ANACÁSSIA FONSECA DE LIMA

AVALIAÇÃO DA ATIVIDADE ANTIMICROBIANA E CICATRIZANTE DO EXTRATO DE *Hymenaea stigonocarpa* Mart. ex Hayne

RECIFE, 2011

Anacássia Fonseca de Lima

Avaliação da atividade antimicrobiana e cicatrizante do extrato de *Hymenaea stigonocarpa* Mart. ex Hayne

Dissertação apresentada ao Programa de Pós-Graduação em Patologia do Centro de Ciências da Saúde da Universidade Federal de Pernambuco, para obtenção do título de Mestre em Patologia.

Orientadora: Eulália Camelo Pessoa de Azevedo Ximenes
Co-orientadora: Maria Bernadete de Sousa Maia

Recife
2011

Lima, Anacássia Fonseca de
Avaliação da atividade antimicrobiana e cicatrizante do extrato de *Hymenaea stigonocarpa* Mart. ex Hayne / Anacássia Fonseca de Lima. – Recife: O Autor, 2011.
110 folhas: il., fig., ; 30 cm
.
Orientador: Eulália Camelo Pessoa de Azevedo Ximenes

Dissertação (mestrado) – Universidade Federal de Pernambuco. CCS. Patologia, 2011.

Inclui bibliografia e anexos.

1. *Hymenaea stigonocarpa. 2.* Diabetes. 3. *S. aureus*. 4.Ferida. 5. Cicatrização I. Ximenes, Eulália Camelo Pessoa de Azevedo. II.Título.

UFPE
616.96 CDD (20.ed.) CCS2011-061

Universidade Federal de Pernambuco
Centro de Ciências da Saúde
Programa de Pós-Graduação em Patologia

Av. Prof. Moraes Rego s/n - Cidade Universitária - CEP: 50670-901 - Recife – PE
Prédio da Pós-graduação do Centro de Ciências da Saúde (CCS) - térreo
Fone/Fax: (81) 2126.8529
http://www. pgmap@ufpe.br http://www.pospat.ufpe.br

DISSERTAÇÃO DEFENDIDA PARA OBTENÇÃO DO TITULO DE MESTRE EM PATOLOGIA.

AUTORA: **ANACÁSSIA FONSECA DE LIMA**

ÁREA DE CONCENTRAÇÃO: **PATOLOGIA**

NOME DA DISSERTAÇÃO: **"AVALIAÇÃO DA ATIVIDADE ANTIMICROBIANA E CICATRIZANTE DO EXTRATO DE *Hymenaea stigonocarpa* Mart. ex Hayne".**

ORIENTADORA: **DRA. EULÁLIA CAMELO PESSOA DE AZEVEDO XIMENES**

CO – ORIENTADORA: **DRA. MARIA BERNADETE DE SOUSA MAIA**

DATA DA DEFESA: **25 DE FEVEREIRO DE 2011**

BANCA EXAMINADORA:

Mônica C. P. A Albuquerque

Prof.ª Drª. **Mônica Camelo Pessoa de Azevedo Albuquerque**

Teresinha G. Silva

Profª. Drª. **Teresinha Gonçalves da Silva**

Liriane Baratella Evêncio

Profª. Drª. **Liriane Baratella Evêncio**

Anacássia Fonseca de Lima

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Dissertação aprovada em 25 de fevereiro de 2011

TITULARES:

_____

Profª Drª Liriane Baratella Evêncio

_____

Profª Drª Monica Camelo Pessoa de Azevedo Albuquerque

_____

Profª Drª Teresinha Gonçalves da Silva

SUPLENTES:

_____

Profª Drª Rejane Pereira Neves

_____

Prof Dr Almir Gonçalves Wanderley

Recife

2011

AGRADECIMENTOS

Dedico esse trabalho a minha família. Meu pai por sempre ser um homem exemplar, me servir de exemplo quanto a sua integridade e ser o alicerce da nossa família. Minha mãe por ser uma mulher incrível sem a qual teria sido impossível aguentar a saudade de casa. As minhas lindas irmãs por me animarem quando pensava que não conseguiria e ao meu irmão por ser um grande amigo.

Agradeço primeiramente a Deus, por me fortalecer sempre e me surpreender com Suas bênçãos quando eu desanimava. As minhas orientadoras: a professora Eulália Ximenes por ter me orientado no desenvolvimento do trabalho e me ajudado ao longo dele com sua sabedoria e experiência e a professora Bernadete Maia por me orientar nas etapas *in vivo* e no estágio em docência permitindo minha iniciação nessa área que sempre foi meu objetivo e sonho.

Agradeço aos colaboradores desse trabalho. A professora Teresinha Silva por ter gentilmente cedido a estreptozotocina para a indução ao diabetes tipo 1. A professora Liriane Baratella por ter me instruído quanto ao desenvolvimento da análise morfométrica. Ao professor Nicodemos Telles, por ter cedido o microscópio com sistema de captura de imagens, essencial para a obtenção das fotos da morfometria. A Marcia Silva pela coorientação da aluna Carla Soares e pelos conhecimentos adquiridos em fitoquímica. A professora Carmem Chaves por ceder as gaiolas para o experimento *in vivo*. Ao Leonardo Aquino por ter me ajudado no desenvolvimento de todo o trabalho, sem o qual seria impossível realizá-lo. Ao Eryvelton Franco por ter me ensinado tanto no desenvolvimento do experimento in vivo. Ao Gustavo Dimech e Carlos Brasileiro por terem realizado os exames hematológicos. Ao Tiago Gomes pela colaboração na avaliação da sensibiliza antimicrobiana. A Sidiclei e Silvânia por terem preparado as lâminas dos tecidos das feridas.

Aos meus amados amigos da graduação por estarem presentes em tantas etapas da minha vida acadêmica, vocês são incríveis e vou sentir muita falta da nossa convivência. Aos meus amigos de mestrado em especial Juliana, Ana Paula, Lívia e Rafaella por serem ótimas amigas.
Aos meus queridos amigos do laboratório pelo ensinamento, carinho e apoio.

A minha amiga Rafaela Fernandes, pelas muitas noites em claro de estudo. Amiga você é um presente de Deus!

Agradeço ainda a todos os meus amigos, irmãos em Cristo e familiares pelo apoio.

“Pouco conhecimento faz com que as pessoas se sintam orgulhosas. Muito conhecimento, que se sintam humildes.”

Leonardo da Vinci

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RESUMO

O reino vegetal é uma das principais fontes de substâncias orgânicas de interesse terapêutico. A espécie *Hymenaea stigonocarpa* Mart. ex Hayne, família Leguminosae-Caesalpinioideae conhecida popularmente como Jatobá-do-cerrado é uma "madeira de lei" utilizada na medicina popular como antibiótico e antiinflamatório. Os resultados promissores a cerca da fotoquímica e farmacologia de espécies do gênero Hymenaea inspiraram o presente estudo, que teve por objetivo avaliar a atividade antimicrobiana do extrato de *Hymenaea stigonocarpa* Mart. ex Hayne frente a 28 cepas de *S. aureus* com fenótipo de resistência a vários antimicrobianos. Os resultados *in vitro,* concentração inibitória mínima das 28 cepas, revelaram que tanto o extrato acetato de etila quanto o hidroalcoólico apresentaram atividade antimicrobiana, sendo o último o mais eficaz, e escolhido para a avaliação da atividade antimicrobiana *in vivo*. Para isso, foi empregado o modelo de ferida aberta infectada utilizando grupos de ratos diabéticos e não-diabéticos. As feridas foram infectadas com uma cepa de *S. aureus* resistente a meticilina e tratadas diariamente com um gel hidrofílico a 2% do extrato hidroalcoólico por 14 dias. Todas as feridas dos animais tratados com o gel de *H. stigonocarpa*, sendo eles diabéticos ou não, cicatrizaram ao fim do experimento. Os animais não-diabéticos tratados com o gel de *H. stigonocarpa* apresentaram um percentual de cicatrização maior que os controles e esta diferença foi estatisticamente significativa. Embora as feridas dos animais diabéticos tratados com o gel não tenham apresentado uma diferença estatisticamente significativa percentual da área de cicatrização em relação aos controles, os resultados histológicos das áreas das feridas revelaram não apenas reepitelização, como também formação de tecido em fase de maturação mais avançada que os controles diabéticos. A avaliação da toxicidade dérmica aguda do gel do extrato de *H. stigonocarpa* não revelou ação tóxica ou irritante. Nosso estudo aponta a eficácia microbiológica dos extratos de *H. stigonocarpa* e desperta para a necessidade de estudos adicionais que contribuam para o desenvolvimento de um novo medicamento.

Palavras-chave: *Hymenaea stigonocarpa,* Diabetes, *S. aureus,* ferida, cicatrização.

SUMÁRIO

1. APRESENTAÇÃO

O reino vegetal é uma das principais fontes de compostos orgânicos de interesse terapêutico, isso ocorre devido à capacidade que estes organismos têm de sintetizar os mais variados tipos de estruturas moleculares (SALISBURY e ROSS, 1992; LORENZI, MATOS, 2002).

O Brasil apresenta uma grande diversidade de plantas, dentre elas as "madeiras de lei". A espécie *Hymenaea stigonocarpa* Mart. ex Hayne, família Leguminosae-Caesalpinioideae conhecida popularmente como Jatobá-do-cerrado é uma madeira de lei que é utilizada na medicina popular como antibiótico e antiinflamatório. (LORENZI, MATOS, 2002). Sua utilização popular pode ser justificada pela presença, de substâncias bioativas conhecidas como metabólitos secundários. Os metabólitos secundários são compostos micromoleculares evolutivamente selecionados que servem para conferir vantagens adaptativas às plantas. Ao longo do processo evolutivo as plantas criaram mecanismos de defesa para a sua sobrevivência, através do desenvolvimento de rotas biossintéticas para a produção de substâncias nocivas e tóxicas aos inúmeros parasitas e predadores (SALISBURY, ROSS, 1992; LORENZI, MATOS, 2002).

A constatação da presença de substâncias antibióticas nas "madeiras de lei" com função de conferir as plantas resistência contra microrganismos surgiu a partir dos resultados obtidos por pesquisadores europeus sobre os antibióticos pinossilvina e tuiaplicina de cerne de madeiras como *Pinus silvestres* (Pino Silvestre) e *Thuja plicata* (Tuia) (LIMA, 1959).

Estudo fitoquímico dos compostos presentes no alburno de *Hymenaea stigonocarpa* Mart. ex Hayne permitiu a identificação de metabólitos secundários como flavonóides, esteróides e terpenos, os quais atuam na defesa das plantas. Um exemplo é a apigenina, um flavonóide com ação anticancerígena, e da α-amirina, um esteróide antiinflamatório (VALENTIN, 2006).

É do conhecimento público que doenças como AIDS, diabetes, câncer, tuberculose e hanseníase causam depressão do sistema imunológico. Pacientes imunodeprimidos acabam sendo acometidos por infecções causadas por microrganismos da própria microbiota, principalmente nos casos de feridas onde *Staphylococcus aureus* é o microrganismo isolado com maior frequência (MAIA, 2006).

Devido aos componentes observados durante a abordagem fitoquímica da *H stigonocarpa* acreditou-se que seus extratos apresentavam atividade antimicrobiana, antiinflamatória e cicatrizante. Diante disso, a avaliação da atividade antimicrobiana "in vivo" e "in vitro" do extrato de *Hymenaea stigonocarpa* Mart. ex Hayne possibilita a investigação dessa atividade biológica para o desenvolvimento de numa formulação farmacêutica baseada no extrato obtido dessa planta.

2. REVISÃO DE LITERATURA

Desde o princípio da civilização humana o reino vegetal foi usado para cura e alívio das enfermidades através de ingestão de infusões, uso tópico de ervas, tinturas e cataplasmas. Essa pode ter sido a primeira forma de utilização de produtos vegetais, além do seu papel como fonte de alimento, materiais para vestuário e habitação (LIMA *et al*., 2001).

Os primeiros relatos do uso medicinal das plantas que se tem registro estão na obra chinesa Pen T'são, no papiro de Ebers e em alguns livros da Bíblia, sendo o mais antigo relato atribuído ao imperador chinês Shen Nung (2838-2698 a.C.) em sua obra Pen T'são Ching (A grande fitoterapia) que contem uma lista de 365 ervas considerada a base da medicina tradicional chinesa (TOMAZZONI, NEGRELLE, CENTA, 2006).

Diversas culturas têm feito uso de plantas medicinais, como a principal ou mesmo a única matéria prima para a elaboração de remédios sendo o maior exemplo a medicina tradicional chinesa. Atualmente os conhecimentos desta medicina milenar são utilizados para estudo do isolamento de princípios ativos e entendimento do seu mecanismo de ação (VIEGAS JR., BOLZANI, BARREIRO, 2006).

Os recursos vegetais utilizados de maneira artesanal foram mundialmente as principais alternativas terapêuticas utilizadas para o tratamento e cura das doenças até meados do século XX. A partir desta época, as plantas começaram a ser estudadas com a finalidade de utilização das suas substâncias ativas isoladas, princípios ativos (FOGLIO *et al.*, 2006). Dentre essas substâncias podemos citar a quinina e a artemisina extraídas, respectivamente da *Cinchona spp*. (Rubiaceae) e *Artemisia annua* L. (Asteraceae) utilizadas para o tratamento da malária (KRETTLI *et al.*, 2001); os alcalóides lidocaína e triptamina extraídos da espécie *Arundo donax* L (Poaceae) com ação analgésica que atuam sobre o sistema nervoso central ou periférico; a

atropina extraída da *Atropa beladona* L (Solanaceae) e a morfina extraída da *Papaver somniferum* L. (Papaveraceae). Outro grupo de substâncias importantes são os flavonóides, a exemplo da daidzeina e genisteina extraídos da *Glycine max* (L) Merr. (Fabaceae) que tem ação antioxidante, sendo indicado para aliviar os sintomas da menopausa, além de prevenir a aterosclerose, doenças inflamatórias crônicas e neoplasias do sistema reprodutor feminino (SHEU, LAI, YEN, 2001; SCHENKEL, GOSMAN, PETROVICK, 2004).

A descoberta e o desenvolvimento dos processos de síntese orgânica a partir de moléculas isoladas de produtos naturais para a produção de fármacos culminaram com a introdução no mercado de diversos medicamentos. Entretanto, devido aos efeitos colaterais destas moléculas sintéticas e dos altos custos, houve um incremento da fitoterapia, resgatando assim o conhecimento popular como uma fonte alternativa na terapêutica principalmente em países em desenvolvimento (VOLAK, STODOLA, 1990).

Em alguns países é utilizado o fracionamento biomonitorado, visando o isolamento de princípios ativos que podem ser utilizados tanto como fitofármacos, quanto para o desenvolvimento de protótipos semi-sintéticos modificados ou análogos sintetizados, cujo objetivo é intensificar sua eficácia e minimizar os efeitos colaterais (DREYFUS, CHAPELA, GULLO, 1994; PHILLIPSON, 2000). Nesse contexto, 30% dos medicamentos produzidos nestes países são provenientes de recursos naturais.

No período de 1981 até 2002, dos 90 novos fármacos analisados pela *Annual Reports of Medicinal Chemistry*, 61 foram derivados semi-sintéticos de plantas e nove oriundos de produtos naturais (SIXEL, PECINALLI, 2002; NEWMANN *et al*, 2003).

O Brasil é o país com a maior biodiversidade do planeta. Apesar de possuir um número considerável de cientistas trabalhando com produtos naturais uma política governamental que incentive o processo integrado e coordenado da pesquisa, do desenvolvimento, do produto e

repasse para a indústria ainda é insipiente. O desenvolvimento de equipes multidisciplinares seria a garantia da qualidade do produto, uma vez que cada profissional estaria envolvido em uma parte do processo, que vai da identificação botânica aos testes clínicos (PINTO *et al.*, 2002).

O que acontece, na maioria dos casos é a manipulação de fitoterápicos fundamentada basicamente no uso popular das plantas sem comprovação cientifica não sendo, portanto, competitivo nacionalmente e muito menos internacionalmente (YUNES, PEDROSA, CECHINEL FILHO, 2001; FREITAS, 2007). Mesmo assim, acredita-se que metade dos casos clínicos nos postos de saúde poderia ser tratada com plantas medicinais. Programas com bases na utilização da medicina popular aplicada a área de saúde pública no Brasil, bem como incentivo a pesquisa científica, reduziriam o custo do tratamento (MARJORA, 1999; BASTOS, 2008).

Estima-se que um quarto dos oito bilhões de dólares de faturamento da indústria farmacêutica nacional em 1996, foram resultantes de medicamentos derivados de plantas. É importante ressaltar que apenas 8% das espécies vegetais brasileiras foram estudadas em busca de moléculas bioativas (AMORIM *et al.*, 2003).

Diversas pesquisas têm comprovado a utilização de várias plantas medicinais no tratamento de infecções (SAVI *et al.*, 1996-1997; COWAN, 1999; LIMA, 2001; SCHLEMPER, *et al.*, 2001; NADINIC *et al.*, 2002; ZACCHINO *et al.*, 2003; COELHO DE SOUZA *et al.*, 2004). Estas pesquisas têm se intensificado no Brasil devido à diversidade de espécies presentes na flora brasileira e a necessidade de busca de novos agentes antimicrobianos no combate a microrganismos multirresistentes (ADEBAJO, OLOREK, ALADESANMI, 1989; BRITO e BRITO, 1993; MIGUEL *et al.*, 1996; LIMA, 2001). Estima-se que aproximadamente 50% dos fármacos empregados para o tratamento de infecções (antibacterianos, antifúngicos, antiparasitários e antivirais), são de origem natural ou semi-sintética e 19,4% utilizaram produtos naturais como protótipos para obtenção de medicamentos sintéticos (NEWMANN *et al.*, 2003).

A família Leguminosae está entre as maiores famílias de angiospermas, compreende aproximadamente 727 gêneros com cerca de 19325 espécies espalhadas por todo o mundo e distribuídas nas subfamílias Caesalpinioideae, Faboide (Papilionoideae) e Mimosoideae (LEWIS *et al*., 2005). No entanto, o taxonomista Bandel em 1972 considerou famílias independentes: Fabaceae, Caesalpinaceae e Mimosaceae alegando serem estas distintas entre si, levando em conta a base filogenética, estrutural e a bioquímica dos grupos.

O gênero *Hymenaea* família Leguminosae-Caesalpinioideae é considerado de distribuição antiatlântica (LEE, LANGENHEIM, 1975). Tem predominância neotropical com 16 espécies distribuídas desde o México até a América do Sul; apenas uma espécie ocorre na costa leste da África. No Brasil ocorrem em todo o território nacional tanto em matas pluviais quanto em matas secas, perfazendo 13 espécies, sendo destas, três encontradas em Mato Grosso do Sul (LANGENHEIM, LEE, 1974; CORRÊA, 1984; RIZZINI, 1985; LEWIS, 1987; DUBS, 1998; KODAMA, SARTORI, 2007).

Todas as espécies deste gênero têm hábito arbóreo, que vão desde árvores com 3 metros de altura como *H. stigonocarpa*, até representantes com mais de 40. Ocorrem em uma diversidade de habitats, variando desde a mata equatorial como *H. courbaril*, *H. parvifolia*, *H. stigonocarpa* no cerrado e na caatinga, *H. eriogyne* (LEE, LANGENHEIM, 1975; LEWIS *et al.* 2005).

A espécie *Hymenaea stigonocarpa* Mart ex Hayne é característica de formações abertas do cerrado e campo cerrado sendo popularmente conhecida como jatobá do campo ou do cerrado. A resina obtida a partir desta planta, chamada "jutaicica" ou "copal", é utilizada na indústria de vernizes (Figura 1). Na etnofarmacologia estas resinas são utilizadas para o tratamento de cistite e como lambedor para problemas respiratórios (ALMEIDA *et al*., 1998).

Figura 1. Foto do tronco de *Hymenaea stigonocarpa* Mart ex Hayne.

Estudos fitoquímicos das espécies do gênero Hymenaea, conhecidas como jatobá, revelaram a presença predominantemente de terpenos, flavonóides e taninos (ISHIBASHI et al., 1999; NOGUEIRA et al., 2001; PETTIT et al., 2003; CARAMORI, LIMA, FERNANDES, 2004). Diante da grande variedade de metabólitos secundários com propriedades farmacológica presentes no gênero Hymenaea algumas espécies como a *Hymeneae courbaril* L., a *Hymeneae martiana* e a *Hymeneae palustris* são alvo de muitas pesquisas quanto à composição química, a atividade biológica e a utilização etnofarmacológico (MATUDA, NETO, 2005).

Paiva e Machado (2008) estudando a morfologia de *H. stigonocarpa* verificaram a presença de protuberâncias intercelulares pécticas que interligam as células facilitando o transporte apoplástico. Especula-se que tais protuberâncias tenham várias funções biológicas dentre elas a hidratação da parede celular, o armazenamento de substâncias nutricionais, adesão celular e a defesa. Estes autores também estudaram as alterações celulares que ocorrem no nectário da planta durante as diferentes fases do desenvolvimento floral a fim de conhecer a melhor época para obtenção da resina. Há ainda quatro estudos que analisaram as sementes da *Hymenaea coubaril* (LIMA, et al, 1995; FRANCO et al., 1996; BUCKERIDGE et al., 1997; ANDRADE et al., 2010).

Uma resistência natural de *H. stigonocarpa* à degradação pelo fungo *Phanerochaete chrysosporium* e aos cupins devido à presença de taninos, flavonóides e terpenos foi observada por Oliveira et al.(2010) e Santana et al. (2010).

Em um estudo etnofarmacológico sobre o uso de plantas medicinais na região de Alto Paraíso de Goiás, Souza e Felfili (2006) observaram que a casca de *H. stigonocarpa* era utilizada como antiinflamatório, estimulante de apetite e fortificante rico em ferro. No estado de Pernambuco a casca de *Hymenaea spp.* é utilizada no tratamento de feridas e como fortificante (ALMEIDA, ALBUQUERQUE, 2002; ALBUQUERQUE, ANDRADE, 2002).

Alguns exemplos de atividade biológica de extratos de outros exemplares do gênero Hymenaea foram relatados. A *H. courbaril* L. exerceu alta atividade antiinflamatória mas especificamente sobre a enzima 5-lipoxigenase, e a resina das suas folhas apresentou propriedades tóxicas, o que a torna eficaz na defesa contra herbívoros (STUBBLEBINE, LANGENHEIM , 1977; BRAGA *et al.*, 2000). O extrato da casca de *H. courbaril* também tem sido usado na indústria de cosméticos, devido à presença de várias policatequinas que tem a função de hidratar e clarear a pele (ABE et al, 2008). A fração lipofílica do extrato bruto da casca

e das folhas dessa espécie apresenta atividade antimalárica (KÖHLER, et al, 2002) e é relatada também potente atividade antimicrobiana contra as bactérias gram-positivas *Staphylococcus sp* e estreptococos do grupo mutans (FERNANDES, SANTOS e PIMENTA, 2005)

Ramos, Lemos-Filho e Lovato (2008) estudando a estrutura filogeográfica *de Hymenaea courbaril* e sua relação com a *Hymenaea stigonocarpa* observaram haver compartilhamento de haplótipos e similaridade genética entre as duas espécies demonstrando a forte relação que há entre elas. As duas espécies têm um ancestral comum e as diferenciações genéticas que as originaram podem ser associadas com as mudanças climáticas durante o período glacial (RAMOS et al., 2007).

O extrato da casca do caule de *H. martiana* Hayne Arzeik apresentou efeitos na indução de contrações do útero de rato e íleo de suínos (CALIXTO, YUNES e MEDEIROS, 1992). Em um estudo desenvolvido por Souza (2010), dessa vez com o extrato bruto do tronco e da casca de *H. martiana,* foi verificado atividade contra *Cryptococcus neoformans* pela fração hidroalcoólica, e pelas frações metanólica e butanólica sobre *Trichophyton rubrum*, *Trichophyton mentagrophytes* e *Microsporum canis*.

Os flavonóides isolados da *Hymeneae palustris* apresentaram atividade antimicrobiana contra *Neisseria gonorrhoeae* e *Enterococcus spp,* mas não foram eficazes na inibição do crescimento de *Staphylococcus aureus, Streptococcus pneumoniae, Micrococcus luteus, Enterobacter cloacae, Escherichia coli, Stenotrophomonas maltophilia, Cryptococcus neoformans* e *Candida albicans* (PETTIT et al., 2003). Xiloglucanos da fração aquosa das sementes de *H. courbaril* apresentaram atividade imunomoduladora frente a macrófagos peritoneais (ROSÁRIO et al., 2008; 2010)

A gravidade da infecção depende de vários fatores como: A patogenicidade da bactéria infectante, o desenvolvimento da resistência bacteriana aos fármacos disponíveis, o sítio de

infecção e a imunidade do hospedeiro. Pacientes imunodeprimidos são mais suscetíveis a infecções causadas tanto por bactérias patogênicas quanto por oportunistas, muitas vezes presentes na microbiota normal (CRUSE e FOORD, 1980; HIERHOLZER, 1997; SANTOS, 1997; TRABULSI *et al.* 1999; PADRÃO *et al.* 2010).

Várias doenças podem levar a imunodepressão com consequente suscetibilidade a infecções oportunistas, dentre estas doenças o diabetes. Esta doença é caracterizada por um distúrbio metabólico de etiologia múltipla. Ela é considerada um dos mais importantes problemas de saúde na atualidade, tanto em termos de número e mortalidade prematura, quanto em relação aos custos envolvidos no controle e tratamento de suas complicações (DINIZ, 2000).

Do total de casos de diabetes 90% são não-insulino-dependentes (tipo 2), 5% a 10% do tipo insulino-dependentes (tipo 1) e 2% do tipo secundário ou associado a outras síndromes (DINIZ, 2000).

Nos Estados Unidos, é de quatro milhões o número de pacientes com esta afecção e no Brasil estima-se que o número chegue a cinco milhões de diabéticos, dos quais metade desconhece o diagnóstico. A prevalência do diabetes no Brasil, na população urbana de 30 a 69 anos, é de 14,92%, pacientes diabéticos com úlceras infectadas representam a maior parte dos pacientes diabéticos nos hospitais brasileiros (MOTTA NETO, 2007).

Estudos realizados por Abdulrazak e colaboradores. (2005), ao analisar 86 úlceras infectadas de pacientes diabéticos que faziam uso de agentes antimicrobianos a mais de 30 dias, mostraram que o *S. aureus* fora o agente etiológico prevalente e o mais resistente.

Goldstein e colaboradores (1996) isolaram *Staphylococcus aureus* de úlceras infectadas de pacientes diabéticos e não hospitalizados em 76% dos casos avaliados, 20% foram cepas resistentes à Meticilina (MRSA).

Shankar e colaboradores (2005), traçaram o perfil epidemiológico e de resistência de pacientes diabéticos infectados no sul da Índia e constataram que os que apresentaram uma maior frequência foram os *S. aureus* e *Pseudomonas aeruginosa.*

As bactérias pertencentes ao gênero *Staphylococcus* são patógenos humanos e de outros mamíferos causadoras de diversos tipos de infecções. A gravidade destas infecções vai desde uma intoxicação alimentar ou infecção cutânea de pouca importância, até infecções graves e potencialmente fatais. Quando as infecções estão localizadas na pele ou mucosas podem causar furúnculos, conjuntivite, impetigos, foliculite, pústulas ou abscessos subcutâneos. Podem causar também infecções sistêmicas profundas como pneumonias, abscessos profundos, osteomielites, endocardites, flebites, mastites, meningites e septicemia. São associadas também a infecções relacionadas à utilização de dispositivos cardiovasculares e válvulas cardíacas (JAWETZ, MELNICK, ADELBERG, 1998; MIMS *et al*., 1999; TRABULSI *et al.,* 1999).

Isolados clínicos de *S. aureus* são com frequência resistentes a muitos antibióticos. Desde a primeira vez que se utilizou a penicilina, cepas de *S. aureus* demonstraram uma marcante habilidade de adaptação. A resistência a penicilina foi desenvolvida rapidamente, após seu lançamento no mercado. Atualmente várias cepas são resistentes a agentes antimicrobianos utilizados na clínica. Este quadro é preocupante, uma vez que são necessários vários anos de estudo para o desenvolvimento de novos antibióticos (SHIOTA *et al.*, 1999; FERENSIS *et al*., 2001).

A resistência aos agentes antimicrobianos é geralmente adquirida por mutações genéticas, mas muitas vezes pode não ser a mutação o fator responsável pela resistência, como ocorre no compartilhamento de plasmídios com genes de resistência. Os mecanismos de resistência relevantes clinicamente incluem a síntese de enzimas que inativam a droga, a prevenção do

acesso ao sítio alvo (inibição da absorção ou aumento da excreção) ou modificações do sítio alvo (BLACK, 1996; SCHAECHTER *et al*., 2002).

O quadro encontrado em pacientes diabéticos que apresentam feridas abertas infectadas por *S. aureus* multirresistente é a dificuldade na cura destas feridas inspira a busca por novos antimicrobianos, cuja biodiversidade brasileira pode ser o celeiro para a solução deste problema.

2.1. Artigo de revisão

Aceito pela revista SÃO PAULO MEDICAL JOURNAL
Qualis – B3 Medicina II

Protocol: SPMJ000049/2010 (6th submission: December 6, 2010)

Systematic review

Interventions for wound healing among diabetic patients infected with *Staphylococcus aureus*: a systematic review

Intervenções para a cicatrização de feridas de pacientes diabéticos infectados com *Staphylococcus aureus*: uma revisão sistemática

Centro de Ciências da Saúde, Universidade Federal de Pernambuco (CCS-UFPE), Recife, Pernambuco, Brazil

Autores

Anacássia Fonseca Lima[I], Lívia Bandeira Costa[II], Joás Lucas da Silva[III], Maria Bernadete Sousa Maia[IV], Eulália Camelo Pessoa Azevedo Ximenes[V]

[I]BSc. Biomedical Scientist, Microorganism Physiology and Biochemistry Laboratory, Universidade Federal de Pernambuco (UFPE), Recife, Pernambuco, Brazil.

[II]BSc. Biomedical Scientist, Cell Culture Laboratory, Department of Histology and Embryology, Universidade Federal de Pernambuco (UFPE), Recife, Pernambuco, Brazil.

[III]MSc. Biomedical Scientist, Bioactive Product Biotechnology Laboratory, Universidade Federal de Pernambuco (UFPE), Recife, Pernambuco, Brazil.

[IV]PhD. Associate Professor, Bioactive Product Pharmacology Laboratory, Universidade Federal de Pernambuco (UFPE), Recife, Pernambuco, Brazil.

[V]PhD. Associate Professor, Microorganism Physiology and Biochemistry Laboratory, Universidade Federal de Pernambuco (UFPE), Recife, Pernambuco, Brazil.

KEY WORDS:

Wound healing.

Infection.

Staphylococcus aureus.

Diabetic foot.

Leg ulcer.

Foot ulcer.

Ulcer.

ABSTRACT

CONTEXT AND OBJECTIVE: *Staphylococcus aureus* is the most frequent agent isolated in diabetic foot infections and may be associated with changes to wound healing times. The aim of this study was to perform a systematic review of the literature, including studies that assessed the efficacy of any clinical or surgical intervention, as well as oral or topical therapy for diabetic ulcers infected with *S. aureus*.

DESIGN AND SETTING: Systematic review with a search conducted in databases.

METHODS: We conducted a systematic review with a comprehensive search in the Lilacs, SciELO, PubMed/Medline, Old Medline, Embase and Cochrane Library databases, for articles published from 1966 to 2010. The articles selected were limited to studies on diabetic patients with wounds infected with *S. aureus* for whom their healing was followed up, with the use of either antibiotics or experimental treatments. Animal studies and those that did not report the wound healing, as well as review articles, were excluded.

RESULTS: Eight studies that met the inclusion and exclusion criteria were analyzed.

CONCLUSIONS: There are few studies reporting the healing of wounds infected with *S. aureus* in diabetic patients, although this is the most commonly found pathogen in this type of wound and it frequently consists of methicillin-resistant *S. aureus* (MRSA). There is insufficient evidence to support early use of broad-spectrum antibiotics against MRSA to promote healing of diabetic ulcers, since antibiotic resistance may develop from such treatment. This highlights the need for further studies on the subject.

PALAVRAS-CHAVE:

Cicatrização de feridas.

Infecção.

Staphylococcus aureus.

Pé diabético.

Úlcera da perna.

Úlcera do pé.

Úlcera.

RESUMO

CONTEXTO: *Staphylococcus aureus* é o agente mais frequentemente isolado nas infecções de pé em pacientes diabéticos e pode estar associado a mudança no tempo de cicatrização de feridas. O objetivo deste estudo foi realizar uma revisão sistemática da literatura, incluindo estudos que avaliaram a eficácia de qualquer intervenção clínica, cirúrgica, bem como terapia oral ou tópica para o tratamento de úlceras diabéticas infectadas com o *S. aureus*.

TIPO DE ESTUDO E LOCAL: Revisão sistemática com busca realizada em bancos de dados.

MÉTODOS: Realizamos uma revisão sistemática com uma busca abrangente nos bancos de dados Lilacs, SciELO, PubMed/Medline, Old Medline, Embase e no banco de dados da biblioteca Cochrane, publicados entre 1966 e 2010. Os artigos selecionados foram limitados aos estudos com feridas infectadas por *S. aureus* de pacientes diabéticos, que tiveram cicatrização relatada, quer pela utilização de antibióticos ou por substâncias experimentais.

Foram excluídos os estudos com animais e os que não relataram a cicatrização das feridas, bem como artigos de revisão.

RESULTADOS: Foram analisados cinco estudos que obedeceram aos critérios de inclusão e exclusão.

CONCLUSÕES: Raros estudos relataram cicatrização de feridas infectadas com *S. aureus* em pacientes diabéticos, embora este seja o patógeno mais comumente encontrado neste tipo de ferida, sendo frequentemente resistente à meticilina MRSA (*methicillin-resistant S. aureus*). Não há evidências suficientes que suportem a utilização precoce de antibióticos de amplo espectro contra MRSA para promoção da cicatrização de úlceras diabéticas, uma vez que o desenvolvimento de resistência a antibióticos pode decorrer desse tipo de tratamento. Isso evidencia a necessidade de novos estudos sobre o assunto.

Corpo do texto

INTRODUCTION

Ischemia, neuropathy and infection are the three pathological components that lead to diabetic foot complications, and they frequently occur together as an etiological trio. Infection of foot ulcers is commonly seen in diabetic patients and is a substantial morbid event.[1] *Staphylococcus aureus* (*S. aureus*) is, by far, the most frequent pathogen isolated in diabetic foot infections, either singly or as a component of mixed infection.[2]

Diabetes also causes structural and functional changes within the arteriolar and capillary systems, notably with thickening of the basement membrane.[3] This thickened membrane impairs leukocytes migration and hampers the normal hyperemic or vasodilatory response to injury, thus simultaneously increasing the susceptibility to injury while also blunting the typical manifestations of such an injury.[4] Because of this blunted neuroinflammatory response, diabetic patients lack a crucial component of the body's natural first line of defense against pathogens and thus are more susceptible to an ensuing foot infection.[5] The present study will provide knowledge of interventions that lead to healing of wounds infected with *S. aureus.*

OBJECTIVE

The aim of this study was to perform a systematic review of the scientific literature, including studies that assessed the efficacy of any clinical or surgical intervention, as well as

oral or topical therapy for diabetic ulcers infected with *S. aureus*.

METHODS

Searches to locate articles relating to the healing of wounds infected with *S. aureus* in diabetic patients were conducted in the Medline/PubMed (Medical Literature Analysis and Retrieval System Online), Lilacs (Literatura Latino-Americana e Caribe em Ciências da Saúde), SciELO (Scientific Electronic Library Online), Old Medline, Embase (Excerpta Medica) and Cochrane Library databases. Searches were restricted to the period from 1966 to 2010.

The databases were searched using a comprehensive strategy (**Table 1**), along with MeSH (Medical Subject Headings) and text words, including the following exhaustive list of synonyms: wound healing, infection, *S. aureus*, diabetic foot, leg ulcer, foot ulcer and ulcer. Bibliographic references in relevant review articles were also examined for eligible trials. In addition, thesis databases were searched manually, references of references were searched, specialists were consulted and contacts were made with the pharmaceutical industry. Searches were also carried in Clinical Trials.gov and in the Current Controlled Trials. References, and any relevant studies identified were scrutinized for additional citations.

The selection of trials, data abstraction and data synthesis were performed by two authors independently. Disagreements were solved by discussion.

The articles selected were limited to studies on diabetic patients with wounds infected with *S. aureus* that were treated for infection using any clinical or surgical intervention, as well as oral or topical therapy for diabetic ulcers infected with *S. aureus*. We excluded animal studies, studies on non-diabetic patients, studies that did not report wound healing and literature review articles.

RESULTS

Among all the articles initially identified through the electronic search, six items from the Medline/PubMed database and two from Embase relating to the healing of wounds infected with *Staphylococcus aureus* in diabetic patients published between 1999 and 2010 were fully recovered for further evaluation. In cases of repeated studies, only one search source was taken into consideration. There were no randomized clinical trials on this subject.

All the studies were observational in nature. The search strategy is shown in **Figure 1**. This systematic review included a total of eight studies.

In **Table 2**, the studies have been displayed in chronological order emphasizing the type of study, number of patients used in each study, treatment administered to the wounds, healing time and conclusion obtained.

DISCUSSION

Few studies have reported the healing of wounds infected with *S. aureus* in diabetic patients. The eight studies examined here were all conducted over a similar time span. The first study was performed in 1999;[6] the second was a continuation of the first, in 2003;[7] the third was in 2004;[8] the fourth was in 2007;[9] another two articles appeared in 2008;[10,11] and the last and most recent two appeared in 2010.[12,13] This small number of studies may be due to difficulty in monitoring diabetic patients from the onset of the infection until wound healing, thus making it complex to obtain a homogeneous sample. It is also due to difficulties in obtaining a diagnosis of infection. It can be very difficult to define the infection, especially in the presence of peripheral ischemia, and there are no clear criteria available for distinguishing infection from non-pathogenic colonization.[14]

The samples in the studies analyzed were of significant size, with numbers of patients with wounds exceeding 50, with the exception of one study.[9] This study evaluated wound healing in 10 diabetic patients, among whom seven were infected with *S. aureus*. The small sample in that study can be explained by the experimental features of the treatment used. Dermal grafts were used, with meshes of autologous fibroblasts. This treatment proved to be very promising and the authors encouraged further studies with a higher number of patients.

Another article reported on 30 wounds infected with *S. aureus* among 75 patients studied: 18 of these were methicillin-sensitive *S. aureus* (MSSA) and 12 were methicillin-resistant *S. aureus* (MRSA).[6] Dang et al.[7] reported on wound development in 63 patients with diabetes, of whom 26 had MSSA and 19 had MRSA. Hartemann-Heurtier et al.[8] studied 180 patients who developed wounds: 64 colonized by MSSA and 29 by MRSA. Cavallini[9] did not reported on the susceptibility of *S. aureus* to methicillin. Kim et al.[10] used modified resection arthroplasty to completely remove the infected phalangeal bone and to suture the dorsal wound and extensor tendon of 52 diabetic patients: 23 of these were infected by *S. aureus*,

including seven cases of MRSA. Richard et al.[11] followed up 188 diabetic patients, among whom 62 had wounds infected with MSSA and 37 with MRSA. In a prospective cohort study, ElMakki Ahmed et al.[12] assessed the risk factors associated with hallux ulceration and the incidence of healing or amputation in 122 diabetic patients, of whom 56 were infected by *S. aureus*. Nagoba et al.[13] investigated susceptibility to citric acid *in vitro* and in ulcers of different Wagner grades infected with a variety of bacteria (47 with *S. aureus*) from 115 diabetic patients. The Wagner grade is determined based on the depth of the skin lesion and the presence or absence of infection and gangrene, and is divided into six grades ranging from grade zero to grade five.[15] The last two authors did not report the susceptibility of *S. aureus* to methicillin.

All the authors found that *S. aureus* was the most frequent microorganism infecting wounds in diabetic patients, but there was disagreement over whether the presence of the organism influenced the healing time. The high prevalence may be due to the fact that this microorganism is a skin colonizer that becomes opportunistic in immunocompromised people such as diabetic patients. The large number of wounds infected with MRSA can be correlated with previous use of broad-spectrum antibiotics.[16]

In the articles selected, the antibiotic treatments used were similar: clindamycin and amoxicillin/clavulanic acid were used in most of the studies. Tentolouris et al.[6] and Dang et al.[7] stated that specific antibiotic therapy for MRSA encouraged microbial resistance and was unnecessary since, according to these authors, MRSA could be eradicated by means of regular debridement, topical treatments and isolation in the foot clinic without the requirement for treatment with specific antibiotics (**Table 2**). However, MRSA was associated with a longer time for healing. ElMakki Ahmed et al.[12] did not report whether there was any association between healing time and susceptibility to infecting microorganisms. Nonetheless, even though these authors advocated that medical therapy alone was the most effective method, they stated that surgical debridement with removal of all the infected area was essential for wound healing.

Hartemann-Heurtier et al.,[8] Richard et al.[11] and ElMakki Ahmed et al.[12] administered similar antibiotic therapies. All of them started their patients on broad-spectrum treatment for infections, and the antibiotic therapy was adapted based on the results from microbiological studies, so that it would cover the most likely pathogenic organisms. Hartemann-Heurtier et

al.[8] agreed that indiscriminate use of broad-spectrum antibiotics promoted the emergence of resistance, but they argued that MRSA was acquired more often from cross-transmission than from antibiotic overuse. According to Richard et al.,[11] isolation of multidrug resistant microorganisms (MDRO) seemed to have no significant impact on healing time when early aggressive treatment of wound infection, including immediate broad-spectrum antibiotics (active against MRSA) were administered, followed by adjustment according to culture results.

With regard to healing time, Tentolouris et al.[6] and Dang et al.[7] reported significant differences between ulcers infected with MRSA and with MSSA. The healing times observed by the first authors were 17.8 weeks (MSSA) and 35.4 weeks (MRSA), while the second authors reported a healing time of 12 weeks, regardless of the infecting pathogen. Hartemann-Heurtier et al.[8] and Richard et al.[11] ranked MRSA among MDRO. Hartemann-Heurtier et al.[8] found healing times of 4.6 ± 8.4 months (MDRO+) and 6.7 ± 13 months (MDRO–), and Richard et al.[11] found 14 weeks (MDRO+) and 10 weeks (MDRO–). ElMakki Ahmed et al.[12] did not report whether there was any association between healing time and susceptibility to infecting microorganisms, and the mean healing time was 16 ± 8 weeks for all wounds in the study.

Kim et al.[10] reported that no antibiotics were used in conjunction with the surgical technique, but showed that with the use of modified resection arthroplasty, it was possible to salvage most of the toes with infected wounds and avoid amputation, thereby leading to a mean healing time of 25.6 ± 6.2 days. Nagoba et al.[13] observed that citric acid treatment promoted healing after 16-34 applications, and that this was highly effective for controlling infections and for successfully managing diabetic foot ulcers without deep osteomyelitis.

Among the eight studies analyzed in this review, two were related to the presence of methicillin-resistant *Staphylococcus aureus* (MRSA)[6,7] with longer healing times. Another two articles that were analyzed claimed that there was no relationship between MRSA and increased healing time[8,10] and four papers did not study this association.[9,10,12,13] Three articles[7,10,12] advocated that surgical debridement with removal of all the infected area was a very appropriate treatment, certainly because ulcers heal more quickly if their surfaces are clean and if sinuses are laid open.[17]

Most of the studies used antibiotic therapy as the priority treatment, except for three.

Cavallini[9] used a combination of antibiotic therapy and debridement with dermal grafts using meshes of autologous fibroblasts, Kim et al.[10] used the surgical technique of modified resection arthroplasty and Nagoba et al.[13] used a treatment with citric acid. These articles show that alternative therapies may be an effective alternative to the indiscriminate use of antibiotics.

The studies that did not observe any relationship between the presence of MRSA and longer healing time had the common feature of the use of early aggressive treatment of wound infection. This feature may explain this finding, and justify the use of this type of treatment, since using specific therapy can enhance the healing of infected wounds. The Brazilian National Sanitary Surveillance Agency (Agência Nacional de Vigilância Sanitária; Anvisa) advocates the use of vancomycin or amoxicillin-clavulanic acid for treating MRSA, since this requires specifically targeted antibiotic therapy.[1] Moreover, the studies that reported an association between healing time and presence of MRSA showed indiscriminate use of antibiotics and a predisposing factor for increased infection by this pathogen.

We did not find any systematic review that was similar to what has been presented here. We found systematic reviews on diabetic foot, chronic wounds and *S. aureus* colonization with conclusive results,[18-27] but no reviews reporting the healing of wounds infected with *S. aureus*, in diabetic patients.

There are few studies reporting the healing of wounds infected with *S. aureus* in diabetic patients, although this is the most commonly pathogen found in this type of wound and it sometimes consists of MRSA. Studies that used early and aggressive treatment against MRSA infections reported that such therapy can make the healing time similar to that found in other infections. Surgical debridement with removal of all the infected area was also observed to be an important tool for wound healing. Alternative therapies for wound treatment, such as the use of meshes with dermal grafts of autologous fibroblasts, the surgical technique of modified resection arthroplasty and treatment with citric acid are promising. We could not find any work reporting the use of natural substances for treating wound infections in diabetic patients. Thus, the possibility arises that there may be opportunities to find widespread naturally occurring substances with antimicrobial activity that could serve as alternative treatments.

Wound infection in diabetic patients is a public health problem. Finding a balance

between effective antibiotic therapy and control over promotion of bacterial resistance is a challenge. Alternative treatments that can be used in combination with antibiotic therapy may be a way to solve problems relating to long periods of hospitalization, since a prolonged stay in hospital just contributes towards infection with multidrug-resistant strains, either through inadequate antibiotic therapy or through cross-contamination.

CONCLUSIONS

There is insufficient evidence to support the use of early and aggressive antimicrobial therapy against MRSA to promote healing of diabetic ulcers, since potentially serious development of antimicrobial resistance can result from such treatment. This highlights the need for a randomized controlled trial on this subject.

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Address for correspondence:

Anacássia Fonseca de Lima
Rua Tomé Gibson, s/nº — edifício Rio Negro — apto 102
Pina — Recife (PE) — Brasil
CEP 51011-480
Tel. (+55 81) 30331247
E-mail: cassialima3@hotmail.com

Table 1. Search strategy

Database	Date	Search filters
Embase/Lilacs/SciELO/Cochrane	06/09/10	Wound [Text Word] AND Diabetes [Text Word] AND *Staphylococcus aureus* [MeSH]
Embase/Lilacs/SciELO/Cochrane	13/09/10	Wound healing [MeSH] AND Diabetes [Text Word] AND *Staphylococcus aureus* [MeSH]
Embase/Lilacs/SciELO/Cochrane	17/09/10	Wound healing [MeSH] AND Diabetic foot [MeSH] AND Wound [Text Word] AND Leg Ulcer [MeSH] AND Foot Ulcer [MeSH] AND Ulcer [MeSH] AND Diabetes [Text Word] AND *Staphylococcus aureus* [MeSH]
Medline/PubMed	19/09/10	("wound healing"[MeSH Terms] OR ("wound"[All Fields] AND "healing"[All Fields]) OR "wound healing"[All Fields]) AND ("diabetic foot"[MeSH Terms] OR ("diabetic"[All Fields] AND "foot"[All Fields]) OR "diabetic foot"[All Fields]) AND ("wounds and injuries"[MeSH Terms] OR ("wounds"[All Fields] AND "injuries"[All Fields]) OR "wounds and injuries"[All Fields] OR "wound"[All Fields]) AND ("leg ulcer"[MeSH Terms] OR ("leg"[All Fields] AND "ulcer"[All Fields]) OR "leg ulcer"[All Fields]) AND ("foot ulcer"[MeSH Terms] OR ("foot"[All Fields] AND "ulcer"[All Fields]) OR "foot ulcer"[All Fields]) AND ("ulcer"[MeSH Terms] OR "ulcer"[All Fields]) AND ("diabetes mellitus"[MeSH Terms] OR ("diabetes"[All Fields] AND "mellitus"[All Fields]) OR "diabetes mellitus"[All Fields] OR "diabetes"[All Fields] OR "diabetes insipidus"[MeSH Terms] OR ("diabetes"[All Fields] AND "insipidus"[All Fields]) OR "diabetes insipidus"[All Fields]) AND ("staphylococcus aureus"[MeSH Terms] OR ("staphylococcus"[All Fields] AND "aureus"[All Fields]) OR "staphylococcus aureus"[All Fields]) AND ("humans"[MeSH Terms])

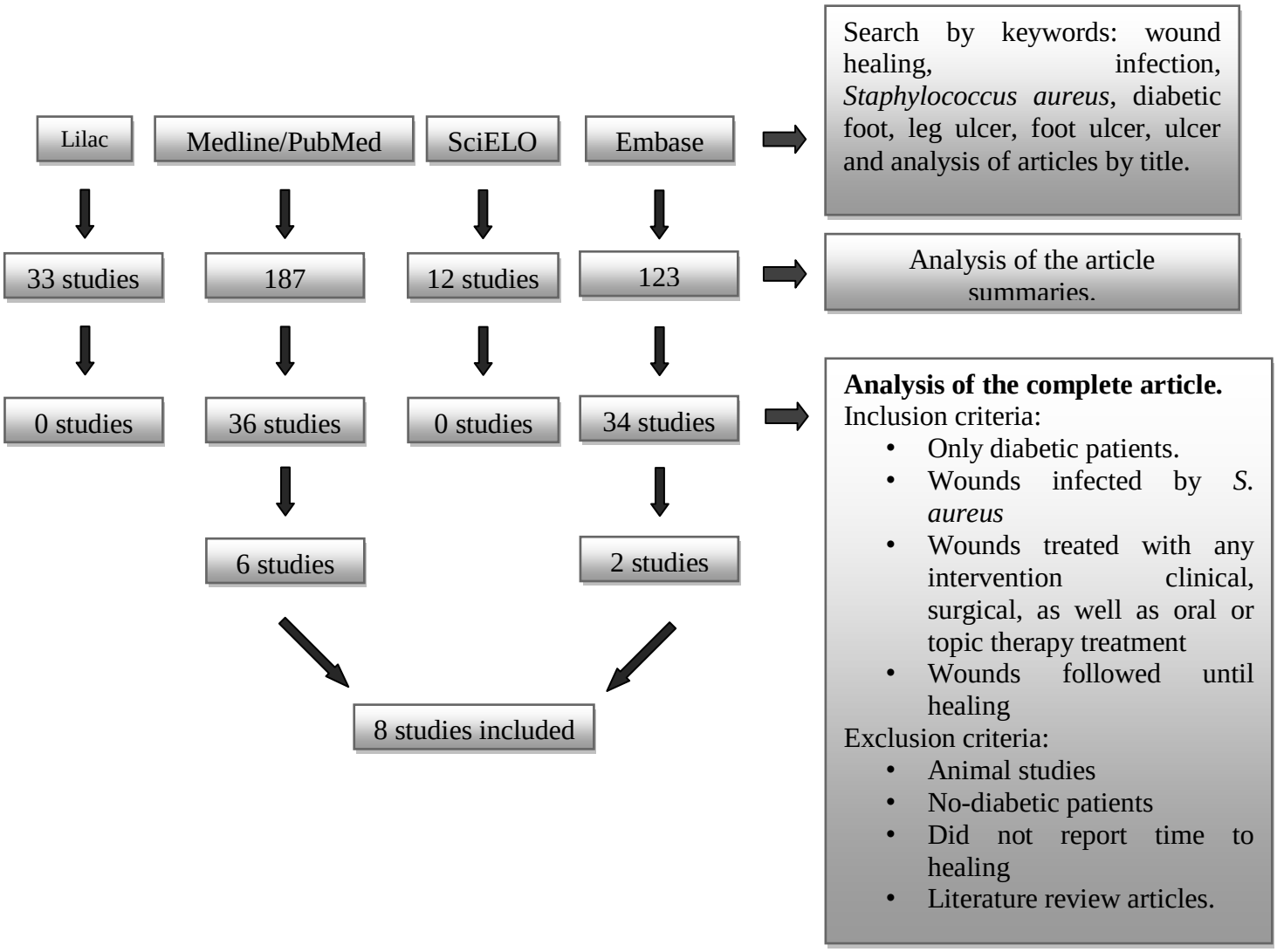
Table 2. Analysis of selected studies

Author (year)	Type of study	Sample	*Staphylococcus aureus* Wound Infection (n)	Treatment	Healing time (mean)	Conclusion
Tentolouris et al.[6]	Prevalence	75 diabetic patients	MSSA (18) MSRA (12)	Antibiotic therapy (clindamycin or amoxicillin/clavulanic acid)	17.8 weeks (MSSA) 35.4 weeks (MRSA)	Methicillin-resistant *S. aureus* (MRSA) infection is common in diabetic foot ulcers and is associated with previous antibiotic treatment and prolonged time to healing.
Dang et al.[7]	Prevalence	63 diabetic patients	MSSA (26) MSRA (19)	Antibiotic therapy(clindamycin and amoxicillin/clavulanic acid)	12 weeks (all ulcers of the study)	MRSA infection is associated with slower ulcer healing and is likely to have impact on the treatment cost. The problem of MRSA continues to increase despite precautions taken to prevent MRSA spread. There is a need for a multi-center study looking into the prevalence of MRSA in diabetic foot ulcer and how this can be reduced in the diabetic foot clinic.
Hartemann-Heurtier et al.[8]	Longitudinal	180 diabetic patients	MSSA (64) MSRA (29)	Antibiotic therapy (amoxicillin/clavulanic acid and aminoglycoside)	4.6 ± 8.4 months (MDRO+) 6.7 ± 13 months (MDRO–)	About one-third of patients with a history of previous hospitalization for the same wound and 25% patients with osteomyelitis had MDRO-positive specimens. Positive MDRO status is not associated with a longer time to healing.
Cavallini[9]	Longitudinal	10 diabetic patients	*Staphylococcus aureus* (7)	Surgical debridement, antibiotic therapy and dermal graft with meshes of autologous fibroblasts	7 patients healed at 14.2 weeks	Autologous fibroblast grafts should be considered to be a correct approach for treating

						chronic and complicated deep ulcers, given that when accompanied by antibiotic therapy, wound healing is optimized.
Kim et al.[10]	Longitudinal	52 diabetic patients	MSSA (16) MSRA (7)	Modified resection arthroplasty	25.6 ± 6.2 days	Modified resection arthroplasty for toe deformities with chronic infected ulcers in diabetic patients is a good treatment alternative to toe amputation.
Richard et al.[11]	Longitudinal	188 diabetic patients	MSSA (62) MSRA (37)	Antibiotic therapy (fluoroquinolone, rifampicin and clindamycin)	14 weeks (MDRO+) 10 weeks (MDRO–)	MDRO presence seems to have no significant impact on healing time if early aggressive treatment is adopted, including empirical broad-spectrum antibiotic treatment, later adjusted according to microbiological findings.
ElMakki Ahmed et al.[12]	Prospective cohort	122 diabetic patients	*Staphylococcus aureus* (56)	Surgical debridement and. antibiotic therapy (amoxicillin/clavulanic acid)	16 ± 8 weeks	Outcome from diabetic foot is dependent on degree of limb ischemia, size of the ulcer, and severity of sepsis, and it can be optimized through debridement and removal of infected bone fragments, in conjunction with antibiotic therapy.
Nagoba et al.[13]	Retrospective	115 diabetic patients	*Staphylococcus aureus* (47)	3% citric acid gel daily	106 cases healed with 16-34 applications	These results indicate that citric acid treatment is highly effective for controlling the infecting organism, which is paramount to the success of healing.

n = number of infected wounds; MSSA = methicillin-sensitive *Staphylococcus aureus*; MRSA = methicillin-resistant *Staphylococcus aureus*; MDRO = multidrug resistant microorganisms.

Figure 1. Methodological flow of search strategy.



3. OBJETIVO

3.1. Objetivo Geral

- ✓ Avaliar a atividade antimicrobiana *in vivo* e *in vitro* dos extratos acetato de etila e hidroalcoólico da casca do tronco de *Hymenaea stigonocarpa* Mart ex Hayne.

3.2. Objetivos Específicos

- ✓ Estudar a atividade antimicrobiana através da determinação da concentração inibitória mínima dos extratos acetato de etila e hidroalcoólico de *Hymenaea stigonocarpa* Mart ex Hayne, frente a 28 cepas de *Staphylococcus aureus* multirresistentes e de coleção.

- ✓ Avaliar a toxicidade dérmica aguda do gel a 1% e 2% do extrato hidroalcoólico de *Hymenaea stigonocarpa* Mart ex Hayne.

- ✓ Avaliar a atividade antimicrobiana e de cicatrização do gel a 2% de *H. stigonocarpa* utilizando um modelo experimental de ferida aberta em animais normais e diabéticos do tipo I.

4. PROCEDIMENTOS METODOLÓGICOS

4.1. Ensaio antimicrobiano *in vitro*

4.1.1. Microrganismos

Neste experimento foram utilizadas 28 cepas de *Staphylococcus aureus* algumas delas obtidas a partir de espécimes de pacientes acometidos por infecções ou isolados de alimento com um fenótipo de resistência para diversos agentes antimicrobianos. Todos os microrganismos pertencentes à coleção do Departamento de Antibióticos da Universidade Federal de Pernambuco (UFPE) (Tabela 1).

Cepas bacterianas	Fenótipo de resistência dos isolado clínico
Am 01	PEN G
Am 02	PEN. G; TET
Am 03	PEN. G
Am 04	CLI; ERI; GENT; OXA; PEN. G
Am 05	ERI
Am 06	TET; PEN. G; TET
Am 07	ND
Am 09	CLI; ERI
Am 13	ND
BA 01	ND
Am 18	ND
Am 19	CFZ; AMP; OXA; PEN. G
Am 20	AMP; CFZ; CIP; CLI; ERI; LEV; OXA; PEN. G.
Am 21	AMP; CFZ; CIP; CLI; ERI; LEV; OXA; PEN. G
IC 012	ND

IC 17	ND
IC 155	ND
IC 247	ND
IC 404	CIP
ATCC 25 923	ND
FI 13	ND
FI 14	ND
FI 15	IMP; SZT
FI 16	ND
IC 27	IMP; SZT
ATCC 6538	NIT; LMX; VAN; NET; RIF; AZI; SUT; OFX
IC 311	IMP; SZT
IC 138	CTX; ERI; CLO

AMP: Ampicilina 10 µg; AZI: Azitromicina 15 µg; CFZ: Cefazolina 30 µg; CIP: Ciprofloxacina 5 g; CLI: Clindamicina 2 µg; CLO: Cloranfenicol 30 µg; CTX: cefotaxima 30 µg; IIE: Eritromicina 15 µg; IPM: Imipenem 10 µg; Levítico: Levofloxacina 5 µg; LMX: Lomefloxacina 10 µg; NET: Netilmicina 30 µg; NIT: Nitrofurantoína 30 µg; OFX: Ofloxacin 5 µg; OXA: Oxacylin 1 µg; PEN G: Penicilina G 10 µg; RIF: Rifampicina 40 µg; SUT: Sulfazotrim 25 mcg; SZT: 25 g Sulfazotrim; TET: Tetraciclina 30 µg; VAN: Vancomicina 30 µg. ATCC: American Type Culture Collection, CI: isolado clínico; ND: Não determinado, FI: isolado de alimento.

4.1.2. Material Botânico

O material vegetal (tronco) da *Hymenaea stigonocarpa* foi coletado na cidade de Caxias, Estado do Maranhão, Brasil e identificado pelo Dr. Gonçalo Moreira Conceição. Uma amostra foi depositada com o número 055 no herbário Professor Aluízio Bittencourt, da Universidade Estadual do Maranhão (UEMA).

4.1.3. Obtenção dos extratos

A casca do tronco foi moída, o pó de *H. stigonocarpa* (890g), foi extraído exaustivamente com etanol P.A. Posteriormente, o extrato foi seco sob pressão reduzida a cerca de 40 ° C e o resíduo foi redissolvido com água / etanol (1:1) e submetido a sucessivas partições com acetato de etila (AcOEt). Os solventes orgânicos foram removidos usando rotaevaporador a vácuo. Os extratos concentrados foram armazenadas em geladeira (4 ° C) até serem utilizado nos testes biológicos.

4.1.4. *Determinação da Concentração Inibitória Mínima dos extratos de H stigonocarpa* **Mart. Ex Hayne**

O ensaio de microdiluição em placa foi realizado em microplacas estéreis de 96 cavidades com fundo em forma de "U".

Um volume de 200 µL dos extratos foi depositado nas colunas de 1 a 9 da linha A. Os demais orifícios foram preenchidos com 100 µL de caldo Mueller-Hinton duas vezes mais concentrado.

Em seguida, uma alíquota de 100 µL do extrato contido nos orifícios da linha A foi transferido para os orifícios da linha B, e após homogeneização, o mesmo volume foi transferido para a linha C. Este procedimento foi repetido até a linha H e o excesso da diluição foi desprezado. Dessa forma foram obtidas assim as concentrações 1 mg/mL e assim por diante. Os inóculos bacterianos padronizados em 10^8 UFC/mL (tubo 0,5 da escala de MacFarland) foram diluídos 1/10 em solução salina esterilizada (0,9%) e desta diluição um volume de 5 µL (10^7

UFC/mL) foi depositada em todos os orifícios das linhas A-H. Os orifícios da coluna 11 foram destinados à avaliação intrínseca do sistema de solventes, bom como da esterilidade da placa.

As microplacas foram incubadas em estufa bacteriológica a 35°C por 24 horas. Decorrido este intervalo de tempo foi adicionado a cada um dos orifícios 20 µL de uma solução aquosa de TTC (cloreto de 2,3,5-trifeniltetrazólio) a 0,5% e as microplacas foram novamente incubadas por mais três horas a 35°C. Após esta última incubação a presença de uma coloração vermelha nos orifícios foi interpretada como prova negativa do efeito inibitório do extrato, ou seja, não houve inibição do crescimento bacteriano. As bactérias viáveis são capazes de reduzir o cloreto de 2, 3, 5- trifeniltetrazólio formando um complexo insolúvel vermelho (Figura 2). A CIM é definida como a menor concentração do extrato em mg/mL capaz de impedir o crescimento microbiano.

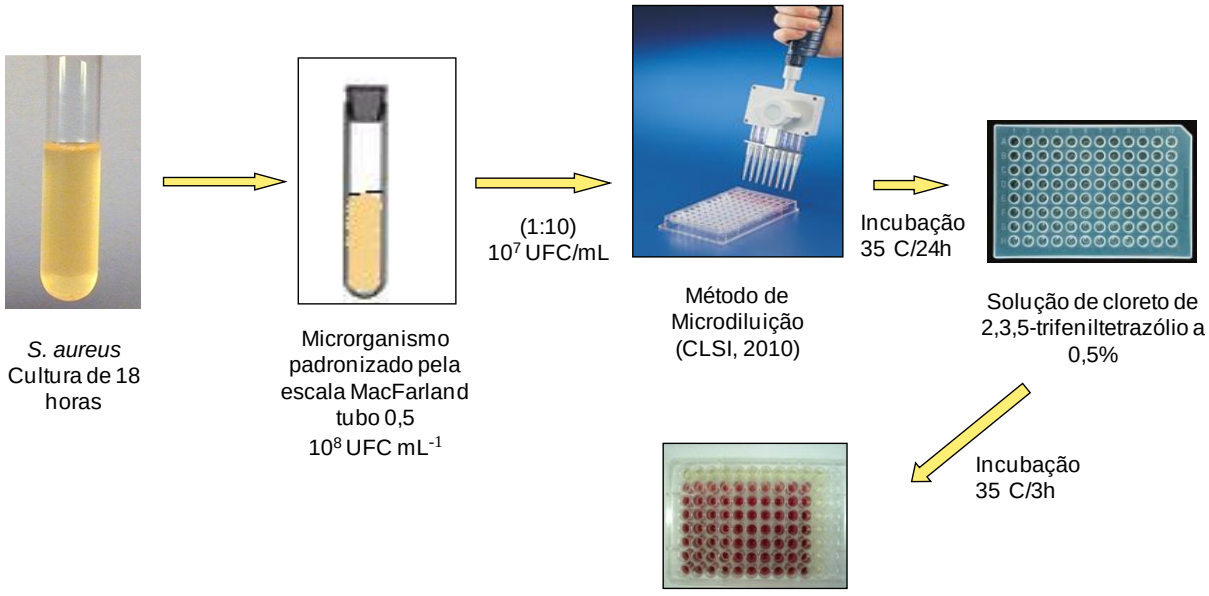


Figura 2. Protocolo experimental para a determinação da Concentração Inibitória Mínima.

4.2. Determinação das atividades antimicrobiana e cicatrizante do gel de Hymenaea stigonocarpa Mart. ex Haine

O extrato que apresentou os melhores resultados no ensaio antimicrobiano foi usado para a preparação do gel usado no experimento *in vivo*. Um gel a 2% do estrato hidroalcoólico de *H. stigonocarpa* foi formulado utilizando Carbopol 940 ® (Vetec) na concentração de 1%.

4.2.1. Animais

Para esta etapa do trabalho foram utilizados ratos Wistar machos, com peso compreendido entre 200 e 300g e camundongos Swiss de ambos os sexos (25 e 30g) procedentes do biotério do Departamento de Antibióticos da UFPE. Os animais foram mantidos em condições padrões no biotério do CCS. Este trabalho foi aprovado pelo comitê de ética em experimentação animal do Centro de Ciências Biológicas da UFPE com número de protocolo (23076.005142/2009-21).

4.2.2. Toxicidade dérmica aguda

Para determinação da toxicidade dérmica aguda foram utilizados grupos de camundongos de ambos os sexos (n=06 animais/grupo), sendo 3 grupos de animais tratados com difenrentes concentrações do estrato hidroalcóolico veiculado em gel hidrofílico (1g/ml, 2g/ml e 4g/ml) e um grupo de animais tratados apenas com o gel hidrofílico. Os animais tiveram a pele do dorso (em torno da linha mediana) depilada e foram acomodados em gaiolas individuais durante 24 horas. Depois disso, os grupos experimentais foram tratados por via tópica com o extrato de *Hymenaea*

stigonocarpa Mart. ex Hayne veiculado em gel hidrofílico em concentrações escolhidas com base nos valores da concentração inibitória mínima (CIM, duas vezes a CIM e quatro vezes a CIM) e tiveram a região tratada imediatamente ocluída com gaze protegida com fita adesiva cirúrgica. Vinte quatro horas após a aplicação, a gaze foi cuidadosamente removida e os grupos avaliados após 30, 60, 120, 240 e 360 minutos. Após este período os animais foram observados a cada 24 horas durante 14 dias.

Os critérios utilizados para a avaliação da toxicidade dérmica aguda foram os mesmos descritos por Malone, 1977, que incluiram observação macroscópica da pele e pêlo adjacente a área da aplicação, olhos, salivação, tremores convulsões, atividade motora, resposta a estímulos sensoriais, bem como outros sinais clínicos de toxicidade ou morte. No final do ensaio, os animais sobreviventes foram eutanasiados em câmara de gás e foi realizado o exame macroscópico das seguintes vísceras: coração, pulmões, rins, fígado, estômago, ovários, úteros testículos (MILLER e TAINTER, 1944).

4.2.3. Modelo experimental de rato diabético tipo I

Para a determinação da atividade cicatrizante do gel de *H. stigonocarpa* sobre feridas abertas em ratos (n=36), os experimentos forma divididos em cinco etapas: Indução do diabetes, processo cirúrgico para indução de ferida no dorso dos ratos, infecção das feridas com *Staphylococcus aureus*, tratamento e avaliação histológica.

Os ratos foram mantidos em jejum por 24 horas antes da indução do diabetes. Esta indução foi realizada por injeção intraperitoneal de 42 mg/kg de uma solução de estreptozotocina Sigma® preparada em tampão citrato pH 4,5, sendo a dose de 0,1ml/100g de peso do animal. No terceiro dia após este procedimento foi realizada a dosagem de glicose sanguínea para

monitoramento da hiperglicemia. Valores de glicemia de jejum entre 200mg/dL e 600mg/dL que persistiram até o sétimo dia confirmaram a indução do diabetes (DELFINO et al., 2002).

4.2.4. Modelo experimental de ferida aberta

O modelo de ferida aberta em ratos diabéticos foi adaptado de Galiano *et al*. (2004). Os animais foram pesados e em seguida anestesiados individualmente por injeção intraperitoneal de Ketamina (75mg/kg), Xilazina (15 mg/kg) e Acepromazina (25 mg/kg). Todos os animais foram pesados e em seguida colocados em decúbito ventral e imobilizados para depilação da região dorsal média. Após antissepsia da região, foi feito com um molde de metal um desenho circular e com o auxílio de uma tesoura estéril, uma ferida de 10 mm na linha média dorsal até o nível do tecido aponeurótico. Um molde de silicone circular, nas dimensões da ferida, foi suturado para conter a contração do músculo dorsal. Em seguida as lesões foram contaminadas com 0,5 mL de suspensão do microrganismo *Staphylococcus aureus* (1,5 x 10^6 UFC/mL), cepa IC 17 e ocluídas (Figura 3).

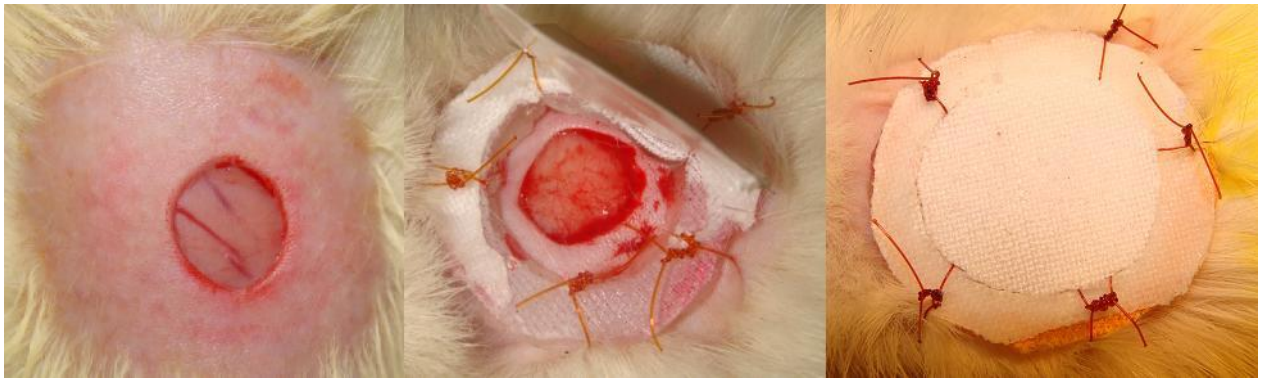


Figura 3. Modelo experimental de ferida aberta.

No segundo dia após a contaminação a oclusão foi retirada e o exsudado de todas as feridas foi coletado utilizando um swab esterilizado. Os exsudado purulentos foram inoculados em meio líquido Mueller-Hinton e semeados em ágar manitol-salgado. Após incubação por 24h a 37°C a viabilidade bacteriana foi observada e a infecção confirmada (Figura 4).

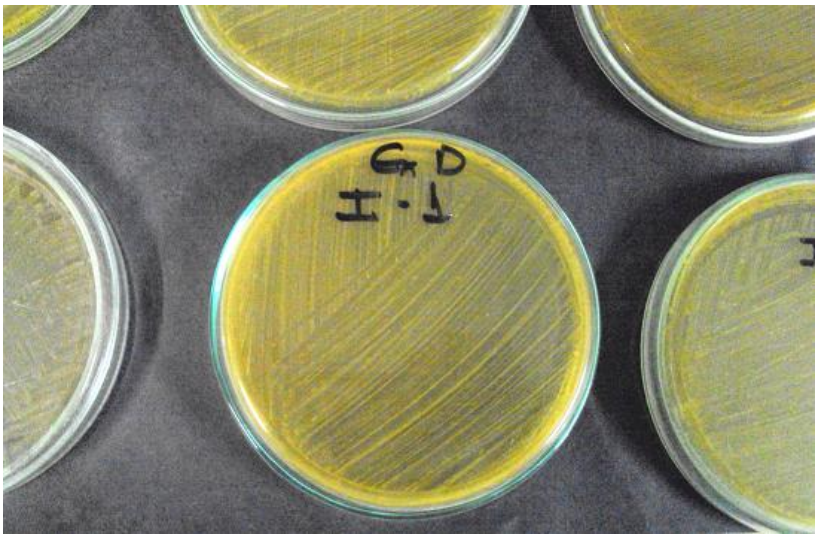


Figura 4. Cultura de *S. aureus* isolado, de ferida após a infecção, cultivado em ágar Manitol Salgado (Merck).

Nessa etapa do trabalho, a pesquisa foi dividida em dois experimentos: O experimento A realizado com animais normoglicêmicos e experimento B com animais diabéticos. Os quais receberam tratamento com insulina (6U) para manter a glicemia controlada. A glicemia foi mensurada ao quarto, oitavo e décimo segundo dia. Esse procedimento foi adotado para manter nos animais glicemia de até 120mg/dL garantindo que a diabetes não estivesse descompensada, pois na diabetes descompensada há uma redução na migração e da quimiotaxia dos neutrófilos (SCORTEGAGNA, 2010) (Figura 5).

Após 24 horas da infecção das feridas com *S. aureus*, animais dos grupos experimentais A e B foram tratados por via tópica com o gel de *H stigonocarpa* a 2%. Os animais do grupo

controle 1 dos experimentos A e B receberam somente o veículo gel Carbopol 940® a 1% sem o principio ativo. Os animais do grupo controle 2 dos experimentos A e B receberam polivinilpirrolidona (Figura 4). As aplicações foram realizadas diariamente até a eutanásia no 14° dia.

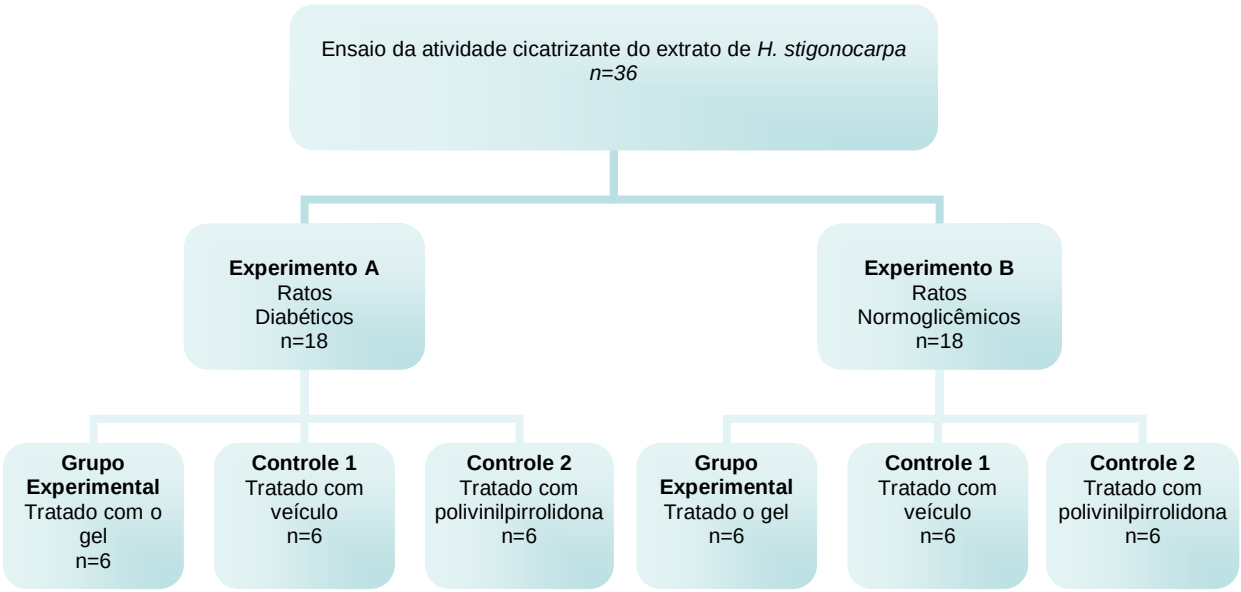


Figura 5. Organograma da distribuição dos animais no ensaio da atividade cicatrizante do extrato hidroalcoólico de *H. stigonocarpa*.

O fechamento das feridas foi avaliado pela medida dos seus diâmetros com o auxílio de um paquímetro digital, realizado sempre pelo mesmo avaliador. O diâmetro maior e menor foi medido a partir das bordas da incisão na pele e a área (S) calculada a partir da seguinte equação $S = \pi ab$, onde a e b correspondem à metade do diâmetro maior e do menor, respectivamente (Figura 6).

A cicatrização foi definida como uma redução da área da ferida e expressa em porcentagem. O percentual de fechamento da ferida foi calculado como [(S inicial - S final) / S inicial] X 100 (SENTHIL KUMAR, 2006).

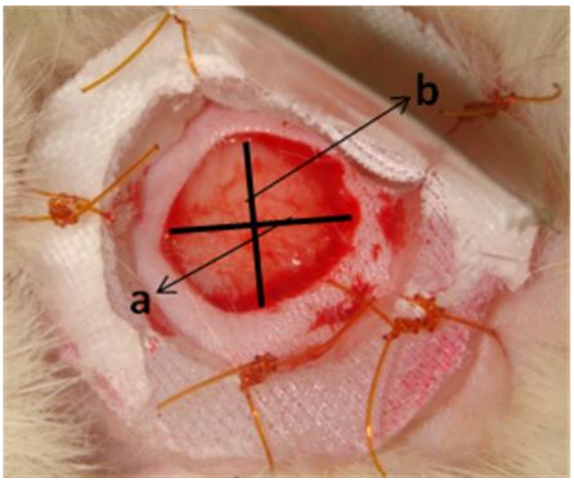


Figura 6. Representação dos diâmetros maior (a) e menor (b) de uma ferida.

Ao décimo dia foram coletadas amostras do tecido de cicatrização dos animais onde o processo cicatricial não havia se completado para verificar a presença de bactérias. Esse tecido foi removido e com um swab esterilizado foi coletada amostra da base de cada ferida. As amostras foram incubadas em meio BHI (brain heart infusion) por 24h a 37°C e em seguida semeadas em ágar manitol-salgado. Após 24 horas de incubação foi observado crescimento bacteriano

Antes da eutanásia, os animais foram anestesiados por injeção intraperitoneal de Ketamina (75 mg/kg) e Xilazina (15mg/kg). Assepsia do tórax com polivinilpirrolidona foi realizada e em seguida, aproximadamente 1 mL do sangue foi coletado via punção cardíaca. O sangue foi utilizado para realização de exame hematológico. Após 14 dias do tratamento seis animais de cada grupo foram eutanasiados em câmara de CO_2.

. Após a eutanásia, os animais tiveram o tecido de cicatrização da ferida e o tecido adjacente dissecados. Este tecido foi lavado em solução salina a 0,9% esterilizada, cortes transversais foram realizados, de modo a facilitar a penetração da substância fixadora, a formalina tamponada a 10% (pH=7,0). Após a fixação, por aproximadamente 1 hora, foram

obtidos fragmentos de tecido de 0,5 cm de espessura. As peças passaram pelo processamento de histologia clássico para preparação das lâminas usadas na análise morfométrica.

4.2.5. Análise histológica

Para a análise histológica os cortes foram examinados ao microscópio acoplado a uma câmera de digital *e* um sistema de análise de imagem (Motic Imagens Plus ® 2.0). Para cada amostra de ferida dos animais, independente da quantidade de preparações histológicas confeccionadas e analisadas morfologicamnte, foram fotografados 10 campos em uma objetiva de 40x. As imagens capturadas foram analisadas com o uso do programa Mesurim® para mensuração dos seguintes parâmetros: contagem de células inflamatórias, vasos sanguíneos, fibrócitos e fibroblastos.

4.3. Análise estatística

Os resultados foram expressos em média e desvio padrão. Os resultados dos extratos na CIM foram comparados estatisticamente pelo teste t de Student, No experimento in vivo diferenças estatísticas entre os grupos tratados e os controles foram determinadas pelo teste Análise de Variância *one-way* (ANOVA) usando o software estatístico GraphPad Prism 5.0, com nível de significância estabelecido em $P < 0,05$.

5. RESULTADOS E DISCUSSÃO

5.1. Artigo original

A ser submetido à revista Wound Repair and Regeneration

Qualis B1

Evaluation of antimicrobial and wound healing activities of the extract of *Hymenaea stigonocarpa* Mart. ex Hayne

Anacássia Fonseca Lima, Maria Bernadete Sousa Maia, Liriane Baratella Evêncio, Teresinha Gonçalves da Silva, Leonardo de Aquino Linhares, Eryvelton de Souza Franco, Tiago Gomes Fernandes, Carmen de Castro Chaves, Carla Michaela Leal Soares, Gustavo Santiago Dimech, Márcia Silva do Nascimento, Eulália Camelo Pessoa de Azevedo Ximenes.

KEYWORDS: *Hymenaea stigonocarpa*, Diabetes, *S. aureus, wound healing*.

ABSTRACT

The plant kingdom is a major source of organic substances of therapeutic interest. The species *Hymenaea stigonocarpa* Mart. ex Hayne, Leguminosae-Caesalpinioideae commonly known as Jatoba-do-cerrado, is a "hardwood" used in folk medicine as anti-inflammatory and antibiotic. Given the promising results about the photochemistry and pharmacology of the genus Hymenaea several researchers rehearsed introduce it as anti-infective agent. The aim of present study was to evaluate the antimicrobial activity *in vivo* and *in vitro* extract from *H. stigonocarpa* Mart. ex Hayne against 28 strains of *S. aureus* with phenotypes of resistance to several antibiotics. In vitro results showed that both extracts, the ethyl acetate and the hydroalcoholic, showed antimicrobial activity, the latter being the most effective, and chosen for the evaluation of antimicrobial activity *in vivo*. For this evaluation was used the wound mode in normoglycemic and diabetic rats. Wounds were infected with a *Staphylococcus aureus* Methicillin resistant strain and treated, once a day, with the *H. stigonocarpa* hydroalcoholic extract gel (2%) for 14 days. All the wounds of animals treated with the *H. stigonocarpa* gel, diabetic and non-diabetic, healed at the end of the experiment. Healing rate of non-diabetic animals treated with the *H. stigonocarpa* gel was higher than controls and this difference was statistically significant. Although the wounds of diabetic animals treated with the gel do not have shown a statistically significant percentage of wound healing compared to controls, histological results of wound areas revealed not only reepithelialization, but also tissue formation in more advanced stage of maturation than controls. The evaluation of acute dermal toxicity of the *H. stigonocarpa* extract gel of revealed no toxic or irritant action. More studies on the active secondary metabolites of *H. stigonocarpa* and investigation of its biological functions may support the development of a new drug.

INTRODUCTION

Brazilian savannas known as 'Cerrado' have a diverse flora with several species used in traditional medicine, many of them possessing antibacterial and antifungal properties and are heavily used by low-income population. [1]

Plants from the genus Hymenaea (Fabaceae family) are commonly used in traditional Brazilian medicine to treat inflammatory process and infectious diseases.[2,3] This plants are characterized by the production of resins of commercial value[4], edible fruits[5] and abundant quantities of floral[6] and extrafloral nectar.[7] The ethanolic extract from resin is used locally in folk medicine for treatment of bronchitis and stomach disorder.[8]

Hymenaea stigonocarpa, also known by its common name 'jatobá-do-cerrado', is a very typical tree of the Brazilian. This species produces large flowers and large quantities of resins that are popularly used as medicine in the treatment of urinary tract disease and respiratory.[5]

The high prevalence of bacterial infections in humans has increased interest in the research and development of new antibacterial agents once these infections are associated with increase mortality and morbidity, especially in the case of chronic diseases like diabetes.[9]

Diabetes *mellitus* is characterized by a metabolic disorder of multiple etiologies. It is considered one of the most important health problems today, both in terms of number and premature mortality, as compared to the costs involved in the control and treatment of its complications.[10] One of the major complications in diabetic patients are infections of the lower limbs by *S. aureus*[11,12] often resistant to conventional treatments.[13,14] This kind of infection complicates the healing process and may progress to osteomyelitis that is responsible for a large number of amputations.

Phytochemical studies of the genus Hymenaea revealed the predominate presence of terpenes, flavonoids and tannins.[15,16,17,18] These substances play varied roles in plant defense and can be used in development of phytotherapy medicines..[19]

In folk medicine the *H. stigonocarpa* resins are used for the treatment of cystitis and as syrup for respiratory problems. Due to this ethnopharmacological importance the aim of this study was to evaluate in vitro and in vivo antimicrobial and wound healing activities of this plant.

MATERIALS AND METHODS

Plant Material and Extraction

The plant material (trunk bark) of the *Hymenaea stigonocarpa* was collected in the Caxias city, state of Maranhão Brazil (04°55'S 43°20'W) and was identified by Dr. Gonçalo Moreira Conceição. A voucher specimen was deposited with the number 055 at the herbarium Prof. Aluízio Bittencourt of Universidade Estadual do Maranhão (UEMA).

Extraction

Air-dried and powdered trunk bark (890g) of *H. stigonocarpa* was extracted exhaustively by ethanol P.A. Subsequently, the extract were dried under reduced pressure at about 40°C and the residue was redissolved with ethanol/water (1/1) and submitted to successive partition with ethyl acetate (EtOAc). The organic solvents were removed in vacuum using rotatory evaporator. The concentrated extracts were stored in a refrigerator (4 °C) until used in biological tests.[20]

Experimental animals

The animals experiment was performed according to the Association for Assessment and Accreditation of Laboratory Animal Care and was approved by the Local Ethical Committee for animal experimentation (Comitê de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco – UFPE) at the number of protocol 23076.005142/2009-21.

Male Wistar albino rats (200-250 g) and mice (30-25 g) were used. The animals were fed with commercial diet (Labina, Agribrands Purina do Brasil Ltda, Brasil) and had free access to water. The animals were housed individually in metabolic cages, maintained under standard conditions (12 h light/12 h dark cycle; 25 °C ± 3°C).

Antibacterial Assay

In this study were used a 28 multi drugs resistant *Staphylococcus aureus* isolated from clinical specimens and food: Am 01, Am 02, Am 03, Am 04, Am 05, Am 06, Am 07, Am 09, Am 13, BA 01, Am 18, Am 19, Am 20, Am 21, IC 012, IC 17, IC 155, IC 247, IC 404, ATCC 25 923, FI 13, FI 14, FI 15, FI 16, IC 27, ATCC 6538, IC 311, IC 138. All of these were maintained on Mueller-Hinton agar slants at refrigerated temperature.

Minimal inhibitory concentration (MIC) of the ethyl acetate and hydroalcoholic extracts was determined by the microdilution method with modifications.[21]

The dried extracts of *H. stigonocarpa* were weighed and solubilized in DMSO/tween80/H2O (1:1:8) to obtain standard solutions of concentration equal to 1000 µg mL^{-1}. These solutions were sterilized by filtration on a membrane Millipore® 0.22µm.

The test was performed in Mueller-Hinton broth. A serial doubling dilution of the extracts were performed in a 96 well microtitre plate (TRP-92096, Switzerland) over the range with dilutions from 1000 to 7.81 μg mL^{-1}.

Overnight broth cultures were prepared in Mueller broth and adjusted so that the final concentration in each well following inoculation was approximately 10^4 UFC mL^{-1}. The concentration of each inocula was confirmed previously by viable counts colonies on Mueler-Hinton Agar (MHA). The solvent system DMSO/Tween 80/H_2O(1/1/8) was used as negative control. One well was used as control of the growth of the *S. aureus* in the medium and one inoculated well free of extract was used to assure the sterility of the medium.

The antibacterial activity was detected by adding 20μL of 0.5% 2,3,5-Triphenyl Tetrazolium Chloride (TTC) Vetec® aqueous solution. The TTC indicates cellular growth, once live cells turn red. Thus, colorless indicated where the extract was active.

The plates were incubated aerobically at 37º C for 24 hours. The MIC was determined as the first well in ascending order which did not produce a red color or that caused complete inhibition of bacterial growth as indicated by the TTC staining. All assays were performed in duplicate. MIC was considered the lowest concentration of the extract that inhibited the bacterial growth, appearing red coloring.[21]

Preparation of gel formulation

The extract which had the best results in antimicrobial assay was used for preparation of gel formulation used in the experiment *in vivo.* A 2% gel of *H. stigonocarpa* hydroalcoholic extract (4x MIC) was formulated using Carbopol 940® (VETEC) in the concentration of 1%.

Acute dermal toxicity

With the aim of further research on antimicrobial and cicatrizant activities of the *H. stigonocarpa* and ensure that the extract was not toxic an acute dermal toxicity test was performed. To determine the acute dermal toxicity groups of mice of both sexes had the dorsal skin shaved (1 cm) and were accommodated in individual cages for 24 hours. Thereafter, the experimental groups were treated topically with gel (1mL) formulation based in hydroalcoholic extract at a concentration equal to MIC, 2x MIC and 4x MIC, obtained on the more resistant microorganism, the treated area was immediately occluded with gauze secured with surgical tape. Twenty four hours after application, the gauze was carefully removed and the groups were carefully observed at 30, 60, 120, 240 and 360 minutes and then every 24 hours for 14 days.

The observations included evaluation of skin and hair surrounding the area of application, eyes, salivation, tremors, convulsions, motor activity, response to sensory stimuli, as well as other clinical signs of toxicity[22] or death. After the test, surviving animals were euthanized and examination was made of the organs.[23]

Experimental induction of type 1 diabetes

Type 1 diabetes was induced by a single intraperitoneal (i.p) injection of streptozotocin (42 mg kg^{-1}) Sigma-Aldrich® (STZ) extemporaneously dissolved in sodium citrate buffer (pH 4.5, 0.1M). The development of the pathology was confirmed by measuring plasma glucose concentration 72 h after injection of STZ. Rats with plasma glucose level 350±150 mg dL^{-1} were used in this study. Treatment with insulin was started on the day after confirmation of the diabetes.[24]

***In Vivo* Wound Healing Activity**

Animals were divided into 2 groups diabetic and non-diabetic, each group was subdivided into three subgroups, three of diabetic animals and three non-diabetic: two subgroups treated with 2% *H. stigonocarpa* gel (HS diabetic and HS non-diabetic groups), two control groups treated with 1% Carbopol 940® gel (diabetic and non-diabetic controls) and two groups treated with polyvinylpyrrolidone (Povidonex® Topic PVPI 10%) POL diabetic and POL non-diabetic groups.

Rats were individually anesthetized using an i.p. injection of ketamine hydrochloride (75,0 mg/kg), xylazin hydrochloride (15,0 mg/kg), and acepromazine (2.5mg/kg). The dorsal surface was shaved and clean with alcohol 70%, full thickness wounds (10x10 mm) extending through the panniculus carnosus were made. A donut-shaped splint with a diameter two times that of the wound and a cover was fashioned from a 0.5mm thick silicone sheet. The splint was sutured so that the wound was centered with in the splint[25].

The wounds were infected with a standardized culture (10^6 CFU mL^{-1}) of *S. aureus* strain CI 17 methicillin-resistant, allowed to infect with the cover closed. The infection was confirmed after 24 hours with culturing of the exudate sample in Brain Heart Infusion medium (BHI).

In the HS groups, rats were topically treated, once a day, with 1mL of the *H. stigonocarpa* hydroalcoholic extract gel 2%, while the control rats were treated only with the vehicle (1% Carbopol 940®, VETEC) and the POL groups with polyvinylpyrrolidone. On the tenth day, exudate samples from wounds were collected to verify persistence of infection. The wounds had the crusts removed and exudate samples were collected with a sterile swab from the base of each wound, the samples were placed incubated in BHI for 24 hours at 37 ° C and then inoculated on mannitol-salt agar. After 24 hours of incubation bacterial growth was observed.

Wound Healing Rates

The diameter of the wounds was measured daily using a digital Paquimetre. Wound closure was determined by using the initial and final areas, with percentage wound closure calculated as [(initial-final)/initial] X 100.[26]

Histological evaluation

Animals were euthanized in the fourteenth day, using a CO_2 chamber. Wound tissues were excised in full depth, including a margin of 5 mm of healthy skin around each wound. Tissues were collected and fixed for 48h in 10% neutral buffered formalin. The formalin fixed tissues were dehydrated through grades of alcohol and cleared in xylene and then embedded in paraffin wax (58–60°mp). The molds were 5 to 7 μm sections deparaffinized and stained with Hematoxylin-Eosin (HE) for overall assessment of tissue sections, and the Masson Trichrome (MT), to assess the presence of collagen fibers and fibrosis exist in interstitial sites.

Computerized image capture system (Motic Images Plus®2.0) was used to perform the morphometric analysis. All morphometric measurements were done from sections through the center of the wounds so as to obtain maximum wound diameter. Healing of wounds was analyzed by measuring the following parameters count inflammatory cells, blood vessels, fibrocytes and fibroblasts. All counts were performed in tem high power fields (40 × objective), midway in the wound.[27,28]

Temperature, weight and hematologic indices

Parameters such as temperature and body weight of animals were followed over the 14 days of experiment. Before euthanasia, on the last day of the experiment, blood was collected for evaluation of hematologic indices through a hemogram and a leucogram.

Statistical Analysis

The results were expressed as mean ± SD. *In vivo* experiment statistical differences between several treatments while their respective control was determined by one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison Tukey tests. *In vitro* experiment result was compared statistically by Student's t test using GraphPad Prism 5.0 software. The level of significance was set at $P < 0.05$.

RESULTS

Antibacterial activity

The results of the antibacterial activity of *H. stigonocarpa* extracts are presented in Table 1. The ethyl acetate extract of *H. stigonocarpa* demonstrated a moderate degree of *in vitro* antibacterial activity against 18 bacterial tested, it were found to be sensitive between 250 and 1000 μg ml^{-1}. The MIC tests revealed that 18 out of 28 *S. aureus* isolates are sensitive to hydro-alcoholic extract between 7,81 and 125 μg ml^{-1}. The biological screening of extracts showed that the ethyl acetate extract have relatively less antimicrobial activity compared with the hydro-alcoholic.

From the result of the MIC hydroalcoholic extract was selected for evaluation of acute dermal toxicity, antimicrobial and wound healing activities in vivo. The concentration of 4x MIC (2000 μg ml^{-1}) was chosen from the results of acute dermal toxicity test. This concentration did not induce any noxious effect on the dermis of mice. All animals survived and organs were analyzed showing no macroscopic changes.

Wound healing activity

When compared to controls diabetic group showed no statistically significant difference in the percentage of wound healing over the 14 days ($P > 0.05$) (Figure 1). In the non-diabetic groups on day 4 the wound closure ratio in the control group was 31,54%, 25,67% in POL group and 59,35% in the HS group (Figure 2). The HS non-diabetic group was statistically significant compared to the control group and the POL group ($P \leq 0.05$). On the twelfth day, wound closure ratios of 97,47%, 98,75%, and 100% were observed in control, POL and HS non-diabetic groups, respectively. Wound closure ratios were higher in HS groups than control and POL groups ($P < 0.05$) (Figure 3 and 4). On the fourteenth day, all wounds of HS groups diabetic and non-diabetic were closed and completely reepithelialized, what does not happen with the control and POL groups.

The cultures of the HS groups showed no bacterial growth. There was growth in the cultures of POL and control groups.

The temperature of the animals remained constant at an average of 34.60 ± 0.31ºC in all groups over the 14 days. As for the weight difference was not statistically significant between diabetic animals (236 ± 16.40 g) and non-diabetic (245 ± 28.70). Diabetic animals showed

polyuria and polydipsia characteristic of the metabolic disorder. There was no change in hematological indices of the animals tested in non-diabetic or diabetic groups.

Histometric analysis

On the fourteenth day, treatment of rats wounds with extract gel led to reduced congestion, edema, inflammatory cells infiltration and necrosis (Figures 5 and 6). A complete reepithelization of the wound was observed in HS diabetic and non-diabetic groups. The granulation tissue showed a higher number of fibrocytes that fibroblast in diabetic HS group when compared to control and POL groups, indicating the change in the fibroblast cell for more mature cells, the fibrocytes. In the non-diabetic groups, morphometric analysis revealed decrease fibroblasts, fibrocytes, blood vessels and immune cells compared to control and POL groups (Figure 7). This shows the maturation of the tissue with replacement of cells by collagen fibers, which was evidenced by reepithelialization. Collagen deposition was visually assessed by MT staining of the wound tissue (Figure 8 and 9). An increase in blue staining fibers was observed in HS diabetic and non-diabetic groups as compared to control and POL groups.

DISCUSSION

Gel formulation of the plant *H. stigonocarpa* was therefore, studied for its wound healing activity in normal as well as in diabetic animals. The selection of this plant was based on its traditional medicinal use and reported pharmacological activities of the genus Hymenaea.[17,29,30,31,32,33]

Thanks to its wide availability, its efficacy and absence of undesirable side effects products derived from plants are potential agents wound healing, and this activity has been proven by several studies.[33]. A study demonstrated that 80% of the world population uses traditional medicine for the treatment of skin diseases; this shows the global dependence of such therapies. [35].

Due to the many uses of plants in folk medicine, in the last years has arisen a great interest of scientists in studying the chemical composition of plants and their biological properties, in order of recognizing their pharmacological activities and take advantage of their therapeutic potential.[2,22,24,26,28,29,35,36 ,] Plants were used for the development of several drugs such as morphine (Papaver somniferum), emetine (Psychotria ipecacuanha) and rutin (Fagopyrum esculentum) that are used in clinical procedures. The complexity of the plants extracts has an advantage in use as antimicrobial agents, makes it difficult the adaptation of microorganisms leading to a lower risk of developing resistance.[37]

Plants of the genus Hymenaea are used in the folk medicine as antimicrobial and antinflammatory[30,35,32]. These antimicrobial properties have been assayed and proven in extracts of *H. courbaril*[29], *H. martiana*[33] and *H. palustris*[17].

The results obtained in this study corroborate with the literature, when showed the strong anti-staphylococcal property of the hydro-alcoholic extract of *H. stigonocarpa*, evidenced by small MIC value and effectiveness in inhibiting the microbial growth of *S. aureus*. These data are promising and could encourage further researches on toxicological and pharmacological aspects of *H. stigonocarpa* by-products in order to support their possible rational use in the antimicrobial therapy, particularly, in anti-*S. aureus* therapy.

The tissue repair process occurs through a series of cellular, biochemical and physiological processes that are complex and are closely related. This process has three phases: inflammation, where there are chemotaxis of polymorphonuclear and lymphocyte, attracted by soluble mediators that facilitate endothelial adhesion and transmigration, playing a key role by secreting cytokines and growth factors; the proliferative phase in which there is angiogenesis, collagen deposition, epithelialization and wound contraction; and the modeling phase which is characterized by a reduced number of cells and remodeling of collagen and other cellular matrix proteins to form the mature tissue.[38] This is especially noticeable when there are healing by secondary intention, which occurs in wounds that cover a large surface area where the borders are apart. Therefore, the model of wound healing is effective for assessing the various parameters involved in the biology of healing.

Diabetic patients end up suffering from diabetic neuropathy and circulatory disorders, this leads to a delayed healing. It occurs mainly due to the difficulty that cells, such as the immune system and those responsible for healing, have in getting to the wound site. This special condition requires the use of agents which facilitate the healing and containing the infections that may have been installed on site.[39]

An important factor related to morbidity and mortality in diabetic patients is the delayed wound healing. The diabetic wounds are difficult to solve, this occurs especially due to an impaired cellular infiltration by an ineffective blood flow, which leads to inadequate formation of granulation tissue. In addition there are changes in the patterns of apoptosis and tissue repair. The presence of fibroblasts is an important indication of an efficient cellular repair, with the evolution of cicatricial process these cells transform into fibrocytes, and begin to deposit collagen fibers. These mature fibers modeling the new tissue.[39]

So presence of fibroblasts and fibrocytes is essential for the production of collagen fibers and maturation of the tissue. *H. stigonocarpa* gel formulation treatment resulted in wounds healing of animals in groups treated (HE groups), this healing activity was stronger in non-diabetic animals being expressed as an increase in the percentage of healing over the first 10 days, which ended in complete healing.

The cicatricial activity was evidenced in morphometry as an increased in the number of fibrocytes in wound of streptozotocin induced diabetic rats, showing that H. stigonocarpa gel formulation enhanced the change in the cell due to the maturation of fibroblasts. This characterizes a phase transition from proliferation to modeling. Animals in the non-diabetic HS group showed a decrease in the number of cells that characterized the modeling phase and was consistent with the evolution of the percentage of healing. Epithelisation was also remarkably better in HS groups as compared to control and POL groups.

In their study, Kietzmann (1999) observed the early closure of wounds of HS non-diabetic group in the tenth day has been shown in morphometric analysis by a decrease in the number of cells and blood vessels, along with the maturation of collagen fibers..

All these findings coupled with the absence of acute toxic activity suggest the therapeutic potential of this plant, particularly regarding to wound healing and antimicrobial activity demonstrated in this study.

CONCLUSION

In conclusion, wound healing and antimicrobial activities of the gel from hydro-alcoholic extract from the *Hymenaea stigonocarpa* were evaluated, and our results revealed that *H. stigonocarpa* possesses significant wound healing and antimicrobial activities.

ACKNOWLEDGMENTS

This paper was financially supported by CNPq through award of a scholarship.

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LIST OF ABBREVIATIONS AND OTHER FOOTNOTES

EtOAc	Ethyl acetate
MIC	Minimal Inhibitory Concentration
DMSO	Dimethyl Sulfoxide
MHA	Mueler-Hinton Agar
TTC	2,3,5-Triphenyl Tetrazolium Chloride
MIC	Minimal inhibitory concentration
2 x MIC	Twice the Minimal Inhibitory Concentration
4 xs MIC	Four times the Minimal Inhibitory Concentration.
STZ	Streptozotocin
i.p	Intraperitoneal
HS diabetic	Groups diabetic treated with 2% *Hymenaea stigonocarpa* gel
HS non-diabetic	Groups non-diabetic treated with 2% *Hymenaea stigonocarpa* gel
POL diabetic	Groups diabetic treated with polyvinylpyrrolidone
POL non-diabetic	Goups non-diabetic treated with polyvinylpyrrolidone
CFU	Colony Forming Units
BHI	Brain Heart Infusion medium
HE	Hematoxylin-Eosin
MT	Masson Trichrome

TABLE

Table 1. Minimum inhibitory concentration (µg/ml) of *Hymenaea stigonocarpa* extracts.

Bacterial strains	Ethyl acetate	Hydro-alcoholic	Phenotype of resistance
Am 01	250	125	PEN G
Am 02	250	125	PEN G; TET
Am 03	250	250	PEN G
Am 04	250	125	CLI; ERI; GENT; OXA; PEN G
Am 05	250	250	ERI
Am 06	250	250	TET; PEN G; TET
Am 07	250	500	ND
Am 09	500	125	CLI; ERI
Am 13	500	250	ND
BA 01	1000	500	ND
Am 18	1000	500	ND
Am 19	250	125	CFZ; AMP; OXA; PEN G
Am 20	250	125	AMP; CFZ; CIP; CLI; ERI; LEV; OXA; PEN. G.
Am 21	250	125	AMP; CFZ; CIP; CLI; ERI; LEV; OXA; PEN G
IC 012	250	125	ND
IC 17	250	125	ND
IC 155	7,81	7,81	ND
IC 247	250	250	ND
IC 404	7,81	7,81	CIP
ATCC 25 923	500	500	ND
FI 13	62,5	62,5	ND
FI 14	125	125	ND
FI 15	125	62,5	IMP; SZT
FI 16	7,81	250	ND
IC 27	62	125	IMP; SZT
ATCC 6538	125	125	NIT; LMX; VAN; NET; RIF; AZI; SUT; OFX
IC 311	7,81	7,81	IMP; SZT
IC 138	7,81	7,81	CTX; ERI; CLO

AMP: Ampicillin 10 µg; AZI: Azithromycin 15 µg; CFZ: Cefazolina 30 µg;CIP: Ciprofloxacin 5 g; CLI: Clindamycin 2 µg; CLO: Chloramphenicol 30 µg; CTX: Cefotaxime 30 µg; ERI: Erythromycin 15 µg; IPM: Imipenem 10 µg; LEV: Levofloxacin 5 µg; LMX: Lomefloxacina 10 µg; NET: netilmicin 30 µg; NIT: 30 µg Nitrofurantoin; OFX: Ofloxacin 5 µg; OXA: Oxacylin 1 µg; PEN G: Penicillin G 10 µg; RIF: Rifampicin 40 µg; SUT: Sulfazotrim 25 µg; SZT: 25 sulfazothrim g; TET: Tetracycline 30 µg; VAN: vancomycin 30 µg. ATCC: American Type Culture Collection, CI: Isolate clinic; ND: Not determined, FI: Food isolate.

FIGURE LEGENDS

Figure 1. Percentage of wound closure in diabetic rats treated with gel formulation *H. stigonocarpa* over 14 days of treatment. There was no statistically significant difference in the percentages of wound closure in the three groups ($p > 0.05$).

Figure 2. Percentage of wound closure in non-diabetic rats treated with gel formulation *H. stigonocarpa* over 14 days of treatment. *There was statistically significant difference in the percentages of wound closure in the HS group when compared with control and POL groups over the 14 days of treatment ($p < 0.05$).

Figure 3. Wound pictures of diabetic groups POL (a) control (b) and HS (c) in the first, seventh and fourteenth days.

Figure 4. Wound pictures of non-diabetic groups POL (a) control (b) and HS (c) in the first, seventh and fourteenth days**.**

Figure 5. Photomicrograph of histological sections from surgical wounds in diabetic animals of POL (a), control (b) and HE (c) groups stained with hematoxylin eosin at a 40x objective. 1 - blood vessels, 2 - fibroblasts, 3 - fibrocytes, 4 - immune system cells.

Figure 6. Photomicrograph of histological sections from surgical wounds in non-diabetic animals of POL (a), control (b) and HE (c) groups stained with hematoxylin eosin at a 40x objective. 1 - blood vessels, 2 - fibroblasts, 3 - fibrocytes, 4 - immune system cells.

Figure 7. Morphometric analysis of non-diabetic and diabetic animals on the fourteenth day. 1- Number of fibroblasts, 2- Number of fibrocytes, 3- Number of blood vessels, 4- Number of immune cells. (c/b) In the diabetic animals was statistical difference between the number of fibrocytes of the HS group compared to control and POL groups, there were also differences in the number of fibroblasts from groups HS and POL.(a/d/e). The non-diabetic animals showed statistically significant differences in the number of fibroblasts, blood vessels and immune cells between the HS group compared to the control and POL groups ($p<0,05$).

Figure 8. Photomicrograph of histological sections from surgical wounds in diabetic animals of POL (a), control (b) and HE (c) groups stained with Masson Trichrome at a 40x objective.

Figure 9. Photomicrograph of histological sections from surgical wounds in diabetic animals of POL (a), control (b) and HE (c) groups stained with Masson Trichrome a 40x objective.

FIGURES

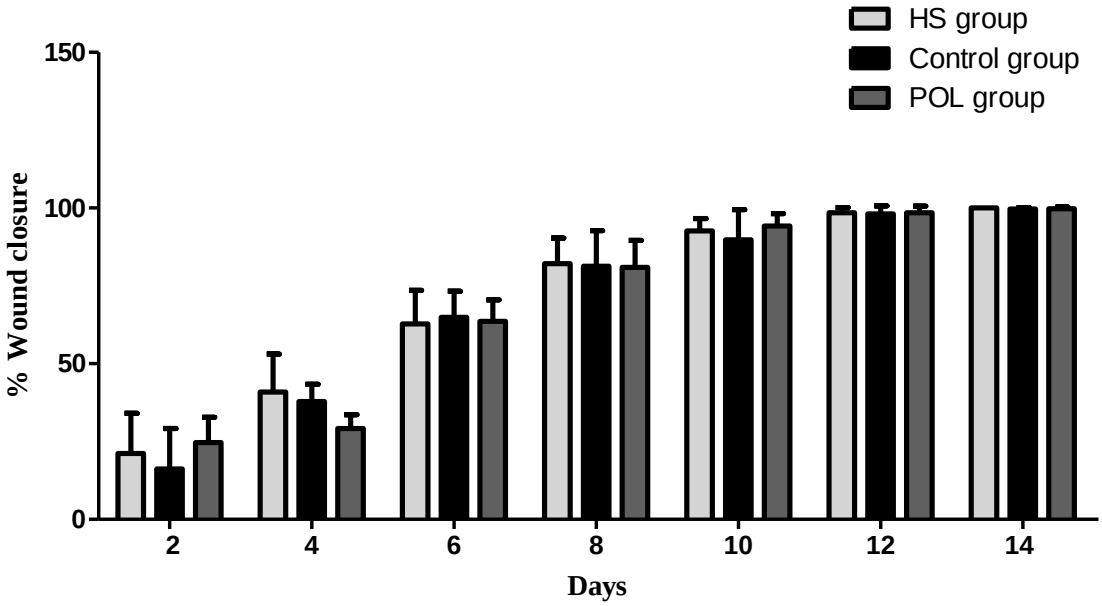


Figure 1. Percentage of wound closure in diabetic rats treated with gel formulation *H. stigonocarpa* (1g/ml) over 14 days of treatment. There was no statistically significant difference in the percentages of wound closure in the three groups ($p > 0.05$, ANOVA).

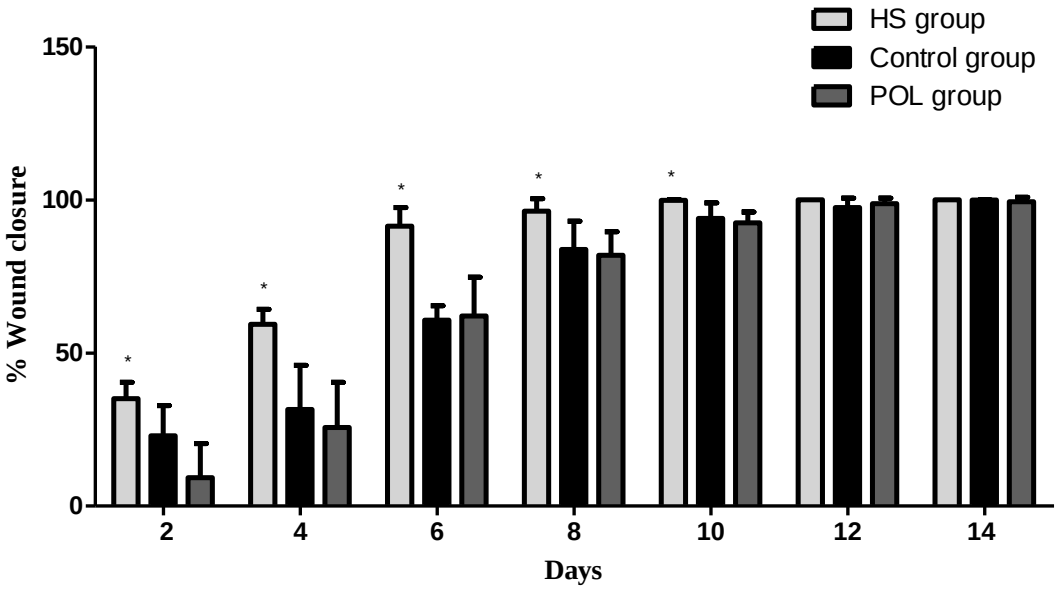


Figure 2. Percentage of wound closure in non-diabetic rats treated with gel formulation *H. stigonocarpa* (1g/ml) over 14 days of treatment. *There was statistically significant difference in the percentages of wound closure in the HS group when compared with control and POL groups over the 14 days of treatment ($p < 0.05$, ANOVA).

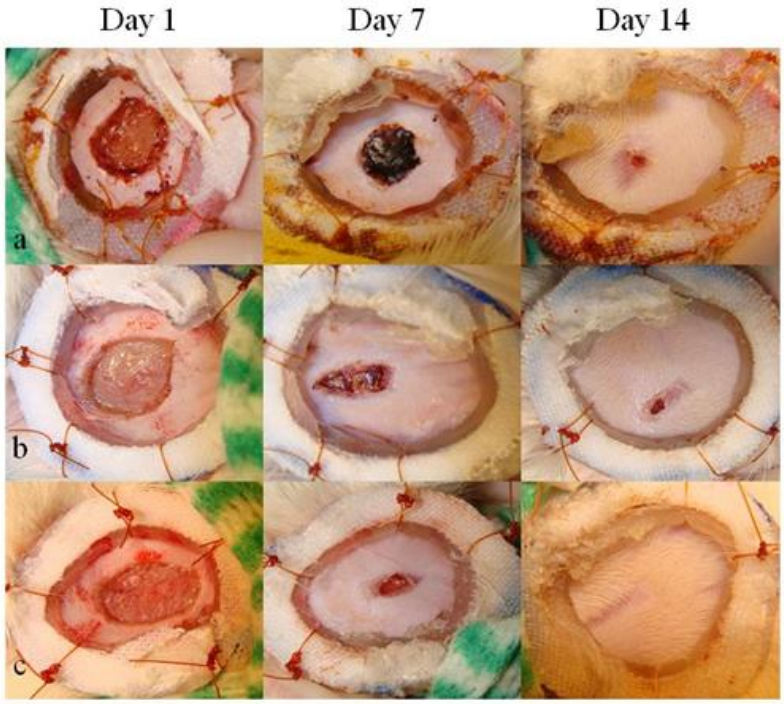


Figure 3. Wound pictures of diabetic groups POL (a) control (b) and HS (c) in the first, seventh and fourteenth days.

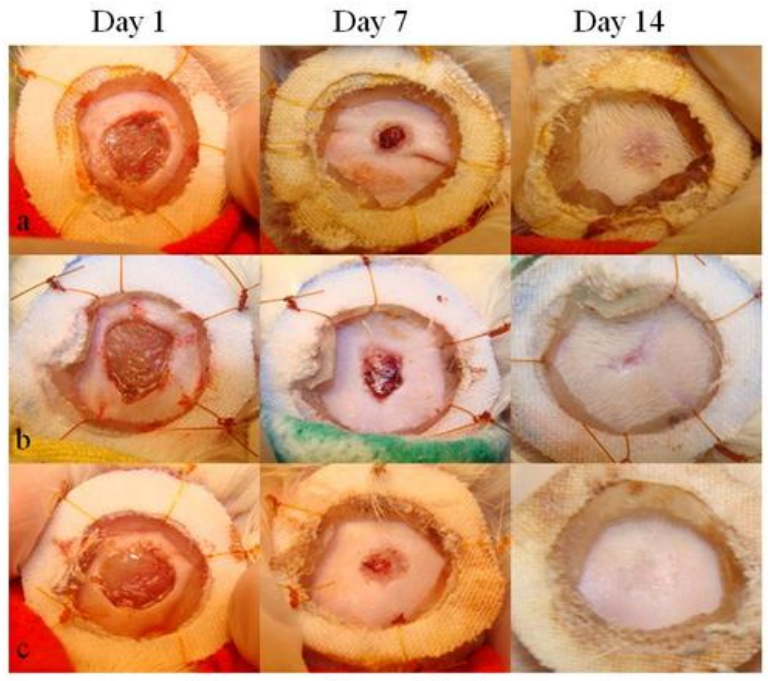
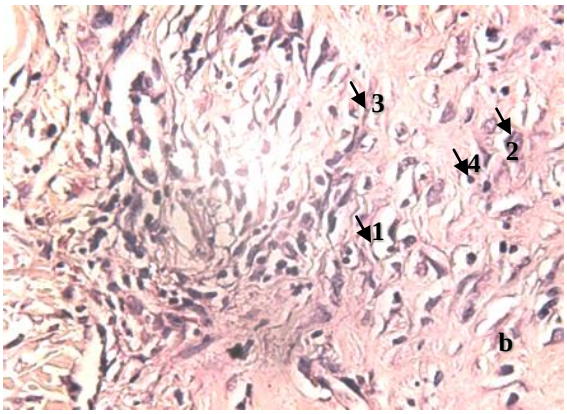
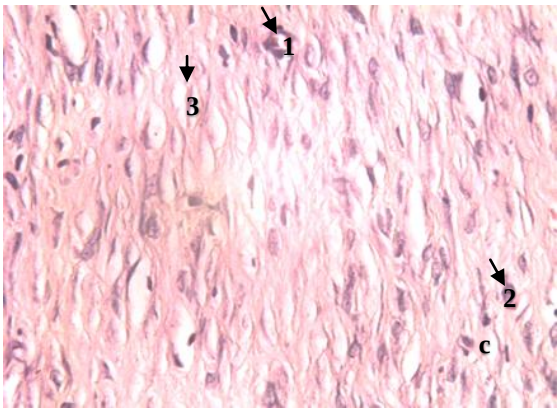


Figure 4. Wound pictures of non-diabetic groups POL (a) control (b) and HS (c) in the first, seventh and fourteenth days.





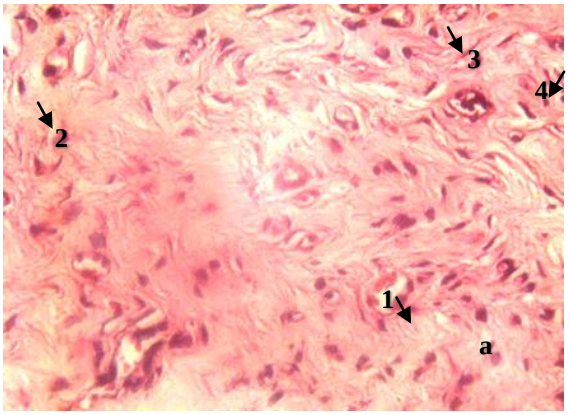
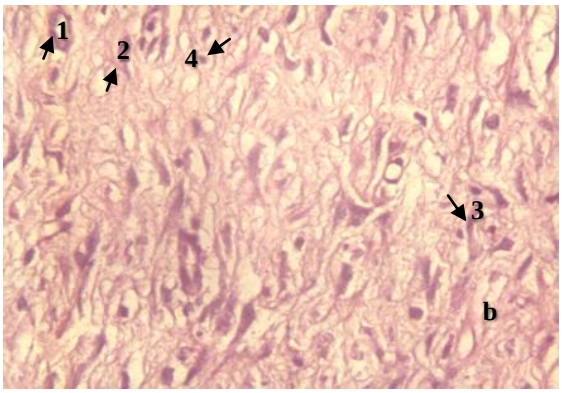
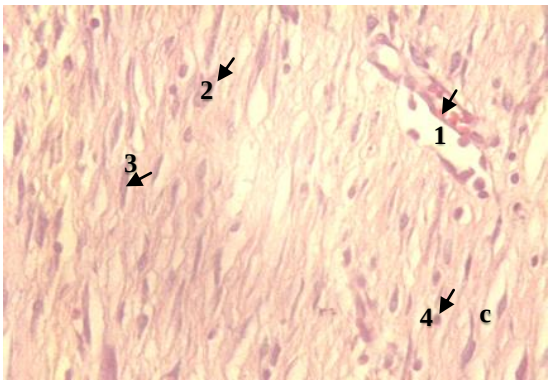


Figure 5. Photomicrograph of histological sections from surgical wounds in diabetic animals of POL (a), control (b) and HE (c) groups stained with hematoxylin eosin at a 40x objective. 1 - blood vessels, 2 - fibroblasts, 3 - fibrocytes, 4 - immune system cells.





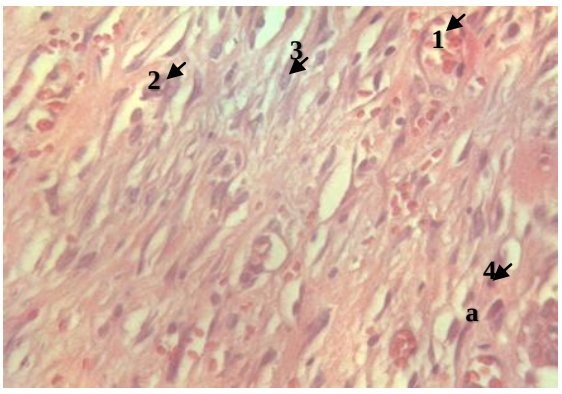


Figure 6. Photomicrograph of histological sections from surgical wounds in non-diabetic animals of POL (a), control (b) and HE (c) groups stained with hematoxylin eosin at a 40x objective. 1 - blood vessels, 2 - fibroblasts, 3 - fibrocytes, 4 - immune system cells.

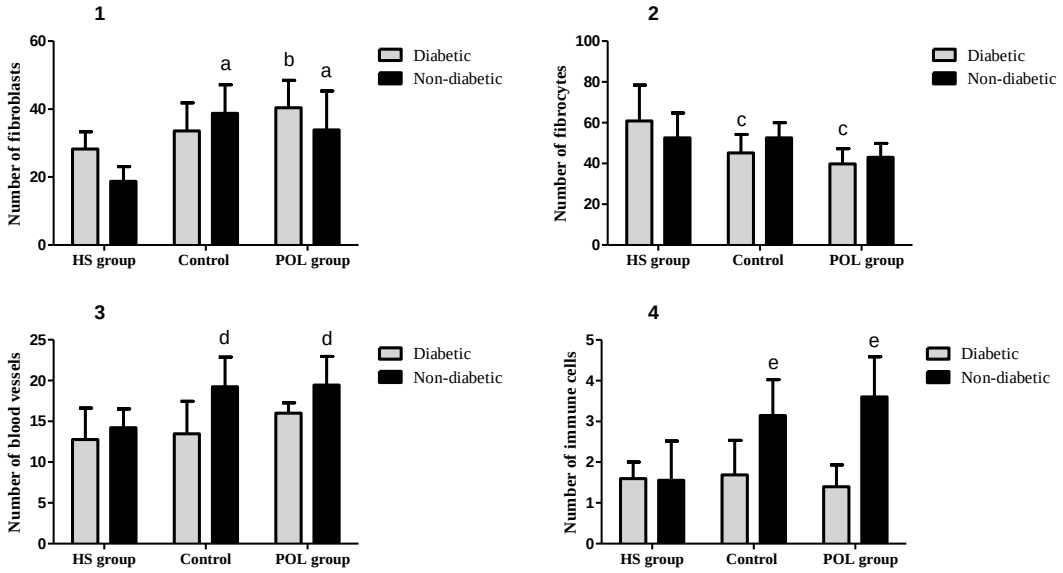


Figure 7. Morphometric analysis of non-diabetic and diabetic animals on the fourteenth day. 1- Number of fibroblasts, 2- Number of fibrocytes, 3- Number of blood vessels, 4- Number of immune cells. (c/b) In the diabetic animals was statistical difference between the number of fibrocytes of the HS group compared to control and POL groups, there were also differences in the number of fibroblasts from groups HS and POL.(a/d/e). The non-diabetic animals showed statistically significant differences in the number of fibroblasts, blood vessels and immune cells between the HS group compared to the control and POL groups ($p<0,05$, T test).

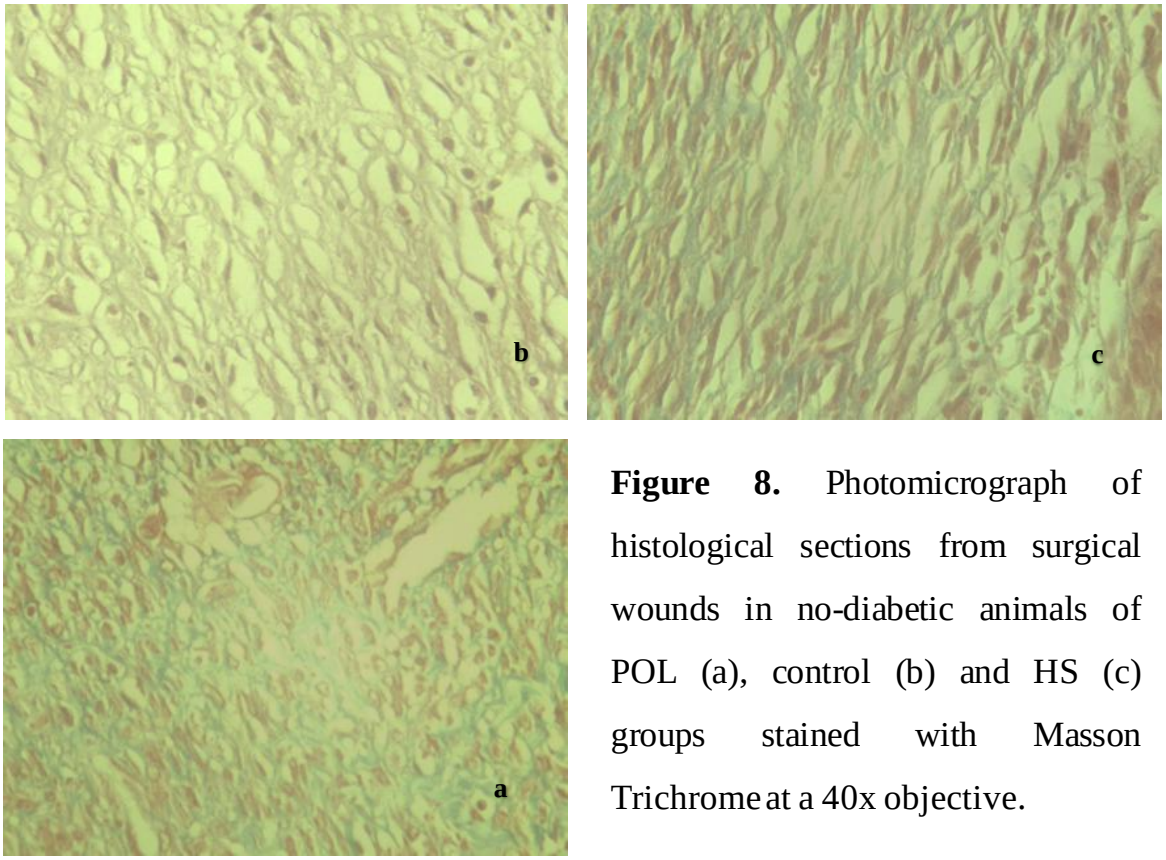


Figure 8. Photomicrograph of histological sections from surgical wounds in no-diabetic animals of POL (a), control (b) and HS (c) groups stained with Masson Trichrome at a 40x objective.

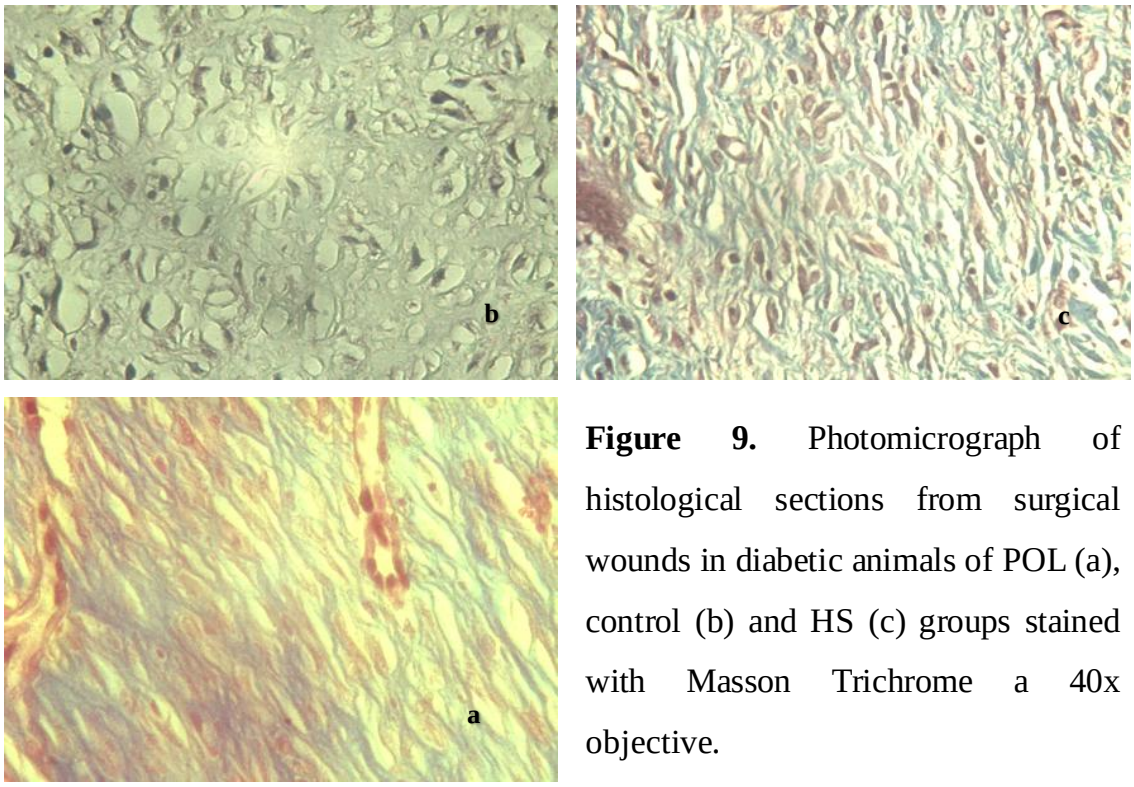


Figure 9. Photomicrograph of histological sections from surgical wounds in diabetic animals of POL (a), control (b) and HS (c) groups stained with Masson Trichrome a 40x objective.

6. CONCLUSÃO

Tanto o extrato hidroalcoólico quanto o acetato de etila da *Hymenaea stigonocarpa* apresentaram significativa atividade antimicrobiana. Essa atividade foi confirmada *in vivo*, tanto nos animais diabéticos quanto nos normoglicêmicos. A atividade cicatrizante da formulação farmacêutica a base do extrato hidroalcoólico foi ressaltada tanto em ratos diabéticos quanto em normoglicêmicos, sendo que os últimos apresentaram maior capacidade de reepitelização. A avaliação da toxicidade dérmica aguda da formulação em gel do extrato de *H. stigonocarpa* revelou não haver ação toxica ou irritante. Mais estudos dessa espécie com o isolamento dos princípios ativos e investigação de suas funções biológicas poderiam servir de suporte para o desenvolvimento de novos medicamentos.

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7. ANEXOS

Normas da revista São Paulo Medical Journal

INSTRUCTIONS TO AUTHORS

Aim and editorial policy

The **São Paulo Medical Journal** , founded in 1932, is one of the oldest medical publications in Brazil. Its articles are indexed in Medline, Lilacs and SciELO, Science Citation Index Expanded e Journal Citation Reports/Science Edition. Published bimonthly by the Associação Paulista de Medicina, the journal accepts articles in the field of clinical health science (internal medicine, gynecology and obstetrics, mental health, surgery, pediatrics, and public health). Articles will be accepted in the form of original articles (experimental studies), literature reviews or updating papers, case reports, short communications and letters to the Editor. Papers with a commercial objective will not be accepted.

The manuscript and types of articles

The manuscript must be sent in English. Nonetheless, it must also include a summary and five key words both in Portuguese (or Spanish) and in English. Texts may be sent in digital form (3 1/2" disk/CD/Zip), in ".doc" or ".rtf" extensions (no other will be accepted) with one printed copy, to the Publications Unit (address below), or via the internet, to revistas@apm.org.br.

Papers submitted must be original and be accompanied by a declaration, signed by all the authors, that the text has not and will not be published in any other journal. Research articles involving human beings must be submitted together with a copy of the authorization from the Ethical Committee of the institution in which the work was performed.

Papers submitted must comply with the editorial standards

established in the Vancouver Convention (Uniform Requirements for Manuscripts Submitted to Biomedical Journals)[1] and the rules for reports on clinical trials,[2] and systematic reviews.[3]

The São Paulo Medical Journal supports the clinical trial registration policies of the World Health Organization (WHO) and the International Committee of Medical Journal Editors (ICMJE) and recognizes the importance of these initiatives regarding the registration and internationally dissemination of information on clinical studies, with open access. Thus, from 2007 onwards, clinical research papers will only be accepted for publication if they have received an identification number from one of the Clinical Trial Registers that have been validated in accordance with the criteria established by WHO and ICMJE. The addresses for these registers are available from the ICMJE website (http://www.icmje.org/). The identification number should be declared at the end of the abstract.

The paper (original articles, short communications and case reports) must be structured so as to contain these parts: introduction, methods, results, discussion and conclusion. Literature reviews may be freely structured, but the text must contain a final part for a conclusion or final considerations.

Abbreviations may not be used, even those in common use. Drugs must be referred to by their generic names, avoiding unnecessary mention of commercial or brand terms. Any product cited in the Methods section, such as diagnostic and testing equipment, reagents, instruments, utensils, prostheses, orthoses and intraoperative devices must be described together with the manufacturer's name and place of manufacture. Medications administered must be described using their generic names followed by the dosage used and posological data.

Grants and any other financial support for the work must be mentioned separately, on the first page. Acknowledgments, if necessary, must be placed after the references.

Original articles (experimental studies)

The text must not exceed 5,000 words (excluding tables, figures and references) and must include a structured abstract with a maximum of 250 words[4]. The structure of the text should whenever possible follow the format laid out below:

1) Introduction: specify the reasons for carrying out the study,

describing the present state of knowledge of the theme. Do not include here any results or conclusions of the study. Use the last paragraph to specify the principal question of the study, and the principal hypothesis tested, if there is one.

2) Objective: described briefly what the main objective of the study was.

3) Methods
3.1) *Type of study*: describe the design of the study specifying, if appropriate, the use of randomization, blind studies, diagnostic test standards and the time direction (retrospective or prospective). For example: randomized clinical trial, double-blind, controlled placebo, or accuracy study.
3.2) *Setting*: indicate where the study was carried out, including the healthcare ranking (for example: primary or tertiary; private or public institution).
3.3) *Sample (participants or patients)*: describe the selection procedures, inclusion criteria and the number of patients at the beginning and end of the study.
3.4) *Procedures* (intervention, diagnostic test or exposition, if necessary): describe the principal characteristics of any intervention, including the method and duration of its administration.
3.5) *Main measurements*: describe the method of measuring the primary result, in the way it was planned before data collection. If the hypothesis reported was formulated during or after data collection, this fact needs to be specified.
3.6) *Statistical methods*: describe the sample size calculation method, the planned statistical analysis, statistical tests used and significance levels, and some post hoc analysis.

4) Results: describe the principal results. If possible, these should be accompanied by their 95% confidence interval and the exact level of statistical significance. For comparative studies, the confidence interval must be stated for the differences between the groups.

5) Discussion: emphasize the new and important factors encountered in the study, which will form part of the conclusion. Do not repeat data presented in the introduction or results in detail. Mention any limitations of your findings that should be noted and possible implications for future research. Relate any observations from other relevant studies.

6) Conclusions: specify only the conclusions that can be sustained

by the results, together with its clinical significance (avoiding excessive generalization), or whether additional studies would be necessary before the information could be put into practice. The same emphasis should be placed on studies with positive and negative results.

Short communications or case series and case reports

Short communications and case reports must be limited to 1,000 words and five references, including an abstract with a description of the case and a pertinent discussion, and key words. The text must be divided in parts as described for original articles.

Review or updating articles

Review and updating articles have free format, provided they present references in the Vancouver Style.[1] Systematic review articles or meta-analyses must comply with the rules for reports on systematic reviews.[3]

Research letters

All research letters published are peer reviewed, with separate statistical review where appropriate. The **São Paulo Medical Journal** wishes to be flexible but ideally a research letter should have no more than 900 words, and a maximum of five references and two tables or figures. An unstructured summary of not more than 100 words is required. The signatures and description of the contributions of all authors are required. A proof will be provided, usually by fax, and the authors must respond immediately because the journal may wish to publish quickly. Authors will be told of non-acceptance.

Letter to the editor

In this category the text must not exceed 500 words and five references.

Format

First page

The first page must contain: 1) the title of the paper in English and Portuguese (os Spanish), which must be short but informative; 2)

the type of paper (original article, review or updating article, short communication, letter to the editor); 3) the name of each author (do not abbreviate), his/her highest academic title attained and the institution where he/she works; 4) the place where the work was developed; 5) the meeting, date, and place where the paper was presented, if applicable; 6) the complete address, e-mail and telephone number of the author to be contacted by the Publication Unit regarding the manuscript; and the addresses and telephone numbers of the main author for publication (which may or may not be the same); 7) sources of support in the forms of finance, equipment or drugs, and the grant numbers; 8) description of any conflicts of interest held by the authors.

Second page: abstract and key words

The second page must include an abstract[4] structured in parts in accordance with the classification of the article. For original articles, there are eight items: 1) context and objective; 2) design and setting (where the study was performed); 3) methods (described in detail); 4) results and 5) conclusions.

This page should also contain five key words. These English terms must be chosen from the Medical Subject Headings (MeSH) list of Index Medicus, which is available on the internet.[5]

References

The references (in the "Vancouver style", as stated by the International Committee of Medical Journal Editors, 1997) should be laid out on the final pages of the article and numbered in the order of citation. References cited in legends of tables and figures must maintain sequence with references cited in the text. All the authors must be listed if there are less than six; if there are six or more, the first three should be mentioned and followed by "et al.". Some examples of the most common types of references:

Article in journal
- Lahita R, Kluger J, Drayer DE, Koffler D, Reidenber MM. Antibodies to nuclear antigens in patients treated with procainamide or acetylprocainamide. N Engl J Med. 1979;301(25):1382-5.

Chapter of book
- Reppert SM. Circadian rhythms: basic aspects and pediatric implications. In: Styne DM, Brook CGD, editors. Current concepts

in pediatric endocrinology. New York: Elsevier; 1987. p. 91-125.

Text on the internet
- Morse SS. Factors in the emergence of infectious diseases. Available from URL: http://www.cdc.gov/ncidod/EID/eid.htm. Accessed in 1996 (Jun 5).

Last page

The last page must contain an abstract written in Portuguese or Spanish, followed by at least five key words ("palavras chave") chosen from among the Subject Descriptors created by Bireme, which are available on the internet.6

Figures and tables

Images must have good resolution (minimum of 300 DPI) and be recorded in ".jpg" or ".tif" format. Do not attach images inside Microsoft PowerPoint documents. If photographs are attached to a Microsoft Word file, send the images separately as well. Graphs must be prepared in Microsoft Excel (do not send them in image formats) and must be accompanied by the tables of data from which they have been generated. The number of illustrations must not exceed half the total number of pages minus one.

All figures and tables must contain legends or titles that precisely describe their contents and the context or sample from which the information was obtained (i.e. what the results presented are, what the kind of sample or setting was). The legend or title sentence should be short but comprehensible without depending on reading the article.

Procedures of the journal

After receipt of the article by the Scientific Publications, the authors will be provided with a protocol number. This number serves to maintain good understanding between the authors and the Publications Unit. Following this, the article will be read by the Editor, who will verify whether it is consonant with the journal's policy and interests, i.e. whether the field of the research or review is within the areas of health or public health.

Next, the Scientific Publications will verify whether the text complies with the journal's Instructions for Authors. If the text is incomplete or if it is not organized as required, the authors will be

asked to resubmit their text after resolving such problems. When its form is acceptable, the Publications Unit will submit the manuscript to open peer review.

Open peer review means that reviewers sign their judgment and that they know the names of the authors. The reviewers are professionals or researchers working in the same field as dealt with by the manuscript. The main objectives of the review are to detect problems in the methodological design and see whether the conclusions are focused on the results presented.

Authors will then receive the reviewers' judgment and will be asked to resolve all the problems pointed out. Once the Publications Unit receives the manuscript again, the text will be sent to the scientific editor and the proofreader, who will point out problems with phrase construction, spelling, bibliographical references and others. Authors should then provide all further information required.

When the text is considered acceptable for publication, and only then, it will enter the queue for publication. The Scientific Publications will provide a proof, including any tables and figures, for the authors to approve. No article is published without this last procedure.

Documents cited

1. International Committee of Medical Journal Editors. Uniform Requirements for Manuscripts Submitted Biomedical Journals. Ann Intern Med 1997; 126:36-47. Available from URL: http: //www.icmje.org. Access in 2003 (Sep 12).

2. Begg C, Cho M, Eastwood S, et al. Improving the quality of reporting of randomized controlled trials. T CONSORT statement. JAMA 1996;276:637-9. Available from URL: http://www.consort-statement.org

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Normas da revista

Wound Repair and Regeneration

The official journal of The Wound Healing Society, The European Tissue Repair Society, The Japanese Society for Wound Healing, and The Australian Wound Management Association

Edited by:
Patricia A. Hebda, Ph.D.

Print ISSN: 1067-1927
Online ISSN: 1524-475X
Frequency: Bi-monthly
Current Volume: 18 / 2010
ISI Journal Citation Reports® Ranking: 2009: Cell Biology: 90 / 161; Medicine, Research & Experimental: 31 / 92; Dermatology: 12 / 48; Surgery: 25 / 166
Impact Factor: 2.781

Top**Author Guidelines**

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ISI Journal Citation Reports® Ranking: 2005: 85/153 (Cell Biology); 8/39 (Dermatology); 31/72 (Medicine, Research & Experimental); 26/139 (Surgery)
Impact Factor: 2.204

Author Guidelines

Wound Repair and Regeneration: The International Journal of Tissue Repair and Regeneration is the official publication of the Wound Healing Society, the European Tissue Repair Society, the Japanese Society for Wound Healing, and the Australian Wound Management Association. This Journal publishes original scientific and/or clinical papers on the broadly defined topics of wound healing and tissue regeneration. Articles that significantly advance the knowledge of processes involved with wound healing and regeneration in all tissues and organisms, or that provide new insights into clinical therapies will be given highest priority. Manuscripts that describe product evaluations will be considered but will receive lower priority. The Journal also welcomes articles that provide the reader with a thorough understanding of a specific methodology or technique pertinent to wound healing and regeneration studies. These articles will be subjected to the same peer review as regular research articles. Manuscripts will be accepted from any country but must be written in idiomatic English, and will be subject to copyediting before publication. Authors may be required to have manuscripts edited for English, either on their own or through the English Language editing services, which may be found on the Author Services webpage (http://www.blackwellpublishing.com/bauthor/english_language.asp).

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1. Cover Letter with Assurances; 2. Title Page; 3. Abstract; 4. Introduction; 5. Materials and Methods; 6. Results; 7. Discussion; 8. Acknowledgments; 9. Footnotes; 10. References; 11. Tables; 12. Figure Legends; 13. Figures; 14. Supporting Information (if applicable)

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This letter from the submitting author must provide written assurance that the paper has not been previously published and that no other submission or publication of the original work has been or will be made. Abstracts or oral or poster presentation are not considered to constitute prior publication. The submitting author must further assure that every author listed meets the qualifications for authorship (see below) and has had the opportunity to read and comment upon the submitted manuscript.

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The title page should include (a) the title of the article, which should be concise but informative; (b) first name, middle initial, and last name of each author, with highest academic degree(s) and institutional affiliation; (c) name of department(s) and institution(s) to which the work should be attributed; (d) name, address, telephone number, fax number, and email address of corresponding author; (e) name and address and email address of the author to whom requests for reprints should be addressed, (f) short running title, and (g) key words.

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The second page should contain an abstract of not more than 200 words. The abstract should state the purpose of

the investigation, basic procedures, main findings (BE SPECIFIC), and the principal conclusion. Emphasize new or unique aspects of the investigation. Abbreviations should not be used in the abstract. Generally, the abstract should be a single paragraph and should NOT be structured into separate sections with headings.

Text

The text of the manuscript should be divided into the following sections with headings: Introduction, Methods, Results, Discussion, Acknowledgments. Longer articles may be further divided with appropriate subheadings. For original research papers, the body of the text including title page should not exceed 21 pages, and the number of references should be limited to 40 or fewer. Tables and figures should be used to support all reported results, but should not be redundant and should be limited to those necessary for data presentation and interpretation. For perspective articles, the manuscript length and number of references may be greater, and will be determined by the Editor.

Introduction

State the purpose of the article; for original research, the statement of a hypothesis to be tested is appropriate. Summarize the rationale for the study, giving only pertinent references, and do not review the subject extensively. Do not include data or conclusions in this section from the work to be reported.

Materials and Methods

Identify the methods, apparatus (include manufacturer's name, city and state or country in parentheses), and procedures in sufficient detail to allow other workers to reproduce the results. Give references for established methods; provide references and brief descriptions for methods that have been published but are not well known; and describe in greater detail new or substantially modified methods (if deemed necessary, a diagram or flow chart may be used for complex procedures). Identify precisely all drugs and chemicals used, including generic name(s), dose(s), and route(s) of administration.

Ethical Considerations

Human Investigations: Manuscripts reporting data obtained from research conducted in human subjects must comply with the ethical rules for human experimentation that are stated in the 1975 Declaration of Helsinki, including approval by the institutional review board - or human experimentation committee. Authors must disclose this compliance within the Materials and Methods section.

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Study protocols must be in compliance with the institution's guidelines or the National Research Council's criteria for humane care as outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, Revised 1985, http://books.nap.edu/catalog/5140.html). Researchers from countries other than the US are encouraged to consider guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care, international (http://www.aaalac.org/index.cfm) and to recommend membership in this organization to their institutions. A statement of assurance of the humane treatment of research animals must be provided within the Materials and Methods section.

Statistics

Statistical methods must be described in sufficient detail to enable a knowledgeable reader with access to the original data to verify the reported results. Whenever possible, quantify findings and present them with appropriate indicators of measurement error or uncertainty. Statistical probability (p) should be reported in tables, figures, and figure legends at only one of the following levels: p

Results

The narrative of the text should take the reader through a logical progression of data consideration and interpretation. Present results in a logical sequence in the text, tables, and illustrations. DO NOT repeat in the text all the data in the tables or illustrations; emphasize or summarize only important observations.

Discussion

Emphasize the new and important aspects of the study and the conclusions that follow from them. DO NOT repeat in detail data or other material given in the Introduction or Results sections. Include in the Discussion section the implications of the findings and their limitations, including implications for future research. It is appropriate to briefly discuss how the results fit into (or deviate from) the larger body of published work on the topic. Link the conclusions with the goals of the study, and avoid unqualified statements and conclusions not supported by the data. State the hypotheses when warranted but clearly label them as such.

Acknowledgments
This section should contain one or more statements that specify (a) contributions that need acknowledgment but do not justify authorship; (b) acknowledgment of technical help; (c) acknowledgments of financial material support (specify the nature of the support); (d) financial relationships that may pose a conflict of interest.

List of Abbreviations and Other Footnotes

All nonstandard abbreviations should be grouped in alphabetical order into one footnote, with all footnotes placed on a separate page of the manuscript following the acknowledgments. Footnotes in the text should be denoted with a superscript Arabic numeral.

References
Number references consecutively in the order in which they are mentioned in the text. Identify references in text, tables, and figure legends by Arabic numerals in parentheses. References cited only in tables or figure legends should be numbered last. Use the style of the following examples, which are based with slight modification on the formats set forth in 'Uniform Requirements for Manuscripts Submitted to Biomedical Journals,' (http://www.nlm.nih.gov/bsd/uniform_requirements.html). The titles of journals should be abbreviated according to the style used in Index Medicus. 'Unpublished observations' and 'personal communications' may not be used as references but should be inserted in parentheses in the text. Include among the references papers accepted but not yet published; designate the journal and add 'In press.' Examples of correct reference styles are given below:

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Figure Legends

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Aprovação do comitê de ética

Universidade Federal de Pernambuco
Centro de Ciências Biológicas
Av. Prof. Nelson Chaves, s/n
50670-420 / Recife - PE - Brasil
fones: (55 81) 2126 8840 | 2126 8351
fax: (55 81) 2126 8350
www.ccb.ufpe.br

Recife, 12 de maio de 2009

Ofício nº 146/09

Da Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Profª. Eulália Camelo Pessoa de Azevedo Ximenes**
Departamento de Antibióticos-CCB
Processo nº 23076.005142/2009-21

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram seu projeto de pesquisa intitulado ***"Avaliação antimicrobiana in vitro e in vivo do extrato de Hymenaea stignocarpa em ratos diabéticos, para análise ."***

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEEA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais realizados.

Atenciosamente,

Profa. Maria Teresa Jansem
Presidente do CEEA
UFPE

Observação: Aluna do mestrado: Anacassia F. Lima
Origem dos animais: Biotério do Departamento de Antibióticos; Animal; Ratos albinos Wistar; Sexo: Machos; Idade: 60 dias ; Número de animais previsto no protocolo: 45 animais.

CCB: Integrar para desenvolver

Aprovação do artigo de revisão

São Paulo Medical Journal/Evidence for Health Care – Volume 129(3) maio e junho de 2011 **São Paulo Medical Journal/Evidence for Health Care, vol. 129, edition number 3, May and Junel 2011**			
Protocolo	**Título do artigo**	**Autores/local/e-mail**	**Classificação do artigo**
8) SPMJ000049	**Interventions for wound healing among diabetic patients infected with *Staphylococcus aureus*: a systematic review.** Intervenções para a cicatrização de feridas de pacientes diabéticos infectados com*Staphylococcus aureus*: uma revisão sistemática.	**Autores:** Anacássia Fonseca Lima, Lívia Bandeira Costa, Joás Lucas da Silva, Maria Bernadete Sousa Maia, Eulália Camelo Pessoa Azevedo Ximenes **Local:** Centro de Ciências da Saúde, Universidade Federal de Pernambuco (CCS-UFPE) E-mail: cassialima3@hotmail.com	**Systematic review** **Revisão sistemática**