thermo resistant lectin isolated from *Eugenia uniflora* seeds demonstrated a remarkable non-selective antibacterial activity [73]; the lectin strongly inhibited the growth of *Staphylococcus aureus, Pseudomonas aeruginosa* and *Klebsiella* sp. with MIC of 1.5 μg/mL while was less effective in inhibiting the growth of *Bacillus subtilis*, *Streptococcus* sp. and *Escherichia coli* (MIC of 16.5 μg/mL). Bactericide activity was mainly detected for *S. aureus, P. aeruginosa* and *Klebsiella* sp. (MBC of 16.5 μg/mL); the authors suggested the use of lectin for clinical microbiology and therapeutic purposes.

The antibacterial activity of *N*-acetyl-D-glucosamine-binding lectin isolated from *Araucaria angustifolia* seeds on phytopathogenic bacteria was revealed by reduction in the colony forming units. The lectin was more effective against the Gram-positive *Clavibacter michiganensis* (80% of reduction) than on Gram-negative *Xanthomonas axopodis* (60% of reduction). Electron microscopy revealed that treatment with *A. angustifolia* lectin promoted morphologic alterations including presence of pores in the Gram-positive bacteria membrane and bubbling on the Gram-negative bacteria cell wall [74].

Bark of *Sebastiania jacobinensis*, used by people as medicine to treat infections, contains antifungal lectin of glycan-complex carbohydrate specificity group [75]. The effect of lectin on growth of *Aspergillus niger*, *Candida albicans*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *Fusarium moniliforme* and *Trichoderma viride* was investigated and the lectin was active only on *Fusarium* species. The lectin was not toxic for *Artemia salina* and embryos of *Biomphalaria glabrata* and it was suggested that this fact is interesting for perspective of its biotechnological use as antifungal agent.

A stable, ion dependent and chitin-binding lectin isolated from *Opuntia ficus indica* cladodes was able to affect the growth of *Colletrotrichum gloesporioides*, *Candida albicans*, *Fusarium oxysporum* and *Fusarium solani*; the lectin showed high activity against *C. albicans*, reducing the fungal growth in 59% [77].

Mannose-binding lectins with antifungal activity have been described. The lectin isolated from *Ophiopogon japonicus* rhizomes was an antifungal agent against the phytopathogens *Gibberella saubinetii* and *Rhizoctonia solani* but not on *Penicillium italicum* [76]. The lectin of *Pisum sativum* seeds inhibited the growth of *Aspergillus flavus*, *Fusarium oxysporum* and *Trichoderma viride* [81].

3. Conclusion

Plant tissues contain secondary metabolites and lectins with antibacterial and antifungal activities and thus are sources of natural bioactive molecules to control pathogens that cause diseases in plants and humans. The ability of lectin selectively to bind microrganisms makes them potential tools to study pathogen species.

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7. CONCLUSÕES

- As lectinas de folha (SteLL) e entrecasca (SteBL) de *S. terebinthifolius* e de casca (MuBL), cerne (MuHL) e folha (MuLL) de *M. urundeuva* foram isoladas por cromatografia em coluna de quitina com elevada atividade hemaglutinante e em quantidade suficiente para realização dos bioensaios propostos;
- O inibidor de tripsina de entrecasca de *S. terebinthifolius* (SteBI) foi isolado por cromatografia em coluna de quitina com elevada atividade inibidora de tripsina.
- SteLL, uma lectina ligadora de quitina purificadaem quantidades miligramas, mostrou atividade antimicrobianacontra bactérias e fungos de importância médica.
- O estudo confirma a ação antimicrobiana da infusão de entrecasca *S. terebinthifolius*, usada pela população no tratamento de infecções genito-urinárias, e identifica SteBL e fenóis como agentes bacterianos e SteBI como fungicida.
- Lectinas de *M. urundeuva* foram ativas contra patógenos de humanos e peixes com efeitos no crescimento, sobrevivência, aderência e invasão de bactérias em células humanas e de peixes.
- A afinidade das lectinas por N-acetilglicosamina, componente da quitina presente em paredes celulares de fungos e bactérias, pode estar relacionada à ação antimicrobiana.
- Tecidos de planta contém metabólitos secundários, lectinas e inibidores de tripsina com atividade antibacteriana e antifúngica, sendo fonte de moléculas bioativas no controle de patógenos que causam doenças em plantas e animais, incluindo os humanos.