VANESSA PASSOS BRUSTEIN

# CARACTERIZAÇÃO DA LECTINA DE *Eugenia malaccensis* L. (EmaL): AVALIAÇÃO DE ATIVIDADES BIOLÓGICAS

RECIFE, 2006

**UNIVERSIDADE FEDERAL DE PERNAMBUCO**

**CENTRO DE CIÊNCIAS BIOLÓGICAS**

**DEPARTAMENTO DE BIOQUÍMICA**

**MESTRADO EM BIOQUÍMICA**

**VANESSA PASSOS BRUSTEIN**

# CARACTERIZAÇÃO DA LECTINA DE *Eugenia malaccensis* L. (EmaL): AVALIAÇÃO DE ATIVIDADES BIOLÓGICAS

Dissertação apresentada ao Mestrado em Bioquímica do Centro de Ciências Biológicas da Universidade Federal de Pernambuco, UFPE, como requisito para obtenção do título de Mestre em Bioquímica.

**Orientadora:** Profa. Dra. Maria Tereza dos Santos Correia

**Co-Orientadora:** Profa. Dra. Luana Cassandra Breitenbach Barroso Coelho

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**FICHA DE APROVAÇÃO**

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**Orientador:**

**Dra. Maria Tereza dos Santos Correia**

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**(UNIVERSIDADE FEDERAL DE PERNAMBUCO)**

**Examinadores:**

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2 Examinador

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3 Examinador

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Data de aprovação: 23 de fevereiro de 2006

Ata da defesa de dissertação da Mestranda **Vanessa Passos Bustein,** realizada em 23 de fevereiro de 2006, como requisito final para obtenção do título de Mestre em Bioquímica.

Às 14:10 horas, do dia 23 de fevereiro de 2006, foi aberto, no Auditório Prof. Marcionilo Barros Lins, Depto. de Bioquímica/CCB/UFPE, o ato de defesa de dissertação da mestranda **Vanessa Passos Bustein**, aluna do Curso de Mestrado em Bioquímica/CCB/UFPE. Iniciando os trabalhos a Profa. Dra. Patrícia Maria Guedes Paiva, Vice-Coordenadora do Curso supra citado, em substituição a Profa, Vera Lúcia de Menezes Lima, Coordenadora, por motivo de saúde, fez a apresentação da aluna, de sua orientadora Profa. Dra. Maria Tereza dos Santos Correia, de sua Co-orientadoras, Profa. Dra. Luana Cassandra B.B. Coelho e Profa. Dra. Maria das Graças C. da Cunha, e da Banca Examinadora composta pelos professores doutores: Maria Tereza dos Santos Correia, na qualidade de Presidente, Eduardo Isidoro Carneiro Beltrão, ambos do Depto. de Bioquímica/CCB/UFPE, Adriana Carla Cavalcante Malta Argolo, Pesquisadora do Depto. de Bioquímica/CCB/UFPE e Maria Inës Sucupira Maciel, do Depto. de Ciências Domésticas/UFRPE. Após as apresentações, a Profa. Dra. Patrícia Maria Guedes Paiva passou a palavra a Presidente da Banca que convidou a aluna para a apresentação de sua dissertação intitulada: **"Caracterizacão da Lectina de *Eugenia malaccensis* L. (LmaL): Avaliacão de Atividades Biológicas",** e informou que de acordo com o Regimento Interno do Curso, a candidata dispõe de até 50 (cinqüenta) minutos para apresentação do trabalho e o tempo de argüição para cada examinador, juntamente com o tempo gasto pelo aluno para responder às perguntas será de 30 (trinta) minutos. A aluna procedeu a explanação e comentários acerca do tema em 30 minutos. Após a apresentação da mestranda, a Sra. Presidente convidou a Banca Examinadora para ocupar seus lugares e passou a palavra ao primeiro examinador, Profa. Dra. Maria Inës Sucupira Maciel que agradeceu o convite, fez alguns comentários e deu algumas sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Da[i a Sra. Presidente passou a palavra para a Profa. Dra. Adriana Carla Cavalcante Malta Argôlo, que agradeceu o convite, fez alguns comentários e deu algumas sugestões, iniciando sua argüição. Ao final, a referida professora deu-se por satisfeita. Em seguida, a Sra. Presidente passou a palavra para o Prof. Dr. Eduardo Isidoro Carneiro Beltrão, que agradeceu o convite, fez alguns comentários e sugestões, e iniciou sua argüição. Ao final, o referido professor deu-se por satisfeito. Em seguida, a Sra. Presidente passou a palavra as Co-orientadoras Profa. Dra. Luana Cassandra B,B, Coelho, que agradeceu o convite e teceu palavras elogiosas ao trabalho apresentado, e em seguida, tendo sido passada a palavra para a Profa. Dra. Maria das Graças C. da Cunha, a mesma apresentou suas congratulações pelo relevante trabalho, extensivo também à orientadora e, finalizando a Sra. Presidente usou da palavra para tecer alguns comentários, agradecer à Banca Examinadora e parabenizar a candidata. Finalmente, a sessão foi suspensa para proceder ao julgamento pela Banca Examinadora, a qual se reuniu na Secretaria do Curso. Após alguns comentários, a Banca decidiu, por unanimidade, conceder a menção "**Aprovada por distinção".** Nada mais havendo a tratar, lavrei a presente ata que vai assinada por mim, Secretário, e demais membros da Banca Examinadora. Recife, 23 de fevereiro de 2006.

Nascestes no lar que precisava;

Vestistes o corpo físico que merecias;

Moras onde melhor Deus te proporcionou;

De acordo com teu adiantamento;

Possuis os recursos financeiros coerentes de acordo com as tuas necessidades, nem mais, nem menos, mas o justo para as tuas lutas terrenas;

Teu ambiente de trabalho é o que elegeste espontaneamente para a tua realização;

Teus parentes, amigos são as almas que atraíste, com tua própria afinidade;

Portanto, teu destino está constantemente sob teu controle;

Tu escolhes, recolhes, eleges, atrais, buscas, expulsas, modificas tudo aquilo que te rodeia a existência;

Teus pensamentos e vontade são a chave de teus atos e atitudes...são as fontes de atração e repulsão na tua jornada e vivência;

Não reclames nem te faças de vítima. Antes de tudo, analisa e observa;

A mudança está em tuas mãos;

Reprograma tua meta;

Busca o bem e viverás melhor;

Embora ninguém possa voltar atrás e fazer um novo começo, qualquer um pode começar agora e fazer um Novo Fim.

Chico Xavier

Um trabalho que temos a graça e a oportunidade de fazer é nossa realização. Dedicar a alguém é demonstrar, reconhecer que eles também ajudaram de algum modo. A Deus pela dádiva da vida e todas as graças alcançadas. Aos meus pais, Solon Sales Passos e Feiga Brustein, meus irmãos, e meu namorado Natan pela torcida e apoio constantes.

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# RESUMO

Lectinas são proteínas ou glicoproteínas de origem não imune cuja ligação reversível a carboidratos resulta em aglutinação celular. A *Eugenia malaccensis L.* pertence à família Mirtaceae. Uma lectina de sementes de *E. malaccencis*, EmaL, foi purificada usando fracionamento com sulfato de amônio (F 0-80), seguida por cromatografia de afinidade em Sephadex G-50. A atividade hemaglutinante (AH) de EmaL foi avaliada em presença de soluções de íons ($Ca^{2+}$ e $Mg^{2+}$), de soluções de carboidratos e glicoproteínas, diferentes valores de pH (2 – 12) e tratamento com diferentes temperaturas. As massas moleculares da proteína nativa e de suas subunidades foram determinadas pelo sistema ÄKTAFPLC em coluna Sephacryl S-300 e por SDS-PAGE, respectivamente. A atividade antimicrobiana de EmaL foi avaliada com amostras de bactéria Gram-positivas e Gram-negativas pela metodologia de difusão em disco. A cromatografia em Sephadex G-50 apresentou um único pico (EmaL) após eluição com glicose, resolvido em três picos protéicos de 112, 28 e 14 kDa pela cromatografia em Sephacryl S-300 e uma banda de 14 kDa por SDS-PAGE. A AH de EmaL é totalmente inibida por glicose, caseína, ovoalbumina e fetuína, não é dependente de íons, é dependente de pH e é totalmente perdida após aquecimento a 30 °C. EmaL inibe o crescimento de bactérias Gram positivas e negativas; o melhor resultado (26.5 mm ± 1.2 halo) foi obtido com *Staphylococcus aureus*, apresentando uma concentração mínima inibitória (CMI) de 1,5 μg/ml e uma concentração mínima bactericida (CMB) de 15 μg/ml. Um nova lectina (EmaL) glicose específica foi obtida, com potencial uso como agente antimicrobiano e de amplo espectro de ação.

# ABSTRACT

Lectins are proteins or glycoproteins of non immune origin which reversible and specific carbohydrate binding results in cellular agglutination. *Eugenia malaccensis* L. belongs to the Myrtaceae family. A lectin from *E. malaccensis* seeds, EmaL, was purified using ammonium sulphate fractionation (F0-80), followed by affinity chromatography in Sephadex G-50 column. Hemmaglutinating activity (HA) was evaluated in presence of ions ($Ca^{2+}$ and $Mg^{2+}$), carbohydrates, glycoproteins, or after treatment with different temperatures and pH values (2 – 12). Antimicrobial activity of EmaL was investigated by the disc diffusion method against Gram-positive and Gram-negative bacteria. Chromatography in Sephadex G-50 column showed a protein peak biospecific eluted using glucose, exhibiting three protein peaks whith molecular masses of 112, 28 and 14 kDa by gel filtration using a ÄKTA*FPLC* system. The purified lectin showed a main protein band in SDS-PAGE (14 kDa). EmaL HA is totally inhibited by glucose, casein, ovoalbumine and fetuin, is not dependent of ions, is dependent of pH and is totally reduced by heating at 30 °C. EmaL inhibited growth of tested microorganisms; best result (26.5 mm ± 1.2 halo) was obtained with *Staphylococcus aureus*, with minimal inhibitory concentration (MIC) of 1.5 μg/ml and minimal bactericide concentration (MBC) of 15 μg/ml. In conclusion, EmaL is a powerful antimicrobial agent of low cost with wide spectrum of action.

# INTRODUÇÃO

## 1. LECTINAS

### *1.1 Generalidades*

O estudo de lectinas teve início no século IX com a descoberta de que extratos de certas plantas além de serem tóxicos para homens e animais poderiam também aglutinar eritrócitos. Acreditava-se que este efeito tóxico ocorria devido à contaminação por toxinas bacterianas. Esta hipótese foi desacreditada quando Bruylants e Vennerman em 1884 demonstraram que a toxicidade da semente de *Abrus precatorius* devia-se a uma fração protéica que podia ser precipitada com álcool a partir de um extrato aquoso da semente (Moreira *et al.*, 1991).

Hermann Stilmark em 1988, observou pela primeira vez que a ricina, proteína extraída da planta *Ricinus communis*, era responsável pela hemaglutinação de diferentes espécies de eritrócitos e ampliou o estudo de aplicações destas proteínas (Gabor *et al.*, 2001). Na década seguinte, Ehrlich utilizou a ricina e a abrina, esta última, uma proteína obtida da planta *A. precatorius*, ambas com atividade hemaglutinante (AH), como potencial modelo antigênico (Sharon e Lis, 1988).

As proteínas que aglutinavam eritrócitos em plantas foram inicialmente denominadas aglutininas, hemaglutininas, fitoaglutininas ou fitohemaglutininas (Sharon e Lis, 1988).

O termo lectina (originado do latim, *"lectus"*) foi proposto por Body e Shapleigh (1954) em virtude da habilidade de algumas proteínas em ligar-se a

carboidratos, aglutinando seletivamente eritrócitos de um grupo sangüíneo particular (Matsui *et al.*, 2001). Lectinas são proteínas ou glicoproteínas que possuem a habilidade de se ligar especificamente a mono ou oligossacarídeos de forma reversível (Sato *et al.*, 2000; Hong *et al.*, 2001; Souza *et al.*, 2001). As lectinas constituem um grupo heterogêneo de proteínas de origem não imunológica de distribuição ubíqua na natureza, contendo dois ou mais sítios de ligação a carboidrato e sua caracterização físico-química é importante para explicar seu comportamento em diferentes processos biológicos (Sharon e Lis, 2001).

A ênfase que é dada quanto à origem não imune das lectinas serve para distingui-las de anticorpos anticarboidratos que aglutinam células. Enquanto os anticorpos são estruturalmente similares, as lectinas diferem entre si quanto à composição aminoacídica, requerimentos de metais, peso molecular e estrutura tridimensional. Além disso, as lectinas não são apenas encontradas em animais, mas também em outros organismos que não possuem sistema imune, como plantas e bactérias (Moreira *et al.*, 1991).

As lectinas estão amplamente distribuídas na natureza. No reino das plantas, as sementes de leguminosas são a principal fonte de lectinas, mas estas também são abundantes em outros tecidos vegetais tais como: raiz, folha, talo, vagem, frutas, flores e até mesmo casca (Coelho e Da Silva, 2000; Ratanapo *et al.*, 2001; Kim *et al.*, 2003). A maior quantidade de lectinas de plantas é encontrada nos órgãos de estoque, nas outras partes da planta as quantidades são bem menores, não sendo necessariamente idênticas em relação à estrutura ou especificidade de carboidratos com as lectinas dos órgãos de estoque. Dependendo da planta, os órgãos de estoque apresentam diferentes localizações como, por exemplo, nas leguminosas, estes órgãos

encontram-se nas sementes e, na batata, encontram-se no tubérculo (Rüdiger, 1998).

O papel fisiológico das lectinas de plantas não está claramente definido, mas o crescente estudo sugere que lectinas são proteínas de defesa que podem protegê-las contra ataques de predadores como vírus, fungos e insetos (Cavada *et al.*, 1998; Ratanapo *et al.*, 2001; Sacchettini *et al.*, 2001). Existem várias outras hipóteses sobre o papel fisiológico das lectinas de plantas como, por exemplo, reconhecimento celular, simbiose, estoque de proteínas (Van Damme *et al.*, 1997) e também na estimulação da proliferação e crescimento celular da planta (Wititsuwannakul *et al.*, 1998). O papel das lectinas nos fungos continua desconhecido (Kawagishi *et al.*, 2001), para bactérias e protozoários foi sugerido que estas lectinas têm uma função importante facilitando sua adesão no epitélio intestinal. Para as lectinas de vírus foi sugerido que em humanos elas se ligam a eritrócitos e outras células pelo reconhecimento do ácido N-acetilneuramínico presente na superfície celular e que esta ligação é um pré-requisito para o início da infecção (Singh *et al.*, 1999).

## ***1.2 Detecção e Especificidade***

O ensaio de hemaglutinação (Figura 1 a) é usado rotineiramente para a detecção da presença de lectinas em uma fonte biológica (Sharon e Lis, 2001). Este ensaio é realizado através de uma diluição seriada da lectina e posterior incubação com eritrócitos (Coelho e Da Silva, 2000). Os eritrócitos utilizados podem ser de humanos ou de animais, onde estes podem ser tratados enzimaticamente (tripsina, papaína, entre outras) ou quimicamente

(glutaradeído ou formaldeído) aumentando ou não a sensibilidade das células a lectina (Correia e Coelho, 1995; Coelho e Da Silva, 2000; Mo *et al.*, 2000). Para assegurar que o agente aglutinante é uma lectina, são necessários ensaios subseqüentes de inibição da AH utilizando uma solução do carboidrato ligante (Cavada *et al.*, 2000; Kawagishi *et al.*, 2001), sendo as reações de aglutinação por lectinas inibidas por seus carboidratos específicos (Figura 1 b).

A determinação da especificidade de uma lectina é dada pelo monossacarídeo que, em menor concentração, possua maior habilidade para inibir sua atividade de hemaglutinação ou de precipitação de polissacarídeos ou glicoproteínas (Gabor *et al.*, 2001; Ng e Yu, 2001). As lectinas podem se ligar a açúcares livres ou a resíduos de açúcares de polissacarídeos, glicoproteínas ou glicolipídeos, onde estes podem estar livres ou ligados à membrana da célula.

Existem lectinas que possuem especificidade para mais de um carboidrato, aglutinando células de diferentes espécies. Também existem lectinas que só aglutinam as células em que houver a presença de um determinado carboidrato (Gabor *et al.*, 2001). Peumans e Van Damme (1998) observaram que as lectinas de plantas exibem uma ampla especificidade para carboidrato, sendo que muitas apresentam maior afinidade para oligossacarídeos do que para açucares simples ou têm especificidade direcionada contra glicanos estranhos (que não são próprios da planta), além disto, lectinas estruturalmente diferentes podem reconhecer o mesmo carboidrato.

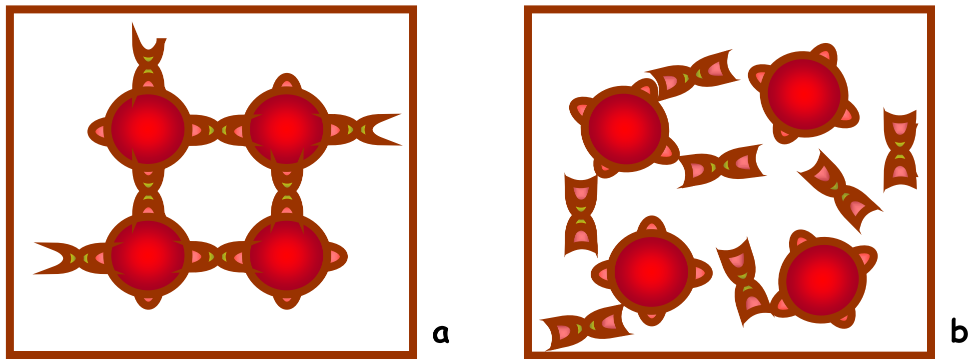


Figura 1. Atividade hemaglutinante (a) e inibição da atividade hemaglutinante (b).

### *1.3 Classificação*

Por representar um grupo heterogênio de proteínas, as lectinas diferem fortemente em relação à: estrutura molecular, especificidade ao carboidrato e atividades biológicas.

Existem vários critérios de classificação de lectinas, como por exemplo, elas podem ser agrupadas dentro de famílias distintas de proteínas homólogas que apresentam propriedades estruturais comuns, sendo a família das leguminosas a mais bem estudada e caracterizada (Sharon, 1993; Cavada *et al.*, 1998). As lectinas de planta podem, ainda, ser classificadas de acordo com sua especificidade de interação com carboidratos em: lectinas glicose/manose específicas (Correia e Coelho, 1995; Nomura *et al.*, 1998), galactose específicas (Coelho e Da Silva, 2000), ácido-siálico específicas (Bhowal *et al.*, 2005), manose-específicas (Koike *et al.*, 1995), entre outras.

## *1.4 Purificação e Caracterização Estrutural*

Para a purificação de lectinas em diferentes fontes, tem sido realizada, como primeiro passo, a preparação de extratos com solução salina ou tampão (Kawagishi *et al.*, 2001; Mladenov *et al.*, 2002). Após a extração, muitos extratos com atividade lectínica são submetidos a purificações parciais através de técnicas convencionais para proteínas, incluindo fracionamento salino com sulfato de amônio e diálise exaustiva (Coelho e Da Silva, 2000; Yeasmin *et al.*, 2001). O uso de técnicas cromatográficas é bastante comum para a purificação de lectinas. Dentre estas técnicas, a cromatografia de afinidade é a mais utilizada na purificação, embora, as vezes, seja necessária a utilização de outros métodos cromatográficos para que se possa ter um elevado grau de pureza (Correia e Coelho, 1995; Sampietro *et al.*, 2001).

A caracterização de lectinas envolve vários métodos, tais como técnicas eletroforéticas (Laemmli, 1970), que servem para indicar basicidade ou acidez de uma lectina, assim como para determinar sua estrutura quanto ao número de subunidades, massa molecular (Correia e Coelho, 1995) ou ainda para caracterizá-la como glicoproteína através da coloração com reativo de Shiff (Coelho e Silva, 2000).

Testes para a determinação da estabilidade térmica são importantes para delinear a capacidade de lectinas de suportar determinadas temperaturas, mantendo sua atividade biológica, e para indicar as condições térmicas ideais para se trabalhar com a molécula, de forma que esteja apresentando sua melhor atividade na interação com carboidratos. Também, testes quanto a modificações de pH e de soluções tampão são importantes para

a determinação da estabilidade das moléculas, quanto à sua propriedade de ligação a carboidratos e glicoconjugados.

Outro ensaio importante na caracterização é a determinação da dependência ou não de tais moléculas por íons metálicos porque, algumas lectinas precisam da presença destes íons para promover sua atividade biológica (Konozy *et al.*, 2003), outras não.

A imunodifusão dupla tem sido bastante utilizada para a caracterização de soros antilectinas, servindo igualmente para estudos preliminares de homologia entre lectinas de mesma espécie ou de espécies diferentes (Ashford *et al.*, 1982). O sequenciamento aminoacídico é outra ferramenta valiosa no estudo das moléculas, como foi verificado para a lectina de *Erytrina indica*, sendo verificado que modificações no aminoácido tirosina desta lectina causam uma inativação parcial da sua função (Konozy *et al.*, 2003). O sequenciamento da lectina de *Cratylia mollis* forneceu informações a cerca de sua estrutura terciária (De Souza *et al.*, 2003).

### *1.5 Aplicações*

As lectinas mostram ser importantes ferramentas para a investigação em diversos processos médicos, químicos e biológicos (Karasaki *et al.*, 2001, Ohba *et al.*, 2003). A distribuição de um grande número de lectinas com diferentes especificidades para carboidratos tem levado à sua utilização como reagentes para explorar carboidratos, sendo este, o ponto mais importante no avanço de numerosas áreas da biologia celular. A aplicação das lectinas pode ser ampla e variada, devido as suas propriedades biológicas, como por exemplo, na investigação estrutural e funcional de carboidratos complexos,

especialmente glicoproteínas, na observação de mudanças que ocorrem na superfície celular durante os processos fisiológicos e patológicos, desde a diferenciação celular ao câncer (Sharon e Lis, 2001), na avaliação de toxicidade para células e animais, bem como no efeito imunossupressor *in vivo*. Em estudos recentes, com lectinas, foi observada: a indução de apoptose em tumores de células humanas (Karasaki *et al.*, 2001); interação com células Du-145 do câncer de próstata (Gabor *et al.*, 2001); aglutinação de células bacterianas (Gaidamashvili *et al.*, 2002, Tasumi *et al.*, 2004); inibição da proliferação de fibroblastos oculares e contração de colágeno (Batterbury *et al.*, 2002); produção dos chamados medicamentos inteligentes, onde estes diferem dos tradicionais por atuarem em células específicas do organismo evitando efeitos colaterais, do tipo provocado pela quimioterapia (Woodley *et al.*, 2001); atividade mitogênica (Banerjee *et al.*, 2004); além de atividade antimicrobiana. A afinidade das lectinas por glicoproteínas de superfície celular tem sido usada para a caracterização epidemiológica da *Neisseria gonorrhoeae* e diferenciação de outras espécies de *Neisseria* (Wu *et al.*, 2001). Vários trabalhos mostraram que determinadas bactérias produzem lectinas específicas para certos carboidratos, e fazem uso das mesmas para se aderir ao tecido hospedeiro como primeiro passo em um processo infeccioso. Ao submeter o organismo infectado com a bactéria a injeções de carboidratos, a colonização é reduzida devido à diminuição de sua adesão ao tecido, sendo o bloqueio aos locais de ataque das bactérias, um caso claro de terapia antiadesiva contra doenças microbianas. Esta forma de aplicação das lectinas é alvo de intensas pesquisas pelas indústrias farmacêuticas como método contra infecções (Sharon e Lis, 1993, Rudiger *et al.*, 2000). Ainda, lectinas de diferentes especificidades foram imobilizadas em suportes inertes

e usadas como matriz de afinidade para fins bastante variados (Lima *et al.*, 1997, Ohba *et al.*, 2003, Bakalova e Ohba, 2003).

### 1.6 *Eugenia malaccensis*

A espécie *Eugenia malaccensis* L. (sinonímia *Syzygium malaccense* Merr. & Perry; Figura 2) pertence à família Myrtaceae. Conhecida popularmente no Brasil como jambo vermelho, jambo roxo e jambo encarnado, faz parte das plantas considerada medicinais, sendo utilizada principalmente pelas populações de baixo poder aquisitivo (Campelo, 1988).

O jambeiro (que produz o jambo) é uma árvore conhecida e bem adaptada às condições do nordeste do Brasil. Chega a mais de 16 m de altura, com copa de forma cônica, densa e com ramificação abundante. Possui folhas de cor verde-brilhante, flores grandes, aromáticas, que quando caem, formam sob as árvores um lindo tapete purpúreo, e podem variar de brancas a róseo-purpúreas de acordo com a espécie. O jambo é uma fruta de aparência exótica, de casca bem fina, forma ovóide, vermelho por fora e muito alvo por dentro, tem um sabor doce e a polpa, apesar de consistente, é muito suculenta e envolve semente globosas. O jambeiro é reconhecido como uma árvore de muita beleza e boa sombra, desenvolve-se em qualquer tipo de solo, desde que permeáveis e profundos. É cultivado em quase todo Brasil, em regiões de clima quente e úmido. A propagação se dá por sementes. Pode produzir por mais de 20 anos, frutificando de janeiro a maio. Estima-se ocorrer grandes perdas de jambo na época da safra, em virtude da alta produtividade, do curto período da safra e da pequena vida útil do fruto *in natura*.

Em diversas partes do mundo tem sido empregado na medicina popular para o tratamento de coceira, diabete, catarro no pulmão, tosse, dor de cabeça, inflamações e hipertensão (Morton, 1987). Estudos realizados no Departamento de Medicina Tropical da Escola de Medicina do Havaí, comprovaram a atividade antiviral seletiva do extrato aquoso do caule de *E. malaccensis* frente ao vírus Herpes Simplex-1 (HSV-1) e vírus Herpes Simplex-2 (HSV-2), da Estomatite Vesicular e HIV-1 (do inglês *human immunodeficiency vírus*, sorotipo I). O extrato aquoso das folhas atuou ainda como inibidor do crescimento do *Staphylococcus aureus* e do *Streptococcus pyogenes*. (Locher *et al.*, 1995; 1996).

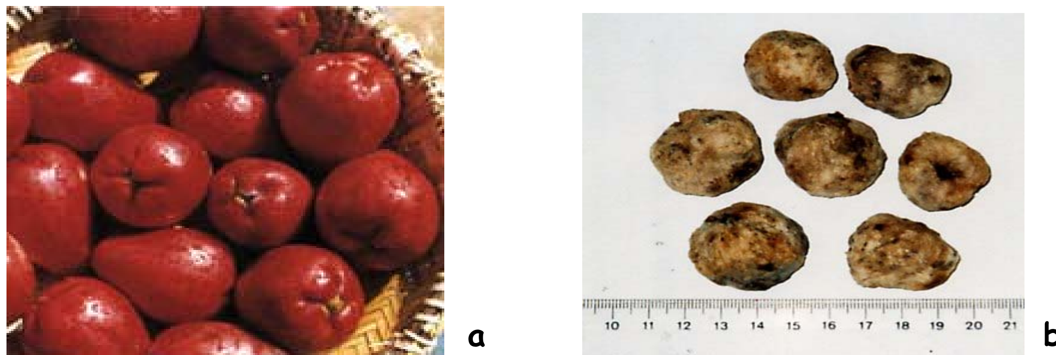


Figura 2. Visão geral da planta *Eugenia malaccensis*: Frutos (a) e sementes (b).

## 2. ATIVIDADE ANTIMICROBIANA

Uma substância apresenta efeito antimicrobiano quando o microrganismo responsável pela infecção é sensível a ela. Então o

microrganismo é considerado sensível a um antimicrobiano quando o seu crescimento é inibido "*in vitro*" por uma concentração três ou mais vezes inferior àquela que o antimicrobiano atinge no sangue. Se a concentração inibitória é igual ou superior àquela que o antimicrobiano atinge no sangue, o microrganismo é considerado resistente (Trabulsi, 1991).

Muitas substâncias, inclusive proteínas, estão sendo avaliadas quanto ao seu efeito antimicrobiano. As proteínas antimicrobianas, em animais, constituem parte do sistema imume inato. Peptídeos e pequenas proteínas com atividade antimicrobiana são usados contra inúmeros microrganismos perigosos. Por causa dos distintos mecanismos de ação, houve um crescente interesse no uso de peptídeos e proteínas antimicrobianos como antibióticos para o controle de patógenos (Wang *et al.*, 2002). Em plantas as proteínas antimicrobianas estão envolvidas com mecanismo de defesa (Lee *et al.*, 2002).

A disponibilidade de um grande número de lectinas com diferentes especificidades para carboidratos tem levado à sua extensiva utilização como reagentes para estudar carboidratos simples e complexos em solução e sobre superfícies celulares (Lis e Sharon, 1986). A lectina de *Canavalia ensiformis*, concanavalina A (Con A) demonstrou aglutinação para certas espécies de micobactérias, sendo específica para a $\alpha$-arabinogalactona presente na superfície de *Mycobacterium bovis* (Goldstein *et al.*, 1970). A capacidade das lectinas em aglutinar as bactérias tem por finalidade estudar a constituição sacarídica da superfície de bactérias, para a tipagem de bactérias e para a determinação de receptores para bacteriófagos (Archibald *et al.*, 1972).

Conseqüentemente as lectinas são moléculas que atuam no sistema de defesa imunológico desde que elas podem seqüestrar várias bactérias, outros

microinvasores celulares, bem como substâncias que eles secretam (Yeaton *et al.*, 1981). A opsonização que ocorre quando a lectina se liga com a bactéria, é considerada a primeira etapa que promove a aderência, ingestão e subseqüentemente a degradação do microrganismo. Considerando sua especificidade para o conteúdo de carboidratos na estrutura, muitas análises têm caracterizado os componentes estruturais das bactérias pelo uso de lectinas que possuem especificidade para açúcares neutros. Apenas poucas investigações têm mostrado a especificidade de lectinas para ácido siálico (Doyle *et al.*, 1994).

Muitas lectinas de planta têm sido estudadas por suas interações com várias bactérias e a específica simbiose entre plantas e bactérias. No entanto pouco tem sido sugerido sobre a ação em reduzir a infectividade das bactérias patogênicas. Algumas lectinas como, lectina 1 de *Anguilla japonica* (AJL-1) apresentou atividade aglutinante frente à bactéria patogênica *Streptococcus difficile*, atuando como fator de defesa (Tasumi *et al.*, 2004). Uma lectina manose-dependente isolada do soro de peixe (salmão) do Atlântico teve efeito antibacteriano contra a bactéria Gram-negativa patógena, *Aeromonas salmonicida,* através da associação com macrófagos (Ottinger *et al.*, 1999) e outra lectina de peixe (*Trichogaster trichopterus*) associada a macrófagos, também, apresentou ação antibacteriana frente a *Aeromonas hydrophila* (Fock *et al.*, 2001). Lectinas parcialmente purificadas a partir de sete plantas medicinais do Sul da África foram avaliadas quanto ao efeito antibacteriano frente às bactérias *Staphylococcus aureus e Bacillus subitilis* através de método de aglutinação, apresentando efeito inibitório no crescimento das mesmas (Gaidamashvili e Staden, 2002). Con A, aglutinina da *Bauhinia purpurea* (BPA), aglutinina da *Lens culinaris* (LCA),

aglutinina de *Germem de trigo* (WGA) bloquearam a adesão dos conídios de *Colletotrichum graminicola*, indicando que o material associado à aderência do conídio é composto de glicoproteínas (Mercure *et al.*, 1995).

Um peptídeo catiônico isolado de sementes de *Robinia pseudoacacia* foi testado contra sete bactérias (*Corynebacterium michiganense*, *Staphylococcus aureus*, *Bacillus subitilis*, *Erwinia carovora subsp Carotovora*, *Pseudomonas syringae pv syringae*, *Xanthomonas campestris pv campestris* e *Escherichia coli*). O peptídeo inibiu a maioria das cepas testadas, sendo que o *Staphylococcus aureus* demonstrou ser o mais sensível para o peptídeo (Talas-Ogras *et al.*, 2005). Também a lectina de *Morus alba* (MLL1) foi estudada por sua ação antibacteriana contra *Pseudomonas syringae pv syringae*, onde a MLL1 induziu a aglutinação de *P. syringae pv mori*, sendo inibida por N-glicoilneuranímico, N-acetilgalactosamina e mucina de submaxilar bovino (Ratanapo *et al.*, 2001).

# OBJETIVOS

## Objetivo Geral

Purificação e caracterização da lectina de sementes de *Eugenia malaccensis* L. (EmaL), bem como avaliação da atividade antimicrobiana.

## Objetivos Específicos

Avaliação da atividade hemaglutinante em extratos de sementes de *E. malaccensis* para a extração de lectinas;

Purificação da lectina de sementes de *E. malaccensis* (EmaL) utilizando processos cromatográficos convencionais e de alta resolução;

Avaliação da atividade hemaglutinante de EmaL frente a diferentes temperaturas, íons e a variações de pH;

Determinação da especificidade de EmaL para carboidratos e glicoproteínas;

Caracterização de EmaL utilizando métodos eletroforéticos;

Avaliação da atividade antimicrobiana de EmaL através do teste de difusão em disco;

Determinação da concentração mínima inibitória (CMI) e da concentração mínima bactericida (CMB);

Determinação da concentração mínima aglutinante (CMA).

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## ARTIGO SUBMETIDO AO PERIÓDICO BIOLOGICALS

# PURIFICATION AND CHARACTERIZATION OF *Eugenia malaccensis* SEED LECTIN WITH ANTIMICROBIAL ACTIVITY

# PURIFICATION AND CHARACTERIZATION OF *Eugenia malaccensis* SEED LECTIN WITH ANTIMICROBIAL ACTIVITY

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## Abstract

Lectins, carbohydrate binding proteins of non immune origin, agglutinate cells and glycoconjugates. A lectin from *Eugenia malaccensis* (EmaL) was purified from a 10% (w/v) seed extract in 0.15 M NaCl. In brief, proteins were precipitated using ammonium sulphate fractionation (F0-80), followed by affinity chromatography in Sephadex G-50 column to obtain EmaL. The purified protein showed a main polypeptide band in SDS-PAGE (14 kDa) in the presence of 2-mercaptoethanol and agglutinated human as well as animal erythrocytes. EmaL exhibited three protein peaks whith molecular masses of 112, 28 and 14 kDa by gel filtration using an ÄKTA*FPLC* system. The lectin activity was stimulated under pH values of 2, 3 and 7 while was completely inactivated at pH 10 to 12. The activity was also totally reduced by heating at 30 °C for 30 min. EmaL HA was totally inhibited by casein, ovoalbumine and fetuin. The antimicrobial activity of EmaL was investigated by disc diffusion method using Nutrient Agar (NA). Warmed medium (100 ml, 43˚C) and inoculum (0.5 ml) were added; the solution was distributed in sterile Petri plates in portions of 10 ml and allowed to solidify. Afterwards, 15 µl of lectin solution were impregnated on sterile paper discs (ϕ = 6 mm) and placed on agar. EmaL inhibited growth of tested microorganisms; best result (26.5 mm ± 1.2 halo) was obtained with *Staphylococcus aureus*, with minimal inhibitory concentration (MIC) of 1.5 µg/ml and minimal bactericide concentration (MBC) of 15 µg/ml. In conclusion, EmaL was a powerful antimicrobial agent of low cost with wide spectrum of action.

*Keywords*: *Eugenia malaccensis*, Lectin Characterization, Lectin Purification, Antibacterial Lectin, Gram-negative bacteria, Gram-positive bacteria.

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## Introduction

Lectins are proteins or glycoproteins with ubiquitous distribution in nature, which have at least one carbohydrate or derivative binding site without catalytic function or immunological characteristic [1], could have one additional hydrophobic site [2] and bind reversibly to mono or oligossacharides of eukaryotic glycoconjugates [3]. They are purified from different species [4, 5, 6, 7] and in plants, they are mainly obtained from legume seeds [8], but could also be obtained from different vegetative tissues such as leaf [9], bulb [10], bark [11], root [12] and pod [13]. The interactions of plant lectins with human pathogenic bacteria have been extensively studied [14, 15]. These proteins have been purified and characterized mainly from mature seeds of leguminous, not only as a single molecule but also as multiple molecular forms [16, 17].

The ability of plant lectins to react when exposed to carbohydrates in the microorganism surfaces has promoted the identification of pathogenic bacteria based on the reaction of selective agglutination between lectins and bacteria [18, 19, 20, 21]. The study of lectin interaction with cellular wall carbohydrates from Gram-positive and Gram-negative bacteria and specialized forms, can demonstrate the binding capacity of these proteic molecules to a wide variety of complex carbohydrates such as teicoic acid, teicuronic acids, peptidoglycans and lipopolysaccharides present in cellular walls [22].

*Eugenia malaccensis* L. (Mirtaceae family) is a tree very common in tropical climate regions, abundant in Brazil, with large quantity of fruits (*jambo*) easily croped; this plant has been used to treat hypertension, diabetes, sore throat, cuts, thrush, venereal disease, tuberculosis and digestive tract disorders [23, 24]. Extracts of *E. malaccensis* showed selective anti-viral activity against Herpes Simplex Virus-1 and 2 and Vesicular Stomatitis Virus, anti-fungal activity and anti-bacterial activity against *Staphylococcus aureus* and *Streptococcus pyogenes* [25, 26].

This work reports the purification and characterization of an antibacterial lectin (EmaL) obtained from *E. malaccensis* seeds.

## Materials and methods

### *Purification of EmaL*

The lectin was purified from a 10% (w/v) seed extract in 0.15 M NaCl (crude extract, CE). In brief, proteins were precipitated using 0-80% ammonium sulphate fractionation (F0-80), followed by affinity chromatography in Sephadex G-50 column. The column was equilibrated and developed with 0.15 M NaCl at 10 ml/h; bound proteins were biospecifically eluted using 0.3 M glucose in 0.15 M NaCl and dialyzed (EmaL). Hemmaglutinating activity (HA) was evaluated in presence of erythrocytes (human and rabbit), carbohydrates, glycoproteins, ions, or after treatment with different temperatures and pH values. Protein concentration was measured according to Lowry *et al*. [27] and samples were stored at -20 °C.

### *Hemagglutination activity and inhibition assays*

Fresh erythrocytes from human (A, B, O and AB types) and rabbit were obtained as described by Bukantz *et al*. [28], and glutaraldehyde treated according to Bing *et al*. [29]. Haemagglutinating activity (HA) was evaluated as described by Correia and Coelho [30] defined as the lowest sample dilution showing haemagglutination; specific HA (SHA) corresponded to HA divided by protein concentration. Carbohydrate binding specificity was determined by HA inhibition using several sugars (D(+)-glucose, D(+)-mannose, D(+)-lactose, methyl-α-D-mannopyranoside and methyl-α-D-glucopyranoside) and glycoproteins (bovine fetal serum, casein, tyroglobulin, ovoalbumin, fetuin, ferritin, aldolase, heparin and peroxidase from Sigma, USA) as described by Coelho and Silva [9]. Rabbit erythrocytes were chosen for subsequent assays.

### *Effect of pH, temperature and metal ions on hemagglutinating activity*

The effects of pH and temperature on HA were evaluated by incubating EmaL samples (200 μg) at different pH values for 1 h at room temperature in selected buffers (10 mM citrate phosphate buffer, pH 2 – 7 and 10 mM Tris-hydrochloric acid buffer, pH 8 - 12) or at 30, 40, 50, 60, 70, 80, 90 or 100 °C for 30 min. The effect of $Mg^{2+}$ and $Ca^{2+}$ was performed with incubation (15 min) in the same volume of a solution containing

either metal ions $CaCl_2$ and $MgCl_2$ (5, 10 and 20 mM) in 0.15 M NaCl and EmaL preparation. An aliquot (50 µl) of the mixture was distributed in microtitre plate wells and the HA was proceeded as described to inhibition assays.

***Molecular mass determination***

Molecular mass of EmaL (2 ml,1 mg/ml) was determined in a ÄKTA*FPLC* system (Pharmacia Fine Chemicals) using a Sephacryl S-300 (Pharmacia Biotech) column (120 ml, 60 x 16mm). Elution was performed with 0.5 M NaCl using 3 ml of fractions, 1 ml/min and a total volume of 168 ml. A mixture of bovine serum albumin (66 kDa), fetuin (64 kDa), ovalbumin (43 kDa), ovomucoid (28 kDa) and trypsin (25 kDa) were used as standard proteins.

***Polyacrylamide gel electrophoresis (PAGE) of denatured protein***

Denatured and reduced samples were evaluated as described by Laemmli [31]. The standard marker proteins were bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibition (20.1 kDa) and α-lactalbumin (14.4 kDa), purchased from Pharmacia Fine Chemicals (Pharmacia Biotechnology, Uppsala, Sweden). The gel was stained with silver stain according to Merril *et al*. [32].

***Double Immunodiffusion***

Immunodiffusion was carried out according to Ashford *et al*. [33] using 1 % agarose gels prepared with 0.15 M NaCl, containing 0.02 % $NaN_3$ and 0.1 M glucose. Samples (20 µg) of isolectin 1 were obtained from *Cratylia mollis* seeds (Cramoll 1) according to Correia and Coelho [30]. F 0-80 and EmaL were applied in a circular distribution, around the central well containing IgG developed against Cramoll 1 (IgG anti-Cramoll 1), allowed to diffuse for 48 h at room temperature and the precipitated lines were detected by 0.005 % (w/v) Coomassie Brilliant Blue staining.

***Test organisms***

Gram-positive bacterial strains of *Staphylococcus aureus*, *Streptococcus* sp. and *Bacillus* sp. and Gram-negative bacterial strains of *Klebsiella* sp., *Pseudomonas*

*aeruginosa*, *Corinebacterium* sp. and *Escherichia coli* were use as test organisms. The bacteria were maintained in Nutrient Agar (NA) in stationary culture and were stored at 4 °C. For agglutination studies and evaluation of antimicrobial activity of EmaL, bacteria were cultured in Nutrient Broth (NB) and incubated under permanent shaking at 37 °C overnight. The concentrations of cultures were adjusted turbidometrically at a wavelength of 600 nm to $10^5$-$10^6$ colony forming units (CFU) $ml^{-1}$.

***Antimicrobial activity assay***

Antibacterial activity of EmaL was investigated by the disc diffusion method [34]. Warm NA (100 ml, 43 ˚C) and 0.5 ml of inoculum ($10^5$-$10^6$ colony/ml) were added; the solution was distributed in sterile Petri plates (90 x 15 mm) in portions of 10 ml and allowed to solidify. Afterwards, 15 µl of sterile EmaL solution (0.2 mg/ml) in 0.15 M NaCl were impregnated on sterile paper discs 6 mm diameter and placed on agar; negative control disk and positive control disk containing 0.15 M NaCl and Amoxicilin (1 mg/ml), respectively. Plates were incubated at 37 ˚C for 24 h. A transparent ring around the paper disc reveals antimicrobial activity. Zones of growth inhibition around discs were measured in mm.

***Minimal inhibitory concentration (MIC) and Minimal bactericide concentration (MBC)***

Seriate dilutions of EmaL in NaCl 0.15 M were prepared and added to culture medium with $10^7$ cell/ml of the microorganisms in the exponential phase of growth to certain the MIC according to Courvalin *et al*. [35]. Samples were incubated by 24 h at 37 ˚C and a series of assay tubes (13 x 100 mm) containing 1.8 ml of NB and 0.2 ml of microorganism suspensions with 1.5 x $10^8$ colony forming units (CFU) $ml^{-1}$ (turbidity equivalent to 0.5 of McFarland scale) were prepared. To the first tube 0.2 ml of lectin (1.5 mg/ml) solution was added, resulting in a final concentration of 150 µg/ml. After homogenation, successive dilutions were preceded to obtain the same final volume of 2.0 ml in all tubes. Control tube contained NB medium and microorganism. MIC corresponds to the smallest lectin concentration capable to inhibit the visible growth of microorganism.

Minimum bactericidal concentration (MBC) was performed starting from the coming tubes MIC assay. Dilutions (1:10.000) of the content of each tube were preceded

and aliquots (10 μl) were removed and sowed in Petri plates containing NA medium. The readings were made through the count of CFU grown in plates. The low bactericidal concentration corresponds to the smallest concentration of the sample capable to reduce the number of CFU to 0.1 % from the initial inoculum [35].

***Bacterial agglutination test***

For quantitative determination of agglutinating activity the minimum agglutinating concentration (MAC), and minimum concentration of lectin which promotes bacterial aggregation, was recorded. Overnight bacterial cultures were diluted at a ratio of 1:100 with NB. Agglutination assay were performed in 96 well microtiter plates with two-fold serial dilutions of lectin in 0.15 M NaCl. To each well 100 μl of diluted bacterial suspension were added to a final volume of 200 μl. MAC was determined by visual agglutination after overnight incubation of plates at 37 °C. EmaL carbohydrate inhibition of induced microorganism agglutination was performed in microplates. The lectin (50 μl) was mixed with equal volume of diluted carbohydrate (200 mM). After incubation at room temperature for 30 min, 50 μl of microorganism were added and the mixture was left at rest for more 30 min.

## Results and Discussion

Several lectins have been purified from leguminous tissues and used in different biological applications. To search for new pure lectins is interesting to amplify the knowledge about this class of proteins as well as if they are responsible by pharmacological activity presented by plant extracts popularly used. Different *E. malaccensis* extracts used in popular medicine [23, 24], waked the interest in to isolate and purify lectins from this plant *E. malaccensis* showed to be a potential tissue to obtain lectin due to highest HA. *E. malaccensis* had broad biological activity since it inhibited four species of viruses, three species of fungus and had some effect on both the alternative and classical pathways of complement activation. In addition to inhibiting the growth of *Streptococcus pyogenes*, inhibition of the classical pathway of complement activation showed that there is a biological basis for the use of *E. malaccensis* extracts in the ethnobotanical treatment of

sore throat by killing bacteria associated whith sore throat and by reducing inflammation in the mucosa [25, 26].

Preliminary experiments indicated that D-glucose inhibited F0-80 activity, and a chromatographic step of F0-80 on Sephadex G-50 column was used to purify EmaL. Affinity chromatography showed a protein peak biospecific eluted using 0.3 M glucose (Figure 1); repetitions were performed, with the same pattern. Results revealed lectin purification with an increase of specific activity (2133.3) and a purification time of 15 (Table 1). The use of affinity chromatography for lectin purification is mainly based on the protein's ability to bind carbohydrates in a specific and reversible way. Lectins have the advantage of not modifying the compounds with which they interact, and binding is not strong and is reversible [36]. Specific lectins to glucose/mannose or their derivatives may use Sephadex with distinct exclusion limits as their matrices. The most commonly used are G-50–G-200 [37]. Concanavalin A (Con A) a lectin obtained from *Canavalla ensiformis* seeds binds to Sephadex G-50, -75, -100 and 200, but Sephadex G-50 was the most appropriate technique for purification [37].

When EmaL was chromatographed in Sephacryl three protein peaks were obtained, with 112, 28 and 14 kDa. Under denatured and reduced conditions EmaL showed only one polypeptide band with molecular mass of approximately 14 kDa (Figure 2). SDS-PAGE and gel filtration assays showed that EmaL could be a monomer of approximately 14 kDa, and each monomer aggregates/associates with each other to give 14 kDa multiple polypeptides, during or not its purification steps. Similar results were obtained to *Grifola frondosa* lectin [38]. Con A, the most known lectin, exist as dimmers (below pH 6.0) or tetrameters (above pH 7.0) of a 26.5 kDa polypeptide [39].

EmaL agglutinated rabbit and human (A, B, AB and O) erythrocytes however, rabbit erythrocytes were more intensely agglutinated (Table 2). The activity of EmaL was stimulated under pH values of 2.0, 3.0 and 7.0; the pH value interval of 10.0 – 12.0 completely inactivated the lectin (Figure 3). The reduction or inactivation was irreversible even though the pH value was returned to neutral. The activity was also totally reduced by heating at 30 °C for 30 min. Divalent tested ions, did not affect or increase activity of *E. malaccensis* lectin. A lectin from *Macrophomina phaseolina* was active between pH values of 4.0 and 10.0, with the maximum of activity when the pH ranged between 6.0 and

7.2. The hemagglutinating activity was nearly unaffected between 10 and 30 °C. Thereafter, its activity decreased gradually with the increase of temperature and became inactive after 90 °C [40].

The determination of which glycans are well-recognized by lectins is important to characterize the lectins's carbohydrate binding sites. EmaL HA was totally inhibited by monosaccharide (glucose) and glycoproteins (casein, ovoalbumine, fetuin, aldolase and bovine fetal serum); thyroglobuline, heparin and ferritin inhibited partially EmaL HA (Table 3). Isolectins from *Acacia constricta* and *Phaseolus vulgaris* were inhibited only by complex carbohydrates present in fetuin and thyroglobulin [41], glycoproteins which inhibited EmaL activity. These findings characterize EmaL and the isolectins from *Acacia constricta* and *Phaseolus vulgaris* as to belong to complex lectins's class.

IgG anti-lectins may also be used to evaluate, in a preliminary approach, the homologies among lectins of the same or distinct species [42, 43]. Immunodiffusion was performed to observe reactivity between F0-80 and EmaL against IgG anti-Cramoll; Cramoll 1 is a glucose/mannose specific lectin [30], structurally similar to Con A [44], the most studied glucose/mannose lectin. In spite of these lectins being specific for glucose/mannose crossed immunoreactivity was not observed among Cramoll 1, F0-80 and EmaL (Figure 4); Con A and Cramoll 1 showed a semi-crossed immunoreactivity. *Cratylia* belongs to the Fabaceae family, while *Eugenia* belongs to the Mirtaceae family. Rougé and Dug [45] observed crossed immunoreactivity among isolectins from *Lathyrus ochrus*, among several lectins of the *Lathyrus* genus, and belonging to the same tribe but, the reaction was negative to lectins belonging to other tribes.

EmaL (0.2 mg/ml) exhibited an antibacterial action on *S. aureus*, *Streptococcus* sp., *Bacillus* sp., *P. aeruginosa*, *E. coli*, *Corinebacterium* sp. and *Klebsiella* sp. revealing the binding ability of this lectin to bacterial wall (Figure 5). The inhibition halos obtained through the diffusion assay in disk are summarized in Table 4. The number of lectins described with antibacterial activity is relatively small [46, 47, 21, 22]. The ability of plant agglutinins to bind human bacteria and inhibit their motility and/or growth has not been related to their physiological activities. Nevertheless, the fact that agglutinins were mostly isolated from storage parts of plants, also suggests their possible contribution to plant defense mechanisms [21].

The MAC of EmaL with the tested bacteria was expressed as a degree of precipitation of the solution lectin-bacteria, in which it was read the titer of the activity in agreement with the bacterial control. Activity observed after incubation overnight indicated the minimum lectin concentration capable to agglutinate bacteria. The differences in MAC were in agreement with the types of bacteria however, in this study the largest MAC went to the bacteria *S. aureus*, *Streptococcus* sp. and *P. aeruginosa* that exhibited a MAC of 0.48 μg/ml (titer of 2048). It is known that lectins do not just bind with sugars of glycoprotein reduced terminals, but some of them, also react with internal components of carbohydrate chains or with non carbohydrates [48]. Inhibition of EmaL and bacteria agglutination using glucose showed that EmaL bound specifically to tested bacteria through surface carbohydrates (Table 5). A lectin of *Morus alba* was studied by exhibited an antibacterial activity against *P. syringae pv mori* and its agglutination was inhibited by fetuin and thyroglobulin [22].

The necessary concentrations to determined the MIC and MBC values are summarized in Table 6. EmaL exhibited a prominent antibacterial activity against Gram-positive and Gram-negative bacteria and the results are in agreement with the diffusion assay. The highest MIC values were obtained to *S. aureus* and *Streptococcus* sp. Equivalently the results indicate that EmaL have bactericide activity against tested bacteria. *Robinia pseudoacacia* seed lectin demonstrated action against *S. aureus*, *B. subtilis* and *E. coli* exhibiting MIC of 20, 40 and 120 μg/ml respectively [49], larger than the data obtained for EmaL. A lectin from the fish *Sebastes schlegeli* [50] demonstrated antibacterial action for *B. subtilis* and *E. coli* with MIC > 200 μg/ml. The mechanism of action of peptides is not very elucidated, but it has been proposed that proteins with antibacterial activity form a channel on the cell membrane and these for its time die due to exit of the cellular content, being this mechanism different from the antibiotics [49].

## Conclusions

In the present study a glucose/mannose lectin (EmaL) was obtained and purified in a unique step by affinity chromatography, immunologically distinct from glucose/mannose lectin belonging to Con A family. The results evidenced a potent antibacterial lectin in

*E. malaccensis* seeds demonstrated by *in vitro* growth inhibition of some important pathogenic bacteria. Future researches of the lectin applications obtained from a medicinal plant, in biological systems, can be of great importance for clinical microbiology and possible therapeutic actions.

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Table 1. Summary of EmaL purification.

| Sample | Volume (ml) | Total protein (vol x mg/ml) | Specific HA (titre/protein) | Purification (times) |
|---|---|---|---|---|
| Crude extract | 200 | 2208.0 | 92.7 | 1 |
| F 0-80 | 15 | 54.0 | 142.2 | 1.5 |
| (EmaL) | 10 | 0.3 | 2133.3 | 15* |

The protein was measured according to Lowry *et al*. [27]. Hemagglutinating activity (HA) was made with 2.5 % (v/v) suspension of glutaraldehyde treated rabbit erythrocytes.

* In relation to F 0-80 (5 ml) applied in Sephadex G-50 column.

Table 2. Specific hemagglutinating activity (SHA) of EmaL with different erythrocytes.

| **Erythrocytes** | **SHA** |
|---|---|
| Rabbit | 2133.3 |
| A | 1066.6 |
| AB | 533.3 |
| B | 1066.6 |
| O | 1066.6 |

Hemagglutinating activity (HA) was performed with 2.5 % (v/v) suspension of glutaraldehyde treated erythrocytes. SHA, specific HA (titer/protein).

Table 3. Inhibition of EmaL activity with glycoproteins.

| Inhibitor | SHA |
|---|---|
| Bovine fetal serum | 0 |
| Casein | 0 |
| Thyroglobulin | 533.3 |
| Ovoalbumin | 0 |
| Fetuin | 0 |
| Ferritin | 533.3 |
| Aldolase | 0 |
| Heparin | 533.3 |
| Peroxidase | 2133.3 |

Hemagglutinating activity (HA) was performed with 2.5 % (v/v) suspension of glutaraldehyde treated rabbit erythrocytes. SHA, specific HA (titer/protein), in 0.15 M NaCl was 2133.3. Peroxidase did not inhibit EmaL activity.

Table 4. Antimicrobial activity of EmaL.

| **Microorganism** | **Diameter of clearing zone (mm)*** |
|---|---|
| *Staphylococcus aureus* (+) | 26.50 ± 1.2 |
| *Streptococcus* sp. (+) | 23.00 ± 0.8 |
| *Bacillus* sp. (+) | 13.00 ± 0.8 |
| *Escherichia coli* (-) | 19.70 ± 0.9 |
| *Corinebacterium* sp. (-) | 13.75 ± 0.5 |
| *Klebsiella* sp. (-) | 12.75 ± 0.9 |
| *Pseudomonas aeruginosa* (-) | 12.00 ± 1.4 |

Gram – positive (+); Gram – negative (-) bacteria. Mean of four assays.

* Diameter of paper disc

Table 5. Agglutination and inhibition activity of EmaL on bacterial cells.

| Microorganism | MAC (μg/ml of EmaL) | Agglutination EmaL (titer$^{-1}$) | Inhibition EmaL (titer$^{-1}$)* |
|---|---|---|---|
| *Staphylococcus aureus* (+) | 0.48 | 2048 | 32 |
| *Streptococcus* sp. (+) | 0.48 | 2048 | 32 |
| *Bacillus* sp. (+) | 500 | 2 | 0 |
| *Klebsiella* sp. (-) | 3.9 | 256 | 16 |
| *Pseudomonas aeruginosa* (-) | 0.48 | 2048 | 64 |
| *Corinebacterium* sp. (-) | 0.97 | 1024 | 64 |
| *Escherichia coli* (-) | 250 | 4 | 0 |

Gram – positive (+); Gram – negative (-) bacteria. Lectin initial concentration = 1 mg/ml.

* Numbers represent the titer of agglutination activity inhibition by glucose (200mM).

Table 6. MIC and MBC of EmaL against bacteria.

| | **MIC value (μg/ml)** | **MBC value (μg/ml)** |
|---|---|---|
| | **Gram positive** | |
| *Staphylococcus aureus* | 1.5 | 15 |
| *Streptococcus* sp. | 1.5 | 15 |
| *Bacillus* sp. | 15 | 150 |
| | **Gram negative** | |
| *Klebsiella* sp | 15 | 150 |
| *Pseudomonas aeruginosa* | 15 | 150 |
| *Corinebacterium* sp. | 15 | 150 |
| *Escherichia coli* | 15 | 150 |

Lectin initial concentration = 1.5 mg/ml

Figure 1. Emal isolation by affinity chromatography in Sephadex G-50 column.
A sample (7.2 mg of protein) was applied at flow rate of 10 ml/h and 2 ml fractions were collected. Arrows indicated elution with 0.15 M NaCl (1), followed by 0.3 M glucose in 0.15 M NaCl (2) and 1M NaCl (3). Absorbance at 280 nm ( □ ); log of HA ( • ). Each bar represents the mean ± S.D. of three experiments.

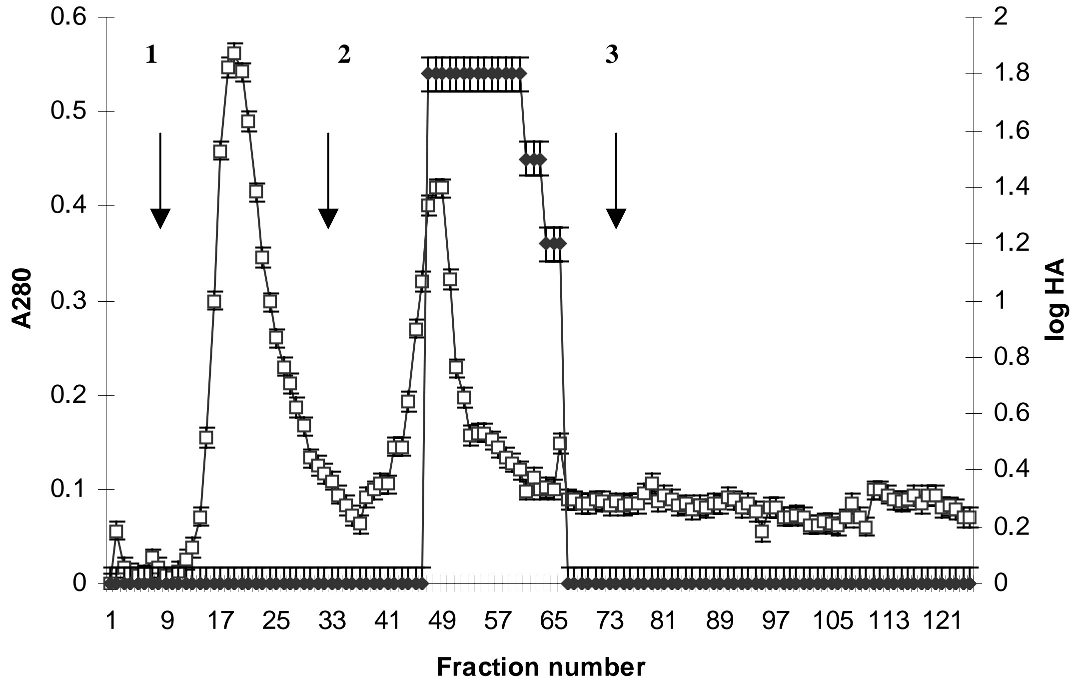
Figure 2. SDS-PAGE of EmaL. Protein was stained with silver stain according to Merril *et al*. (1981).

Figure 3. Influence of the pH on hemagglutinating activity of EmaL.
Log HA of EmaL in 0.15 M NaCl with rabbit erythrocytes was 2.7. Each bar represents the mean ± S.D. of three experiments.

Figure 4. Double Immunodiffusion of EmaL with IgG anti-Cramoll 1.
(1) IgG anti-Cramoll 1; (2) EmaL; (3) Cramoll 1; (4) F 0-80.

Figure 5. Diffusion assay of EmaL against bacteria (a) *Staphylococcus aureus*, (b) *Streptococcus* sp., (c) *Bacillus* sp., (d) *Escherichia coli*, (e) *Corinebacterium* sp., (f) *Klebsiella* sp, (g) *Pseudomonas aeruginosa*.

**Figure 1**



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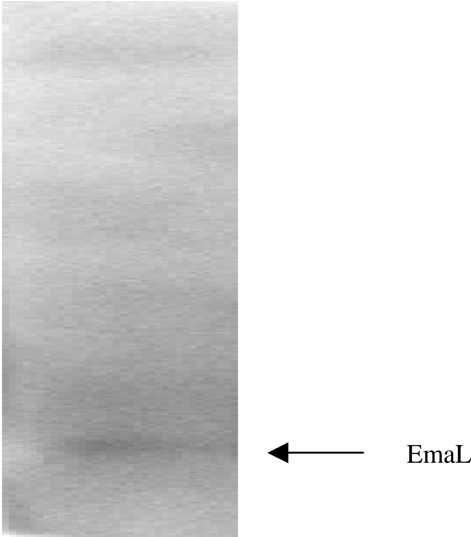

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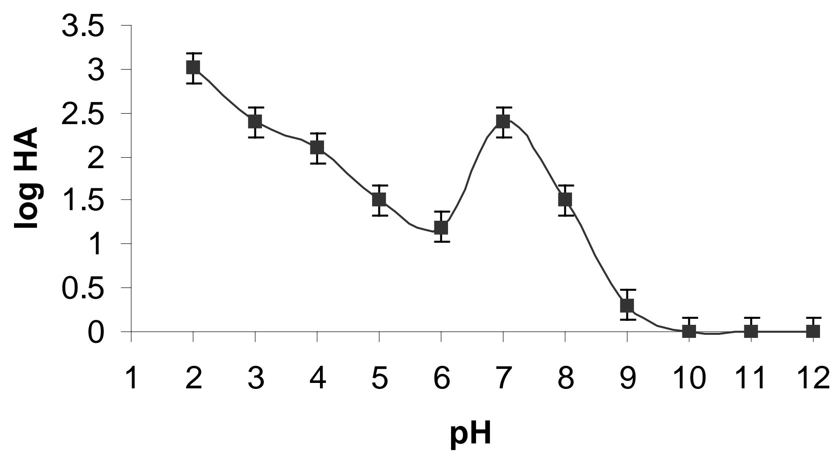

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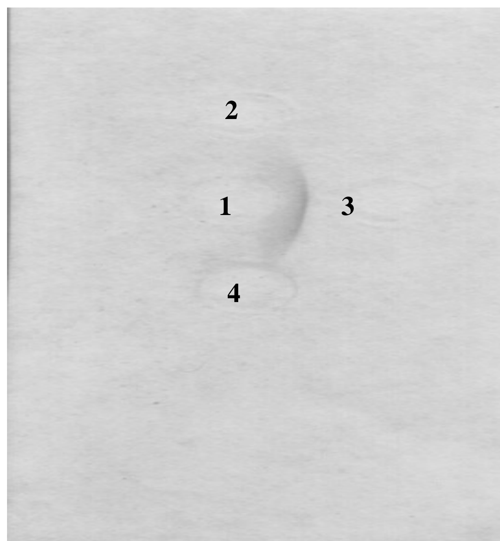

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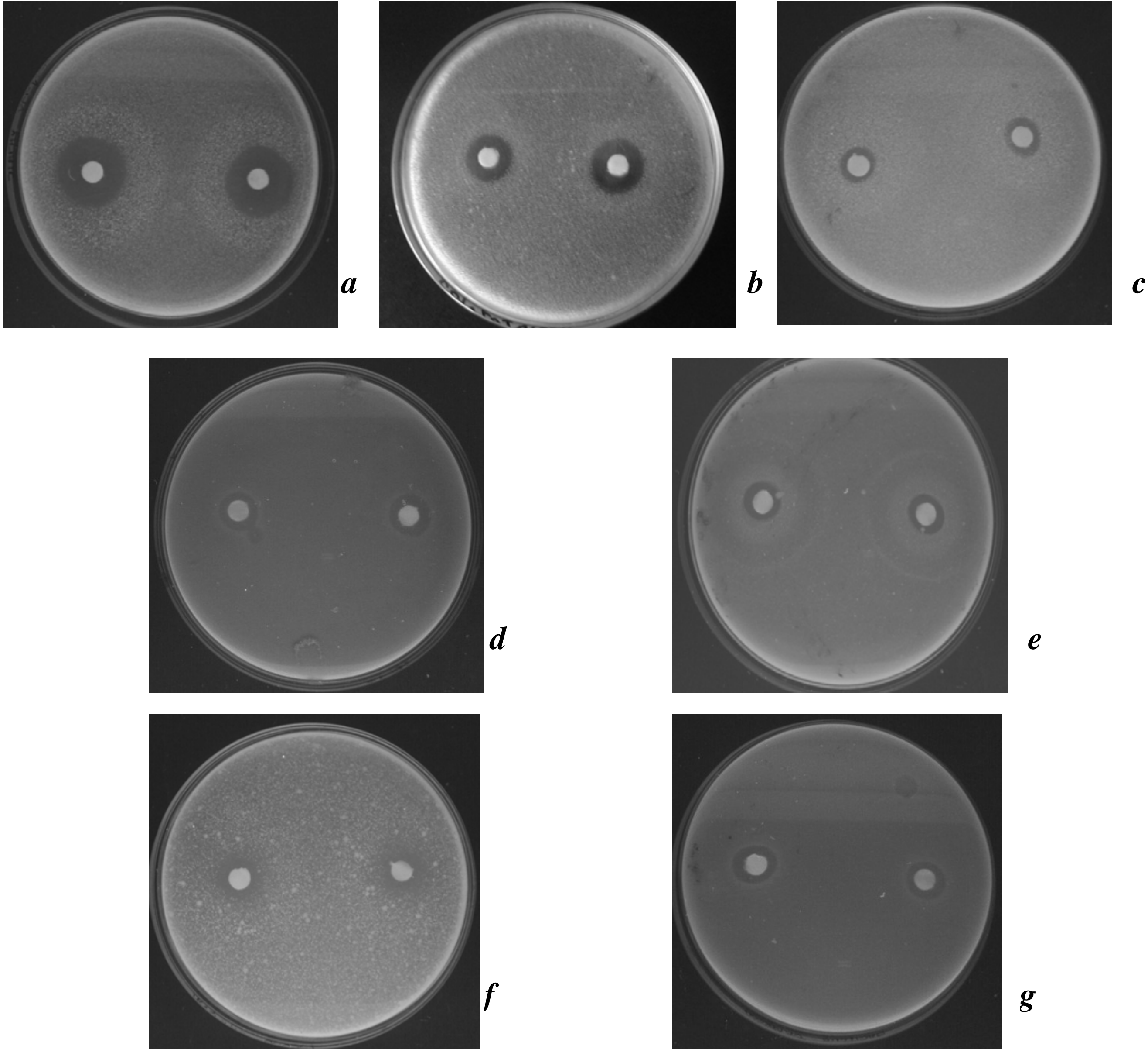


# CONCLUSÕES

Sementes de *E. malaccensis* apresentam uma lectina (EmaL) purificada por cromatografia de afinidade em Sephadex G-50 com um fator de 15 vezes;

EmaL é uma lectina específica para glicose, imunologicamente distinta de Cramoll 1, e que reconhece com alta afinidade as glicoproteínas caseína, ovoalbumina e fetuína;

EmaL permanece ativa após variações de pH (2 a 8) mas é sensível à temperatura;.

SDS-PAGE revelou a purificação de EmaL apresentando peso molecular de 14 kDa;

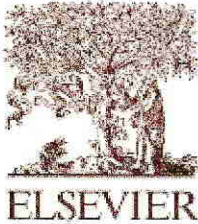
EmaL é constituída por subunidades de 14 kDa que podem formar dímeros de 28 e agregados de 112 kDa;

EmaL apresentou capacidade de aglutinar bactérias via interação com as células bacterianas;

A aglutinação das bactérias testadas por EmaL ocorre pelo sítio de interação da lectina para o carboidrato demonstrada pela total inibição da reação por glicose;

EmaL apresentou atividade antibacteriana de amplo espectro, podendo ser considerada um potente agente antimicrobiano.

# ANEXOS

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•BCG Bacille Calmette-Guerin
•CCID50 median cell culture infective dose
•CCTD50 median cell culture toxic dose
•CF complement fixation
•cfu colony forming units
•cpe cytopathic effect

- cpm counts per minute
- DEAE-cellulose diethylaminoethyl-cellulose
- DF degrees of freedom
- DNA deoxyribonucleic acid
- eop efficiency of plating
- HA haemagglutination
- HAI haemagglutination inhibition
- IgA immunoglobulin A
- ID50 median infective dose
- IgE immunoglobulin E
- IgG immunoglobulin G
- IgM immunoglobulin M
- ImD50 median immunizing dose
- IU International Unit
- Lf Flocculation unit
- LD50 median lethal dose
- MIC minimum inhibiting concentration
- NCTC National Collection of Type Cultures
- PAGE polyacrylamide gel electrophoresis
- P probability
- PD50 median paralytic dose
- pfu plaque forming unit
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- RNA ribonucleic acid
- SDS sodium dodecyl sulphate
- SD standard deviation
- SEM Standard error of the mean
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[3] Mettam GR, Adams LB. How to prepare an electronic version of your article. In: Jones BS, Smith RZ, editors. Introduction to the electronic age, New York: E-Publishing Inc; 1999, p. 281-304

Note shortened form for last page number. e.g., 51-9, and that for more than 6 Authors the first 6 should be listed followed by "et al." For further details you are referred to "Uniform Requirements for Manuscripts submitted to Biomedical Journals" (J Am Med Assoc 1997;277:927-934) (see also http://www.nlm.nih.gov/tsd/serials/terms_cond.html).

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