DÉBORA LIMA SALES

ANÁLISE QUÍMICA, ATIVIDADE ANTIBACTERIANA E TOXICIDADE DA SECREÇÃO GLANDULAR DE *Rhinella jimi* (STEVAUX, 2002)

Crato, CE

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Tese apresentada ao Programa de Pós-Graduação em Etnobiologia e Conservação da Natureza da Universidade Federal Rural de Pernambuco como parte dos requisitos para obtenção do título de Doutor.

Orientador: Prof. Dr. Waltécio de Oliveira Almeida

Universidade Regional do Cariri - URCA

Crato, CE

2017

BANCA EXAMINADORA

ANÁLISE QUÍMICA, ATIVIDADE ANTIBACTERIANA E TOXICIDADE DA SECREÇÃO GLANDULAR DE *Rhinella jimi* (STEVAUX, 2002)

Tese defendida, avaliada e aprovada em 08 de dezembro de 2017 pela Comissão Julgadora para obtenção do título de Doutora em Etnobiologia e Conservação da Natureza.

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Dedico este trabalho ao meu avô Francisco Sales (*in memoriam*) exemplo de bom humor, de amor à leitura e maior incentivador de novas conquistas. Minha alegria após cada viagem era ouvir “Por aqui nunca foi tão bom, mas me conte como foi na Veneza nordestina”.

“Vô Salim, eu não consegui te levar em Recife para rever os lugares de suas lembranças, mas dedico a você um pouquinho do que fiz por lá! Não tem ideia da falta que nos faz!”

AGRADECIMENTOS

Aos meus pais Fernando Bezerra Sales e Francisca Paulo de Lima Sales (Cinete), por serem exemplos de luta e superação, por serem amor e paciência comigo. Às minhas irmãs Fernanda Lima Sales e Sarah Lima Sales por serem companheiras e amigas, estando sempre presentes, mesmo quando eu estava longe. Obrigada por tentarem entender minhas ausências e meus estresses, meus sufocos e minhas angústias! À minha sobrinha Ana Maria por me mostrar que um amor puro e uma saudade grande podem transformar uma pessoa. Ao meu sobrinho Bernardo por trazer ainda mais alegria à nossa casa nas férias. Estou tentando fazer algo para melhorar nossos dias! Amo muito vocês!

À minha avó Francisa Bezerra Sales por estar sempre na torcida pelo sucesso de seus netos. Ao meu avô Francisco Sales (*in memoriam*) e minha tia Maria José Paulo de Lima (*in memoriam*), pela alegria de sempre, pelos bons momentos de alívio que proporcionaram nos dias em que estivemos juntos. Hoje são os dois motivos de nossas festas juninas serem dias tristes, das fogueiras terem novo significado.

Ao meu Orientador Doutor Waltécio de Oliveira Almeida, por ser guia na vida acadêmica. E, como um grande amigo, ser apoio em todas as horas difíceis, vibrar comigo em cada conquista, e mostrar o lado bom das coisas, quando nem parecia existir. Obrigada por ter acreditado em mim! Obrigada por tudo, sempre!

À minha amiga Maria Flaviana Bezerra Morais Braga, grande companheira de Doutorado, pelos dias sofridos e os divertidos que tivemos. Obrigada por me acordar nas noites que só tínhamos duas horas para dormir e pela companhia naquelas que nem podíamos descansar. Seminários e apresentações difíceis, semanas de testes, ensinamentos, trocas, e por cada café gostoso que tomamos juntas na tentativa de aliviar as tensões ou ficar um pouco mais acordadas. Fizemos uma boa dupla!

Aos Professores Doutores Henrique Douglas Melo Coutinho, José Galberto Martins da Costa, Irwin Rose Alencar de Menezes e Francisco Bezerra da Cunha pelo suporte nos laboratórios e discussões importantes sobre o trabalho.

Ao Dr. Daniel Carvalho Pimenta e seu aluno Hugo Vigerelli pelo suporte dado no Instituto Butantan e realização das análises químicas.

Aos colegas e amigos do Laboratório do Zoologia João Antônio Araújo Filho, Adonias Aphoena Martins Teixeira, Diego Alves Teles, Nayane Kely Silva Sampaio, Érica Gomes da Silva, Maria Érika Pereira dos Santos, Monique Celião de Oliveira, Cícero Leonardo de Morais Pinto, Rivanilda Gonçalves Duarte, Diógenes Dias Queiroz e os Doutores Samuel Cardozo Ribeiro (Goiaba), Samuel Vieira Brito (Calango) e Felipe Silva Ferreira, pela ajuda em campo, conversas, ensino e cafés divertidos.

Aos colegas do Laboratório de Microbiologia e Biologia Molecular Antonia Thassya Lucas dos Santos, Antonio Judson Targino Machado e Joara Pereira Carneiro pelo tempo e paciência comigo em todos os testes. Maria Audilene Freitas, Ana Raquel Pereira da Silva, Fábia Ferreira Campina, Maria do Socorro Costa, Gioconda Morais de A. B. Martins por alguns dias de trabalho e muitos dias de sorrisos no LMBM.

Aos Professores do Programa de Pós-Graduação em Etnobiologia e Conservação da Natureza e convidados, pelo tempo dedicado e ensinamentos que contribuíram com minha formação: Dr. Waltécio de Oliveira Almeida, Dr. Henrique Douglas Melo Coutinho, Dr. Felipe Silva Ferreira, Dr. Irwin Rose Alencar de Menezes, Dra. Marta Regina Kerntopf, Dra. Maria Franco Trindade de Medeiros, Dr. Antonio da Silva Souto, Dr. Wedson Medeiros da Silva Souto, Dr. Ângelo Giuseppe Chaves Alves, Dra. Elcida Lima Araújo, Dra. Fernanda Duarte Amaral, Dr. Joabe Gomes de melo, Dr. Nicola Schiel, Dr. Ulysses Albuquerque Paulino.

Aos colegas que conheci no doutorado, com quem passei bons momentos em Recife: Wendy Marisol Torres Aviléz, Flávia Santos Silva, André Sobral, Washington Soares Ferreira Júnior, José Ribamar de Sousa Júnior, Josivan Soares, Andrêsa Suana Argemiro Alves, Taline Cristina da Silva.

Às meninas de Recife: Samara Alves Brito, Jamilly Lopes de Macedo, Gestrudes Nunes de Melo e Maria Audilene Freitas, pela amizade e pelo abrigo.

Aos meus amigos de sempre: Jéssika Dias Andrade, Gestrudes Nunes de Melo, Tamiris Pereira da Silva, Maria Tamires do Nascimento, Kadidja Luciana Tavares Augusto, Aline Santos Lima, Olga Paiva Oliveira, João Adolfo Ribeiro Bandeira, Mário Machado Beserra,

Mikkael Duarte dos Santos. Agradeço por tudo! Cada conversa boba, cada momento em mesa de bar, a presença nos períodos de dor (especialmente durante os últimos dois anos), os sorrisos e as saudades que vocês deixam quando passam muito tempo longe. Minha família escolhida, amo vocês!

Agradeço a quem, de alguma forma, contribuiu para que eu conseguisse chegar até aqui!

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LISTA DE SÍMBOLOS E ABREVIATURAS

%	Porcentagem
<	Menor que
≥	Maior ou igual a
Å	Ångström
ANOVA	*Analysis of Variance*
ATCC	*American Type Culture Collection*
BHI	*Brain Heart Infusion*
C18	Carbono 18 – coluna cromatográfica
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEUA	Comissão de Experimentação e Uso de Animais
CFU	*Colony Forming Units*
CIM	Concentração Inibitória Mínima
CLSI	*Clinical & Laboratory Standards Institute*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
DHF	Dihidrofolato
DNA	Ácido Desoxirribonucleico
ESI-IT-TOF	*Electrospray Ionization - Ion Trap - Timeof-Flight*
et al	Do latim et alii - e outros; e colaboradores
G	Grama (s)
H_2O	Água
HPLC	High Performance Liquid Chromatography
kV	Quilovolt (s)
LMBM	Laboratório de Microbiologia e Biologia Molecular
m/z	Razão massa/carga

MIC	*Minimum Inhibitory Concentration*
MIC/8	*Sub-Inhibitory Concentration*
min	Minuto (s)
mL	Mililitro (s)
mm	Milímetro (s)
Nm	Nanômetro (s)
ºC	Graus Celsius
OMS	Organização Mundial de Saúde
p	Nível de significância
PABA	Ácido Paraaminobenzóico
RNAm	Ácido Ribonucleico Mensageiro
RNAr	Ácido Ribonucleico Ribossômico
RNAt	Ácido Ribonucleico Transportador
RP-HPLC	*Reverse Phase - High Performance Liquid Chromatography*
sp	Espécie
SRJ-A	Secretions of *Rhinella jimi* collected in Aiuaba
SRJ-C	Secretions of *Rhinella jimi* collected in Crato
SRJ-E	Secretions of *Rhinella jimi* collected in Exu
TFA	Ácido Trifluoroacético
THF	Tetrahidrofolato
URCA	Universidade Regional do Cariri
VRJA	Veneno de *Rhinella jimi* coletado no município de Aiuaba - CE
VRJC	Veneno de *Rhinella jimi* coletado no município de Crato - CE
VRJE	Veneno de *Rhinella jimi* coletado no município de Exu - PE
WHO	*World Health Organization*
β	Beta
µg/mL	Microgramas de soluto por mililitro de solvente

µL	Microlitro (s)
µL/min	Microlitro (s) por minuto (s)
µm	Micrômetro (s)
µm	Micrômetro (s)

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RESUMO

Sales, Débora Lima. Universidade Federal Rural de Pernambuco. Drª. Universidade Federal Rural de Pernambuco. 24/02/2017. **Análise química, atividade antibacteriana e toxicidade da secreção glandular de *Rhinella jimi* (Stevaux, 2002).** Waltécio de Oliveira Almeida.

Bufonídeos possuem glândulas parotóides produtoras de secreções importantes no processo de defesa passiva, elas são ativadas através de pressão mecânica, como a mordida de algum predador. Nelas foram identificadas alcaloides, proteínas, peptídeos, esteroides e aminas, com importantes funções farmacológicas. *Rhinella jimi* (Stevaux, 2002) é um anfíbio anuro popularmente conhecidos por "sapo cururu", e estão entre as 47 espécies de anfíbios citadas com uso etnozoológico e zooterápico. Desta forma o presente trabalho tem por objetivo analisar e comparar a composição química da secreção glandular da espécie *Rhinella jimi*; avaliar a sua ação contra bactérias de linhagens padrão e multirresistentes; analisar o efeito da secreção combinado com drogas antibacterianas. Os animais foram capturados por coleta ativa, anestesiados e, então tiveram as glândulas parotóides pressionadas para retirada da secreção. Para identificação da secreção cutânea foi usada a cromatografia líquida de alta eficiência em fase reversa (RP-HPLC) em um sistema HPLC binário, e espectrometria de massas. Foram utilizadas cepas padrões para determinação da Concentração Inibitória Mínima (CIM) e, para a atividade moduladora de antibióticos, os isolados clínicos: *Escherichia coli* 06, *Pseudomonas aeruginosa* 03 e *Staphyloccocus aureus* 10. A atividade moduladora foi avaliada pelo método de microdiluição em caldo e teve como antibióticos alvos aminoglicosídeos (amicacina e gentamicina) e β-lactâmicos (Imipenem e Oxacilina). Os resultados obtidos mostraram perfis semelhantes nas três amostras estudadas, os picos de 205m/z, 203m/z, 401m/z, 403m/z, 387m/z, que correspondem respectivamente a bufotenina, dehidrobufotenina, marinobufagina, telocinobufalina e bufalina. As secreções quando usadas sozinhas não tiveram efeito sobre as linhagens bacterianas testadas, mas em associação com os antibióticos tiveram uma redução significativa da CIM tanto em aminoglicosídeos quanto em β-lactâmicos. A secreção glandular de *Rhinella jimi* demonstrou resultados clinicamente relevantes, à modulação. Isso representa uma alternativa interessante, uma vez que pode servir como ponto de partida para a produção de novas composições farmacêuticas ou complemento para os antibióticos já existentes.

Palavras-chave: Etnobiologia; zooterapia; bufalina; atividade antibacteriana; modulação de antibióticos.

ABSTRACT

Sales, Débora Lima. Universidade Federal Rural de Pernambuco. Dr[a]. Universidade Federal Rural de Pernambuco. 24/02/2017. **Chemical analysis, antibacterial activity and toxicity of glandular secretion of *Rhinella jimi* (Stevaux, 2002).** Waltécio de Oliveira Almeida.

Bufonids have parotoid glands that produce important secretions in the passive defense process, they are activated through mechanical pressure, like the bite of some predator. In them, alkaloids, proteins, peptides, steroids and amines were identified, with important pharmacological functions. *Rhinella jimi* (Stevaux, 2002) is anuran amphibian popularly known as the "cururu toad" and are among the 47 species of amphibians cited with ethnozoological and zooterapeutic use. In this way the present work aims to analyze and compare the chemical composition of the glandular secretion of the species *Rhinella jimi*; Evaluate its action against bacteria of standard and multiresistant strains; To analyze the effect of secretion combined with antibacterial drugs. The animals were captured by active collection, anesthetized and then had the parotoid glands pressed for removal of the secretion. To identify cutaneous secretion, reverse phase high performance liquid chromatography (RP-HPLC) was used in a binary HPLC system, and mass spectrometry. Standard strains were used to determine the Minimum Inhibitory Concentration (MIC) and, for antibiotic modulating activity, the clinical isolates *Escherichia coli* 06, *Pseudomonas aeruginosa* 03 and *Staphylococcus aureus* 10. The modulatory activity was evaluated by the microdilution method in broth and Had aminoglycosides (amikacin and gentamicin) and β-lactams (Imipenem and Oxacillin) as antibiotics. The results showed similar profiles in the three samples studied, the peaks of 205 m/z, 203 m/z, 401 m/z, 403 m/z, 387 m/z, corresponding respectively to bufotenin, dehydrobufotenin, marinobufagine, telocinobufaline and bufalin. Secretions when used alone had no effect on the bacterial strains tested, but in combination with antibiotics they had a significant reduction of MIC in both aminoglycosides and β-lactams. Glandular secretion of *Rhinella jimi* demonstrated clinically relevant results at modulation. This is an interesting alternative since it can serve as a starting point for the production of new pharmaceutical compositions or a complement to existing antibiotics.

Keywords: Ethnobiology; Zooterapia; Buffalin; Antibacterial activity; Modulation of antibiotics.

Introdução Geral

INTRODUÇÃO GERAL

Plantas e animais evoluíram ao longo de séculos e adquiriram mecanismos de defesa específicos e eficientes que lhes permitiram melhor sobrevivência. Embora por muito tempo as plantas tenham sido consideradas as principais fontes de substâncias ativas para pesquisas e produção de novos fármacos, os animais ganharam destaque pelos compostos que produzem.

Sob diferentes abordagens ecológicas, etnográficas e farmacológicas (Clarke, 1997; Alves et al., 2007), animais e seus produtos vêm sendo alvo de estudos, com o intuito de elucidar os compostos presentes em suas secreções, venenos e gorduras, e verificar a eficácia destes na produção de medicamentos. No Brasil, em especial nas regões Norte e Nordeste, vertebrados são os mais citados no uso e comercialização para fins terapêuticos, estando as infecções no topo das categorias de doenças tratadas (Ferreira et al., 2012).

A maior preocupação com infecções está no desenvolvimento de resistência dos microrganismos aos medicamentos, o que compromete a eficácia de muitas drogas, tornando de maior custo e duração o tratamento de pacientes infectados (Brasil, 2007; OMS, 2015). A Organização Mundial da Saúde (OMS) estima que apenas para a União Européia, as bactérias farmacoresistentes causem cerca de 25 mil mortos a cada ano e custe 1,5 bilhão de gastos sanitários e perda de produtividade (OMS, 2015). Um estudo feito pela OMS e Banco Mundial aponta um prejuízo não apenas de saúde, mas uma grande queda na economia, estabelecendo uma projeção otimista, os pesquisadores calculam uma queda no PIB mundial de 1,1% quando comparado a um cenário em que não houvesse crescimento na resistência a antibiótico, isso representa um trilhão de dólares nos próximos dez anos (Adeyi et al., 2017).

Dentre as classes de antibióticos, os aminoglicosídeos e os β-lactâmicos são opções de grande importância clínica, os primeiros devido ao uso mais comum, a exemplo amicacina, gentamicina, neomicina e estreptomicina. E os β-lactâmicos representam uma das classes com maior espectro de atividade e eficácia, em especial os carbapenêmicos, como imipenem, meropenem e doripenem (Drawz & Bonomo, 2010). Os β-lactâmicos atuam prejudicando a parede celular enquanto aminoglicosídeos atuam inibindo a produção de proteínas (Azucena & Mobashery, 2001; Wilke et. al., 2005; Thanbichler & Shapiro, 2008).

Alguns microrganismos produzem β-lactamases que hidrolisam antimicrobianos β-lactâmicos, causando resistência a eles (Santos et al., 2015). Aminoglicosídeos apresentam eficácia excelente, porém, devido à alta toxicidade, não podem ser usados por longos períodos

de tempo. A toxicidade é um fator importante na escolha da medicação a ser adotada em casos de infecção. Com o intuito de diminuir o período de exposição às drogas e/ou potencializar seus efeitos, associações entre medicamentos podem ser uma boa alternativa clínica (Fishbain & Peleg, 2010). Com o mesmo propósito de aumento de eficácia dos tratamentos, produtos naturais também podem ser usados em associações com produtos sintéticos (Coutinho et al., 2008).

Neste sentido, produtos naturais, principalmente plantas tem sido alvos de estudos com resultados promissores no combate a microrganismos, inclusive na modificação da atividade de aminoglicosídeos (Matias et al., 2010; Sousa et al., 2010; Barreto et al., 2014). Produtos animais também foram investigados, como óleos fixos da serpente *Spilotes pullatus* (Oliveira et al, 2014), do quelônio *Phrynops geoffroanus* (Dias et al., 2013), e anfíbios da família Leptodactylidae (Cabral et al., 2013), bem como gorduras e secreções de outros anfíbios (Cunha-Filho et al., 2005; Conceição et al., 2007; Sales et al., 2015; Schmeda-Hirschmann et al., 2016; Nalbantsoy et al., 2016).

Rhinella jimi (Stevaux, 2002) é um anfíbio anuro popularmente conhecido por "sapo cururu" e está entre as 47 espécies de anfíbios citadas com uso etnozoológico e zooterápico no mundo. Estudos etnozoológicos indicaram o uso de indivíduos inteiros, ou partes de *Rhinella jimi* para problemas no trato respiratório, deficiências renais, diarreias, reumatismo, inflamações, artrites, câncer, osteoporose e infecções e machucados na pele em humanos e animais domésticos (Bernarde & Santos, 2009; Ferreira et al., 2009a, b; Alves & Alves, 2011; Alves et al., 2012).

Compostos presentes na gordura desse animal também apresentaram resultados químicos e farmacológicos relevantes, ao modular antibióticos e reduzir edemas inflamatórios em aplicações tópicas (Sales, 2012; Sales et al., 2015). A bioprospecção de substâncias presentes na secreção das glândulas de *R. jimi* mostra grande quantidade de compostos bioativos com atividades farmacológicas comprovadas (Vitor, 2009; Vigerelli et al., 2015). Trabalhos realizados com venenos de outros anfíbios demonstram a importância desses animais como fonte de pesquisas farmacológicas, como efeitos fungistático, antibacteriano, antineoplásico e analgésico (Clarke, 1997; Rozek et al., 2000; Brito et al., 2012a, b).

O objetivo do trabalho é (1) avaliar a composição química da secreção glandular de *R. jimi* (2) avaliar a ação da secreção glandular de *R. jimi* contra bactérias de linhagens padrão e multirresistentes; (3) analisar o efeito da secreção combinado com drogas usadas para

tratamento dessas infecções; e (4) verificar se existe variação de efeito de acordo com localidade de coleta do animal.

Revisão de Literatura

REVISÃO DE LITERATURA

Etnozoologia

A existência das relações do homem com a natureza pode ser explicada pela hipótese da biofilia, proposta por Wilson (1984), a qual sugere que a dependência da espécie humana com os elementos naturais é devido a sua história evolutiva, desenvolvendo com as espécies que teve contato um conjunto de informações sobre as mesmas e o ambiente, traduzidos em saberes, crenças e práticas culturais (Santos-Fita & Costa-Neto, 2007). O comportamento humano frente aos animais segue da mesma forma, sendo constituído por um conjunto de valores, percepções e conhecimentos, bem como pela natureza das interações que os seres humanos mantêm com esses organismos (Drews, 2002).

Assim, a ciência que se dedica ao estudo dessas interações do homem com os componentes naturais é a etnobiologia, que assume ainda o papel de compreender e respeitar as diferentes culturas (Posey, 1987). Segundo Toledo & Barrera-Bassols (2010), cada pessoa transmite às novas gerações suas experiências enriquecidas e refinadas, de forma já adequada às novas circunstâncias, desta forma, a repetição dessa informação adaptada gera um aperfeiçoamento do tradicional. A etnobiologia e alguns de seus ramos trazem uma abordagem holística, bem como desafia paradigmas da ciência convencional (Funtowicz & Ravetz, 1993), criticando o mundo moderno ao buscar o resgate da “memória da espécie”, a memória bio-cultural, reivindicando e valorizando o povo que a mantém viva (Toledo & Barrera-Bassols, 2008).

De acordo com Mason (1899), a etnozoologia era a zoologia da região narrada pelos nativos, mas o termo foi usado apenas no século seguinte por Henderson & Harrington (1914). Décadas mais tarde, em 1990, Overal sugeriu que a etnozoologia dizia respeito ao estudo dos conhecimentos, significados e usos de animais nas sociedades humanas, mas Marques (2002) vai além ao incluir pensamentos, percepções e sentimentos humanos relacionados a animais como objeto de estudo.

Vários temas são abordados pelos etnozoólogos, tais como: percepção cultural e classificação zoológica; importância e presença de animais em mitos, contos e crenças; aspectos biológicos e culturais da utilização dos animais pelas sociedades humanas; formas de obtenção e preparo das substâncias orgânicas extraídas dos animais para diversos fins (ritualístico, medicinal, alimentar, etc.); domesticação, verificando as bases culturais e as

consequências biológicas do manejo dos recursos faunísticos ao longo do tempo; heterogeneidade biológica e processos cognitivos envolvidos no manejo e conservação dos recursos; técnicas de coleta e seu impacto sobre as diferentes populações animais (Lewis, 1991; Digard, 1992; Descola, 1998; Costa-Neto & Oliveira, 2000; Fleck & Harder, 2000; Gunnthorsdottir, 2001; Pessoa et al., 2002; Motta, 2003; Martins & Souto, 2006; Mourão et al., 2006; Souto, 2007).

Os estudos etnozoológicos com ênfase nos conhecimentos tradicionais de comunidades têm ganhado destaque por complementarem e/ou servirem de base para o conhecimento científico em diferentes áreas. Trabalhos como os de Alves (2008, 2009), Alves & Alves (2011), Ferreira et al. (2009b), Santos et al. (2012a, b) e Costa-Neto (1996, 2005) são exemplos de utilização medicinal dos recursos faunísticos.

Assim, Alvez & Souto (2015) consideram etnozoologia como uma área científica fundamental para análise dos aspectos históricos, econômicos, sociológicos, antropológicos e ambientais das relações entre humanos e animais. Os mesmos autores destacam que os conhecimentos populares sobre a fauna local podem facilitar projetos de pesquisa acadêmicos, trazendo inclusive economias quando comparadas aos custos de pesquisas não realizadas com essa abordagem.

A zooterapia é o ramo da etnozoologia que estuda o uso de animais com finalidades terapêuticas, seja na medicina tradicional humana ou veterinária. Os registros terapêuticos das espécies animais abrem um leque de opções de estudos para a etnofarmacologia, pois com as investigações dos compostos bioativos das espécies, pode-se obter um potencial para descoberta de medicamentos (Elisabetsky, 2003).

Dentre os táxons mencionados por Alves & Alves (2011) para uso zooterápico, os anfíbios são o grupo de vertebrados com menor número de espécies citadas pelos entrevistados, sendo 47 espécies a nível mundial e 23 espécies para toda a América Latina, das quais sete espécies da família Bufonidae: *Rhinella schneideri* (Werner, 1894), *R. marina* (Linnaeus, 1758), *R. jimi* (Stevaux, 2002), *R. icterica* (Spix, 1824), *Incilius bocourti* (Brocchi, 1877), *I. macrocristatus* (Firschein & Smith, 1957),, *I. valliceps* (Wiegmann, 1833); três para Leptodactylidae: *Leptodactylus labyrinthicus* (Spix, 1824), *L. vastus* A. Lutz, 1930, *L. troglodytes* A. Lutz, 1926; dois para a família Craugastoridae: *Craugastor laticeps* (Duméril, 1853), *C. glaucus* (Lynch, 1967); quatro para Ranidae: *Lithobates maculata* (Brocchi, 1877), *L. berlandieri* (Baird, 1859), *L. montezumae* (Baird, 1854), *L. spectabilis* (Hillis and Frost,

1985); cinco para Hylidae: *Charadrahyla chaneque* (Duellman, 1961), *Trachycephalus typhonius* (Linnaeus, 1758), *T. resinifictrix* (Goeldi, 1907), *Phyllomedusa bicolor* (Boddaert, 1772), *P. burmeisteri* Boulenger, 1882; um para Microhylidae: *Hypopachus barberi* Schmidt, 1939; e um para Telmatobiidae: *Telmatobius culeus* Garman, 1876. Dessas, apenas nove têm registros de uso para o Brasil (Alves et al., 2013; Alves & Alves, 2011).

É possível atribuir a pouca citação dos anfíbios como recurso medicinal à percepção que as comunidades têm desses animais, que são culturalmente vistos como perigosos, sendo por vezes dispostos em outra classificação zoológica, passando de anfíbios para insetos (Costa-Neto & Pacheco, 2004; Costa-Neto & Resende, 2004; Barros, 2005; Ceríaco, 2012). De acordo com Costa-Neto & Pacheco (2004), o modo como as pessoas se expressam com relação aos animais que compõem a etnocategoria "insetos" (anfíbios, répteis, mamíferos, aracnídeos, além dos próprios insetos da classificação oficial), coloca em evidência os sentimentos e reações de desprezo, medo e nojo ou aversão aos mesmos. Como Lauck (2002) demonstra, o conceito de nojo ou medo ensinado na infância, frequentemente impede que a criança explore a conexão com os animais mais adiante. Na maior parte das vezes, o medo de insetos e outros animais está acompanhado por falta de informação sobre o mesmo, em relação a sua biologia, importância econômica e ecológica (Smith, 1934).

Embora seja pequeno o número de espécies usadas, especialmente quando comparados a outros grupos de vertebrados, as condições tratadas com anfíbios e seus produtos abrangem muitos dos sistemas humanos: incontinência urinária, câncer, cárie dentária, feridas, furúnculos, acne, erisipela, indução de aborto, picada de escorpião, gastrite, raiva, reumatismo, dor de ouvido, dores articulares, dores de cabeça, dores musculares, dores de garganta, alergias e diabetes (Bernarde & Santos, 2009; Ferreira et al., 2009b; Alves & Alves, 2011).

O conhecimento da toxicidade dos sapos remonta a tempos antigos. Na medicina asiática de 3.000 anos atrás, a secreção em pó era usada como remédio para o coração, sendo o costume posteriormente introduzido na Europa e usado para a mesma finalidade (Habermehl, 1995). As secreções da pele dos anfíbios são muito utilizadas por comunidades indígenas para variados fins, como, na ponta das zarabatanas durante as caçadas; ou para dar mais vigor aos caçadores, afastando a fraqueza e má sorte. Porém, no Brasil existem poucos registros das comunidades não-indígenas (Bernarde & Santos, 2009; Ferreira et al., 2009b).

Peles e secreções apresentam grande quantidade de substâncias bioativas já registradas na literatura (aminas biogênicas, peptídeos, alcaloides, esteroides e proteínas),

sendo estas com funções de proteger contra infecções microbianas e do ataque de predadores. Essas substâncias têm algumas ações farmacológicas registradas como citocida, cardioativas, antileishimânia e antitripanossoma (Brito et al., 2012ab; Daly, 1998; Daly et al., 1984; 1992, 2004; Duellman & Trueb, 1994; Maciel et al., 2003; Tempone et al., 2008).

Por viverem tanto em habitats terrestres quanto aquáticos e, portanto, servirem como transportadores de biomassa entre os tipos de habitat, a diversidade e o status dessa população podem refletir a integridade ecológica de determinada área, bem como as consequências das modificações causadas pelo homem. Assim, é hoje considerado o grupo de vertebrados terrestre mais ameaçado devido ao declínio de populações no mundo inteiro, embora haja discordância entre autores (Stuart et al., 2004; Pimenta et al., 2005). As causas podem ser o processo de urbanização que gera destruição/fragmentação de habitats, bem como poluição dos corpos d'água e infecções fúngicas (Hayes et al., 2010).

Segundo Riclefs (2009), a razão de se conservar a biodiversidade depende dos nossos envolvimentos pessoais e valores relacionados aos nossos interesses. No mesmo sentido, Saccaro Júnior (2011), destaca que agregar valores à biodiversidade pode ser a maneira mais efetiva de proteção, assim, uma forma de extrair valor da biodiversidade seria a bioprospecção, definida pelo mesmo autor como a busca sistemática por organismos, genes, enzimas, compostos, processos e partes provenientes de seres vivos em geral, que possam ter um potencial econômico e, eventualmente, levar ao desenvolvimento de um produto.

Rhinella jimi (ANURA: BUFONIDAE)

Pertencente ao grupo *Rhinella marina*, grupo este que já teve duas mudanças taxonômicas em seu gênero nos últimos anos, passando de *Bufo marinus* (sensu Martin, 1972ab), como era inicialmente utilizado, grupo *Chaunus marinus* Frost et al. (2006), e em 2008 Pramuk et al. decidiram por *Rhinella marina*.

Rhinella jimi (Stevaux, 2002) (Figura 1) é um dos 85 bufonídeos descritos para o Brasil (Segalla et al., 2016). Popularmente conhecido por "sapo cururu", ele apresenta pela espessa e coberta por glândulas, distribuídas no antebraço, pés, cloaca, e parte posterior da cabeça, possui hábitos noturnos e está geograficamente distribuído em toda a região nordeste

do Brasil, tanto em lugares de baixas altitudes, como de altitudes elevadas, não havendo especializações entre as populações (Stevaux, 2002; Kwet et al., 2006).

Apesar de sua ampla distribuição geográfica e de sua abundância na região Nordeste do Brasil, existem poucos estudos realizados com substâncias biologicamente ativas extraídas a partir da pele desses anfíbios, porém existem relatos de atividades microbiológicas para extratos obtidos da pele desses animais, que demonstram atividade antileshimânia e antitripanossoma (Tempone et al., 2008).

Figura 1 - Vista dorsal de uma fêmea de *Rhinella jimi* (Stevaux, 2002).

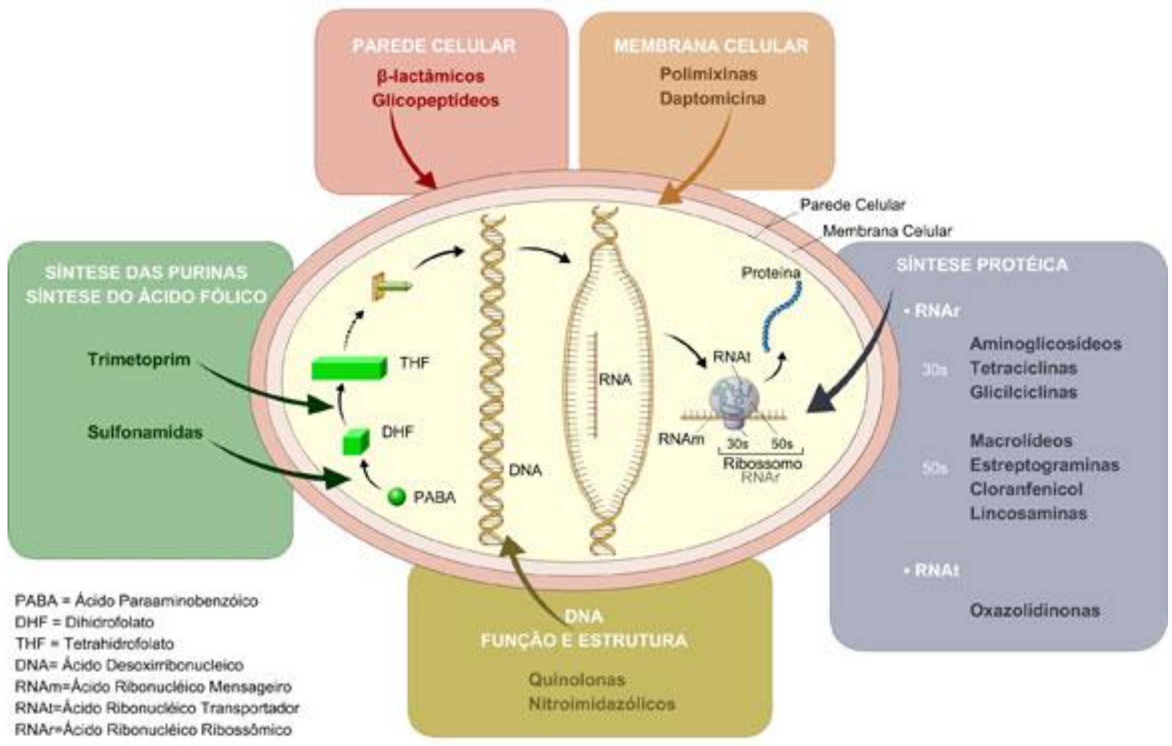
Foto da autora.

Testes buscando esclarecer a atividade microbiológica dos extratos da pele desses anfíbios foram realizados por Brito et al. (2012a) e Tempone et al. (2008). Os resultados dessas pesquisas evidenciaram que o extrato metanólico de *R. jimi* continha alcalóides, terpenos, esteróides e saponinas. Os testes microbiológicos realizados demonstram que os extratos e frações da pele de *R. jimi* interagem de diferentes formas com linhagens de bactérias. Brito et al. (2012b) demonstraram que o extrato metanólico de *R. jimi* produziu um ótimo perfil de atividade citocida, contra diferentes linhagens de células tumorais.

Antimicrobianos e Resistência bacteriana

Para que os antibióticos exerçam sua função na célula ele precisa inicialmente estar na concentração correta e atingir o seu alvo na célula para assim, matar ou inibir o microrganismo. Existem diversas formas de antibióticos interagirem com bactérias (Figura 2), podem atuar modificando aspectos na parede celular ou inibindo a sua síntese; agir na membrana celular, alterando a permeabilidade e possibilitando a entrada e saída de substâncias prejudiciais à célula; alterando ou inibindo processos de síntese proteica e de ácidos nucleicos ou ainda por inibição competitiva (Brasil, 2007).

Figura 2 – Mecanismos de ação de antibióticos.

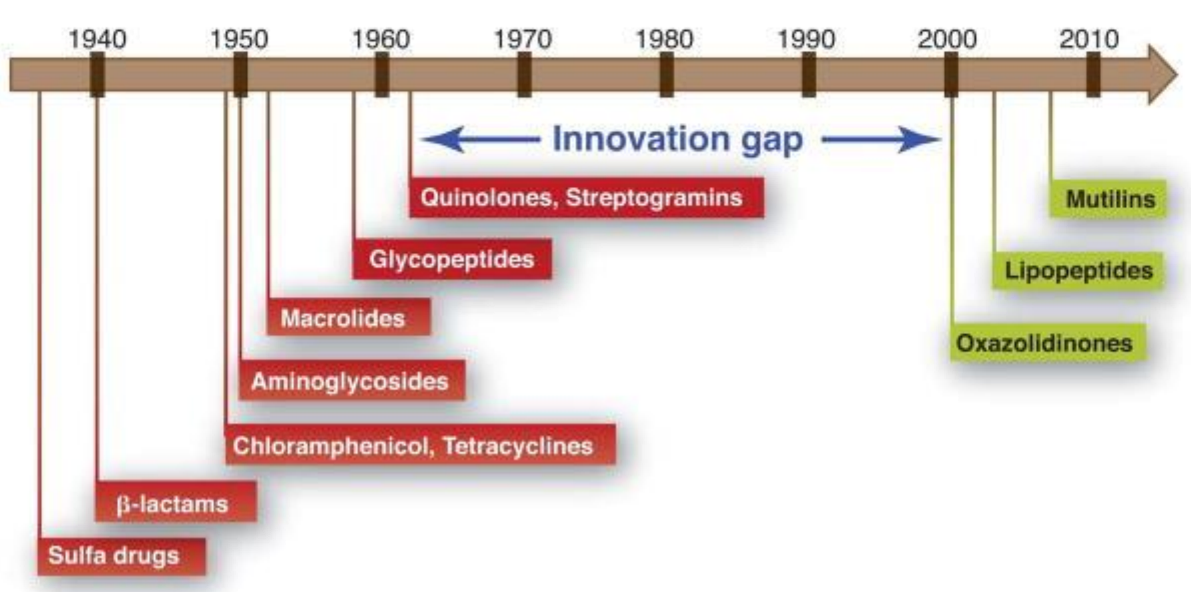


Fonte: (Brasil, 2007) - Anvisa: http://www.anvisa.gov.br/servicosaude/controle/rede_rm/cursos/rm_controle/opas_web/modulo1/pop_mecanismo.htm, acessado em 19/09/2016.

Fischbach & Walsh (2009) afirmam que a descoberta de novas drogas antibióticas deve ser prioridade. Na Figura 3, os autores trazem os períodos em que as principais categorias de antibióticos foram introduzidas, bem como um alerta sobre o déficit de descoberta de novas drogas antibacterianas nos últimos anos. A busca por novas drogas capazes de ação antibacteriana é necessária pois é crescente o surgimento de microrganismos resistentes a múltiplas drogas.

A resistência a antibióticos é um processo evolutivo natural, ocorre quando as bactérias mudam em resposta ao uso destes fármacos. No entanto o uso em excesso desses medicamentos tem levado o problema a níveis alarmantes no mundo inteiro. Segundo a OMS (2015), é necessário um esforço de todos os países no combate ao problema, que vai desde ações educativas e cuidados com higiene até campanhas de incentivo a pesquisa e otimização dos antimicrobianos. Isso explica a urgência na procura de novos agentes antimicrobianos ou compostos que sejam capazes de potenciar a ação de fármacos já comercializados (Bolla et al., 2011).

Figura 3 – Introdução das maiores classes de antibióticos entre 1962 e 2000.



Fonte: Fischbach & Walsh, 2009

Neste cenário, o estudo de produtos naturais e seus derivados tomam espaço, seja no uso isolado ou em combinação de compostos naturais e sintéticos. Novos fármacos produzidos a partir de produtos naturais no período de 1981 a 2014 foram listados por Newman & Cragg (2016), dentre os 140 antibacterianos registrados para o período, onze foram através de produtos naturais sem modificação na estrutura, 71 derivados de produtos naturais, um de origem biológica não determinada e um sintético baseado em estudos e estruturas de produtos naturais. Portanto, além de novos produtos, combinações e melhoramentos em fármacos já existentes pode ser uma alternativa viável e possivelmente mais econômica de contribuição.

Linhagens

O estudo foi realizado com espécies de linhagem padrão e linhagens resistentes a múltiplas drogas para que fosse possível verificar a interação da secreção glandular e o medicamento. Todas as espécies usadas apresentam importância na clínica médica.

Pseudomonas aeruginosa é um agente patogênico oportunista, responsável por um número importante de infecções hospitalares, como pneumonias, infecções no sistema urinário e queimaduras. Gram-negativa com diversos mecanismos de resistência intrínseca, assim como aquisição de genes, transferência horizontal de genes e acumulação de mutações pontuais, alterando o seu material genético (Delgado et al., 2007; Murray 2007).

Outra Gram-negativa estudada foi *Escherichia coli*, uma das principais causas de infecções bacterianas em humanos, responsável por infecções no sistema urinário, colites e diarreias. No Brasil foi considerada como o agente mais freqüentemente isolado em crianças hospitalizadas, no primeiro ano de vida nas décadas de 1980 e 1990 (Oliva et al., 1997). *Staphyloccocus aureus* é comum na pele, podendo causar foliculites e furúnculos, representa uma importante bactéria em crescente resistência a antibióticos convencionais (Freitas, 2003; Catao et al., 2006).

Devido à importância clínica desses agentes biológicos, é recomendada a administração de agentes antimicrobianos associados, devido à alta probabilidade de desenvolvimento de resistência em uma monoterapia. Entre outros tratamentos, é frequente o uso de aminoglicosídeos combinados com β-lactâmicos (Fishbain & Peleg, 2010; Raja & Singh 2007), por este motivo foram as classes de fármacos selecionadas para os ensaios neste trabalho.

Os resultados da pesquisa estão apresentados em dois capítulos distribuídos da seguinte forma: O primeiro capítulo, o artigo submetido à revista Toxicon, traz resultados da composição química da secreção glandular de *Rhinella jimi* coletados em três localidades distintas nos Estados do Ceará e Pernambuco. O segundo capítulo, o artigo aborda a resposta microbiológica da secreção contra as bactérias *Staphyloccocus aureus*, *Escherichia coli* e *Pseudomonas aeruginosa*, além de combinações com antibióticos das classes dos aminoglicosídeos e β-lactâmicos, tendo sido publicado na revista Biomedicine & Pharmacotherapy.

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Artigo 1

Artigo submetido ao periódico Toxicon

Análise química da secreção glandular de *Rhinella jimi* (Stevaux, 2002) (Anura: Bufonidae)

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Resumo

Bufonídeos apresentam glândulas parotóides, que são macroglândulas produtoras de secreções importantes no processo de defesa passiva. Estas são ativadas através de pressão mecânica, como a mordida de algum predador, podendo ter ativação espontânea em algumas espécies. Nelas foram identificadas alcaloides, proteínas, peptídeos, esteroides e aminas, com importantes funções farmacológicas. Desta forma, o presente trabalho tem por objetivo analisar e comparar a composição química da espécie *Rhinella jimi* (Stevaux, 2002) em três localidades diferente. Os animais foram capturados por coleta ativa, anestesiados e, então tiveram as glândulas parotóides pressionadas para retirada da secreção. Para identificação da secreção cutânea foi usada a cromatografia líquida de alta eficiência em fase reversa (RP-HPLC) em um sistema HPLC binário, e espectrometria de massas. Os resultados obtidos mostram perfis semelhantes nas três amostras estudadas, com os picos de 205m/z, 203m/z, 401m/z, 403m/z, 387m/z, que correspondem respectivamente a bufotenina, dehidrobufotenina, marinobufagina, telocinobufagina e bufalina. Embora a presença da última indique semelhanças com o perfil químico das secreções glandulares de *R. schneideri*, os dados morfológicos e distribuição geográfica diferem bastante deixam claro ser uma semelhança casual.

Palavras – Chave: taxonomia química; bufonídeos; sapo*; Rhinella;* glândula parotóide, veneno, secreção glandular.

Introdução

Bufonídeos apresentam glândulas paratóides, que são macroglândulas produtoras de secreções importantes no processo de defesa passiva, elas são ativadas através de pressão mecânica, como a mordida de algum predador, podendo ter ativação espontânea em algumas espécies (Jared et al., 2009; 2011). O veneno liberado é rico em compostos que lhe conferem proteção contra infecções microbianas, como fungos e bactérias (Daly et al., 1984).

Nas peles e secreções de anfíbios foram identificadas alcaloides, proteínas, peptídeos, esteroides e aminas (Maciel et al., 2003; Daly et al., 1984; Duellman e Trueb, 1994). Muitos deles com estudos e comprovações de atividades farmacológicas importantes como efeito fungistático, antibacteriano, antiviral, antineoplásico, analgésico, cardioativos, antileishimânia e antitripanossoma (Brito et al., 2012ab; Daly, 1998; Daly et al., 1984; 1992, 2004; Duellman & Trueb, 1994; Maciel et al., 2003; Tempone et al., 2008; Vitor, 2009; Vigerelli et al, 2014).

Segundo Sebben et al. (1993), a composição e concentração dos compostos da pele de anfíbios pode variar entre espécies, estádio de desenvolvimento, e de distribuição geográfica, dados que corroboram com os resultados encontrados por Cei et al. (1968). Sciani et al. (2013) analisaram e compararam as secreções de sete espécies do gênero *Rhinella* e uma espécie do gênero *Rhaebo*. Com seus resultados eles afirmaram que para estes gêneros, havia uma discordância com os dados de outros autores, uma vez que parte das espécies estudadas por eles compartilham mesmo habitat e dieta e ainda assim, apresentaram diferenças nas composições, sugerindo uma relação espécie-específica.

Por haver discordância entre autores quanto aos compostos presentes na secreção serem ou não espécie-específico, é prudente uma análise mais detalhada da composição química em estudos que se destinam principalmente a informações farmacológicas. Desta forma, foi feita uma análise e comparação do perfil cromatográfico de amostras de *Rhinella jimi* de três localidades diferentes com perfis cromatográficos de *Rhinellas* sp. já conhecidos.

Material e Métodos.

Coleta animal e secreção glandular

Os animais foram coletados em três localidades próximas, sendo duas em ambientes antropizado no município de Crato, estado do Ceará e no município de Exu, estado de Pernambuco, e uma em área de conservação de Caatinga, na Estação Ecológica de Aiuaba, estado do Ceará. As coletas foram devidamente autorizadas pelo Sistema de Autorização e Informação em Biodiversidade – SISBIO, sob número: 39353-2 de 2014. A coleta e manipulação dos animais foi autorizada pela Comissão de Experimentação e Uso de Animais – CEUA da Universidade Regional do Cariri – URCA, sob número: 130/2013.

Os animais foram capturados manualmente, por coleta ativa, foram identificados e anestesiados com lidocaína 10%. Para simular a mordida de um predador possível, foi aplicada uma pressão grande e constante nas glândulas parótidas dos indivíduos vivos, apertando-as lateralmente entre o polegar e o dedo indicador, para que fosse possível a expulsão de veneno, conforme descrito por Jared et al. (2009).

As amostras foram identificadas como "VRJC" para a secreção de animais coletados no município de Crato; "VRJE" para a secreção de animais coletados no município de Exu; e "VRJA" para a secreção de animais coletados no município de Aiuaba. Seguiu o congelamento da secreção a -20ºC até a sua utilização.

Identificação da secreção glandular das espécies coletadas

As secreções foram identificadas pela técnica de cromatografia líquida de alta eficiência em fase reversa (RP-HPLC) em um sistema HPLC binário (10A vp Prominence, Shimadzu Co., Japan). As amostras foram solubilizadas, centrifugadas a 10.000 x g e 5 µL do sobrenadante foi analisado utilizando uma coluna C18 (ACE® 5µm, 100 Å, 250 x 4.6 mm), com solventes (A) ácido trifluoroacético (TFA) / H_2O (1:1000) e (B) TFA / acetonitrila / H_2O (1:900:100) em fluxo constante de 1 mL/min-1a 30°C. O gradiente utilizado foi de 0 a 100% de solvente B em 20 minutos de corrida, após uma eluição isocrática de solvente A, por 5 min. Esta analise foi monitorada pela medida de absorbância da amostra em 214 nm.

Espectrometria de massas.

As análises por espectrometria de massas foram realizadas no laboratório de Bioquímica e Biofísica do Instituto Butantan, em um espectrômetro ESI-IT- TOF (Simadzu Co., Japão). As amostras foram analisadas em modo positivo, com fluxo de 50 µL/min. A voltagem da interface utilizada foi de 4,5 kV e a voltagem do detector, 1,76 kV, com temperatura de 200°C. Os espectros foram obtidos na faixa de 50 a 2000 m/z. Os dados obtidos foram analisados pelo software LCMSsolution (Shimadzu Co., Japão).

Resultados e Discussão

Nos experimentos de cromatografia, os perfis cromatográficos das três amostras foram muito similares um ao outro, variando apenas na intensidade dos picos. Isso pode ser observado nas figuras 1, 2 e 3.

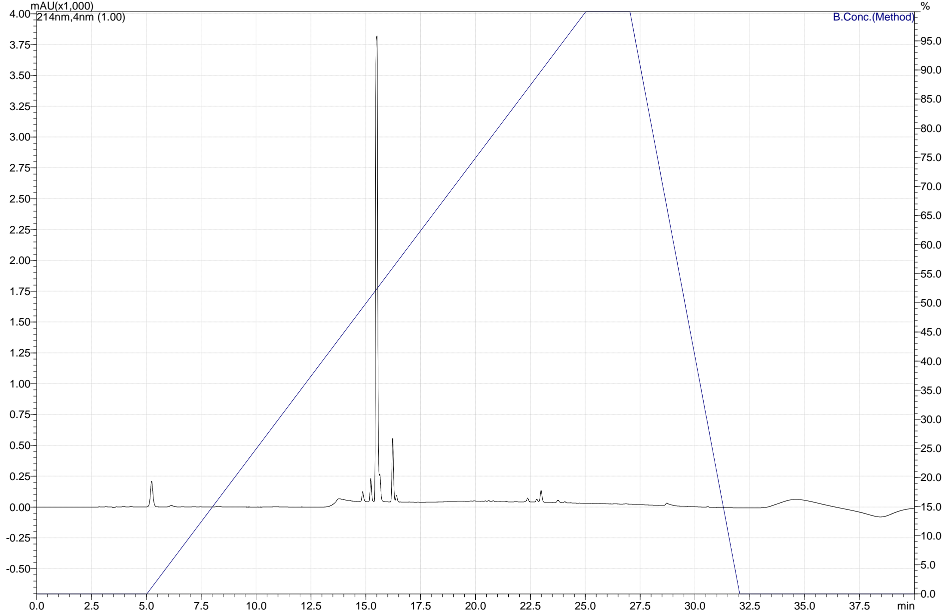

Figura 1 - Perfil cromatográfico da amostra "VRJC".

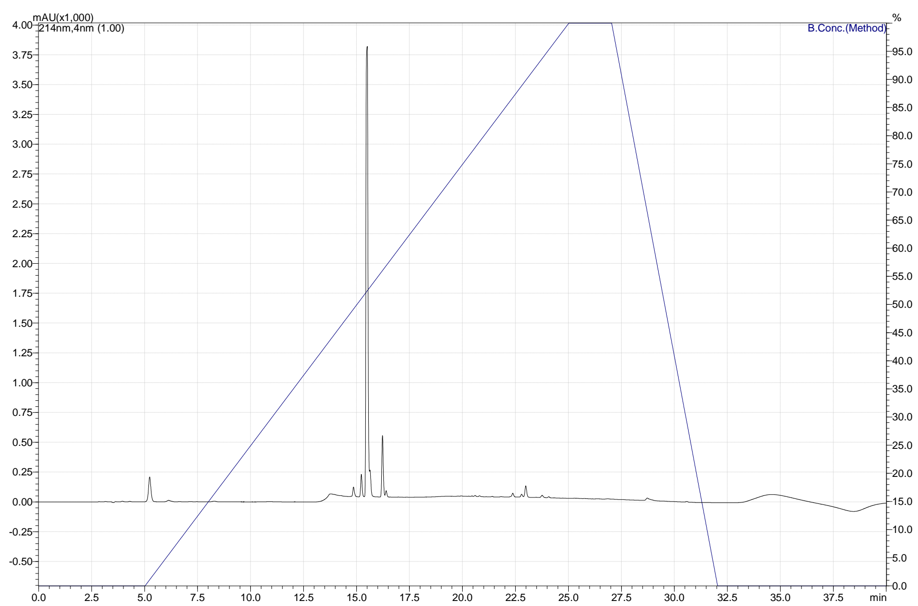

Figura 2 - Perfil cromatográfico da amostra "VRJE".

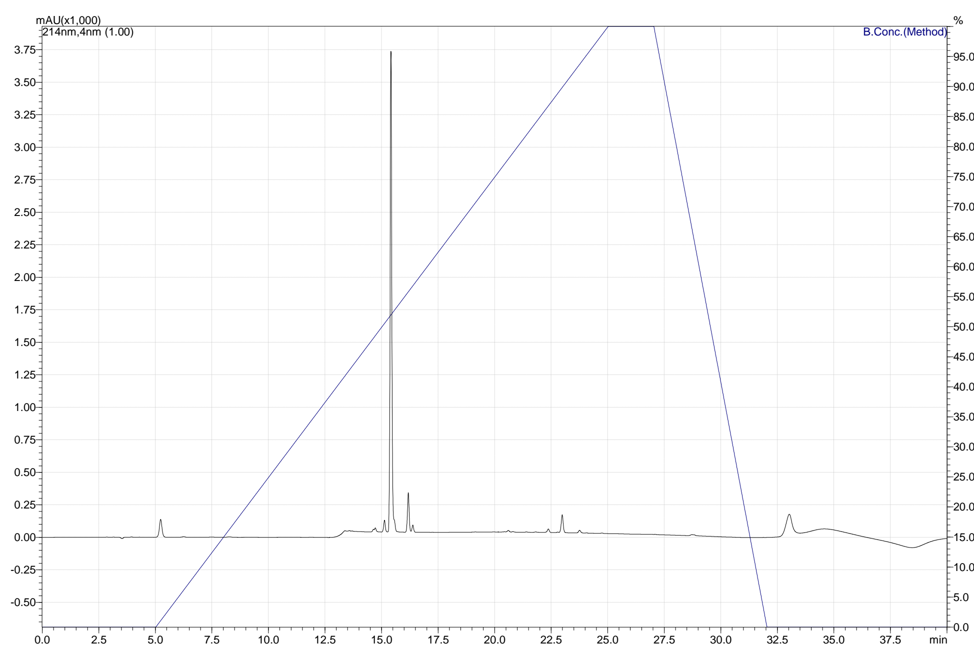

Figura 3 - Perfil cromatográfico da amostra "VRJA".

Isso fica mais evidente ao sobrepor os três perfis e ao fazer zoom dos dois grupos de picos, como mostram as figuras 4, 5 e 6.

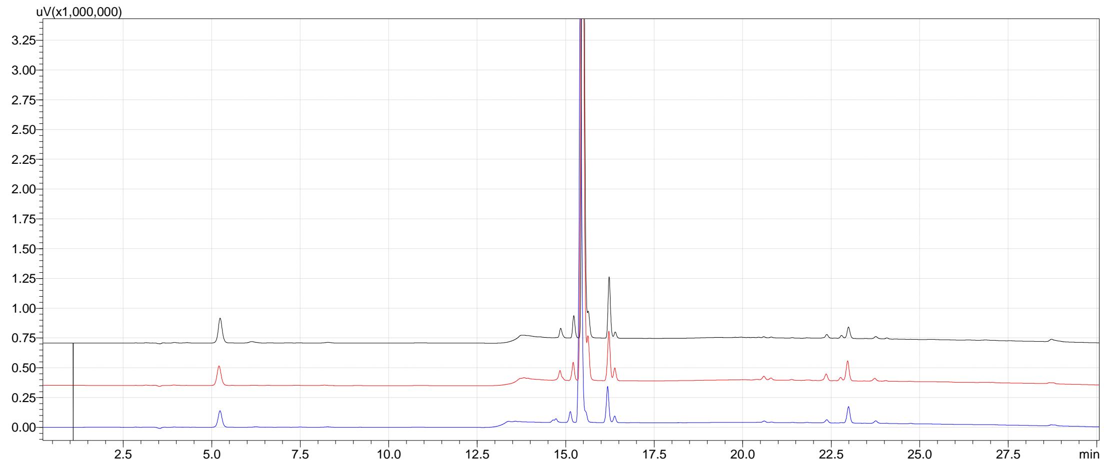

Figura 4 - Sobreposição dos Perfis cromatográficos das amostras "VRJC, VRJE e VRJA".

Perfis cromatográficos das amostras VRJC (preto), VRJE (vermelho) e VRJA (azul).

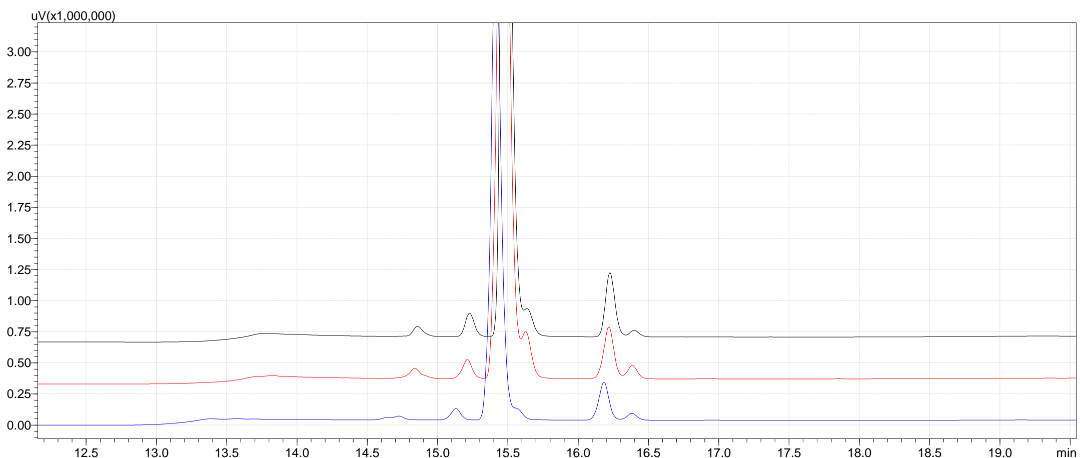

Figura 5 – Zoom do primeiro grupo de picos da sobreposição dos Perfis cromatográficos das amostras "VRJC, VRJE e VRJA".

Perfis cromatográficos das amostras VRJC (preto), VRJE (vermelho) e VRJA (azul).

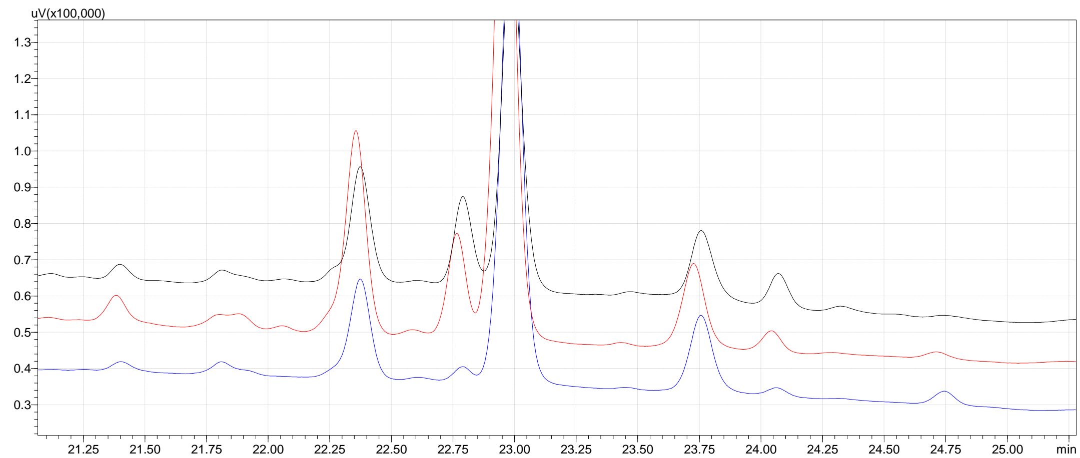

Figura 6 - Zoom do segundo grupo de picos da sobreposição dos Perfis cromatográficos das amostras "VRJC, VRJE e VRJA".

Perfis cromatográficos das amostras VRJC (preto), VRJE (vermelho) e VRJA (azul).

Os perfis foram comparados com os de diferentes secreções parotóides de outros anfíbios do gênero *Rhinella* e um do gênero *Rhaebo* analisados por Sciani et al. (2013). A comparação visual dos perfis mostrou que as secreções analisadas diferem do perfil de *Rhinella jimi* estudado pelos autores. Como forma de experimento complementar, os principais picos foram coletados conforme representado na figura 7 e foram realizados experimentos de espectrometria de massas para avaliar se os picos apresentam as mesmas moléculas nas três amostras diferentes.

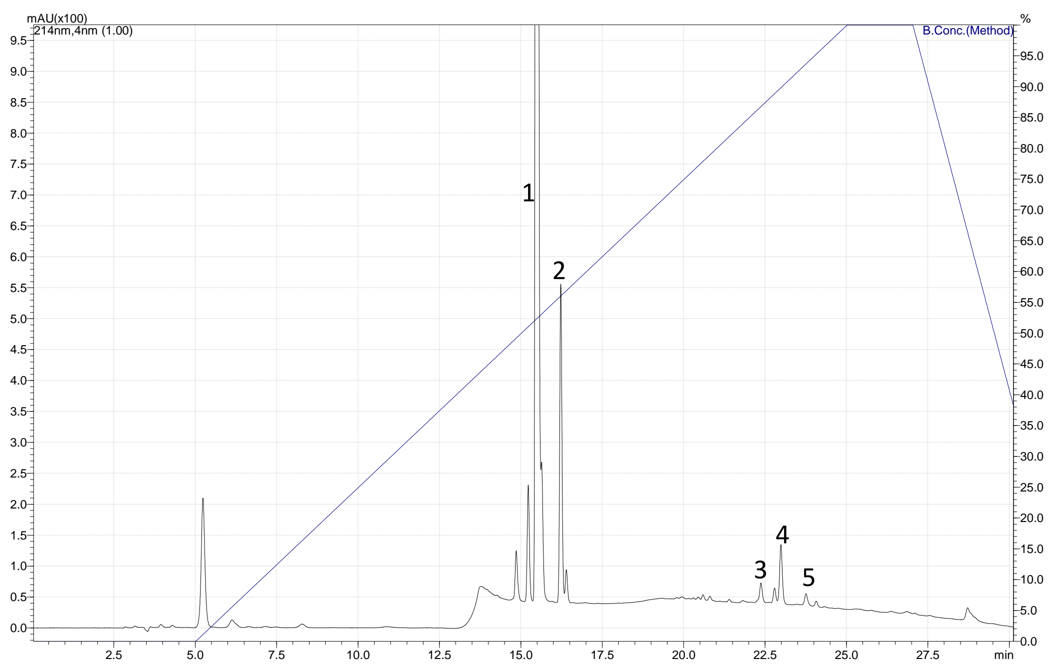

Figura 7 - Representação dos picos coletados para análises por espectrometria de massas

Os experimentos de espectrometria de massas mostraram que, além do mesmo perfil cromatográfico, as três secreções também apresentam a mesma faixa de massas de moléculas nos picos coletados, sendo essas: pico 1 - 205 m/z correspondente a bufotenina, pico 2 – 203 m/z correspondente a dehidrobufotenina, pico 3 – 401 m/z correspondente a marinobufagina, pico 4 - 403 m/z correspondente a telocinobufagina e pico 5 – 387 m/z, correspondente a bufalina. Os perfis de massas de todos os picos estão representados nas figuras 8, 9, 10, 11, e 12.

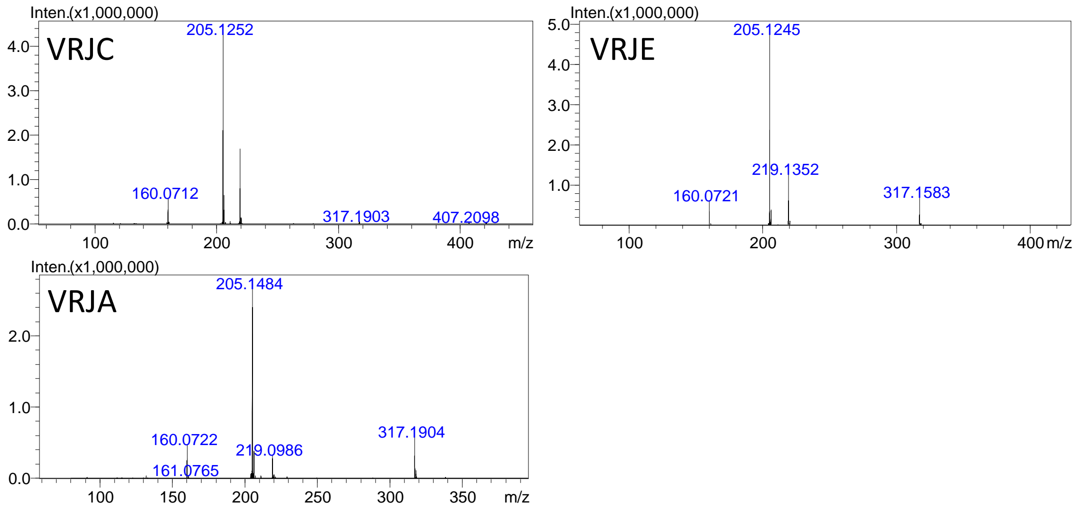

Figura 8 – Perfil de massas do pico 1 com o íon majoritário de 205 m/z.

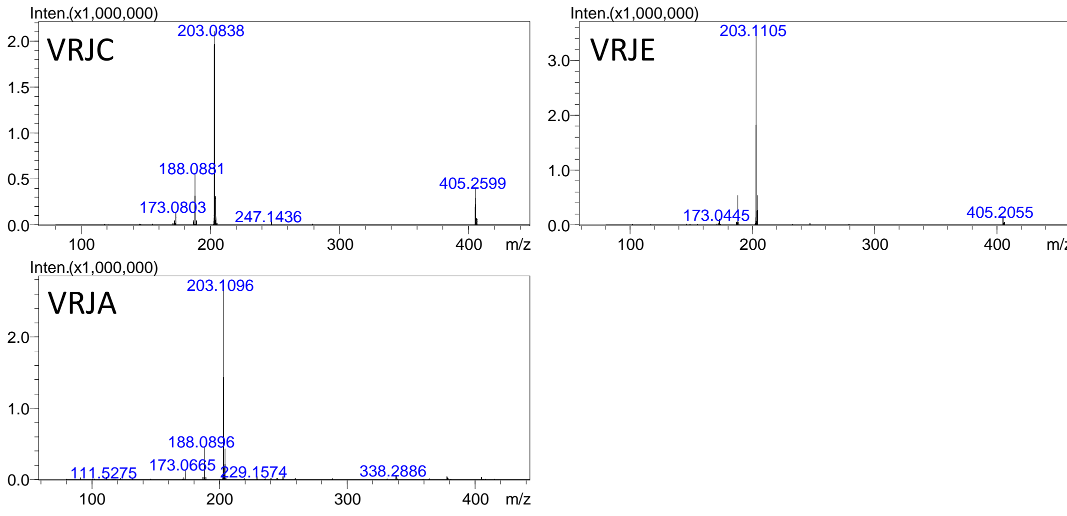

Figura 9 – Perfil de massas do pico 2 com o íon majoritário de 203 m/z.

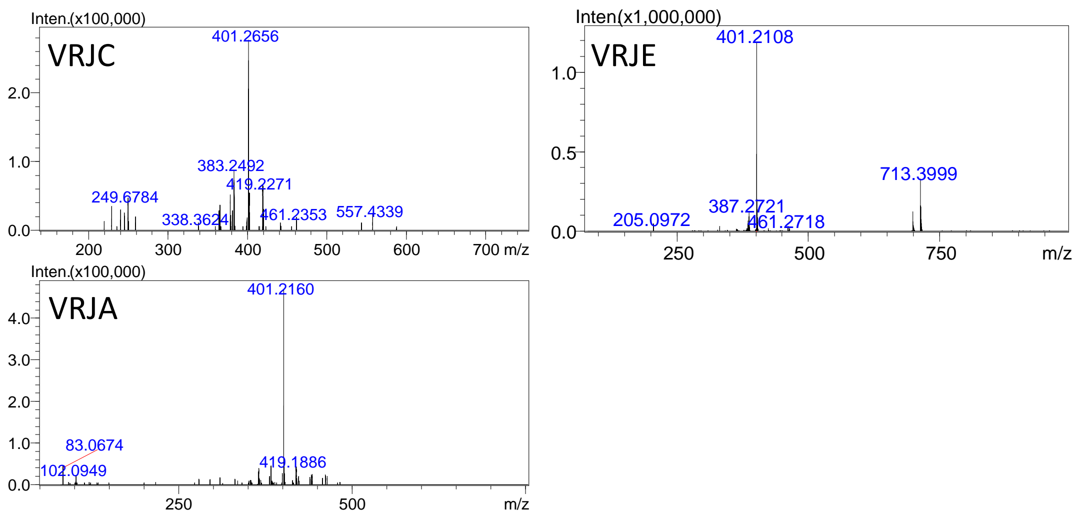

Figura 10 – Perfil de massas do pico 3 com íon majoritário de 401 m/z.

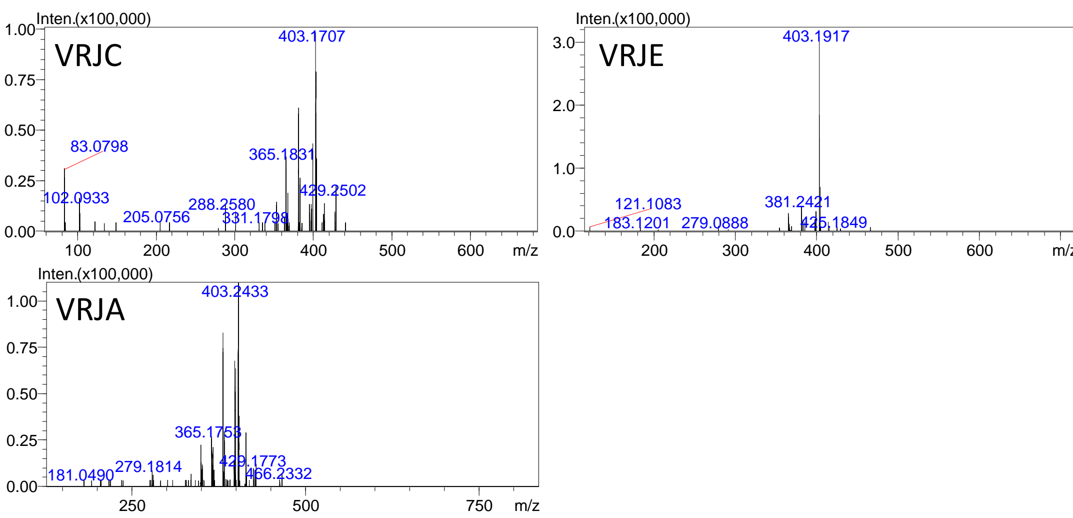

Figura 11 – Perfil de massas do pico 4 com íon majoritário de 403 m/z.

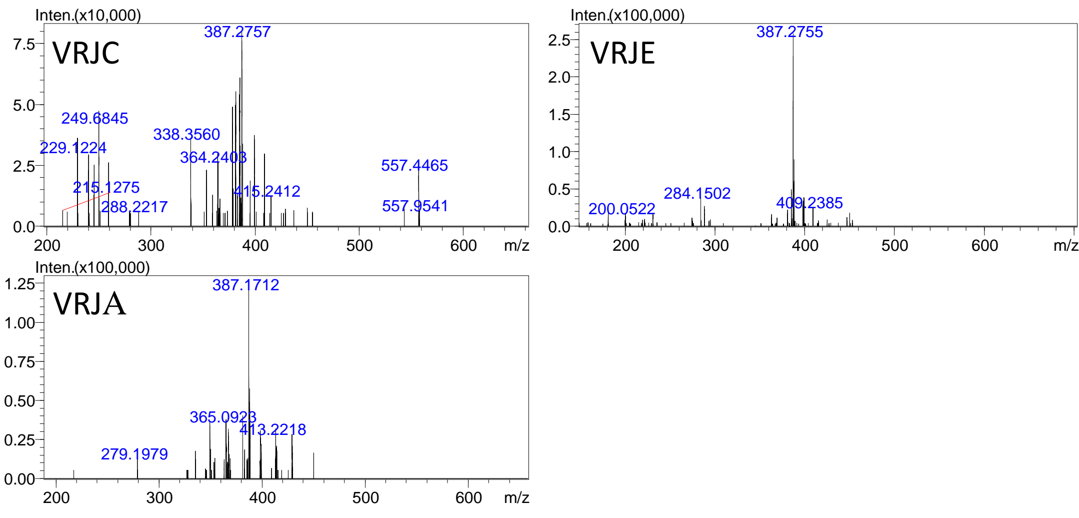

Figura 12 – Perfil de massas do pico 5 com íon majoritário de 387 m/z.

É possível verificar que não houve variação na análise das secreções dos animais entre as localidades, o que pode ser justificado pela proximidade entre os pontos de coleta. A distinção feita entre a presença do íon 387 m/z (Figura 12), correspondente ao esteroide bufalina, é destaque pois, segundo Sciani et al. (2013), esse esteroide está presente na secreção de *Rhinella schneideri*, mas não aparece no perfil de secreção de *Rhinella jimi*. Os autores sugerem uma identificação taxonômica, através da composição química ao caracterizar os venenos de cada espécie do gênero estudada.

Um inventário de anfíbios e répteis para a região do Araripe traz apenas duas espécies da família Bufonidae registradas para área (Ribeiro et al., 2012), *Rhinella jimi* e *Rhinella granulosa*. *Rhinella schneideri*, no entanto, não possui distribuição geográfica abrangendo a região estudada. Além disso, os caracteres morfológicos elegidos por Pramuk (2006) para os bufonídeos sul-americanos, bem como chave proposta por Kwet et al. (2006) foram utilizados para identificar taxonomicamente os espécimes, chegando às características morfológicas da espécie *Rhinella jimi*.

Desta forma, a variação existente entre os dados obtidos neste trabalho com os amostrados por Sciani et al. (2013) corroboram com o trabalho de Daly et al. (2008) no qual afirmam que a variação na composição de toxinas pode ocorrer de acordo com a localidade de

coleta, uma vez que a mesma pode estar diretamente relacionada à alimentação. No mesmo sentido Gao et al. (2010) apontam que esta variação pode ocorrer inclusive dentro a mesma espécie.

Conclusão

Por comparação dos cromatogramas, juntamente com os perfis de espectrometria de massas, pode-se concluir que as amostras analisadas das três localidades não possuem diferenças significativas. A semelhança entre a secreção estudada e a secreção glandular de *Rhinella schneideri* é importante por ressaltar que ocorrem variações entre espécies e na mesma espécie, provavelmente de acordo com a dieta do animal. Deste modo, é necessária cautela para afirmar que características da secreção glandular podem ser usadas como recurso quimiotaxonômico.

Agradecimentos

Os autores agradecem a colaboração do Dr. Samuel Cardozo Ribeiro e Dr. Samuel Vieira Brito pela identificação dos animais. Ao professor Daniel Carvalho Pimenta e Hugo Vigerelli pelo suporte dado no Instituto Butantan e realização das análises químicas.

Financiamento

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), referente a bolsa de Doutorado de Débora Lima Sales e Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, referente à bolsa de Produtividade em Pesquisa de Waltécio de Oliveira Almeida, processo: 302429/2015-8.

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Artigo 2

Journal: Biomedicine & Pharmacotherapy
Aceito em 22 de maio de 2014 conforme Anexo IV

Antibacterial, modulatory activity of antibiotics and toxicity from *Rhinella jimi* (Stevaux, 2002) (Anura: Bufonidae) glandular secretions

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The increase in microorganisms with resistance to medications has caused a strong preoccupation within the medical and scientific community. Animal toxins studies, such as parotoid glandular secretions from amphibians, possesses a great potential in the development of drugs, such as antimicrobials, as these possess bioactive compounds. It was evaluated *Rhinella jimi* (Stevaux, 2002) glandular secretions against standard and multi-resistant bacterial strains; the effect of secretions combined with drugs; and determined the toxicity using two biologic *in vivo* models, and a *in vitro* model with mice livers. Standard strains were used for the determination of the Minimum Inhibitory Concentration (MIC), while for the modulatory activity of antibiotics, the clinical isolates *Escherichia coli* 06, *Pseudomonas aeruginosa* 03 and *Staphylococcus aureus* 10 were used. Modulatory activity was evaluated by the broth microdilution method with aminoglycosides and β-lactams as target antibiotics. The secretions in association with the antibiotics have a significant reduction in MIC, both the aminoglycosides and β-lactams. The toxicity and cytotoxicity results were lower than the values used in the modulation. *R. jimi* glandular secretions demonstrated clinically relevant results regarding the modulation of the tested antimicrobials.

Key-words: poisons; parotoid gland; antimicrobial activity; bacterial resistance; modulation of antibiotics; toxicity; cytotoxicity.

Introduction

The increase in the number of microorganism strains with resistance to medications has caused preoccupation within the scientific community, especially when faced with strains resistant to multiple drugs [1]. Development of resistance is a natural evolutionary process, however it has stood out due to its increase in the last years, which is disproportional to the production of new antimicrobial drugs, which compromises the efficacy of many drugs, thus increasing the cost and duration of infected patient's treatment [2, 3].

This explains the urgency in the search for new antimicrobial agents or compounds which are capable of potentiating the action of already commercialized drugs [4]. Woolhouse and Farrar [5] suggested the creation of an intergovernmental force of action which emerged from the affirmation of the public health problem and assumed a role in the identification of failures for their corrections. In the same manner, the WHO [6], judges the efforts of all countries in combating the problem a necessity, which ranges from education initiatives and hygiene care to research incentive campaigns and optimization of antimicrobials.

Within the antibiotic classes, we emphasize through clinical importance: aminoglycosides due to their common usage, and β-lactams for presenting a wide spectrum of activity and efficacy, in particular carbapenems [7]. β-lactams act by damaging the cellular wall while aminoglycosides act by inhibiting the production of proteins [8, 9, 10].

Some microorganisms produce β-lactamases which hydrolyze antimicrobial β-lactams, causing resistance to these [11]. Aminoglycosides present excellent efficacy, however due to high toxicity, they cannot be used for prolonged periods of time, therefore the association between these can be a good clinical alternative to improve efficacy [12].

In this sense, natural products, especially plants, have been the targets of studies with promising results in microorganism combat, including in the modification of aminoglycoside activity [13, 14, 15, 16]. Animal products have also been investigated, such as fixed oils from the *Spilotes pullatus* serpent [17], the chelonian *Phrynops geoffroanus* [18], and amphibians from the family Leptodactylidae [19].

Amphibians from the family Bufonidae have also been the target of recent studies, these present parotoid glands that produce important secretions in the passive defense process. These are activated through mechanical pressure, such as the bite from a predator, and can have spontaneous activation in some species [20, 21]. The poison released is rich in

compounds which confer it protection against microbial infections, such as fungi and bacteria [22].

The popularly known "sapo cururu", *Rhinella jimi* (Stevaux, 2002) [23] is one of the 84 Bufonidae described for Brazil [24]. It has a thick presentation and is covered in glands distributed across the forearm, feet, cloaca and the posterior part of the head, it possesses nocturnal habits and is geographically distributed in all the northeastern region of Brazil. Compounds present in the fat of these animals also present relevant chemical and pharmacological results by modulating antibiotics and reducing inflammatory edemas in topical applications [25]. Bioprospecting of the present substances in the secretion of *R. Jimi* glands possess a great quantity of bioactive compounds with proven pharmacological activities [26, 27, 28, 29]. Work performed with poisons from other amphibians demonstrate the importance of these animals as sources of pharmacological research with fungistatic, antibacterial, antineoplastic and analgesic effects [30, 31, 32, 33].

However, although there is a record of the traditional use of glandular secretion of Bufonidae in ethnomedicine [for example 34, 35, 36, 37], such as data on important biological activities [27, 28, 29, 32, 33], toxicological studies are mainly performed with isolated compounds from their secretions [38]. Thus, the objective of this work is (1) to evaluate the action of the *R. jimi* glandular secretion against standard and multi-resistant bacterial strains; (2) to analyze the effect of the secretion in combination with drugs used in the treatment of these infections; (3) to verify if variations in effect exist according to the location of the animals; and (4) to evaluate the toxicity using *Drosophila melanogaster* and *Artemia salina* as the models organisms and a *in vitro* model with mice livers.

Materials and Methods

Rhinella jimi Glandular Secretions

Rhinella jimi specimen were collected actively through the months of March to August 2014 in the counties of Aiuaba and Crato, State of Ceará, and Exu, State of Pernambuco. Removal of secretions was performed mechanically by applying pressure in the parotoid glands of the individuals, as described by Jared et al. [20]. The secretions were treated with 70% ethanol, lyophilized and kept refrigerated at 10°C for further analysis. The secretions were identified through the reverse phase high performance liquid chromatography (RP-

HPLC) technique in a binary HPLC system (10A vp Prominence, Shimadzu Co., Japan) and by mass spectrometry, as described in Sciani et al. [39]

Antibacterial Activity and Antibiotic- Modulatory Effect

The following standard bacterial strains were used to determine the Minimum Inhibitory Concentration (MIC): *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25853 and *Staphylococcus aureus* ATCC 25923. For the modulatory activity of antibiotics, bacterial clinical isolates of *Escherichia coli* 06, *Pseudomonas aeruginosa* 03 and *Staphylococcus aureus* 10, with a resistance profile to various drugs, in accordance with Table I, were used.

Table I – Bacterial clinical isolate strains used for tests with their resistance profile to antibiotics and origin. Source: Microbiology and Molecular Biology Laboratory – LMBM – Universidade Regional do Cariri–URCA.

Bacteria	Origin	Resistance profile
Escherichia coli 06	Urine culture	Cephalothin, cephalexin, cefadroxil, ceftriaxone, cefepime, ampicillin-sulbactam
Pseudomonas aeruginosa 03	Uroculture	Amikacin, imipenem, ciprofloxacin, levofloxacin, piperacillin-tazobactam, ceftazidime, merpenem, cefepime
Staphylococcus aureus 10	Rectal swab culture	Cefadroxil, cephalexin, cephalothin, oxacillin, penicillin, ampicillin, amoxicillin, moxifloxacin, ciprofloxacin, levofloxacin, ampicillin-sulbactam, amoxilin / ac. Clavulanic, erythromycin, clarithromycin, azithromycin, clindamycin

To determine the MIC of glandular secretions, the broth microdilution method was used. The concentration of the secretions used in the tests were of 2048 µg/mL, with dilutions performed with distilled sterile water. The inoculum was made in tubes containing saline and the turbidity was compared to the McFarland scale which equated to a concentration of 10^5 CFU/mL. 100 µL of Brain Heart Infusion (BHI) medium and inoculum (10%) were distributed in each well of a 96 well plate. In the first well, 100 µL of the tested product was added and following this a serial microdilution until the penultimate well, with concentrations ranging from 1024 µg/mL to 1 µg/mL, was initiated. The plates were taken to the incubator for 24 hours at 35°C [40]. The revelation of the bacterial MIC was performed using

Resazurin. The MIC was defined as the lowest concentration in which no growth was observed in accordance with the CLSI [41].

The method proposed by Coutinho et al. [42] was utilized for the modulation of drugs test, where a solution of the natural product is tested at a sub-inhibitory concentration (MIC/8). 100 μL of a solution containing 10% BHI, inoculum and natural product was distributed in each well, in the alphabetical order of the plate. Thereafter, 100 μL of the drug (amikacin, gentamycin, imipenem and oxacillin) was mixed in the first well, proceeding a serial microdilution until the penultimate well. The initial concentration of each drug was of 2048 μg/mL, varying gradually in the well from 1024 μg/mL to 1 μg/mL.

All the determinations were performed in triplicates and the results were normalized through calculation of the geometric mean, standard error of the geometric mean and geometric standard deviation. The results were compared by an Analysis of Variance (ANOVA) and the comparison between the geometric means in accordance with Tukey's test, with a $p<0.05$ value being considered significant, using the software Prism 6.0.

In vivo toxicity assays

For the *D. melanogaster* toxicity model the protocol according to Cunha et al. [43] was adopted. Individuals from the Harwich lineage, obtained from the Vivarium of the Regional University of Cariri- URCA were used. The flies were grown in glass flasks measuring 5.5cm in diameter and 15cm in height, containing 8mL of standard media (1% w/v brewer's yeast, 2% w/v sucrose; 1% w/v milk powder; 1% w/v agar; 0.08% w/w nipagin), with constant temperature and humidity (25 ± 1°C, 60% relative humidity, respectively).

Twenty adult flies (males and females) were placed in 40mL flasks, each containing a filter paper at the bottom. For the control group, 1mL of 20% sucrose in distilled water was added to the flask, whereas for the tested groups, the glandular secretion was diluted in 20% sucrose and 1mL of the following concentrations: 1mg/mL, 10mg/mL and 50mg/mL, were added to the flask. All the flasks were maintained in the same temperature and humidity conditions with a photoperiod of 12h. The experiment was carried out in six replicates, counting dead individuals after 3, 6, 12, 24 and 36 h.

In the *Artemia salina* Leach toxicity assays, the methodology recommended by Meyer et al. [44] was used, in which crustacean cysts were hatched in seawater and constant light for 24 hours. In the tested group, test tubes containing 10mL of the secretion diluted in seawater

in different concentrations (250, 275, 300, 325, 350, 375, 400, 425, 450, 475 e 500 μg/mL), followed by a positive control prepared with potassium chromate (K_2CrO_4) in the same concentrations, and a negative control with seawater were used. The assays were carried out in triplicates and 10 individuals were used in each replica of the experiment. After 24 h of constant light and temperature of 25 ± 2°C, counting of dead individuals was performed.

The LD_{50} of both assays were determined with a 95% confidence interval, through the probit analysis method, using the TSK – Trimmed Spearman Karber Method, version 1.5.

In vitro cytotoxicity assay in *Mus musculus* hepatocytes

Female Swiss mice (*Mus musculus*), weighing 25–35 g, were previously housed in standard polypropylene cages under controlled conditions of temperature (22 ± 2 ◦C) and 12-h light/dark cycle, with free access to water and rodent chow (Labina, Purina, Brazil). Mice were allowed to adapt to the laboratory for at least 1 h before testing. Animals were previously anesthetized with ketamine 20 mg/kg i.p. and xylazine 10 mg/kg i.p. Livers were excised and maintained continuously in ice-cold Phosphate Buffered Saline (PBS) pH 7,4 (Moronvalle-Halley et al. [45])

Slices from *Mus musculus* liver (dimensions: 8.3 ± 0.67 mm × 1.3 ± 0.44 mm; 34.8 ± 0.01 mg) were obtained according to the method described by Moronvalle-Halley et al [45], with adaptations, and maintained in PBS pH 7,4 prior to cytotoxicity assay. The effect of glandular secretion of *Rhinella jimi* (SRJ) on cell viability of cultured *Mus musculus* liver slices was evaluated using the MTT assay. Firstly, liver slices were preincubated in microtubes at different concentrations of secretion (1, 10, 100, 1000 and 5000 μg / mL) or vehicle control for 30 minutes at 37 ° C, followed by addition of 50 μl MTT (0.5 mg / mL) and incubation for 20 minutes at 37 ° C. After that, 100 μl of DMSO were added to samples in order to solubilize the reduced formazan and stop the reaction. Samples were vortexed for 10 minutes to allow the releasing of formazan produced by viable cells contained in the tissue. The absorbances were measured at 570 nm wavelength for subsequent LC_{50} calculation.

Results

The Minimum Inhibitory Concentration (MIC) for the specimen secretions collected in Aiuaba (SRJ-A), Crato (SRJ-C) and Exu (SRJ-E) were ≥ 2048 μg/mL without variations

between them. When the action of the secretions (256 µg/mL) in combination with aminoglycosides and β-lactams were evaluated, the results obtained demonstrated an increase in efficacy of the antibiotics against resistant bacterial strains.

For *E. coli* the products reduced the MIC of the drugs Gentamicin, Imipenem and Oxacillin by 46.8%, 60.3% and 7.4%, respectively. For *P. aeruginosa* a reduction in MIC of 99.8%, 93.7% and 50%, respectively, occurred, in the same tested antibiotics. For *S. aureus*, the glandular secretions reached a reduction of 18.3% for amikacin and of 43.8% for gentamicin in MIC. β-lactams had their effects potentiated in both Gram-negative bacteria, which presented with elevated MIC levels of the drugs and a significant reduction, especially for Imipenem, without a difference between the three tested products (Figs 1 and 2). The secretions also presented a considerable MIC reduction for amino glycosides, with an accentuated synergisms with gentamicin, for all the tested bacterial strains (Figs. 1, 2 and 3).

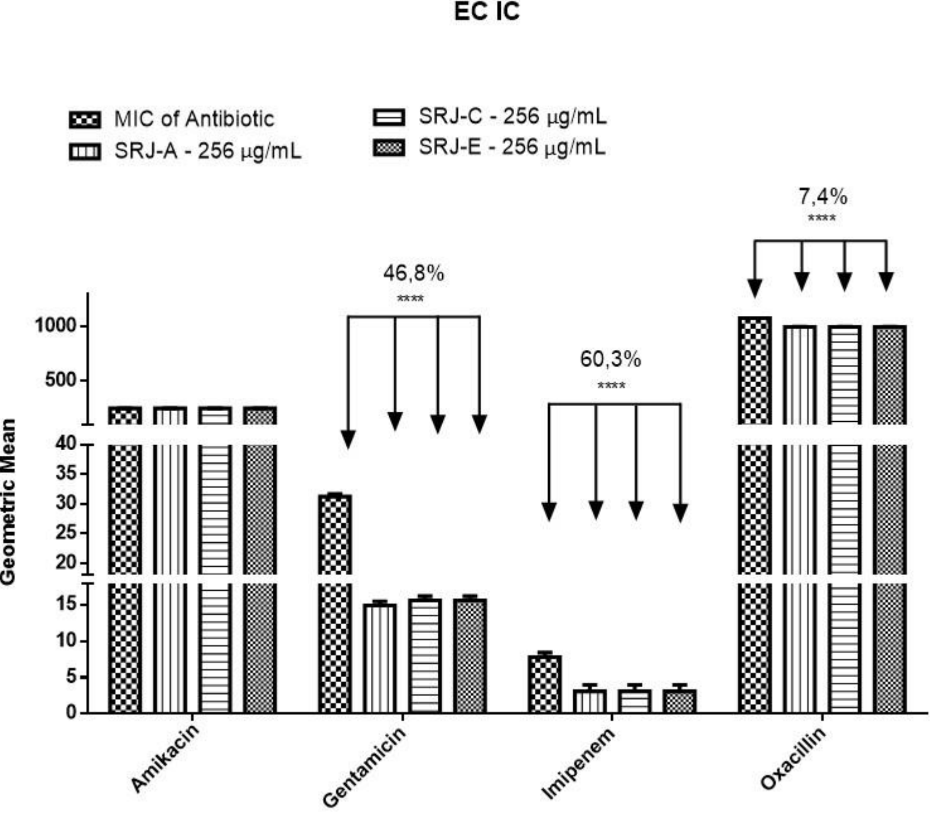

Fig. 1 – Comparative graph of the Minimum Inhibitory Concentration (MIC) of antibiotics and the antibiotic modulatory activity of the products: SRJ-A (*Rhinella jimi* glandular secretion collected from Aiuaba-CE), SRJ-C (*R. jimi* glandular secretion collected from Crato-CE) and SRJ-E (*R. jimi* glandular secretion collected from Exu-PE) in association with aminoglycosides (Amikacin and Gentamicin) and β-lactams (Imipenem and Oxacillin) against *Escherichia coli* 06. **** - Significance with P <0,0001.

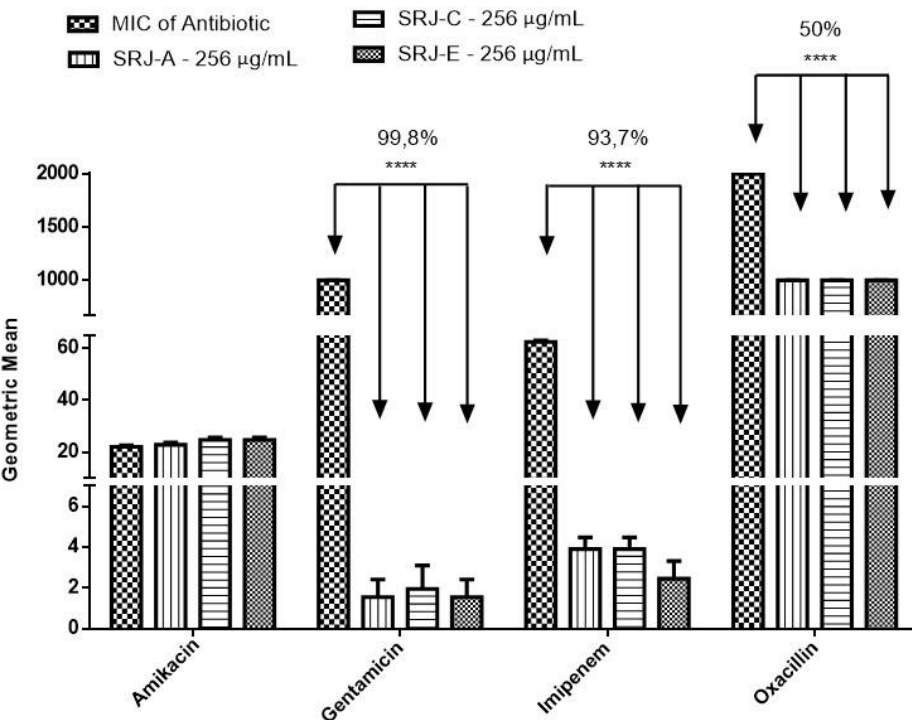

Fig. 2 - Comparative graph of the Minimum Inhibitory Concentration (MIC) of antibiotics and the antibiotic modulatory activity of the products: SRJ-A (*Rhinella jimi* glandular secretion collected from Aiuaba-CE), SRJ-C (*R. jimi* glandular secretion collected from Crato-CE) and SRJ-E (*R. jimi* glandular secretion collected from Exu-PE) in association with aminoglycosides (Amikacin and Gentamicin) and β-lactams (Imipenem and Oxacillin) against *Pseudomonas aeruginosa* 03. **** - Significance with P <0,0001.

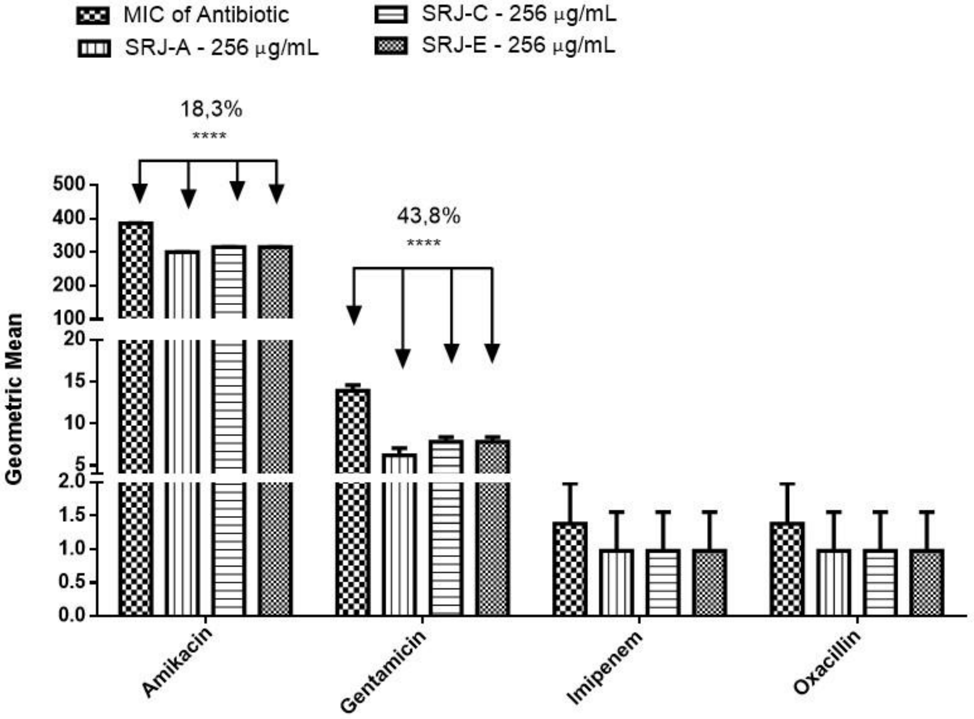

Fig. 3 - Comparative graph of the Minimum Inhibitory Concentration (MIC) of antibiotics and the antibiotic modulatory activity of the products: SRJ-A (*Rhinella jimi* glandular secretion collected from Aiuaba-CE), SRJ-C (*R. jimi* glandular secretion collected from Crato-CE) and SRJ-E (*R. jimi* glandular secretion collected from Exu-PE) in association with aminoglycosides (Amikacin and Gentamicin) and β-lactams (Imipenem and Oxacillin) against *Staphylococcus aureus* 10. **** - Significance with P <0,0001.

The chemical analyzes of the glandular secretions did not present differences between them, with the same chromatographic profile and the same range of molecular masses in the collected peaks (Fig. 4), with the collected peaks presenting molecular masses of 205, 203, 401, 403 and 387, corresponding to bufotenine, dehydrobufotenine, marinobufagin, telocinobufagine and bufalin, respectively.

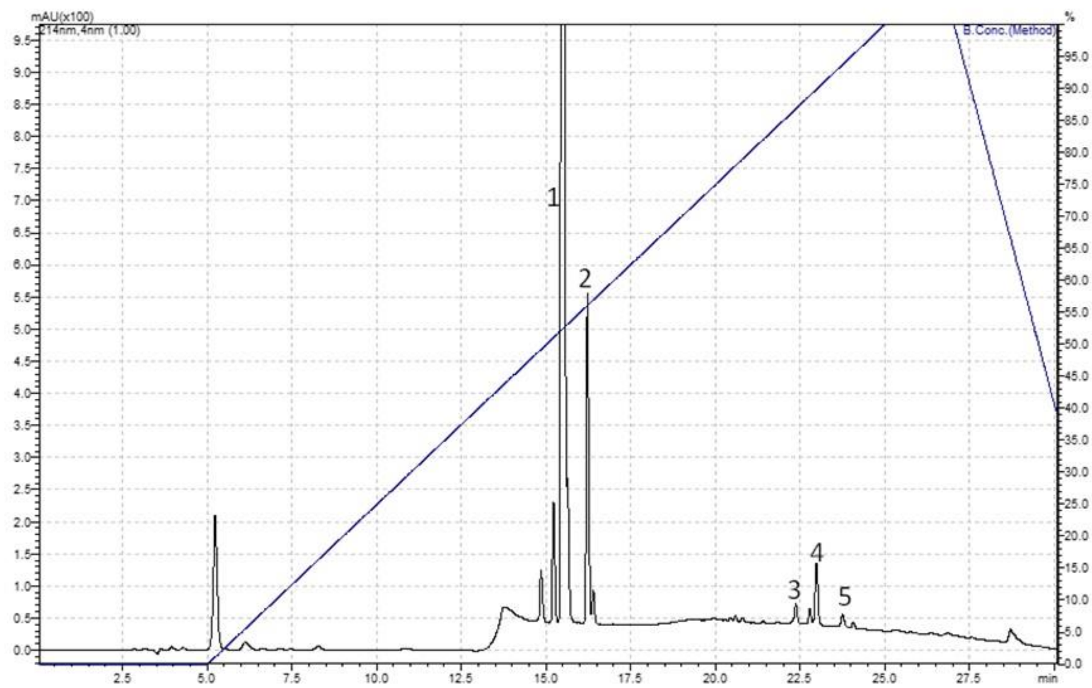

Fig. 4 – Representation of the peaks collected for mass spectrometry analyses. Peak 1 – 205 m/z corresponding to bufotenine, peak 2 – 203 m/z corresponding to dehydrobufotenine, peak 3 – 401 m/z corresponding to marinobufagin, peak 4 – 403 m/z corresponding to telocinobufagine and peak 5 – 387 m/z corresponding to bufalin.

The toxicity assays with *Artemia salina* (Table II) showed that the estimated *R. jimi* (SRJ) glandular secretion dose necessary to kill 50% of individuals (LD_{50}) was of 365.94 µg/mL (with an inferior and superior 95% confidence interval of 338.97 µg/mL and 395.04 µg/mL, respectively). Whereas in the positive control group with potassium chromate, the LD_{50} was of 41.72 µg/mL (with an inferior and superior 95% confidence interval of 32.06 µg/mL and 54.31 µg/mL, respectively).

The assays performed with *Drosophila melanogaster*, showed in Table III, presented an LD_{50} of 12.02 mg/mL (with an inferior and superior 95% confidence interval of 9.57 mg/mL and 15.68 mg/mL, respectively). Despite higher concentrations of SRJ slightly decrease the viability of *Mus musculus* hepatocytes *in vitro* from MTT test in mice liver slices, it was found an LC_{50} > 5000 µg/mL after 30 min of preincubation with SRJ.

Table II – Percentage mortality of *Artemia salina* against *Rhinella jimi* Glandular Secretion (SRJ) and Potassium Chromate in different concentrations (µg/mL), considering the control group with 0% mortality, results from readings taken after 24 h.

Concentration (µg/mL)	**Mortality (%) *Artemia salina***	
	SRJ	Potassium chromate
5	0	7.0
10	3.3	13.0
25	6.6	30.0
50	6.6	43.0
100	20.0	87.0
250	20.0	100
250	20.0	100
275	23.3	100
300	36.6	100
325	40.0	100
350	46.6	100
375	50.0	100
400	53.3	100
425	66.6	100
450	70.0	100
475	73.3	100
500	76.6	100

Table III – Percentage mortality of *Drosophila melanogaster* against *Rhinella jimi* Glandular Secretion (SRJ) in different concentrations (mg/mL), results from readings taken at 3, 6, 12, 24 and 36h.

Concentration (mg/mL)	**Mortality (%) *Drosophila melanogaster***				
	3h	**6h**	**12h**	**24h**	**36h**
Control	0	0	0	0	3
1	0	0.83	1.66	3.33	9.16
10	0	0.86	1.66	10.0	39.1
50	0	1.66	4.16	16.6	90.8

Discussion

It is important to highlight that there was no variation in the chemical composition between the three analyzed samples. For this reason, there was also no statistically relevant variation within these regarding the microbiological results. Moreover, the results for the

Minimum Inhibitory Concentration, not being the crude secretion, therefore, capable of killing or inhibiting the growth of bacteria, were very high.

Similar results were obtained by Brito et al. [32] with methanolic extracts of the skin from *R. jimi* and fractions of these extracts. Freitas [46] found bacterial activity for fractions of the poison, but the data cannot be dully compared due to a different methodology, as the author used disc diffusion tests. Studies indicating the antibacterial action of the secretion were acquired from isolated substances, such as peptides and the steroids telocinobufagine and marinobufagin, but not the brute poison [47, 48].

Regarding the modulation of aminoglycosides and β-lactam antibiotics, the *Rhinella jimi* glandular secretion demonstrated clinically relevant results over multi-resistant bacteria (Figs 1, 2 and 3).

For the Gram-negative *Escherichia coli*, the *Rhinella jimi* secretion promoted a significant reduction in both antibiotic classes. Cunha Filho et al. [48] reported antibacterial activity of the compounds marinobufagin and telocinobufagine isolated from the *Rhinella* genus over *E. coli* and *S. aureus* bacteria, however this was not confirmed in our study for the pure secretion.

P. aeruginosa presents intrinsic resistance mechanisms to diverse antibiotics in clinical use, including aminoglycosides and β-lactams [49]. The results presented here in Figure 02, show the synergistic activity of the glandular secretion to the aminoglycoside Gentamicin and the β-lactams Imipenem and Oxacillin. These associations are fairly representative, as among the mechanisms responsible for resistance in this species the low external membrane permeability, efflux system, production of aminoglycosides inactivating enzymes, alteration of fluoroquinolones targets and the production of β-lactamases stand out [50, 51].

The aminoglycosides are preferentially active against Gram-negative bacilli and Gram-positive cocci [52]. The glandular secretion modulated the action of Amikacin only for *S. aureus* and the action of Gentamicin against all three tested bacterial strains analyzed. The synergistic modulatory activity presented by *S. aureus* is very relevant as species of this genus represent a public health problem due to the growing resistance to conventional antibiotics [53, 54].

Biological activities of compounds present in the secretion of Bufonidae, for example: anesthetic activity for bufalin [55]; cytotoxic activity for bufalin, arenobufagine, other compounds and brute secretions [56, 57, 58, 59], have been reported; in addition to a hallucinogenic power and the ability to inhibit the penetration of rabies virus, which have been described for bufotenine [28, 60]; as well as antibacterial activities which were reported for marinobufagin and telocinobufagine [48]. However, this is the first report on the granular secretion of *Rhinella jimi* being used as a modifier of the action of aminoglycoside and β-lactam antibacterials.

Toxicity studies by Gadelha et al. [61] where *Gallus gallus domesticus* chicks received the *Rhinella jimi* venom orally in single doses varying from 3 to 25 mg/kg, even without clinical signs of toxicity, the animals were necropsied and subjected to pathological exams which revealed dose-dependent damages in the heart, liver and lungs. In the present study an LD_{50} of 12.02 mg/mL was verified only with 36 hours of exposure to the venom and considered, therefore, as chronic toxicity.The results from the toxicity assays with *Artemia salina* show an LD_{50} of 365.94 μg/mL. Assay in vitro from MTT test in mice liver slices, it was found an LC_{50} > 5000 μg/mL after 30 min of preincubation with SRJ.therefore superior to the dose utilized in the antibiotic modulation experiments (256 μg/mL).

Conclusion

The *Rhinella jimi* granular secretion no presented antibacterial activity when tested alone. When used in association with the antibiotics Amikacin, Gentamicin, Imipenem and Oxacillin, it demonstrated a significant reduction in Minimum Inhibitory Concentration of all of these, being, thus, a synergistic action. No variation in the analyses occurred in accordance with the location of animal collection.

The results from the antibiotic modulation are clinically relevant, as the secretions were capable of resulting in a phenotypic change in the tested bacteria from resistant to sensitive for the evaluated drugs, in doses below the established LD_{50} and LC_{50} in this study. Thus they represent an alternative to the combat or reduction of resistance levels, which can serve as a starting point for the production of similar molecules in structure and effect. Above all, studies are still needed to verify the mechanism of action involved in this interaction.

Conflicts of Interest

The authors declare there is no conflict of interest.

Acknowledgements

The authors thank the collaboration of Dr. Robson Waldemar Ávila for the identification of animals. Coordination of Improvement of Higher Level Personnel (Capes), referring to the doctoral scholarship of Débora Lima Sales and National Council of Scientific and Technological Development - CNPq, referring to the Research Productivity grant of Waltécio de Oliveira Almeida, process: 302429/2015 -8.

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Considerações Finais

CONSIDERAÇÕES FINAIS

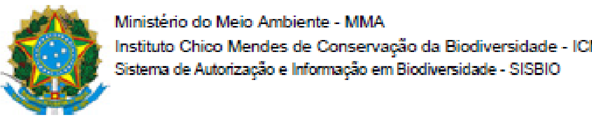
As análises químicas das secreções estudadas indicam que as amostras das três localidades Aiuaba, Crato e Exu não apresentam variações na composição. Por comparação com estudos anteriores, o perfil químico da secreção de *Rhinella jimi* apresenta semelhança casual com perfil de *R. schineideri*, uma vez que as espécies diferem morfologicamente e em distribuição geográfica.

A variação química demonstra um aspecto fenotípico interessante. Serão necessários novos estudos para elucidar a natureza dessa variação, sendo o perfil químico um passo essencial na identificação e exploração terapêutica de agentes bioativos presentes.

Do ponto de vista microbiológico, as secreções não apresentaram efeito antibacteriano frente às linhagens padrão e resistente de *Escherichia coli*, *Pseudomonas aeruginosa* e *Staphyloccocus aureus*, quando testadas sozinhas.

Mostraram, porém, importância clínica ao atuarem como moduladoras de antibióticos aminoglicosídeos e β-lactâmicos frente às mesmas espécies de bactérias com resistência a múltiplas drogas. A secreção glandular de *Rhinella jimi* possui, portanto, substâncias com potencial de utilização em novas composições farmacêuticas que busquem melhorar o potencial de antibióticos já existentes.

Anexos



Autorização para atividades com finalidade científica

Número: 39353-2	Data da Emissão: 24/01/2014 11:11	Data para Revalidação*: 23/02/2015
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Dados do titular

Nome: Débora Lima Sales	CPF: 021.698.813-60
Título do Projeto: ANÁLISE DAS ATIVIDADES BIOLÓGICAS DA SECREÇÃO GLANDULAR DE RHINELLA JIMI (STEVAUX, 2002) E RHINELLA GRANULOSA (SPIX, 1824) (ANURA: BUFONIDAE)	
Nome da Instituição : universidade federal rural de PE	CNPJ: 24.416.174/0001-06

Cronograma de atividades

#	Descrição da atividade	Inicio (mês/ano)	Fim (mês/ano)
1	Identificação dos espécimes	06/2013	06/2014
2	Extração, análise e identificação química da secreção glandular	06/2013	09/2014
3	Extração e preparo dos extratos da pele	10/2013	11/2014
4	Trabalho de Campo - Coleta dos espécimes	02/2014	10/2014
5	Testes de citotoxicidade	06/2014	10/2015
6	Testes de atividade bactericida	12/2014	03/2015

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Outras ressalvas

1	1)Incluida coleta de R.granulosa,pela oportunidade de aplicação do método quimiotaxonômico,para verificar se há a mesma eficiência,por essa ser uma espécie filogeticamente próxima a R.jimi;2)Estudo contempla o Acesso ao Componente do Patrimônio Genético(PG),de cunho científico,não se caracterizando,À PRINCÍPIO,em ACESSO AO PG nos termos da MP nº2186-16/2001,como preconizam as Resoluções CGEN 21/2006 e,28/2007;3)Na evolução do estudo,em se avançando para os campos da BIOPROSPECÇÃO ou DESENVOLVIMENTO TECNOLÓGICO ou seja,finalidade comercial ou industrial,há que se atentar para a necessidade de obtenção de AUTORIZAÇÃO DE ACESSO junto ao CGEN,conforme dispõe legislação em vigor;4)Estudo remete ao Doutorado da proponente,iniciado em 2013,em Etnobiologia e Conservação da Natureza.

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Página 1/4

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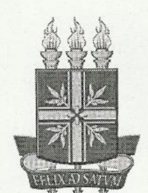

D E C L A R A Ç Ã O

Declaro para os devidos fins que o projeto intitulado "ANÁLISE DAS ATIVIDADES BIOLÓGICAS DA SECREÇÃO GLANDULAR DE RHINELLA JIMI (STEVAUX, 2002) E RHINELLA GRANULOSA (SPIX, 1824) (ANURA: BUFONIDAE)" Processo Nº 130/2013 foi **APROVADO** pela Comissão de experimentação e Uso de Animais – CEUA/URCA. Protocolo.

Roseli Barbosa
Presidente do CEUA/URCA

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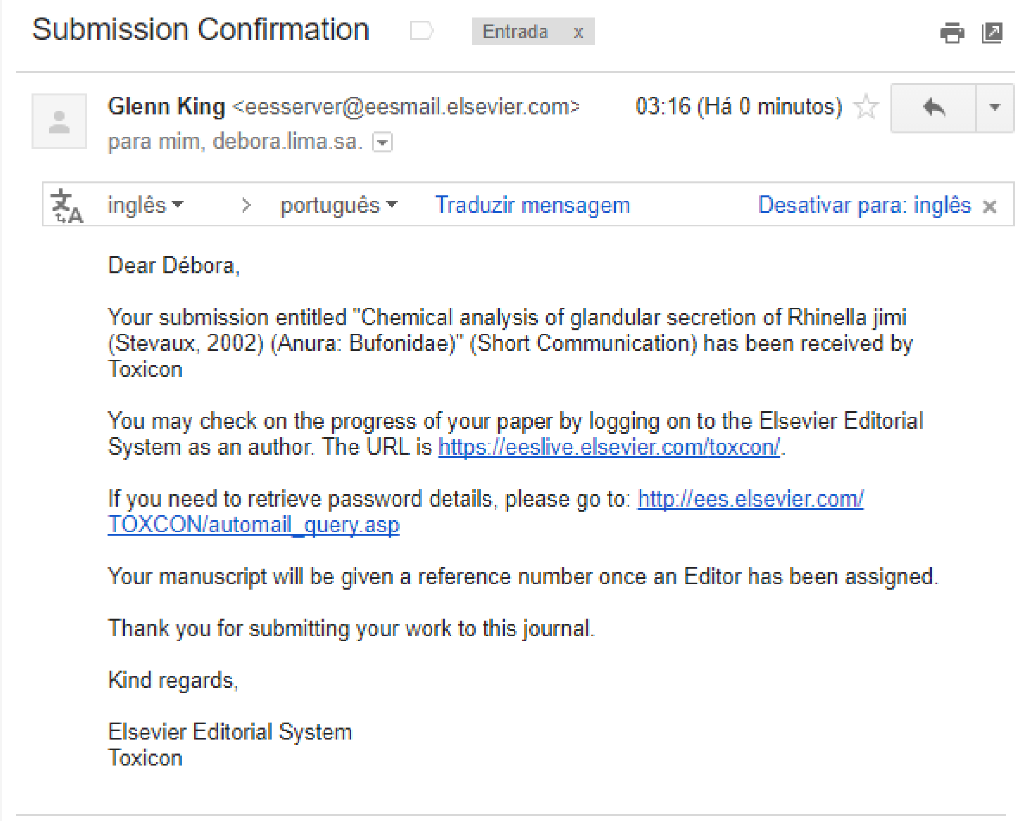
III – Comprovante de submissão do Artigo 1



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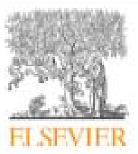

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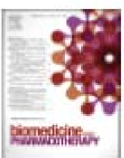

Original article

Antibacterial, modulatory activity of antibiotics and toxicity from *Rhinella jimi* (Stevaux, 2002) (Anura: Bufonidae) glandular secretions

Débora Lima Sales[a,*], Maria Flaviana Bezerra Morais-Braga[b], Antonia Thassya Lucas dos Santos[b], Antonio Judson Targino Machado[b], João Antonio de Araujo Filho[c], Diógenes de Queiroz Dias[a], Francisco Assis Bezerra da Cunha[b], Rogério de Aquino Saraiva[a], Irwin Rose Alencar de Menezes[b], Henrique Douglas Melo Coutinho[b], José Galberto Martins Costa[b], Felipe Silva Ferreira[d], Rômulo Romeu da Nóbrega Alves[e], Waltécio de Oliveira Almeida[b]

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ARTICLE INFO

Article history:
Received 25 December 2016
Received in revised form 19 May 2017
Accepted 22 May 2017

Keywords:
Poisons
Parotoid gland
Antimicrobial activity
Bacterial resistance
Modulation of antibiotics
Toxicity
Cytotoxicity

ABSTRACT

The increase in microorganisms with resistance to medications has caused a strong preoccupation within the medical and scientific community. Animal toxins studies, such as parotoid glandular secretions from amphibians, possesses a great potential in the development of drugs, such as antimicrobials, as these possess bioactive compounds. It was evaluated *Rhinella jimi* (Stevaux, 2002) glandular secretions against standard and multi-resistant bacterial strains; the effect of secretions combined with drugs; and determined the toxicity using two biologic *in vivo* models, and a *in vitro* model with mice livers. Standard strains were used for the determination of the Minimum Inhibitory Concentration (MIC), while for the modulatory activity of antibiotics, the clinical isolates *Escherichia coli* 06, *Pseudomonas aeruginosa* 03 and *Staphylococcus aureus* 10 were used. Modulatory activity was evaluated by the broth microdilution method with aminoglycosides and β-lactams as target antibiotics. The secretions in association with the antibiotics have a significant reduction in MIC, both the aminoglycosides and β-lactams. The toxicity and cytotoxicity results were lower than the values used in the modulation. *R. jimi* glandular secretions demonstrated clinically relevant results regarding the modulation of the tested antimicrobials.
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Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: http://www.tandfonline.com/loi/iphb20

Antimicrobial activity and chemical composition of fixed oil extracted from the body fat of the snake Spilotes pullatus

O. P. Oliveira, D. L. Sales, D. Q. Dias, M. E. S. Cabral, J. A. Araújo Filho, D. A. Teles, J. G. G. Sousa, S. C. Ribeiro, F. R. D. Freitas, H. D. M. Coutinho, M. R. Kerntopf, J. G. M. da Costa, R. R. N. Alves & W. O. Almeida

To cite this article: O. P. Oliveira, D. L. Sales, D. Q. Dias, M. E. S. Cabral, J. A. Araújo Filho, D. A. Teles, J. G. G. Sousa, S. C. Ribeiro, F. R. D. Freitas, H. D. M. Coutinho, M. R. Kerntopf, J. G. M. da Costa, R. R. N. Alves & W. O. Almeida (2014) Antimicrobial activity and chemical composition of fixed oil extracted from the body fat of the snake Spilotes pullatus, Pharmaceutical Biology, 52:6, 740-744, DOI: 10.3109/13880209.2013.868495

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ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, 2014; 52(6): 740–744
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ORIGINAL ARTICLE

Antimicrobial activity and chemical composition of fixed oil extracted from the body fat of the snake *Spilotes pullatus*

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Abstract

Context: Ethnozoological studies have shown that *Spilotes pullatus* Linn. (Colubridae: Ophidia), is associated with medicinal and magic-religious uses in Brazil.
Objectives: This study was designed to determine the chemical composition of the oil extracted from the body fat of *S. pullatus* and to test its antimicrobial properties, alone and in association with aminoglycosides, against fungi and bacterial strains in concentrations ranging between 1024 and 0.5 μg/mL.
Material and methods: The snakes were collected in the Chapada do Araripe, county of Crato, Ceará State, Brazil. The oil was extracted in a Soxhlet apparatus using hexane. The methyl esters of the fatty acids present in the samples were identified using GC-MS. The antimicrobial and drug modulatory activities of oil were tested by microdilution against fungal and bacterial strains.
Results: The chemical composition of the fixed oils of *S. pullatus* identified 10 constituents representing 94.97% of the total sample. The percentages of saturated and unsaturated fatty acids were 33.59 and 61.38%, respectively, with the most abundant components being elaidic (37.26%). The oil did not demonstrate any antimicrobial or antifungal activity when tested alone, presenting MIC values ≥ 1024 μg/mL. However, when associated with antibiotics, it demonstrated synergistic effects with gentamicin against all the bacterial lineages assayed, and antagonistic effects with amikacin and neomycin against strains of *Escherichia coli*.
Conclusions: Oil extracted from the body fat of *S. pullatus* did not demonstrate any inhibitory effects on bacterial or fungal activities, but was effective in modulating the effects of certain antibiotics.

Keywords

Aminoglycosides, antibiotic, ethnozoology, modulatory antibiotic activity

History

Received 22 May 2013
Accepted 18 November 2013
Published online 21 February 2014

Introduction

Spilotes pullatus Linn. (Colubridae), popularly known as "cobra caninana" in Brazil, is a diurnal and semi-arboreal snake that preys on small rodents and birds and is widely distributed throughout of South America to Mexico (Vanzolini et al., 1980).

Globally, 123 snake species are used in traditional folk medicine for therapeutic purposes (Alves et al., 2013) to treat asthma, rheumatism, wounds, and thrombosis. Some studies of ethnomedicinal practices have reported the use of *S. pullatus* to alleviate pain caused by the bites of insects and other snakes, although there is no proof yet of its efficiency in these situations (Alves & Pereira Filho, 2007; Alves & Rosa, 2007; Alves et al., 2007a, 2009). In addition to its medicinal uses, Alves et al. (2010, 2012) reported the use of *S. pullatus* in magic-religious circumstances, as well as the persecution of these snakes in situations of direct contact with humans.

The use of animal resources constitutes an important therapeutic alternative for many populations (Alves et al., 2007b), and they have been cited being used against illnesses apparently caused by pathogenic microorganisms (Aguiar et al., 2008; Lima et al., 2006; Salvagnini et al., 2008). Associated with this fact, microbiological studies indicate the natural products as a weapon against the microbial resistance to drugs (Sousa et al., 2010). Due this fact, zootherapeutic products can be effective in treating human illnesses directly, or in association with synthetic antibiotics – to amplify their action spectrum and minimize undesirable side effects (Gibbons, 2004; Gurib-Fakim, 2006; Salvat et al., 2001; Shin & Pyun, 2004; Sousa et al., 2010). With this objective, several studies using the body fat of animals, as demonstrated in the work of Dias et al. (2013), Cabral et al. (2013), Ferreira et al. (2011, 2009), and Falodun et al. (2008).

Due to the huge use of the animal resources and the traditional medicine and due to the aspects involved in

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the conservation of species against the massive exploitation, the bioprospection of natural products starting from the traditional knowledge represent an important activity to isolate and validate the pharmacological properties of the animal resources (Alves & Rosa, 2005; Hunt & Vincent, 2006), for a possible therapeutic usage (Pieroni et al., 2002) and demonstrate the efficacy or risks in the usage of these products (Alves, 2009).

The present work undertook chemical analyses of the oil extracted from the body fat of *S. pullatus*, tested its antimicrobial activity against standard lines of bacteria and fungi, and examined its effects against multiresistant bacterial strains when combined with antibiotics.

Materials and methods

Zoological material

Four specimens of the snake *S. pullatus* were collected on the slopes of the Chapada do Araripe Range (7°07′ to °49′ S and 38°30′ to 40°55′ W), in the municipality of Crato, in the Cariri region of southern Ceará State, Brazil, between the months of August and October, 2011. The region has a semiarid climate, with average temperatures varying from 24 to 26 °C (IPECE, 2012). *S. pullatus* is distributed on all studied region, occurring in the biomes called ''caatinga'', ''cerrado'', and wet-forest (Ribeiro et al., 2012).

The capture of specimens of *S. pullatus* was authorized by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) by the System of Authorization and Information about Biodiversity (SISBIO), with number 29838-1 (19 August 2011). The project was submitted and approved by the Committee of Ethics in Animal Research of Universidade Regional do Cariri (URCA), with number 16/2012.

Collections were made by actively searching microenvironments favorable to the occurrence of these reptiles (active capture in trees and bushes). The collected specimens were sacrificed using lidocaine and the fat located in the ventral region of their bodies was removed. The animal specimens were then fixed using formol 10% and conserved in 70% alcohol, and subsequently deposited in the Herpetological Collection of the Zoology Laboratory of the Regional University at Cariri (record number URCA-2096).

Extraction of the fixed oils of *S. pullatus*

Oil extraction was performed for 4 h using hexane as a solvent in a Soxhlet apparatus, using 119.56 g of body fat. The hexane was removed in a rotary evaporator and the material subsequently held in a water bath (60 °C) for 2 d. The final recovery was 27.39%, and this material was maintained in a freezer until used.

Fatty acid determinations

The fatty acid content of the fixed oil of *S. pullatus* (FOSP) was determined indirectly using their corresponding methyl esters. The oil was saponified for 30 min by refluxing with a solution of potassium hydroxide in methanol, following the methodology described by Hartman and Lago (1973). After this treatment, the pH was adjusted, and the free fatty acids were methylated with methanol through acid catalysis to obtain the respective methyl esters.

Analyses of the fixed oils of *S. pullatus* using gas chromatography coupled to a mass spectrometer (GC/MS)

Analyses of the volatile constituents of the FOSP were undertaken using a gas chromatograph coupled to a mass spectrometer (GC/MS) (Hewlett-Packard, model 5971, Los Angeles, CA) using a non-polar DB-1 capillary column of fused silica (30 m $\times$ 0.25 mm internal diameter); the carrier gas was helium with a flux velocity of 0.8 mL/min. The injector and detector temperatures were 250 °C and 200 °C, respectively. The column temperature was programmed from 35 °C to 180 °C at 4 °C/min, and followed by 180–250 °C at 10 °C/min. The mass spectra were recorded from 30 to 450 *m/z*. The individual components were identified by comparing their corresponding mass spectra (70 eV) to an accumulated database for the spectrometer (Wiley, 229, San Diego, CA) and with two other databases using retention indices as the pre-selection criteria (Alencar et al., 1984, 1990), as well as by visual comparisons of the fragmentation patterns with those reported in the literature (Adams, 2001; Stenhagen, 1974).

Bacterial material

Minimum inhibitory concentration trials used standard lines of *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 15442, and *Klebsiella pneumoniae* ATCC 4362. Modulation trials used multiresistant clinical isolates of *E. coli* (EC27), *S. aureus* (SA358), and *P. aeruginosa* (P03), whose origins and profile resistances are indicated in Table 1. All the strains were maintained in Heart Infusion Agar (HIA, Difco, St. Louis, MO); before the trials, they were transferred to Brain Heart Infusion (BHI, Difco) for 24 h at 37 °C (Coutinho et al., 2005; Freitas et al., 1999).

Fungal material

Antifungal activity was evaluated using the fungal strains *Candida tropicalis* (6526), *Candida albicans* (40046), and *Candida krusei* (6258), which were maintained in HIA and kept at 4 °C. Before the trials, the cells were cultivated for growth in BHI for 24 h at 37 °C.

Drugs

The antifungal agents, nystatin, amphotericin B, mebendazole, and benzoilmetronidazol, were used at concentrations of 1024 μg/mL. The antibiotics amikacin, gentamicin, and neomycin were used at concentrations of 5000 μg/mL. All the antimicrobial agents were prepared according to the manufacturer's instructions.

Testing for antibacterial and antifungal activities

Testing was undertaken using a solution with 100 mg/mL of the extracted oil solubilized in 1 mL of dimethyl sulfoxide (DMSO – Merck, Darmstadt, Germany), which was then subsequently diluted with distilled water to 1024 μg/mL.

Table 1. Origins of the bacterial strains and their antibiotic resistance profiles.

Bacteria	Origin	Resistance profiles
Escherichia coli 27	Surgical wound	Ast, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Imi, Can, Szt, Tet, Tob
Staphylococcus aureus 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
Pseudomonas aeruginosa 03	Uroculture	Cpm, Ctz, Imi, Cip, Ptz, Lev, Mer, Ami

Ast, aztreonam; Ami, amicillin; Amox, amoxicillin, Ca, cefadroxil; Cfc, cefaclor; Cf, cefalotin; Caz, ceftazidime; Cip, ciprofloxacin; Clo, chloramphenicol; Imi, imipenem; Can, kanamycin; Szt, sulfatrim; Tet, tetracycline; Tob, tobramycin; Oxa, oxacillin; Gen, gentamicin; Neo, neomycin; Para, paromomycin; But, butirosin; Sis, sisomicin; Net, netilmicin; Cpm, cfepime; Ctz, ceftazidime; Ptz, piperacillin-tazobactam; Lev, levofloxacin; Mer, meropene.

BHI suspensions (100 μL) of each of the microbial lineages were placed in each well of a microdilution plate (96-well plates), followed by 100 μL of the oil solution in serial dilutions – with the final oil concentrations varying from 8 to 512 μg/mL. The negative control wells received only DMSO. The microdilution plates were then incubated for 24 h at 37 °C (Javadpour et al., 1996). To determine the MIC of the bacterial strains, resazurin indicator solution (20 μg) was added to each well. Color changes from blue to pink indicated bacterial growth. Fungal growth was determined by observing the formation of turbidity in the wells (Palomino et al., 2002).

Drug susceptibility testing

Suspensions (100 μL) of the microbial lineages were placed in each well of a microdilution plate, followed by 100 μL of serially diluted drugs corresponding to each type of microorganism. The concentrations of the antifungal agents varied from 0.5 to 1024 μg/mL, and antibiotic concentrations varied from 1.22 to 2500 μg/mL. The solutions of fixed oil were subsequently added at the MIC concentrations determined for each microorganism. The plates were then incubated for 24 h at 37 °C (Javadpour et al., 1996). The results were evaluated using the same techniques applied for the MIC.

Results

The analyses of the chemical composition of the fixed oil of *S. pullatus* (FOSP) by GC/MS allowed the identification of the methyl esters of 10 fatty acids that constituted 94.97% of the sample (Table 2). Unsaturated fatty acids represented 61.38% of the 10 principal methyl esters identified, with elaidic acid (37.26%) being the most prevalent, followed by linoleic acid (17.28%). Saturated fatty acids represented 33.59% of the principal methyl esters identified, with palmitic (19.01%) and stearic (10.58%) being the most prevalent.

FOSP did not inhibit fungal or bacterial growth in any of the strains tested, indicating that this product alone was not efficient in controlling microorganisms. Likewise, FOSP in association with antimicrobial agents did not show any inhibitory effect against multiresistant bacterial strains, or inhibitory effects against fungi in association with antifungal agents (using a MIC $\geq$ 1024 μg/mL for all of the microbial strains tested) (Table 3).

FOSP did demonstrate synergistic effects in association with gentamicin against all the microbial lines tested. No clinically significant interaction with *S. aureus* (SA 358) or *P. aeruginosa* (PA 03) was seen when FOSP was combined with the antibiotics amikacin and neomycin. Antagonistic effects were observed, however, with these aminoglycosides against *E. coli* (EC 27) (Table 4).

Table 2. Methyl esters of the fatty acids identified in the fixed oil extracted from the body fat of *Spilotes pullatus* by gas chromatography coupled to a mass spectrometer.

Fatty acid equivalent	RT (min)	(%)	Constituents
Saturated			
Myristic acid	19.48	1.28	Methyl myristate
Palmitic acid	22.97	19.01[a]	Methyl palmitate
Stearic acid	25.37	10.58[a]	Methyl stearate
Nonadecanoic acid	27.07	2.72	Methyl nonadecanoate
Unsaturated			
Palmitoleic acid	22.64	4.02	Methyl palmitoleate
Linoleic acid	25.09	17.28[a]	Methyl linoleate
Elaidic acid	25.12	37.26[a]	Methyl elaidate
Arachidonic acid	26.68	1.48	Methyl arachidonate
Eicosanoic acid	26.85	0.88	Methyl eicosa-7,10,13-trienoate
Docosahexaenoic acid	28.40	0.46	Methyl docosahexaenoate
Total saturated		33.59	
Total unsaturated		61.38	
General total		94.97	

[a]Principal fatty acids.

Table 3. Results of the trials using FOSP as a modulator of the actions of micro-diluted antifungal agents and controls (without FOSP); all results in μg/mL.

	C.T. 13803		C.A. 40046		C.K. 6258	
Antifungal agents	FOSP	Control	FOSP	Control	FOSP	Control
Benzoilmetronidazol	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
Anfotericina B	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
Mebendazol	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024
Nistatina	≥1024	≥1024	≥1024	≥1024	≥1024	≥1024

CT, *Candida tropicalis*; CK, *Candida krusei*; CA, *Candida albicans*.

Table 4. CIM values (μg/mL) of aminoglycosides in the absence and presence of the fixed oil from *S. pullatus*, against *Staphylococcus aureus* 358 (SA 358), *Escherichia coli* 27 (EC 27), and *Pseudomonas aeruginosa* 03 (PA 03).

Antibiotics	SA 358		EC 27		PA 03	
	Control	FOSP	Control	FOSP	Control	FOSP
Amikacin	39.06	39.06	9.76	78.125[a]	156.25	78.125
Gentamicin	39.06	2.44[a]	39.06	9.76[a]	39.06	9.76[a]
Neomycin	9.76	19.53	39.06	156.25[a]	625	312.5

[a]Significant results.

Discussion

Fatty acids can inhibit microbial activity (Agoramoorthy et al., 2007; Nobre et al., 2002). According to Zheng et al. (2005), unsaturated fatty acids are especially effective in this type of microbial control as they impact endogenous bacterial fatty acid synthesis. From this perspective, higher unsaturated fat contents may indicate greater therapeutic efficiencies.

The presence of myristic, palmitic, stearic, palmitoleic, and linoleic acids in the body fat of the reptiles *Crotalus atrox*, *Elaphe obsoleta*, *Python regius*, *Boa constrictor*, *Bitis gabonica*, and *Varanus exanthematicus* has been reported in other studies (McCue, 2008). According to Agoramoorthy et al. (2007), palmitic, linoleic, stearic, and myristic acids are known for their antimicrobial activities, and even though FOSP did not show any antimicrobial activity when used alone, the positive results seen when used in combination with aminoglycosides may be related to the presence of these fatty acids.

Although FOSP did not demonstrate any inhibitory effects on bacterial or fungal activities, the results of the interactions of this oil with antimicrobial compounds indicated that it negatively influenced treatments to control *E. coli* using the antibiotics amikacin and neomycin. In contrast, FOSP did appear to be clinically effective when associated with gentamicin, positively boosting its activity.

The inefficiency of FOSP in controlling microbial activity when used alone corroborates the results of Ferreira et al. (2009), who undertook a microbiological study of the oil extracted from the body fat of *Tupinambis merianae* – with no modulations of the activities of antibiotics being observed in the presence of that oil. Research with *Phrynops geoffroanus* perfomed by Dias et al. (2013) indicates that the oil did not demonstrate clinical efficacy against the bacterial strains alone; however, a MIC = 128 μg/mL was observed against *C. krusei*. When the oil was associated with antibiotics, the oil from *P. geoffroanus* presented a similar behavior with the oil of *S. pullatus*, demonstrating a synergism against *P. aeruginosa* when associated with gentamin. Falodun et al. (2008) tested the fixed oil of *B. constrictor* in similar manners but did not report any antimicrobial activity. Ferreira et al. (2011) complemented this latter study and did report the modulation of antibiotic activity by the oils extracted from *B. constrictor*, but only in terms of the action of gentamicin against *E. coli*. This study demonstrated a higher proportion of unsaturated fatty acids, as observed in the work of Ferreira et al. (2011).

While the oil extracted from the body fat of *S. pullatus* was effective in modulating the effects of certain antibiotics, natural products with few studies or with unsafely manipulating practices, can be important agents of diseases, causing serious health problems (Alves & Rosa, 2005). So, more studies are necessary to evaluate this and other natural products from animals before their usage as possible new drugs or pharmaceutical formulations.

Acknowledgements

The authors would like to thank the ICMBio for the collecting permit; ao com; the Universidade Federal da Paraíba (UFPB) for donating the microbial strains; and Prof. Dr. Robson Waldemar Ávila for identification and catalogation of this zoological material.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article. The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the research productivity grant awarded to Dr. Waltécio de Oliveira Almeida; the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the study grants awarded to Mario E. S. Cabral and Diógenes Q. Dias; the Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico (FUNCAP) for the study grants awarded to Olga P. Oliveira and Débora L. Sales.

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Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: http://www.tandfonline.com/loi/iphb20

Anti-inflammatory potential of zootherapeutics derived from animals used in Brazilian traditional medicine

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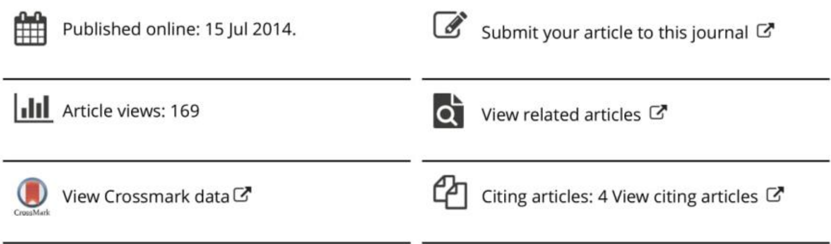
To cite this article: Felipe S. Ferreira, Samuel V. Brito, Débora L. Sales, Irwin R. A. Menezes, Henrique D. M. Coutinho, Emmanuel P. Souza, Waltécio O. Almeida & Rômulo R. N. Alves (2014) Anti-inflammatory potential of zootherapeutics derived from animals used in Brazilian traditional medicine, Pharmaceutical Biology, 52:11, 1403-1410, DOI: 10.3109/13880209.2014.894091

To link to this article: https://doi.org/10.3109/13880209.2014.894091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Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, 2014; 52(11): 1403–1410
© 2014 Informa Healthcare USA, Inc. DOI: 10.3109/13880209.2014.894091

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ORIGINAL ARTICLE

Anti-inflammatory potential of zootherapeutics derived from animals used in Brazilian traditional medicine

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Abstract

Context: Animals are used for the treatment of diseases caused by inflammatory processes, although few studies evaluate their potential for these purposes.
Objectives: To evaluate the anti-inflammatory potential of zootherapeutic products derived from vertebrates used in Brazilian traditional medicine.
Material and methods: The species analyzed were *Tupinambis merianae*, *Iguana iguana*, *Crotalus durissus*, *Boa constrictor*, and *Euphractus sexcinctus*. The methods used in anti-inflammatory assays were ear edema (topical) and paw (systemic).
Results: With regard to topical anti-inflammatory activity, the fat from *T. merianae*, *C. durissus*, *I. iguana*, *B. constrictor*, and *E. sexcinctus* reduced inflammation, while for systemic anti-inflammatory activity, only the fat and the skin of *C. durissus*, the skin of *I. iguana* and the fat from *B. constrictor* reduced inflammation.
Conclusions: Studies should be conducted to evaluate the mechanisms of action for each product that demonstrated anti-inflammatory activity as well as against other inflammatory processes.

Keywords

Anti-inflammatory activity, traditional medicine, zootherapy

History

Received 11 December 2013
Revised 27 January 2014
Accepted 7 February 2014
Published online 15 July 2014

Introduction

Since the beginning, humanity has depended or co-depended on natural resources (Alves & Souto, 2011), experimenting with and benefiting from plants or animals for the production of food, shelter, clothes, methods of transport, fertilizers, aromas, fragrances, and medicines (Cragg & Newman, 2001). The identification and evaluation of biological material found in nature, for the attainment of new products, characterizes bioprospection (Artuso, 2002). The pharmaceutical industry studies compounds derived from numerous species of micro-organisms, plants, and animals.

Strategies to explore the pharmaceutical potential through bioprospection are diverse (Albuquerque & Hanazaki, 2006; Albuquerque et al., 2012). Taking advantage of traditional knowledge is important for researches whose objective is to develop new drugs (Albuquerque et al., 2012). It is estimated that much of the world population are aware of medicinal properties of flora and fauna and use this knowledge for the treatment of diseases (Robinson & Zhang, 2011; WHO, 2002).

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Through this knowledge, several organisms normally used for therapeutic purposes have been tested for the production of drugs (Raskin et al., 2002). Nevertheless, bioprospection studies have given more attention to knowledge associated to medicinal plants (Albuquerque et al., 2012), despite the fact that animals are also widely explored as raw material in various traditional medical systems (Alves et al., 2013; Ferreira et al., 2013). Human societies have accumulated practices and knowledge for the use of animals to treat various diseases and/or symptoms. Like medicinal plants, these resources represent an interesting area of study for bioprospection. Some authors point out the pharmacological potential of medicinal animals (Ferreira et al., 2010a,b). However, bioactive compounds derived from animals have been less studied relative to medicinal plants (Harvey, 2008). Pieroni et al. (2002) reported that chemical constituents and pharmacological actions of medicinal products of animal origin are little known.

In Brazil, Alves et al. (2013) documented at least 354 species that are used for medicinal purposes. Even though a rich faunal diversity is used for medicinal purposes, few zootherapeutic products have been analyzed to verify their pharmacological potential. However, these products are used in traditional communities and sold in public markets in various cities (Ferreira et al., 2013). The most commonly used products are those derived from mammals and reptiles,

and groups that possess a high number of species are used for the treatment of diseases (Ferreira et al., 2013). Examples of species that are broadly used in the region are snakes *Crotalus durissus* (Linnaeus, 1758) and *Boa constrictor* (Linnaeus, 1758); the lizards *Iguana iguana* (Linnaeus, 1758), and *Tupinambis merianae* (Duméril & Bribon, 1839), and the armadillo *Euphractus sexcinctus* (Linnaeus, 1758).

All these species are widely distributed in various regions of Brazil (Feijó & Langguth, 2013; Vanzolini et al., 1980). Of these species, the parts mostly used are the body fat (lard) and skin, products that have been traditionally prescribed for the treatment of osteoporosis, rheumatism, arthritis, osteoarthritis, ulcers, gastritis, and inflammatory conditions (see Ferreira et al., 2013). These diseases involve an inflammatory process, which suggests a potential use as anti-inflammatory agents, making the evaluation of zootherapeutics important for these purposes.

The objective of this study was to evaluate the anti-inflammatory potential of the fat and skin derived from *C. durissus*, *B. constrictor*, *I. iguana*, *T. merianae*, and *E. sexcinctus* through *in vivo* topical models (using oil of croton) and systemic models (application of carragenine).

Materials and methods

Experimental specimens

The zoological materials (fat and skin) used in the experiments were obtained from specimens donated by the Centro de Triagens de Animais Silvestres (Centre of Screening of Wild Animals) (CETAS-IBAMA) in João Pessoa – PB. An individual from each of the species was used.

Extraction of oil from the fat of *C. durissus* (OCD), *I. iguana* (OII), *B. constrictor* (OBC), and *E. sexcinctus* (OES)

For preparation of the oil, 100 g of localized fat was used from the ventral region of the animals. The extraction was done through a Soxhlet apparatus (Sigma-Aldrich, St. Louis, MO) using hexane as a solvent for 4 h. The oil was dried in a water bath set at 70 °C for 2 h and then frozen for later analysis.

Preparation of decoctions from the skin of *T. merianae* (DTM), *C. durissus* (DCD), and *I. iguana* (DII)

Dry skin (200 g) from *T. merianae*, *C. durissus*, and *I. iguana* were used as source materials. The products (skin and thorns) were macerated and boiled in water for 24 h after which they were filtered and frozen and then lyophilized for later analysis.

Determination of species for each trial

Based on the current literature of the use and trade of medicinal animals (Alves & Rosa, 2007a,b; Alves et al., 2007, 2013; Andrade & Costa-Neto, 2006; Costa-Neto, 1999; Costa-Neto & Motta, 2010; Ferreira et al., 2009a,b, 2012, 2013; Freire, 1996; Oliveira et al., 2010; Pinto & Maduro, 2003), a criterion was established to define which products would be used in the ear and paw edema test. The criteria adopted for choosing the species were the following: the number of diseases cited for a given specie and the ethnobiological index (relative importance or the use value). Thus, the species used were the following: (i) most cited species and products for topical inflammations (analyzed from the ear edema), fat from *B. constrictor*, *C. durissus*, *I. iguana*, *T. merianae*, and *E. sexcinctus*; (ii) most cited species and products for inflammations in general (in this case, evaluated from the paw edema), *C. durissus*, *I. iguana*, *T. merianae* (fat and skin), and *B. constrictor* (fat).

Evaluation of anti-inflammatory activity

Animals

Male mice (*Mus musculus*; Swiss), with a body weight of between 25 and 30 g and male rats (*Rattus norvegicus*, Wistar) with a body weight of between 130 and 150 g were used for topical (mice) and systemic inflammation (rats) experiments. Animals were maintained in a polypropylene box in an environment with a controlled temperature and *ad libitum* access to water and specific food. The current study was approved by the Ethical Committee in Research of Universidade Regional do Cariri-URCA (Protocol: no. 00137-2013.1).

Ear edema induced by oil of croton

For the analysis using oil of croton, pure oils from animals were administered (13 mg/ear). In this model, the inflammation was caused using 20 μL of 5% oil of croton (v/v) in acetone inside and outside of the right ears while the left ears received 20 μL acetone (to verify that the vehicle did not interfere with results). Fifteen minutes before the application of the inflammatory agent, the animals were pre-treated with 20 μL of the fat of each animal used and the negative control received 20 μL of saline, while the positive control received dexamethasone (0.08 mg/ear). The edema was evaluated 6 h after the application of the oil of croton (Tubaro et al., 1986). The ear edema evaluation occurred by the measurement of the ear thickness using a digital caliper (Jomarca, Sao Paulo, Brazil).

Ear edema induced by multiple applications of oil of croton

Aiming to evaluate the anti-inflammatory effect of zootherapeutics in an established chronic inflammatory model, we multiple applications of the phlogiston agent. The chronic inflammatory process was induced by the application of 20 μL of oil of croton 5% (v/v) in acetone for 8 d (applied every other day), in mice ($n = 6$/group). The fat and dexamethasone (0.1 mg/ear, positive control) were topically applied for 4 d (twice a day). From the fifth day, evaluations of edema (thickness of the right ear) were performed daily. On ninth day, animals were sacrificed by cervical displacement and samples of ear tissue (6 mm) for analysis were collected from each ear (using a personalized leather metallic perforator) (Stanley et al., 1991). The mass from the fragments of the right ear (m_{re}) and the left ear (m_{le}) was registered and the edema (in mg) of the different groups was calculated according to the formula:

$$\text{Edema} = [(m_{re} - m_{le})/m_{re}] \times 100$$

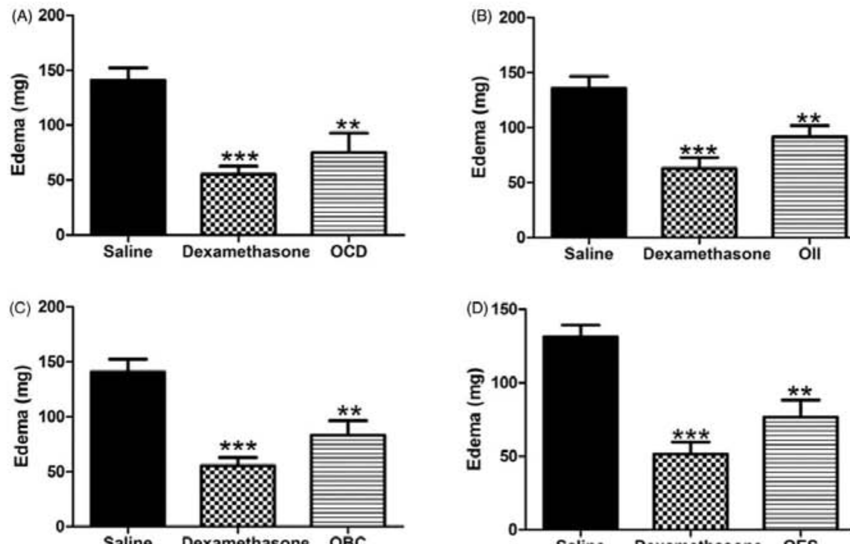

Figure 1. Effect of oil from (A) *Crotalus durissus*, (B) *Iguana iguana*, (C) *Boa constrictor*, and (D) *Euphractus sexcinctus* on single application croton oil-induced mouse ear edema. Dexamethasone was used as a positive control. Statistical analysis: a one-way ANOVA followed by the Student–Newman–Keuls test. $^{**}p<0.01$ and $^{***}p<0.001$ compared with the negative control group.

The calculation of the average inhibitory effect of the inflammation (EIM in %) of each treatment was obtained by the following formula:

$$\mathrm{AIE} = [(a_{\mathrm{cont}} - a_{\mathrm{trat}})/a_{\mathrm{cont}}] \times 100,$$

where $\mathrm{ave}_{\mathrm{cont}}$ represents the average of the edema of the control group while $\mathrm{ave}_{\mathrm{trat}}$ is the average edema of the treated groups (dexamethasone and zootherapeutics).

Statistical significance was analyzed across different groups by a variance analysis (ANOVA) with post hoc Student–Newmann–Keuls ($p<0.05$) using GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA).

Table 1. Edema weight of ear mice sensitized with croton oil single application and the mean inhibitory effect on inflammation after treatment with zootherapeutics, dexamethasone (positive control), and negative control (saline).

Treatment	Edema (mg)	Inhibition (%)
Saline	140.8 ± 11.45	–
Dexamethasone	55.26 ± 7.53***	60.7
OCD	75.05 ± 17.65**	46.6
OII	84.3 ± 8.5**	40.1
OBC	83.1 ± 13**	40.9
OES	76.7 ± 10.6**	45.5

Data expressed as mean ± s.e.m. $^{**}p<0.01$ and $^{***}p<0.001$ compared with the control (ANOVA and the Student–Newman–Keuls test).

Paw edema

Paw edema was induced by injection of 150 µL of carragenine (20% p/v) in sterile saline and administered in the subplantar region of the right paw of the rats (Winter et al., 1962). The oils were administered by intraperitoneal injections at 300 mg/kg (solubilized in tween), while the skin decoction was administered at 5 mg/kg (solubilized in sterile saline). Dexamethasone, used as a positive control, was applied at 10 mg/kg. Zootherapeutics and dexamethasone were applied 1 h before injection of carragenine. Saline injections were used as negative controls for anti-inflammatory potential of the skin decoctions, while Tween was used as the control for evaluating the potential of oils.

Before the application of the carragenine, the right paw was measured to establish the initial size (T_0). To evaluate the edema, 1 h after the application of carragenine, successive measurements were taken (T_1, T_2, T_3, and T_4) hourly. Measurements were performed through the displaced volume using a digital plethysmometer (UgoBasile, Comerio, Italy).

The difference between edemas was confirmed after each measurement (done after the application of carragenine) relative to T_0 (i.e., $\mathrm{AEC}_1 = T_1 - T_0$; $\mathrm{AEC}_2 = T_2 - T_0$; $\mathrm{AEC}_3 = T_3 - T_0$; $\mathrm{AEC}_4 = T_4 - T_0$). Statistical significance was confirmed between groups by an analysis of variance (ANOVA) and a *post hoc* Student–Newmann–Keuls test ($p<0.05$) with GraphPad Prism 5.0 software.

Results

Evaluation of the topical anti-inflammatory activity of fat from zootherapeutics through the ear edema

The fat from *C. durissus*, *I. iguana*, *B. constrictor*, and *E. sexcinctus* demonstrated a significant topical anti-inflammatory activity, reducing the edema caused by the oil of croton (Figure 1 and Table 1). This result appears

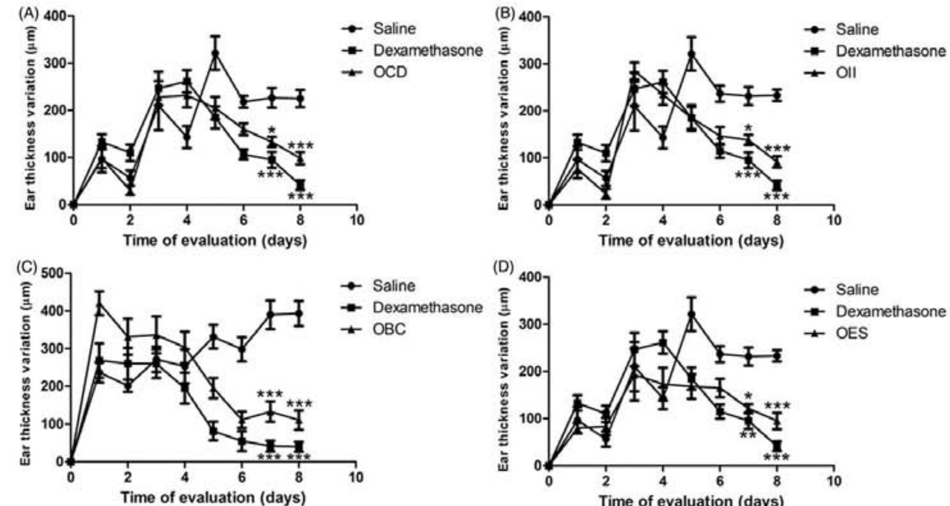

Figure 2. Effect of oil from (A) *Crotalus durissus*, (B) *Iguana iguana*, (C) *Boa constrictor*, and (D) *Euphractus sexcinctus* on croton oil multiple applications-induced mouse ear edema, showing the time–response curve effect during 9 d. Data expressed as mean ± s.e.m. $*p<0.05$, $**p<0.01$, and $***p<0.001$ compared with the negative control group.

Table 2. Edema weight of ear mice sensitized with croton oil multiple applications and the mean inhibitory effect on inflammation after treatment with zootherapeutics, dexamethasone (positive control), and negative control (saline).

Treatment	Edema (mg)	Inhibition (%)
Saline	113.9 ± 7.4	–
Dexamethasone	36.89 ± 7.6***	67.6
OCD	44.3 ± 14.9***	61.1
Saline	144.7 ± 16.4	–
Dexamethasone	36.8 ± 7.6***	74.5
OII	55.1 ± 9.8***	61.8
Saline	150.3 ± 10.4	–
Dexamethasone	29.6 ± 2.7***	80.2
OBC	55.5 ± 13.5***	63
Saline	113.9 ± 7.4	–
Dexamethasone	36.8 ± 7.6***	67.6
OES	64.1 ± 10***	43.6

Data expressed as mean ± s.e.m. $***p<0.001$ compared with the control (ANOVA and the Student–Newman–Keuls test).

to corroborate the traditional use of products from these species, frequently used for the treatment of inflammation.

When the oil of croton was applied, negative controls demonstrated an elevated percentage of edema while the positive control group (dexamethasone) and zootherapeutics demonstrated a significant reduction relative to the negative control (Table 1).

When considering the multiple applications of the oil of croton model, the results suggest a significant topical anti-inflammatory activity, reducing edema from a chronic inflammation (Figure 2 and Table 2). In this model, the negative control showed the highest edema percentage while the groups treated with the positive control (dexamethasone) and zootherapeutics showed low edema relative to the negative control group.

In the two models of topical inflammation used in the current study (simple and multiple applications of oil of croton), a reduction in the percentage of edema was observed when compared with the positive control. We observed that anti-inflammatory activity (%) of the tested products (with the exception of the oil of *E. sexcinctus*) was greater in the chronic inflammatory model (multiple applications) than in the acute model (single application).

Evaluation of the anti-inflammatory activity of zootherapeutics through paw edema caused by carragenine

Against carragenine-induced paw edema, only the fat and skin of *C. durissus*, the skin of *I. iguana*, and the fat from *B. constrictor* displayed an anti-inflammatory activity when compared with the controls. The other products (fat from *I. iguana* and *T. merianae*) did not show significant differences relative to controls.

Dexamethasone treatment presented differences in the volume of edema when compared with the negative control group (Tween and saline in the tests with fat and skin decoctions, respectively, see Tables 3 and 4 and Figures 3 and 4). For experiments with the fat, treatment with dexamethasone presented differences at times 2, 3, and 4 (Figure 3). In the trials with the decoctions, the positive control also showed differences at times 2, 3 and 4 (Figure 4).

Fat and skin from *C. durissus*, fat from *B. constrictor*, and the skin from *I. iguana* showed significant differences relative to the control group (Tables 3 and 4 and Figures 3 and 4). Fat from *C. durissus* showed significant differences only at

time 4. In trials with the skin of *C. durissus*, there were significant differences in the volume of edema at times 3 and 4. From the fat of *B. constrictor*, there were significant differences in the volume of edema when compared with the control only at time 4. Experiments utilizing the skin of *I. iguana* showed significant differences only at time 4.

Table 3. Edema of paw rats sensitized with carragenine application and the mean inhibitory effect on inflammation after treatment with zootherapeutics (decocts of skins), dexamethasone (positive control), and negative control (saline).

Treatment	Edema (ml)	Inhibition (%)
Saline	0.538 ± 0.04	–
Dexamethasone	0.15 ± 0.05***	72.1
DII	0.352 ± 0.04*	34.5
DCD	0.448 ± 0.11*	16.7
DTM	0.468 ± 0.11	–

Data expressed as mean ± s.e.m. $*p < 0.05$ and $***p < 0.001$ compared with the control (ANOVA and the Student–Newman–Keuls test).

Table 4. Edema of paw rats sensitized with carragenine application and the mean inhibitory effect on inflammation after treatment with zootherapeutics (oils), dexamethasone (positive control), and negative control (Tween).

Treatment	Edema (ml)	Inhibition (%)
Tween	1.188 ± 0.176	
Dexamethasone	0.262 ± 0.09***	77.9
OTM	1.066 ± 0.12	–
OII	0.980 ± 0.07	–
OCD	0.716 ± 0.09**	39.7
OBC	0.766 ± 0.03*	35.5

Data expressed as mean ± s.e.m. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the control (ANOVA and the Student–Newman–Keuls test).

Discussion

Animals have been systematically used for the treatment of numerous diseases; however, little is known about their pharmacological potential (Ferreira et al., 2010a). The results from the oil of croton-induced ear edema experiments showed that the traditional use of fat from *C. durissus*, *I. iguana*, *B. constrictor*, and *E. sexcinctus* against skin inflammations may have a pharmacological base. In the traditional medicine, the fat is used in a topical form (similar to an ointment) on skin inflammations or orally administered (Ferreira et al., 2013).

The oil of croton was used as a phlogistic agent to evaluate the topical anti-inflammatory potential of zootherapeutics. The oil of croton induces local inflammation by increasing vascular permeability and vasodilation. This results in the migration of leukocytes, polymorphonuclear cells (especially neutrophils), release of histamine and serotonin, and synthesis of eicosanoids (Badilla et al., 2007). The oil of croton exerts its effect by the activation of the protein kinase C (PKC), which in turn activates down-stream signaling including mitogen-activated protein kinase (MAPK) and phospholipase A2 (PLA_2). This in turn induces the release of platelet aggregation factor (PAF) and arachidonic acid (AA), which consequently trigger the production of inflammatory eicosanoids via cyclo-oxygenase (COX) or lipoxygenase (LOX) (Wang et al., 2000).

Calder (2005) reported that administration of fatty acids can result in the partial substitution of arachidonic acid in the cell membranes, i.e., the eicosapentaenoic and docosahexaenoic acids. This could lead to a decrease in the production of arachidonic acid and its derivatives, consequently attenuating inflammation. Further, Das (2008) asserts that fatty acids (like those described in this study) can directly mediate inflammatory processes by antagonistic activities

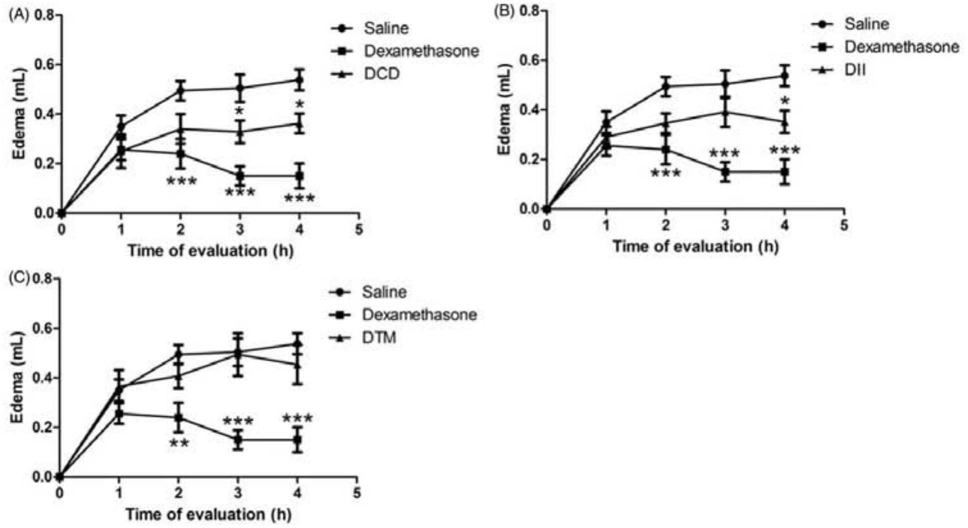

Figure 3. Effect of decoction of skin of (A) *Crotalus durissus*, (B) *Iguana iguana*, and (C) *Tupinambis merianae* on carregenine application-induced paw edema. Data expressed as mean ± s.e.m. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ compared with negative control group.

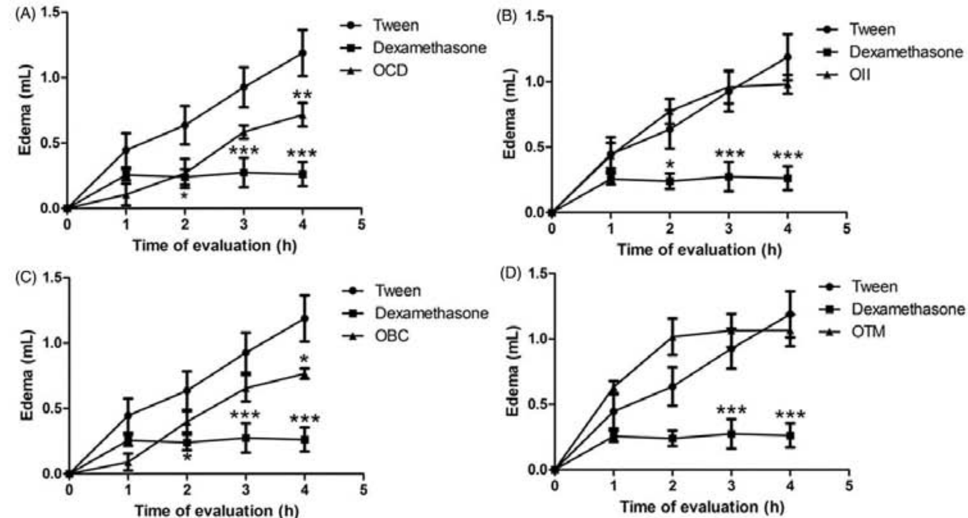

Figure 4. Effect of oils of (A) *Crotalus durissus*, (B) *Iguana iguana*, (C) *Boa constrictor*, and (D) *Tupinambis merianae* on carregenine application-induced paw edema. Data expressed as mean ± s.e.m. $*p < 0.05$ and $**p < 0.01$ and $***p < 0.001$ compared with the negative control group.

on arachidonic acid and its metabolites, conferring a topical anti-inflammatory action.

Studies that evaluate the topical anti-inflammatory potential of products derived from animals using oil of croton induced ear edema are scarce. Ferreira et al. (2010b) evaluated the anti-inflammatory potential of body fat of *T. merianae*, which reduced topical inflammation caused by the oil of croton (39%). Buthelezi et al. (2012) reported that fat from *Crocodylus niloticus* reduces topical inflammation. However, these authors did not state the concentration of the extract or its percentage of inhibition. Yoganathan et al. (2003) evaluated the topical anti-inflammatory activity from the oil of the *Dromaius novaehollandiae* bird through ear edema, which reduced inflammation by 70% relative to controls. Falodun et al. (2008) evaluated the topical anti-inflammatory potential of oil from *B. constrictor* at the different concentrations (3, 6, and 12 mg/ear) and produced a dose-dependent decrease in inflammation (3 mg/ear = 36%; 6 mg/ear = 42%; 12 mg/ear = 65%). The species tested in the current study displayed a similar anti-inflammatory effect (see Tables 1 and 2) to the species tested by Yoganathan et al. (2003) and Falodun et al. (2008).

With regard to experiments utilizing the fat of *B. constrictor* (present study and Falodun et al., 2008), variability between studies could be accounted for by different extraction techniques. All extracts used in the present study were obtained from the species recently sacrificed and properly identified. Conversely, the material used by Falodun et al. (2008) was obtained from public markets in the city of Benin, Nigeria. There is the possibility that the material used was not from the specie (*B. constrictor*) claimed by the sellers. Alves and Rosa (2010) report the misrepresentation of products by sellers in the city of Belém, North of Brazil, in which products of domestic species are sold as wild species that are difficult to obtain (such as manatee and turtles). In addition, the preparation of extracts used by Falodun et al. (2008) to access the fatty acids differed from our methods and probably because of differences caused in the results.

Amongst the zootherapeutics whose systemic anti-inflammatory potential was evaluated, the fat and the skin from *Crotalus dissurus*, the skin of *I. iguana*, and the fat of *B. constrictor* reduced the inflammation caused by carragenine. Paw edema caused by carragenine enables the evaluation of new drugs against acute anti-inflammatory processes (Salvemini et al., 1996). Carragenine is a substance that leads to increased paw volume, vasodilation, and permeability. One hour after application, we observed an increase in paw volume. The mode of action includes release of inflammatory mediators such as vasoactive amines (histamine and serotonin), kinins, and metabolites derived from arachidonic acid (prostaglandins and leukotrienes) (Winter et al., 1962).

Carragenine-induced inflammation is biphasic (Maruyama et al., 2010). Initially edema is characterized by the production of vasoactive amines and increased expression of nitric oxide (via nitric oxide synthase, NOS). Second, nitric oxide is produced by inducible NOS and increased the expression of cyclo-oxygenase (especially COX-2) and prostaglandins (PGE_2) (Rosa & Sorrentino 1971; Salvemini et al., 1996).

Dexamethasone is a well-known anti-inflammatory drug used in the models of inflammation caused by carragenine. Dexamethasone reduces edema in the first hours of evaluation (times 1 and 2) by inhibiting the first phase of inflammation (Wise et al., 2008). Edema treated with fat and skin of *C. durissus*, fat of *B. constrictor*, and the skin of *I. iguana* showed differences relative to the negative control group at time 3 or 4. These natural products appear to act only in the

second phase of inflammation caused by carragenine, inhibiting the expression of cyclo-oxygenases and prostaglandins, consequently attenuating the effects of the inflammation.

Studies that aim to evaluate the anti-inflammatory potential of zootherapeutics via systemic application in Brazil (via the carragenine model) are, until now, non-existent. However, animal studies in other countries illustrate the systemic anti-inflammatory potential of these products. Omar et al. (2012) also evaluated the systemic anti-inflammatory potential of the extracts of oligochaetes *Pheretima hawayana* and *Allolobophora caliginosa*, reducing inflammation caused by histamine. However, the authors did not clarify the percentage inhibition of the extracts. Balamurugan et al. (2007) described an anti-inflammatory activity of extracts of oligochaete *Lampito mauritii* used in the traditional Indian medicine (using a carragenine paw edema model). All the concentrations tested (20, 40, 80, 160, and 320 mg/kg) displayed inhibitory activity ranging from 46% to 64%. Compared with the present study, only the fat (OCD and OBC) displayed higher percentages of inhibition.

In general, biodiversity represents a vast resource with medicinal potential. The search for novel medicinal compounds has driven an intensification research in this area. With little available data about the potential of zootherapeutics, information obtained in the current study contributes to the literature for bioprospection for medical and traditional knowledge of animals. Overall, even with limited data about the pharmaceutical potential of zootherapeutics. Our results suggest that there could be a basis for medicinal use (especially in the experiments to evaluate anti-inflammatory potential) for some species. Nevertheless, more pharmacological studies are needed to evaluate specific models of diseases for the verification of such medicines derived from animals. This is evident when considering the data obtained in the current study and data available in the literature. Results of various studies, i.e., Balamurugan et al., 2009; But et al., 1990, 1991; Falodun et al., 2008; Liu et al., 2008, 2010, 2011; Omar et al., 2012; Wei et al., 2009, report that it is difficult to accept the full effectiveness of products from animal origin as medicines, by taking into consideration three aspects: (i) in the majority of cases, the results of zootherapeutics are not better than standard drugs; (ii) positive controls are not used to compare and confirm the effectiveness of the results; or (iii) zootherapeutics simply do not demonstrate positive results.

Conclusion

Human dependency on biodiversity for procuring medicine is historic. Products derived from the Brazilian fauna are widely used in traditional medicine. However, studies that seek to test the pharmacological effectiveness of these products are rare. Little use has been made of traditional knowledge of zootherapeutics as a source of information for bioprospection despite evidence of their pharmacological potential.

In the current study, we demonstrate that products, derivatives of some species, were effective in reducing inflammation. The current literature regarding the pharmacological potential of animal products provides evidence of a promising source of raw material for the pharmaceutical industry. However, the scarcity of studies restricts solid assumptions of their pharmaceutical potential. Thus, more studies should be conducted to evaluate the mechanisms of each product that demonstrated anti-inflammatory activity as well as against other inflammatory processes.

Acknowledgements

The authors would like to thank the Fundação Cearense de Apoio ao Desenvolvimento Científico and Tecnológico – FUNCAP for the research grant awarded to Felipe S. Ferreira, Samuel V. Brito, Henrique D. M. Coutinho, and Irwin R. A. Menezes; Capes for de Ph.D. scholarship awarded to Débora L. Sales; Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq for the research grant awarded to W. O. Almeida and Rômulo RN Alves.

Declaration of interest

The authors report no conflicts of interest.

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Pharmaceutical Biology

ISSN: 1388-0209 (Print) 1744-5116 (Online) Journal homepage: http://www.tandfonline.com/loi/iphb20

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To cite this article: Débora Lima Sales, Olga Paiva Oliveira, Mário Eduardo Santos Cabral, Diógenes Queiroz Dias, Marta Regina Kerntopf, Henrique Douglas Melo Coutinho, José Galberto Martins Costa, Francisco Roberto Dias Freitas, Felipe Silva Ferreira, Rômulo Romeu Nóbrega Alves & Waltécio Oliveira Almeida (2015) Chemical identification and evaluation of the antimicrobial activity of fixed oil extracted from Rhinella jimi, Pharmaceutical Biology, 53:1, 98-103, DOI: 10.3109/13880209.2014.911331

To link to this article: https://doi.org/10.3109/13880209.2014.911331

Published online: 07 Oct 2014.

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Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, 2015; 53(1): 98–103
© 2015 Informa Healthcare USA, Inc. DOI: 10.3109/13880209.2014.911331

ORIGINAL ARTICLE

Chemical identification and evaluation of the antimicrobial activity of fixed oil extracted from *Rhinella jimi*

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Abstract

Context: The toad *Rhinella jimi* (Stevaux, 2002) (Bufonidae) is used in traditional medicine to treat a number of illnesses (inflammation, infections, and wounds) in humans as well as animals.

Objectives: The present work examined the antimicrobial actions of the extracted oils from the body fat of *R. jimi* (ORJ) against fungi and standard and multi-resistant lines of bacteria, as well as their effects when combined with aminoglycosides.

Materials and methods: The toads were collected in the municipality of Exu in Pernambuco State, Brazil, and their body fat oils extracted in a Soxhlet apparatus using hexane. A gas chromatograph coupled to a mass spectrometer was used to identify the fatty acids, based on their methyl esters. The antimicrobial activities of the oil were analyzed against standard and multi-resistant lines of *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, as well as against fungal lines of *Candida albicans* and *Candida krusei* using the broth micro-dilution method.

Results: The minimum inhibitory concentrations (MIC) of ORJ were 512 μg/mL for *Candida krusei* and ≥1024 μg/mL for the other microorganisms. When associated with amikacin, ORJ demonstrated an increase in its ability to inhibit *E. coli* growth (from 156.25 to 39.06 μg/mL), indicating synergistic interaction. In the same way, when allied with amikacin, gentamicin, and neomycin, the ORJ reduced the MICs meaningly, against *P. aeruginosa*.

Conclusions: These data will enable searches to be made to obtain new products in combination with antibiotics, enhancing the efficacy of these drugs against drug-resistant microorganisms.

Keywords

Conservation, ethnobiology, modulation of aminoglycosides, zootherapy

History

Received 22 November 2013
Revised 9 February 2014
Accepted 29 March 2014
Published online 7 October 2014

Introduction

Many natural products are used in traditional medical systems, and while plants and plant parts represent the majority of products utilized, animals (whole animals, their parts, or derived products) are well-represented (Alves & Rosa, 2005; Scarpa, 1981). Alves and Alves (2011) listed 584 animal species distributed among 13 distinct taxonomic categories being used for therapeutic purposes in Latin America. A total of 354 animal species have been recorded as being used in medicinal practices in Brazil, which demonstrates the importance of the regional fauna to traditional populations (Alves et al., 2013a).

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A large number of animal species are utilized for medicinal purposes throughout the world, including 47 amphibian species, of which six are cited for Brazil (Alves et al., 2013a,b). According to Alves and Albuquerque (2013), these animals gained the attention of researchers because of their pharmaceutical potential and have shown interesting physiological activities that include vasoconstriction, and antitumor cells, anti-inflammatory, cardiotoxic, neurotoxic, antimicrobial, and hallucinogenic activities.

Due to the use of both natural resources and allopathic medicinal substances by many traditional communities, there are now numerous examples of the utilization of synthetic medicines combined with natural products. While there is a growing concern about the increasing resistance of infectious agents to commonly used antimicrobial products, which limits their efficiency against many types of infections (De Backer et al., 2008; Deurenberger et al., 2007; Varaldo, 2002), a number of authors have reported the use of multi-drug treatments (Keith et al., 2005) composed of combinations

between antibiotics, or their association with natural products, to amplifying their action spectra and minimize toxic effects (Salvat et al., 2001; Shin & Pyun, 2004; Sousa et al., 2010).

The amphibian *Rhinella jimi* (Stevaux, 2002) (Bufonidae), popularly known as ''sapo cururu'', is distributed throughout the entire northeastern region of Brazil (Stevaux, 2002). This toad is utilized in traditional medicinal practices for treating illnesses in both humans and animals (inflammation, infections, and wounds) (Ferreira et al., 2009a). Recent studies have reported microbiological activities in extracts obtained from these animals, with demonstrations of antileishmanial and antitrypanossomal actions, in addition to showing antimicrobial effects against different bacterial lines, and cytotoxic properties (Brito et al., 2012a,b; Tempone et al., 2008).

The fats extracted from wild animals have been cited in a number of studies in traditional treatments of many illnesses involving inflammatory processes caused (or not) by infections (Costa-Neto & Alves, 2010; Ferreira et al., 2009a, 2011, 2012). *Rhinella jimi* fat has been specifically reported in the literature as a treatment for a number of infectious and inflammatory illnesses of possible microbial origin, such as infections, toothaches, sore throats, ear infections, and for treating wounds in animals (Costa-Neto & Alves, 2010; Ferreira et al., 2009a, 2011, 2012).

In light of the popular use of this toad species, and of scientific investigations of their biological properties, the present work examined extracts of the body fat of the Bufonidea *Rhinella jimi* to evaluate its antimicrobial action, both alone and in association with aminoglycoside antibiotics.

Materials and methods

Animal collections

Toad specimens were collected between April and June 2011 in the municipality of Exu (7°30′S × 39°43′W), Pernambuco State in Brazil (Figure 1). The 15 live specimens collected were sacrificed by freezing and the fat from the ventral region of their bodies was subsequently removed. After their identification, the animals were fixed in 10% formalin and subsequently conserved in 70% alcohol; testimonial specimens were deposited in the Herpetological Collection of the Regional University of Cariri (collection number 3132). The ethical approval for the study was obtained from the Ethics Committee of Universidade de Fortaleza – UNIFOR (No. of protocol: 006/2011-CEUA).

Extraction and analysis of the fixed oil composition

The fixed oil from the body fat of *Rhinella jimi* (ORJ) were extracted for 4 h in a Soxhlet apparatus using the solvent hexane. The extracted oil was heated in a water bath at 70 °C for 6 h to evaporate the solvent which was then stored in a freezer for subsequent analyses.

The ORJ was saponified by refluxing in a solution of potassium hydroxide and methanol for 2 h, following the method described by Hartman and Lago (1973). The residue was mixed with water and ethyl ether, the aqueous phase separated, and its pH subsequently adjusted by the addition of sulfuric acid. The free fatty acids were then methylated with methanol through acid catalysis to obtain their respective methyl esters (subsequently used to identify the original fatty

Figure 1. The municipality of Exu (7°30′S × 39°43′W), Pernambuco State in Brazil.

acids). The analyses of the chemical composition of the ORJ were undertaken using a Shimadzu gas chromatography system coupled to a *QP5050A* selective mass spectrometer (using an ionizing energy of 70 eV). A DB-5HT capillary column was used (30 m × 0.25 mm internal diameter) with the following specifications: temperatures of 270 °C at the injector and 290 °C at the detector, using helium as a carrier gas (1.0 mL/min); a linear velocity of 47.3 cm/s; a total flux of 24 mL/min; a carrier flux of 24 mL/min; a pressure of 107.8 kPa; the column temperature was program for 60 °C (2 min) to 180 °C (1 min) at 4 °C/min, and 180–260 °C at 10 °C/min (10 min). Component identifications were made by comparisons of their respective mass spectra with standards in the database of the Wiley 229 library, and by comparing their calculated retention indices with indices published in the specialized literature (Adams, 2001; Alencar et al., 1984, 1990; Stenhagen et al., 1974).

Bacterial and fungal lineages

The experiments were undertaken using multi-resistant clinical isolates of *Escherichia coli* 27, *Staphylococcus aureus* 358, and *Pseudomonas aeruginosa* 24. The standard bacterial lineages were *E. coli* ATCC 10536, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 15442, and *Klebisiella pneumonie* ATCC 4362; the fungi used were *Candida albicans* ICB 12 and *Candida krusei* ATCC6258. All the isolates were provided by the Universidade Federal da Paraíba, and were maintained in Heart Infusion Agar (HIA, Difco) until tested, at which time they were transferred into Brain Heart Infusion (BHI, Difco, Detroit, MI) growth media for 24 h at 37 °C (Coutinho et al., 2008; Freitas et al., 1999).

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) of the animal oils were determined by microdilution using bacterial suspensions containing 10^5 cells/mL. To prepare the test solutions, 10 mg of the original oil sample was mixed into 1 mL of dimethylsulfoxide (DMSO – Merck, Darmstadt, Germany) resulting in an initial concentration of 10 mg/mL. This initial solution was successively diluted with sterile water until reaching a concentration of 1024 μg/mL. After preparing wells with the inoculates, 100 μL of ORJ were added to initiate the serial dilutions, and the plates were then incubated for 24 h at 37 °C (Javadpour et al., 1996). The MIC was defined as the lowest concentration of ORJ that inhibited bacterial growth. Bacterial growth was observed using an indicator solution of resazurin, which turns from blue to pink in the presence of growing bacteria (due to the reduction of that dye) (Palomino et al., 2002). Fungal growth was visualized by noting turbidity of the culture solutions.

Drug susceptibility tests

The putative zootherapeutic extracted oils were also tested in association with antibiotics and antifungal compounds to determine their effects on fungi and multi-resistant bacteria. The examination of drug activity modulation was based on the results of the MIC, making it necessary to reduce the test concentrations of ORJ to sub-inhibitory levels (MIC/8). The antibiotics tested were gentamicin, neomycin, and amikacin, all at concentrations of 5000 μg/mL. The antifungal agents used were mebendazol, amphotericin B, nystatin and benzoilmetronidazole at concentrations of 1024 μg/mL. Aliquots (100 μL) of each antibiotic were added, in serial dilutions, to the plates containing the inoculums in 10% BHI with added ORJ, or just the inoculums in 10% BHI (control). The plates were maintained at 37 °C for 24 h, after which growth was measured using turbidity evaluation or by adding resazurine (for anti-fungal and antibacterial tests, respectively).

Checkerboard method

The best results obtained in the drug modulation experiments were retested using the checkerboard method (Eliopoulos & Moellering, 1991), using combinations of the antibiotics (an initial concentration of 5000 μg/mL) and ORJ (an initial concentration of 1024 μg/mL). The results were evaluated by adding 20 μL of resazurine and evaluating any color changes after 1 h. The fractionated inhibitory concentration (FIC) was calculated as the sum of $FIC_A + FIC_O$, where A represents the antibiotic and O represents the substance tested (ORJ). The parameters for the interpretation of the FIC are: synergistic when the FIC is less than 0.5; additive when between 0.5 and 1.0; indifferent when greater than 1.0; and antagonistic when greater than 4.0.

Results and discussion

The analyses of the chemical composition of the ORJ by GC/MS allowed the identification of 12 constituents responsible for 93.7% of the total fatty acid methyl esters present (Table 1). Of this percentage, 55.39% were unsaturated acids, notably oleic, palmitoleic, and linoleic acids; saturated acids represented 44.61% of the total percentage of acids identified, notably palmitic, stearic, and myristic acids.

Table 1. Methyl esters and fatty acids identified in the fixed oil extracted from *Rhinella jimi* specimens collected in Exu – PE (ORJ) using a gas chromatograph coupled to a mass spectrometer (CG/MS), in increasing order of retention/min (RT).

Constituents	RT (min)	(%)	Equivalent fatty acid
Saturated			
Methyl dodecanoate	17.5	0.6	Lauric acid
Methyl tetradecanoate	21.9	2.4	Myristic acid
Methyl pentadecanoate	23.2	1.9	Pentadecanoic acid
Methyl eicosanoate	33.9	0.4	araquídico acid
Methyl octadecanoate	30.6	8.1	Steric acid[a]
Methyl hexadecanoate	25.9	26.8	Palmitic acid[a]
Methyl heptadecanoate	27.8	1.6	Heptadecanoic acid
Unsaturated			
Methyl (Z,Z)-octadeca-9, 12-dienoate	29.5	6.2	Linoleic acid[a]
Methyl (*E*)-octadec-10-enoate	29.8	38.0	Oleic acid[a]
Methyl (*Z*)-octadec-13-enoate	29.9	1.4	Oleic acid[a]
Methyl hexadec-11-enoate	25.3	1.4	Palmitoleic acid
Methyl (Z)-hexadec-9-enoate	25.4	4.9	Palmitoleic acid
Total		93.7	

[a]Principal fatty acids.

Earlier research, as well as the results of the present study, established that unsaturated fatty acids compose the bulk of animal fat (Cabral et al., 2013; Ferreira et al., 2009b, 2011; McCue, 2008), and our results also corroborated these previous research papers in terms of the notable presence of oleic, linoleic, palmitic, and stearic acids.

The application of resazurine to the bacterial culture wells, and the observation of turbidity in the fungal cultures, established the MIC of ORJ as being 512 µg/mL for *Candida krusei* and ≥1024 µg/mL for the other fungi. The MIC results demonstrated that although ORJ is indicated in traditional medicine for treating numerous infections (Costa-Neto & Alves, 2010; Ferreira et al., 2009a, 2012) it did not demonstrate any clinically relevant antimicrobial activity; similar results were found for the body oils of *Tupinambis merianae* (Duméril & Bibron, 1839) (Teiidae) (Ferreira et al., 2009b). However, when ORJ was combined with aminoglycosides, the positive results (Tables 2 and 3) were significantly different from the use of oils extracted from *T. merianae* (which did not demonstrate any increase in anti-microbial efficiency when combined with aminoglycosides).

ORJ did not demonstrate any modulation by antibiotics when acting against *S. aureus* (Table 2). When associated with amikacin, however, ORJ demonstrated a four-fold increase in its ability to inhibit *E. coli* growth, indicating a synergistic interaction. In the same way, when allied with amikacin, gentamicin, and neomycin, the MICs of ORJ were reduced eight-, four-, and four-fold, respectively, against *P. aeruginosa*.

There are very few effective treatments against opportunist infections caused by species of *Candida*, and many antifungal agents have undesirable side effects or can induce fungal resistance (principally in immune-depressed individuals; see Fica, 2004), which can result in superficial or even systemic infections (Shao et al., 2007).

Table 2. Test results of ORJ concentrations as effective modulators of micro-diluted antibiotics. Control = without ORJ. All results in µg/mL.

	S. aureus 358		*P. aeruginosa* 24		*E. coli* 27	
Antibiotics	ORJ	Control	ORJ	Control	ORJ	Control
Amikacin	78.12	39.06	39.06	312.50[a]	39.06	156.25[a]
Neomycin	9.76	9.76	39.06	156.25[a]	39.06	78.12
Gentamicin	2.44	4.88	9.76	39.06[a]	9.76	19.53

[a]Most expressive results.

Table 3. Test results of the use of ORJ concentrations as effective modulators of the actions of micro-diluted anti-fungal agents. Control = without ORJ.

	C. albicans ICB 12		*C. krusei* ATCC6258	
Anti-fungal agent	ORJ	Control	ORJ	Control
Mebendazol	≥1024	≥1024	≥1024	≥1024
Amphotericin B	≥1024	≥1024	64[a]	512[a]
Nystatin	≥1024	≥1024	≥1024	≥1024
Benzoilmetronidazole	≥1024	≥1024	≥1024	≥1024

All results in µg/mL.
[a]Significant results.

There are numerous reports in the literature of the use of industrialized medicines in association with natural products (Calvet-Mir et al., 2008; Shin et al., 2008; Vandebroek, et al., 2008), as well as evidence that animal and plant products can modulate the effects of commercial medicines by increasing or reducing their efficiency (Coutinho et al., 2008; Santos et al., 2012a,b).

Table 3 presents the results of the association of ORJ in sub-inhibitory concentrations (MIC/8) with antifungal agents (mebendazol, amphotericin B, nystatin, and benzoil-metronidazole) against species of *Candida*. No modulation was seen against *C. albicans*, although ORJ reduced by eight the MIC of amphotericin B against *C. kusei*, indicating a synergistic effect between them.

According to Granowitz and Brown (2008), antagonistic effects are often observed with antibiotic combinations, probably due to their mutual chelation – quite different from the present results indicating synergism. The data reported here corroborate Agoramoorthy et al.(2007) and Nobre et al. (2002) who noted that fatty acids can often inhibit microbial activity and that unsaturated varieties are the most effective because they affect the endogenous bacterial synthesis of their own fatty acids (Zheng et al., 2005) (with ORJ being 55.39% unsaturated).

Evidence of drug modulation also was also assessed by FIC – in which it was possible to determine with precision the types of interactions occurring (indifferent, additive, synergistic, or antagonistic). As can be seen in Table 4, modulation was confirmed. ORJ in association with gentamicin demonstrated and additive effect (FIC index of 0.51) against *P. aeruginosa*, indifferent against the same bacterial line when tested with amikacin and neomycin, and synergistic when associated with amikacin (FIC index of 0.25) against *E. coli*.

Clinical evaluations of the bio-activities of animal fats have yielded varying results. The fat of *Tupinambis merianae*, for example, did not demonstrate any clinically relevant antimicrobial activity (Ferreira et al., 2009b), while the body fat of *Boa constrictor* Linnaeus, 1758 (Boidae) demonstrated antimicrobial and anti-inflammatory effects (Falodun et al., 2008) as well as synergism in modulating antibiotic activities (Ferreira et al., 2011).

Table 4. Results of the checkerboard experiments using ORJ with amikacin, gentamicin, and neomycin against the multi-resistant bacteria *Escherichia coli* 27 and *Pseudomonas aeruginosa* 24, and the types of interactions of ORJ with aminoglycosides.

	E. coli 27		*P. aeruginosa* 24	
	MIC	MIC combined	MIC	MIC combined
ORJ_E	1024	4	1024	64
Amikacin	78.12	19.53	39.06	78,12
CIF and type of interaction	0.25 – synergistic		2.06 – indifferent	
ORJ_E			1024	8
Gentamicin			39.06	19,53
CIF and type of interaction			0.51 – additive	
ORJ_E			1024	16
Neomycin			39.06	39,06
CIF and type of interaction			1.01 – indifferent	

MIC, minimum inhibitory concentration; ORJ_E, *Rhinella jimi* oil collected in Exu – PE; FIC, fractionated inhibitory concentration.

Conclusions

The results presented here indicated that the fixed oils extracted from the body fat of *Rhinella jimi* (ORJ) did not demonstrate clinically relevant antimicrobial activity against the microbial lineages used when tested alone. However, the use of ORJ in the traditional medicine can be explained by the symbolic nature of this animal by the traditional communities. According with the work of Ârhem (1989) and Heinrich (1994), there is an important relationship between the communities and the natural resources. This symbolism together with the emotional situation of the patient can be more easy to cure the process and the symbolic efficacy (Tesser & Luz, 2008).

However, when ORJ was tested in combination with antibiotics and antifungal agents, it modulated the action of amphotericin B against *C. krusei*, the action of amikacinin a synergistic manner against *Escherichia coli* 27, and gentamicin in an additive manner against *Pseudomonas aeruginosa* 24. These data will permit new searches for new products to be used in new formulations in association with antibiotics, enhancing the efficacy of these drugs against drug-resistant microorganisms.

It should be noted that the present study examined the effects of ORJ through *in vitro* testing – so that similar studies should be undertaken with live organisms to verify the systemic efficiency and toxicity of the oils extracted from *R. jimi* during continuous use, and elucidate additional related clinical questions.

Acknowledgements

The authors would like to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES for the study grants awarded to Mario E.S. Cabral and Diógenes Q. Dias; the Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico – FUNCAP for the study grants awarded to Débora L. Sales, Olga P. Oliveira, and the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq for the productivity grants awarded to Waltécio O. Almeida (No. 311713/2012-2); and IBAMA for the collecting permit (SISBIO – IBAMA: 154/2007 no. 27486-1, process identification code: 62956869).

Declaration of interest

The authors report no conflicts of interest.

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Saudi Journal of Biological Sciences (2017) 24, 302–313

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ORIGINAL ARTICLE

Phenolic composition and medicinal usage of *Psidium guajava* Linn.: Antifungal activity or inhibition of virulence?

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Received 20 May 2015; revised 15 September 2015; accepted 22 September 2015
Available online 30 September 2015

KEYWORDS

Fungistatic effect;
Inhibition of dimorphism;
Tea;
Tincture

Abstract *Psidium guajava* is a Myrtaceae plant whose medicinal properties are recognized in several locations. The use of teas and tinctures prepared from their leaves has been used to combat infections caused by fungi of the genus *Candida*. In this study, aqueous extracts of leaves and hydroethanolic were tested to verify the antifungal potential and its chemical composition has been investigated. The microbiological assays were performed by broth microdilution to determine the minimum inhibitory concentration (MIC) and from these the minimum fungicidal concentration was performed (MFC) by subculturing on solid media. A cell viability curve was obtained for demonstration of inhibition of fungal growth of strains of *Candida albicans* and *Candida tropicalis*. Tests to check morphological changes by the action of the extracts were performed in microcultive cameras depleted environment at concentrations of MIC/2, MIC and MIC × 2. Extracts analyzed by high performance liquid chromatography demonstrated flavonoids and phenolic acids. The extracts showed fungistatic effect and no fungicide with MIC >8192 μg/mL, MFC above 8192 μg/mL. The IC_{50} was calculated ranging from 1803.02 to 5623.41 μg/mL. It has been found

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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.sjbs.2015.09.028
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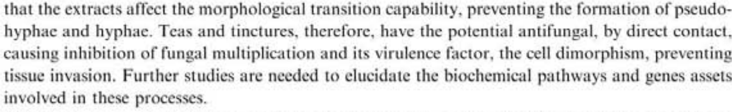

that the extracts affect the morphological transition capability, preventing the formation of pseudo-hyphae and hyphae. Teas and tinctures, therefore, have the potential antifungal, by direct contact, causing inhibition of fungal multiplication and its virulence factor, the cell dimorphism, preventing tissue invasion. Further studies are needed to elucidate the biochemical pathways and genes assets involved in these processes.

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1. Introduction

Microorganisms of the genus *Candida* can be found naturally composing the microbiota of the human organism inhabiting your gastrointestinal tract and mucous membranes (Lu et al., 2014; Shao et al., 2007). Changes in dynamic of the host organism too favor the proliferation of these fungi and the disturbance caused in homeostasis can lead to a range of infections that range in their level and location and can only be superficial, in skin and mucosal (oral, vaginal candidiasis) or systemic, compromising the life of an individual (Mayer et al., 2013; Sardi et al., 2013).

Usually the infections caused by *Candida* spp. in its magnitude are assigned to the species *Candida albicans*, however, illness caused by *Candida* non-albicans (CNAM) had increased incidence over the years and yeasts of *Candida glabrata*, *Candida tropicalis*, *Candida krusei* and *Candida parapsilosis* have been increasingly identified as human pathogens (Sardi et al., 2013; Silva et al., 2012).

Mechanisms of resistance to commercial drugs developed by these microorganisms have been constantly investigated and reported and the continuous evolution for resistance is extremely worrying considering the limited number of antifungal classes currently available (Maubon et al., 2014; Xie et al., 2014). The search for different therapeutic alternatives is a constant and the use of natural products of plant origin often serves as a reference to the search for active compounds and, in this sense, a ethno directed approach has directed pharmaceutical research (Albuquerque and Hanazaki, 2006), in this case, in order to antifungal discovery potential.

Member of the Myrtaceae, *Psidium guajava* Linn. species (guava), plant native to tropical America (Okamoto et al., 2009), has its widespread medicinal use among the world's populations. Being a plant of tropical and subtropical regions, can be found on plantations, in backyards of homes, or naturally in other areas, and could even be considered invasive species (Richardson and Rejmánek, 2011).

The medicinal attributes from all parts of the species are spread over several generations and therefore, make up many lists of ethnobotanical studies, showing great versatility and value in use, being mentioned for the treatment of various types of diseases (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008).

This therapeutic use recorded in different locations includes a significant number of body systems such as disorders of the sensory system: vertigo (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); disorders of the respiratory system: laryngitis, sore throat, colds, coughs, tuberculosis, lung problems, bronchitis, catarrh, rhinitis (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Ogbole and Ajaiyeoba, 2010; Waruruai et al., 2011); disorders of the genito-urinary system: menstrual disorders, vaginal discharge, childbirth, nephritis, premenstrual syndrome, gonorrhea, non-specified venereal diseases, leucorrhea (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Van Vuuren and Naidoo, 2010); disorders of the nervous system: anorexia, epilepsy, cerebral ailments, chorea, convulsions, nervousness (Dakappa-Shruthi et al., 2013; Gómez-Estrada et al., 2011; Gutiérrez et al., 2008); disorders of the digestive system: diarrhea, dysentery, stomachache, digestive problems, gastric insufficiency, ulcers, dyspepsia, gastroenteritis, gastritis, bowel disorders, colic, toothache, constipation (Dakappa-Shruthi et al., 2013; Gómez-Estrada et al., 2011; Gutiérrez et al., 2008); disorders of the circulatory system: piles, swelling, hypertension, edema (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); the musculoskeletal system and connective tissue diseases: gout, spasm, rheumatic pain (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); not defined conditions or pain not defined: aches (Dakappa-Shruthi et al., 2013); skin diseases and tissue subcutaneous: inflamed mucous membranes, mouth – swelling, skin problems, ulcers, itch, scabies, skin sores, wounds, dermatosis, sores, boil, gingivitis (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008); diseases of the endocrine glands, nutrition and metabolism: diabetes (Gutiérrez et al., 2008); infectious and parasitic diseases: cholera, worms, bacterial infections, herpes, mycoses, thrush, pox, measles (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Waruruai et al., 2011); neoplasms: cancer (Alonso-Castro et al., 2011), and disease of the blood and blood-forming organs: hemorrhages, blood cleansing (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008).

The *P. guajava* is popularly used in the treatment of infectious diseases, particularly against those caused by fungi, it is common practice registered in different countries such as Brazil, Cuba and South Africa where it is used to treat thrush, leucorrhoea, and vaginitis, pathologies associated with infections caused by *Candida* spp. (Borba and Macedo, 2006; Fenner et al., 2006; Oliveira et al., 2010; Ramirez et al., 2007; Van Vuuren and Naidoo, 2010).

Considering the pharmacological potential of the species *P. guajava* described in ethnobotanical reports, especially with regard to its therapeutic use in treatments against diseases caused by fungi, this study aims to scientifically validate the antifungal properties of tea and tincture prepared with leaves of guava and evaluate the effect of natural products in virulence strains of *C. albicans* and *C. tropicalis*, particularly its morphological transition process.

2. Material and methods

2.1. Collection area

The collection was realized in the rainy season at the county of Milagres, Ceará, Northeastern region of Brail (07° 17.119′ S

and 038° 51.779′ W, 388 m of altitude; 07° 17.120′ S and 038° 51.778′ W, 389 m of altitude; 07° 17.122′ S and 038° 51.776′ W, 392 m of altitude; 07° 17.119′ S and 038° 51.779′ W, 388 m of altitude) at "Sítio" Malhada. The climate is semi-arid, with temperatures ranging between 24° and 26 °C (IPECE, 2013).

2.2. Plant material

The study was conducted using young, healthy leaves of a *Psidium* species locally known as red guava, which were collected and transported to the Laboratory of Microbiology and Molecular Biology at the Regional University of Cariri – URCA. Twigs with flowers of the species were also collected and vouchers were produced and deposited in the Herbarium Dárdano de Andrade Lima at the university under No. 10935, where the species was identified as *Psidium guajava* Linn. The collection period included January, February, March and April, known as the "wintry block of the Cariri Ceara region." Collections were made between 8:30 and 10:30 am, and the plant material was taken to the laboratory where it was subjected to qualitative screening and cleaning before being weighed and stored under refrigeration. Altogether, there was 2650 g of leaves in perfect condition, and this quantity was divided for preparation of three types of extracts: Aqueous Extract of *P. guajava* Infusion (AEPGI), Aqueous Extract of *P. guajava* Decoction (AEPGD) and: Hydroethanolic Extract of *P. guajava* (HEPG).

2.3. Preparation of extracts

2.3.1. Aqueous extracts

Two types of aqueous extracts were prepared, each using 399.9 g of leaves mixed with 6 L of water (based on a proportion of 10 g/150 mL, equivalent to one cup of tea – 150 cc). The decoction was made by mixing roughly cut leaves in cold water and then boiling for 15 min. Afterward, the tea was allowed to cool, filtered and then stored under refrigeration. As for the infusion, the water was boiled without leaves, which were placed in the water after turning off the heat. The pot was covered with a lid and allowed to stand until the tea cooled down (Matos, 2002), and the preparation was then filtered and stored under refrigeration and both infusion and decoction were frozen (−60 °C) and lyophilized to dryness. The powdered extracts were stored under refrigeration for testing, using 14.46 g (yield 3.62%) and 15 g (yield 3.75%) extract powder from the decoction and infusion, respectively.

2.3.2. Hydroethanolic extract

The hydroethanolic extract (70%) was prepared by trituration with cold extraction, using a total of 1846.5 g leaves in a proportion of 5 g/mL of hydroethanolic solution (Matos, 2002). The leaves were cut to increase contact surface with the solvent, and the mixture was left at room temperature protected from air and light, for a period of 96 h for maximum extraction efficiency. The mixture was then filtered and placed in a rotary evaporator (Q-344B – Quimis – Brazil) at 40 rpm and 60 °C to concentrate the extract. Finally, the crude extract was frozen, lyophilized (50.8 g – yield 2.75%) and then stored under refrigeration.

2.4. Chemical analysis

2.4.1. Chemical, apparatus and general procedures

All chemicals were of analytical grade. Methanol, acetic acid, gallic acid, caffeic acid and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, isoquercitrin, rutin, kaempferol, luteolin, catechin and epicatechin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC–DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A diode array detector and LC solution 1.22 SP1 software.

2.4.2. High performance liquid chromatography with diode array detection (HPLC–DAD)

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% acetic acid (A) and methanol (B), and the composition gradient was: 5% (B) for 2 min; 25% (B) until 10 min; 40, 50, 60, 70 and 80% (B) every 10 min; following the method described by Silva et al. (2014) with slight modifications. *P. guajava* extracts (hydroethanolic – EHEPG, infusion – EAIPG and decoction – EADPG) and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use, the extracts of *P. guajava* were analyzed at a concentration of 20 mg/mL. The flow rate was 0.6 mL/min and the injection volume was 50 μL. The sample and mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standard references were prepared in water: methanol (1:1; v/v) at a concentration range of 0.025–0.300 mg/mL catechin, epicatechin, quercetin, quercitrin, isoquercitrin, kaempferol, luteolin and rutin, and 0.035–0.300 mg/mL for gallic, caffeic and chlorogenic acids. Quantification was carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid, 281 nm for catechin and epicatechin, 327 nm for chlorogenic and caffeic acids, and 366 for quercetin, quercitrin, isoquercitrin, luteolin, kaempferol and rutin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–500 nm). Calibration curve for gallic acid: $Y = 12673x + 1281.9$ ($r = 0.9998$); caffeic acid: $Y = 12943x + 1191.7$ ($r = 0.9996$); chlorogenic acid: $Y = 12083x + 1327.9$ ($r = 0.9995$); catechin: $Y = 11734x + 1306.8$ ($r = 0.9999$); epicatechin: $Y = 12387x + 1239.1$ ($r = 0.9997$); rutin: $Y = 13752x + 1186.5$ ($r = 0.9991$); quercetin: $Y = 11970x + 1181.7$ ($r = 0.9996$); quercitrin: $Y = 11679x + 1251.7$ ($r = 0.9998$), isoquercitrin: $Y = 13759x + 1251.9$ ($r = 0.9993$), kaempferol: $Y = 12659x + 1172.3$ ($r = 0.9997$) and luteolin: $Y = 12507x + 1341.8$ ($r = 0.9990$). All chromatography operations were carried out at ambient temperature and in triplicate.

2.4.3. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent

analytical curves, as defined by Kamdem et al. (2013). LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.5. Antifungal assay

2.5.1. Strains and culture media used

Standard types of strains were obtained from the Culture Collection of Oswaldo Cruz of the Brazilian Institute of Quality Control in Health (INCQS) and clinical isolates of the yeasts *C. albicans* and *C. tropicalis* were provided by Dr. Edeltrudes Oliveira Lima (Mycology Laboratory of Paraiba Federal University), namely CA INCQS 40006, CA LM 62, CA LM 77, CA LM 109, CA LM 111, CA LM 122, CT INCQS 40042, CT LM 18, CT LM 20 and CT LM 23. These strains were inoculated into Sabouraud Dextrose Agar (SDA, KASVI) and incubated for 24 h at 37 °C. Afterward, small aliquots of yeast were transferred to test tubes each containing 3 mL of sterile saline (0.9%). Using the McFarland scale, the concentration of the inoculum was standardized by comparing its turbidity with the 0.5 standard, giving a standard yeast suspension of 1×10^5 cells/mL (NCCLS, 2002). The inocula thus prepared were used to determine the minimum inhibitory concentration (MIC) in Sabouraud Dextrose Broth (SDB, HIMEDIA), double concentrated. Another culture medium was used for analysis of yeast micromorphology. The potato dextrose agar (PDA, DIFCO) was prepared by diluting it more than that recommended by the manufacturer to make it a depleted medium capable of stimulating yeast to produce hyphae. Agar was added to this diluted medium to obtain a solid medium.

2.5.2. Drugs, reagents and preparation of solutions

Dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) was used for dilution of the extracts, and the antifungal fluconazole (Capsule – FLUCOMED), diluted in water, was used as the reference drug. The matrix solutions of the extracts were prepared by weighing 0.3 g of each extract and then diluting in 1 mL of DMSO. To obtain the desired concentration for testing, the extracts were further diluted in sterile distilled water so that the concentration of DMSO in the natural product did not exert any activity in the test cells (Stoppa et al., 2009).

2.5.3. Microbiological screening

Microbiological screening was performed to select the yeasts to be used in microbiological testing. The microdilution broth was chosen to perform this procedure, and this was done by determining the MIC (Javadpour et al., 1996), since the plates prepared to carry out this test would be used later in tests to find the minimum fungicidal concentration, besides facilitating the demonstration of cell viability curve and calculating the IC_{50} of the test products.

2.5.4. Determination of minimum inhibitory concentration (MIC)

This test was performed by the broth microdilution method in 96-well plates. Each well was filled with 100 μL of SDB containing 10% fungal inoculum, and then, 100 μL of the natural product (16,384 μg/mL) or fluconazole (antifungal reference) at the same concentration, was added to the first well, followed by twofold serial dilution. The concentrations in the wells ranged from 64 to 8192 μg/mL. The last well contained no extract or drug and served as the normal growth control (Javadpour et al., 1996). Controls for diluent of the products (using saline instead of inoculum) and the sterile medium were also prepared. All tests were performed in triplicate. The plates were incubated at 37 °C for 24 h and afterward read in an ELISA spectrophotometer (Thermoplate®) at a wavelength of 630 nm. The MIC was defined as "the lower concentration of na antimicrobial agent that inhibit the visible growth of

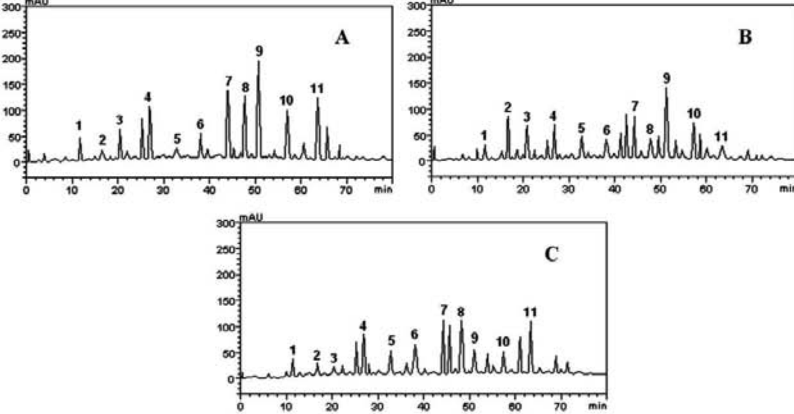

Figure 1 Chromatogram of *Psidium guajava* extracts. High performance liquid chromatography phenolics and flavonoids profile of *Psidium guajava*. (A) Hydroethanolic Extract of *P. guajava* (HEPG); (B) Aqueous Extract of *P. guajava* Decoction (AEPGD); Aqueous Extract of *P. guajava* Infusion (AEPGI); gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), epicatechin (peak 5), rutin (peak 6), quercitrin (peak 7), isoquercitrin (peak 8), quercetin (peak 9), kaempferol (peak 10) and luteolin (peak 11).

Table 1 Phenolics and flavonoid composition of *Psidium guajava*.

Compounds	*Psidium guajava*			LOD µg/mL	LOQ µg/mL
	EHPG mg/g	AEPGD mg/g	AEPGI mg/g		
Gallic acid	3.46 ± 0.01 a	1.57 ± 0.02 a	1.54 ± 0.01 a	0.019	0.062
Catechin	1.57 ± 0.03 b	4.94 ± 0.01 b	1.29 ± 0.02 b	0.008	0.025
Chlorogenic acid	4.39 ± 0.01 c	4.23 ± 0.03 c	1.25 ± 0.03 b	0.024	0.081
Caffeic acid	8.01 ± 0.02 d	4.30 ± 0.01 c	4.73 ± 0.01 c	0.035	0.116
Epicatechin	1.58 ± 0.01 b	3.61 ± 0.02 d	3.58 ± 0.02 d	0.010	0.034
Rutin	3.62 ± 0.01 a	3.52 ± 0.01 d	3.97 ± 0.01 e	0.017	0.056
Quercitrin	11.17 ± 0.03 f	4.95 ± 0.01 b	8.62 ± 0.01 f	0.032	0.105
Isoquercitrin	10.35 ± 0.01 e	3.48 ± 0.03 d	8.59 ± 0.03 f	0.009	0.031
Quercetin	16.81 ± 0.02 g	10.15 ± 0.03 e	3.45 ± 0.03 d	0.025	0.083
Kaempferol	8.26 ± 0.03 d	4.32 ± 0.01 c	3.42 ± 0.01 d	0.018	0.059
Luteolin	10.13 ± 0.01 e	1.69 ± 0.03 a	8.51 ± 0.02 f	0.023	0.075

Results are expressed as mean ± S.E. of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.01$. HEPG: Hydroethanolic Extract of *P. guajava*; AEPGD: Aqueous Extract of *P. guajava* Decoction; AEPGI: Aqueous Extract of *P. guajava* Infusion; LOD: limit of detection; LOQ: limit of quantification.

Table 2 IC_{50} (µg/mL) of *Psidium guajava* extracts against *Candida* strains.

Product tested	*Candida albicans*		*Candida tropicalis*	
	INCQS 40006	LM 77	INCQS 40042	LM 23
AEPGI	3235.94	3890.45	4570.88	4570.88
AEPGD	4797.33	5623.41	5495.41	5128.61
HEPG	1803.02	1905.46	1862.09	1905.46
Fluconazole	76.72	69.09	73.98	58.63

AEPGI: Aqueous Extract of *P. guajava* Infusion; AEPGD: Aqueous Extract of *P. guajava* Decoction; HEPG: Hydroethanolic Extract of *P. guajava*: INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia.

na microorganism in dilution assays" (CLSI, 2002). The results obtained in the ELISA readout were used to construct the cell viability curve and the IC_{50} of the extracts of *P. guajava*.

2.5.5. Determination of minimum fungicidal concentration (CFM)

For this test, a small sterile rod was placed in each well of the MIC test plate (except for sterility control). After mixing the medium in each well, the rod was taken to a large petri dish containing SDA, streaking its surface and transferring the solution (medium + inoculum + natural product) for subculture of yeast and checking cell viability. After 24 h incubation, the plates were inspected for any formation of colonies of *Candida* (Ernst et al., 1999, with modifications). The concentration at which there was no growth of fungal colonies was considered the MFC of the natural product.

2.5.6. Effect of natural products on fungal morphology

To determine if the natural product caused any change in fungal morphology, by inhibiting the development of hyphae, sterile micromorphological chamber slides were prepared for observation of yeasts. Three milliliters of PDA medium depleted by dilution were added to chambers, containing the natural product concentrations MIC/2, MIC and MIC × 2. Aliquots of the inoculi were taken from the petri dishes to make two parallel steaks on the solid medium, which were then covered with a sterile coverslip. The chambers were placed in the incubator for 24 h (37 °C) and inspected under a light microscope using a 40× objective. A camera was attached to the microscope to capture images. A control for yeast growth (hyphae stimulated by depleting medium) was performed, as well as a control with the conventional antifungal fluconazole for comparative purposes and a control with DMSO at 100% and 0.5% (the concentration in the natural products used in the tests). The assays were performed according to Sidrin and Rocha (2010) and Mendes (2011), with some modifications.

2.6. Statistical analysis

The results of the tests were done in triplicate. Data obtained for each sample and concentration were checked for their normal distribution and then analyzed by one-way ANOVA by post hoc Tukey test. EC_{50} values were obtained by nonlinear regression for the purpose of interpolating values from standard curves (using the software Graphpad Prism, v. 5.0) of the % *growth* values plotted against concentration and EC_{50} values are expressed as µg/mL.

3. Results and discussion

The chemical analysis was performed to detect the presence of phenolic acids and flavonoids, verifying the predominance of the latter in two extracts. The hydroethanolic extract showed more efficient as extracting agent, since quantity of compounds able to extract higher. To the aqueous extracts, exposure to different temperature times caused no significant difference in the amount of extracted compounds, however the extract obtained from infusion had a greater abundance of flavonoids as compared to the decoction. Studies show that can occur degradation of flavonoids with temperature rise, however, this

process also depends on the chemical structure and the interaction between them (Baby et al., 2007; Mello et al., 2010). In this sense, the decoction longer exposure to elevated temperature may have been the cause of the reduction of the level of flavonoids. The major compound differed only in the analysis of each extract into the aqueous infusion made, which in this case is the quercitrin, while the other the quercitrin appears to be the most expressive content. The chromatogram extracts of the species are shown in Fig. 1, and the results representing their chromatographic profiles in front the parameters used are detailed in Table 1.

The chemical composition of *P. guajava* has been widely investigated and studies have reported that plant extracts are constituted alkaloids, triterpenoids, tannins, saponins, glycosides, flavonoids, and phenolic compounds and other compounds (Dakappa-Shruthi et al., 2013; Tambe et al., 2014).

A chemical analysis of tea from the leaves of guava (Chang et al., 2013) highlighted a polyphenol profile in which the main components were found: quercetin, myricetins, catechin, gallic and ellagic acids and their derivatives, but the researchers call attention to differences in chemical composition, stating that several factors such as time of collection, form of collection and processing, temperature, among others, may influence the outcome of the chemical prospecting.

The data obtained in microdilution test were used for MIC determination, assembly of cell viability curve and to calculate the IC_{50} for each product. The same procedure was performed with fluconazole. Cell viability curve of microorganisms in contact with different concentrations of natural products showed a similar behavior in the screening, which allowed us a random choice of lines for continuity of the work. Thus, the standard strains and clinical isolates of *C. albicans* were selected (CA INCQS 40006 e CA LM 77) and *C. tropicalis* (CT INCQS 400042 e CT LM 23). Information is shown in Table 2 and Fig. 2. The IC_{50} of products ranged from 1803.02 to 5623.41 μg/mL and cell viability curve image points the hydroethanolic extract of the species as being the most effective because it could reduce a higher percentage of micro-organisms, when compared to the others.

The MIC of the products in this study was determined and standardized as > 8192 μg/mL and cell viability curve, the concentration at which it was observed a marked reduction in the percentage of viable microorganisms. The results of minimum fungicidal concentration showed that, the concentrations tested, no extract showed fungicidal effect, since it failed to remove, but reduce the population of microorganisms of *Candida*. We found that the antifungal effect seen is fungistatic and that minimum fungicidal concentration is > 8192 μg/mL.

One of the main virulence factors of *Candida* is the cell dimorphism, which depends on the environmental conditions in which micro-organisms are found growing. This condition dimorphic is characterized by the ability to alter the cellular morphology, alternating, in a reversible manner, between the shape of yeast and hyphae or pseudohyphae, by issuing a structural extension, a filament, passing a state considered dispersive to invasive, respectively, this being a process commonly observed in candidemia systemic, which can be found in both morphological types, the yeast and filamentous at the same time (Lu et al., 2014).

Based on the results of the MIC, the tests were performed to verify that the extracts influenced the morphological transition of yeast. To this end we prepared a poor environment in nutrients, where it functioned as stressor of microorganisms, stimulating their potential dimorphic. In carrying out the

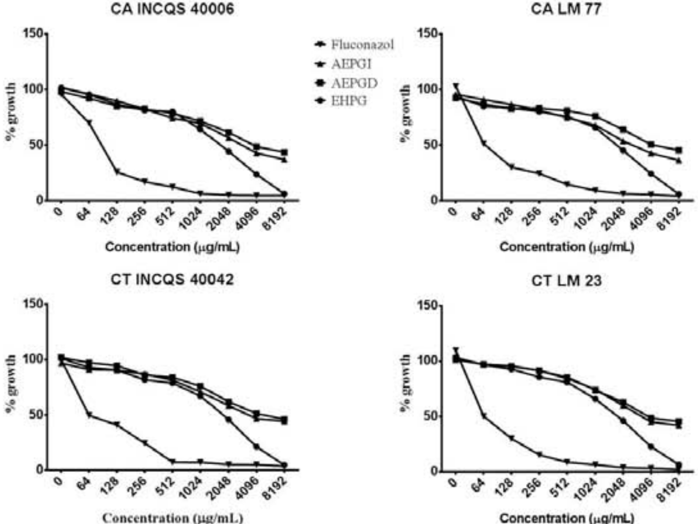

Figure 2 Cell viability curve *Candida* strains under the effect of *Psidium guajava*. CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia; AEPGI: Aqueous Extract of *P. guajava* Infusion; AEPGD: Aqueous Extract of *P. guajava* Decoction; HEPG: Hydroethanolic Extract of *P. guajava*.

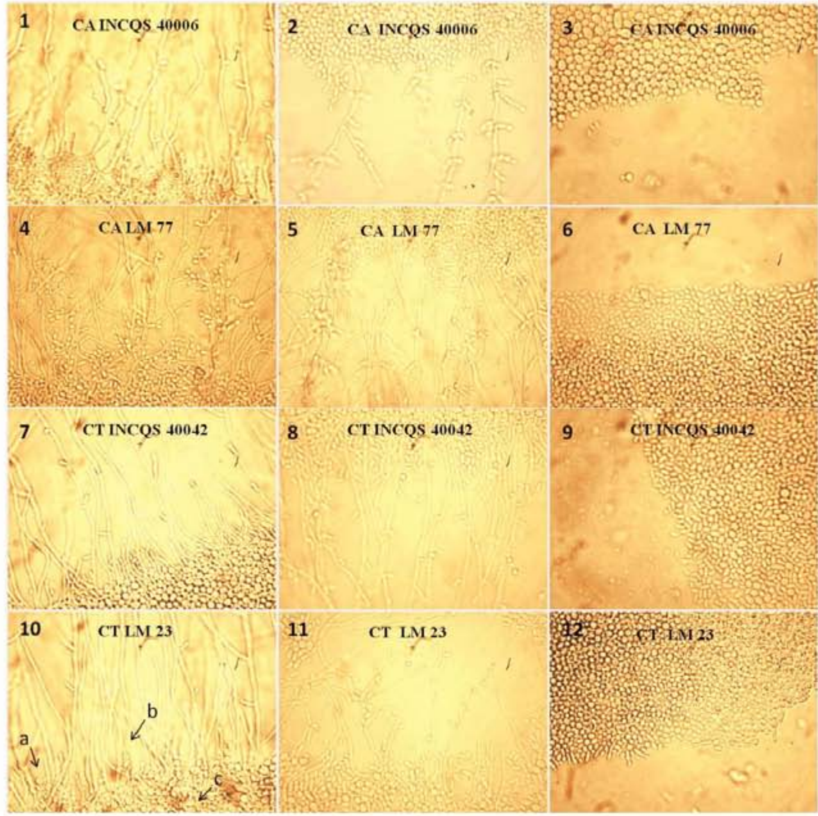

Figure 3 Controls used in micromorphology tests. Cell forms of *Candida*: a: pseudohiphae; b: hiphae; c: yeast; 1, 4, 7 and 10: Growth control; 2, 5, 8 and 11: DMSO 0.05%; 3, 6, 9 and 12: Fluconazole; CA: *Candida albicans;* CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. Images inspected under a light microscope using a 40× objective.

different tests controls were included, one of the growth control, and demonstrated the feasibility of this morphological transition allowed by the nutrient poor environment where the microculture reveals the presence of pseudohyphae, and hyphae. In another control, DMSO was tested at 100% and the maximum concentration is able to prevent the morphological transition (data not shown). However, to show that the change does not exert dimorphic yeast, the DMSO was assayed in concentration contained in dilution of natural products (0.05%), confirmed that the microculture presented microorganisms in accordance with the growth control. The reference drug, fluconazole, was also evaluated and, as the lowest concentration assayed in the test (MIC/2), caused inhibition of emission of filamentous structures. The images of the controls are shown in Fig. 3.

The reading of other microcultivations performed with the extracts in concentrations of 4096, 8192 and 16384 µg/mL showed that they were able to affect the phenotypic plasticity of *C. albicans* and *C. tropicalis* reducing hyphae and pseudohyphae formation process in so far as their concentrations were increased. At higher concentration, the yeast form prevailed so that, or not verified the presence of filaments, or these filaments were significantly reduced, as can be seen in Figs. 4 and 5.

The guava has been used in Brazil for the treatment of oral diseases, where both the leaves as bark are used in the preparation of tea (for infusion or decoction) to be swallowed or swished still warm, with pretensions to combat thrush and mouth sores, which may be caused by *Candida* strains (Borba and Macedo, 2006; Oliveira et al., 2010). In addition, there is no record that various parts of the plant are used not only to treat thrush but also for the treatment of leukorrhea (Fenner et al., 2006), one of the symptoms of vaginal candidiasis. In South Africa the tea from the leaves and roots of guava by infusion is prepared for the treatment of non-specific venereal diseases (Van Vuuren and Naidoo, 2010) and in Cuba, the use of *P. guajava* in folk medicine was also registered against fungi, where parts of the plant

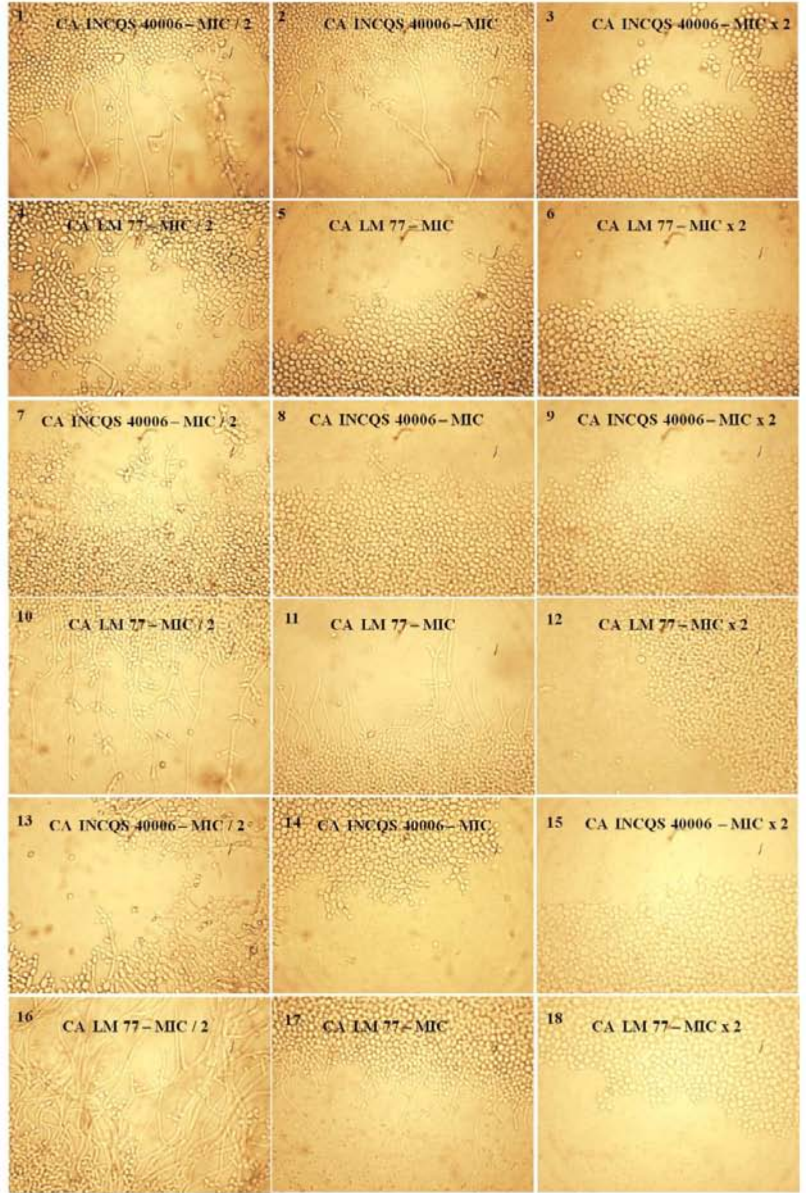

Figure 4 Effect of *Psidium guajava* extracts on the morphology of *Candida albicans*. Concentrations MIC/2, MIC and MIC × 2 (4096, 8192 and 16384 μg/mL, respectively). Aqueous Extract of *P. guajava* Decoction (AEPGD): 1–6; Aqueous Extract of *P. guajava* Infusion (AEPGI): 7–12; Hydroethanolic Extract of *P. guajava* (EHPG): 13–18. INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. Images inspected under a light microscope using a 40× objective.

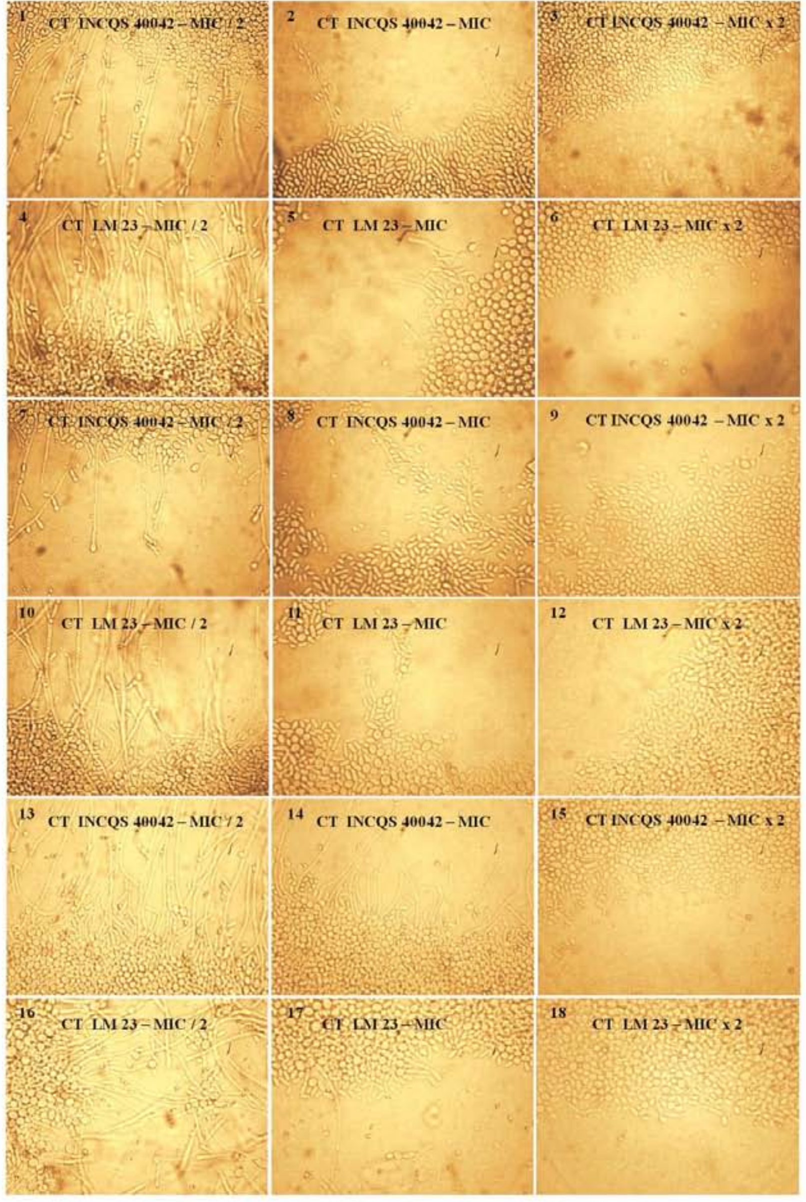

Figure 5 Effect of *Psidium guajava* extracts on the morphology of *Candida tropicalis*. Concentrations MIC/2, MIC and MIC × 2 (4096, 8192 and 16384 μg/mL, respectively). Aqueous Extract of *P. guajava* Decoction (AEPGD): 1–6; Aqueous Extract of *P. guajava* Infusion (AEPGI): 7–12; Hydroethanolic Extract of *P. guajava* (EHPG): 13–18. INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. Images inspected under a light microscope using a 40× objective.

are used in the preparation of dye, powder and elixir (Ramirez et al., 2007). Based on these ethnobotanical reports, we can assume that the main form of therapeutic use *P. guajava* against fungi is the topical use, as the natural product is placed directly on the skin or mucosa, used in mouthwash and gargle, in sitz baths and even in tea administration, which when taken favors the contact of the natural product with the intestinal lumen, where the infection causing microorganisms can be accommodated.

If we consider the context of this research, a cup of tea (made by decoction and infusion) with 150 mL of water and 10 g of fresh leaves, will be contained in this volume, just over 4 times the concentration considered as MIC, was able to reduce the percentage of viable microorganisms by direct contact. The same situation is extended to hydroethanolic extract. In 150 mL of tincture is contained 25 times the minimum inhibitory concentration. If relate to the preparation of virulence potential inhibitor in 150 mL of aqueous extracts of plants have about 2 times the concentration at which the filamentous structures of *Candida* have been reduced. In this same volume hydroethanolic extract is contained 12 times the inhibitory concentration dimorphism. Thus a direct contact of tea or tincture prepared in the above relation not only reduces the percentage of viable microorganisms, but also disturbs the process of morphological yeast transition that remains in place after the addition of home-made preparations, neutralizing one of its virulence factors the ability to invade substrates.

Due to the ethnomedicinal use of *P. guajava* observed both in the traditional medicine as complementary and alternative medicine, the plant is now part of the list of medicinal plants of the World Health Organization (WHO). Based on fundamental criteria such as common use in at least two regions of WHO and satisfactory amount of scientific data, this organization has promoted the development of monographs in which relevant information about this and other species of medicinal relevance, was made available to the public access (WHO, 2009). *P. guajava* also reported in national lists of medicinal plants in some countries and is covered in public policy programs focused on primary health care, as occurs, for example, in Brazil (BRASIL, 2009; RENISUS, 2009).

The belief system of some people, low economic power of users, the medications available at minimal cost, the lack of access to another type of therapeutic resource in conflict areas (especially in poor countries), the fact that they are natural products and considered by some to be more effective than allopathic medicines and cause side effects or milder side effects compared to commercial drugs are some of the reasons given to justify the significant use of medicinal plants (Adnan et al., 2014; Khan et al., 2014; OMS, 2002), including the species under study.

Regarding the popular therapy with *P. guajava*, several factors can influence the final result of a treatment as a contact time of natural product with the infection microorganisms, duration of treatment, methods of use, among others.

Although we are talking about parts of a plant that has its fruit habitually used in nutrition for human populations from different locations, the use of tea fresh leaves for infusion has had its cytotoxic potential investigated. The aqueous extract intragastric administration in rats of both sexes (doses of 0.2, 2.0 and 20.0 g/day) for prolonged period (six months) resulted in signs of hepatotoxicity and renal problems as hydronephrosis in males and pyelonephritis, and nephrocalcinosis in females. The LD_{50} of the extract was more than 20.0 g/kg (Attawish et al., 1995).

Almeida et al. (2006) evaluated in vitro the cytotoxicity of tea made by infusing peritoneal macrophages of mice. The infusion of the leaves was prepared and tested both immediately as a few hours after preparation. Soon after preparation, the infusion was added to the culture environment which exhibited 10% mortality rate, increasing to 31.82% after storage at 4 °C for a period of 48 h. After this period, the index rose to 76.18% revealing that the infusion, therefore, presents an immunotoxic effect. In this sense, taking tea in the same day it is prepared can prevent damage being caused to the cells of the immune system, which, according to the authors, may be due to flavonoids oxidation and subsequent release of their derivatives capable to generate radicals free, which would cause toxicity.

People affected with candidiasis usually present with some impairment of their immune responses and in this sense, would be at serious risk, and may further compromise the body's defense case, for lack of such risks, adopt an inadequate alternative therapy. These studies therefore point to a cautious use of tea as much as the duration of treatment, preparation and storage even as the administration of excessive amounts.

The antifungal effect of the species *P. guajava* reported here may be due to the presence of phenolic compounds in the extracts, since they are able to promote both inhibition of growth of *Candida* lineages (Alves et al., 2014; Barros et al., 2013; Candiracci et al., 2011; Tempesti et al., 2012; Vashisth et al., 2013; Candiracci et al., 2012), as well as their filamentous structures resulting from the transition process (Candiracci et al., 2012; Canonico et al., 2014). The percentage of phenolic compounds of the hydroethanolic extract was more pronounced compared to aqueous extracts as well as their potential inhibitor, as can be seen in cell viability curve. However, further investigations are needed to elucidate the mechanisms by which act the extracts and which in fact, are the phytochemicals contained therein, responsible for the observed effect.

P. guajava, in subsequent studies, had its antifungal potential investigated obtaining results favorable for different methodologies (Assuncсedil et al., 2013; Jebashree et al., 2011; Mailoa et al., 2014; Suwanmanee et al., 2014), but this was the first report which was investigated and verified its influence on a virulence factor of *Candida*.

4. Conclusion

The use of teas, pastes, plasters and sitz baths prepared from leaves of *P. guajava* (red guava) for different populations had, in this study, their potential bioactive scientifically justified through tests with leaf extracts by direct contact, since, besides provoking a decrease in the population of microorganisms of the genus Candida, affected an important fungal virulence factors, morphological transition, and consequently their invasive potential of tissues. The observed antifungal effect is fungistatic and not fungicidal, since he did not kill the fungi. However, it is important to remember that the existing cultural complex systems in these populations allow different forms of therapeutic preparations with amounts of ingredients that may be different from that used in our tests, it is known that there is no standardization when it comes to the use of medicinal plants. Further studies are needed to

understand the genetic and biochemical processes involved in both dynamic fungistatic as in inhibiting emissions of cell extensions of *C. albicans* and *C. tropicalis* in its virulence.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors are grateful to the Brazilian Research agencies: CNPq, CAPES and FUNCAP by the financial and grant support.

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International Journal of Food Properties

ISSN: 1094-2912 (Print) 1532-2386 (Online) Journal homepage: http://www.tandfonline.com/loi/ljfp20

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To cite this article: Maria Flaviana B. Morais-Braga, Joara N. P. Carneiro, Antonio J. T. Machado, Débora L. Sales, Dara I.V. Brito, Rosimeire S. Albuquerque, Aline A. Boligon, Margareth L. Athayde, João T. Calixto Júnior, Djair S. L. Souza, Edeltrudes O. Lima, Irwin R. A. Menezes, José G. M. Costa, Felipe S. Ferreira & Henrique D. M. Coutinho (2016) High-Performance Liquid Chromatography-Diodic Array Detector, Fungistatic, and Anti-Morphogenical Analysis of Extracts from Psidium brownianum Mart. ex DC. Against Yeasts of the Genus Candida, International Journal of Food Properties, 19:8, 1837-1851, DOI: 10.1080/10942912.2015.1079786

To link to this article: http://dx.doi.org/10.1080/10942912.2015.1079786

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International Journal of Food Properties, 19:1837–1851, 2016
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ISSN: 1094-2912 print/1532-2386 online
DOI: 10.1080/10942912.2015.1079786

High-Performance Liquid Chromatography-Diodic Array Detector, Fungistatic, and Anti-Morphogenical Analysis of Extracts from *Psidium brownianum* Mart. ex DC. Against Yeasts of the Genus *Candida*

Maria Flaviana B. Morais-Braga[a], Joara N. P. Carneiro[a], Antonio J. T. Machado[a], Débora L. Sales[a], Dara I.V. Brito[b], Rosimeire S. Albuquerque[a], Aline A. Boligon[c], Margareth L. Athayde[c], João T. Calixto Júnior[d], Djair S. L. Souza[e], Edeltrudes O. Lima[f], Irwin R. A. Menezes[b], José G. M. Costa[b], Felipe S. Ferreira[b], and Henrique D. M. Coutinho[b]

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We assessed extracts from *Psidium brownianum* for antifungal activity and identified the phenolic phytocompounds. Minimum inhibitory concentration was determined by microdilution and IC_{50} was calculated. The minimun fungicidal concentration and the morphology of *Candida* were evaluated. Extracts analyzed by high-performance liquid chromatography demonstrated flavonoids and phenolic acids. The minimum inhibitory concentration was 8192 μg/mL and the IC_{50} varied between 1056 and 5128 μg/mL. Extracts showed fungistatic effect and altered the dimorphism of the strains, being the better result observed using the decoction, that affected the fungal dimorphism of the strain CA ATCC40006 at 4096 μg/mL.

Keywords: Antifungal activity, Dimorphism, *Psidium brownianum*, *Candida*.

INTRODUCTION

Infections caused by *Candida* species have been considered to be one of the biggest problems related to human fungal pathogens. The great and severe impact of the pathogenesis is related to

Received 30 March 2015; accepted 2 August 2015.
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the impairment of the immune system and the use of certain drugs that allow the emergence of invasive candidiasis, as for example, antibiotics against anaerobic microorganisms.[1] *Candida albicans* is a commensal polymorphic fungus and part of the human microbiota[2] with the highest prevalence of isolation either in healthy or diseased organisms.[3] Regarded as a harmless commensal microbe, it may change status, becoming, in favorable conditions, an opportunistic pathogen capable of causing common clinical situations, such as thrush and vaginitis, to severe life-threatening infections, such as systemic infection known as candidemia.[4]

A similar behavior is found in the fungus *Candida tropicalis*, which belongs to the same family (Saccharomycetaceae—Ascomycete) and same clade (CUG) as *C. albicans*.[5] This fungus is responsible for high mortality rates ranging from 40 to 70%,[6] where there are reports stating that in the intestine of cancer patients, it is much more invasive than *C. albicans*.[7] Drug resistance of these microorganisms has been determined and often reported to the point that many classes of antifungals have already been produced. The arsenal of drugs is designed to neutralize the virulence factors expressed by fungi as well as resistance mechanisms additivity or even multiple mechanisms.[8–10]

Some studies have sought alternative sources of compounds able to neutralize virulence factors and mechanisms of fungal resistance, such as those investigating natural products (whether from plants or animals or other organisms) and their bioactive potential against fungi.[11,12] However, many of them have been unable to explain the mechanisms by which these products act. Plants of the genus *Psidium* have been studied with regard to antifungal activity, in particular the species *Psidium guajava*.[13] Besides them, *Psidium sartorianum*,[14] *Psidium acutangulum*,[15] and *Psidium guineense* Sw[16] have also been evaluated. *Psidium brownianum* Mart. ex DC. Occurs as a shrub or tree, reaching up to 0.5 to 8 m. It is a glabrous plant with leathery leaves and usually short petiole. Its flowers are white, and the fruit can vary between elliptical and striated and globose and grooved.[17,18] It can often be found in regenerating areas, and its presence has been recorded in Northeast and Southeast Brazil, inhabiting different geographic areas, such as Caatinga, Cerrado, and Atlantic Forest.[18] The ethnobotanical use of *P. brownianum* has been observed in some places as food (fruit) or medicinal purposes (sprouts), for example, for the treatment of flu.[19] To date, there has been no report on biological activity of this species. The aim of this study was to evaluate the antifungal activity of extracts of *P. brownianum*, comparing the kinds of extracts by their methods of preparation (decoction, infusin, and hydroethanolic), efficacy and effect against the fungal morphology.

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MATERIAL AND METHODS

Collection Area

The area is characterized as an enclave of Cerrado *sensu stricto*[20] it is located in northeast Chapada do Araripe (7° 21.685' S and 39° 28.605' W, at 907 m asl; 7° 21.793' S and 39° 28.605' W, at 902 m asl; 7° 21.787' S and 39° 28.558' W, at 906 m asl) municipality of Crato, Ceara, in Northeast Brazil (Fig. 1). Chapada do Araripe is situated at the border of the states of Ceará, Piauí, and Pernambuco. It is a plateau, with a maximum altitude of 1000 m and minimum of 700 m. The predominant soil type is dystrophic red latosol.[21] The average annual precipitation is about 760 mm, concentrated between the months of January and April (66.3%), and the average annual temperature is 24.1°C.[22]

Plant Material

The study was conducted using young, healthy leaves of a *Psidium* species locally known as araçá de veado, which were collected and transported to the Laboratory of Microbiology and Molecular

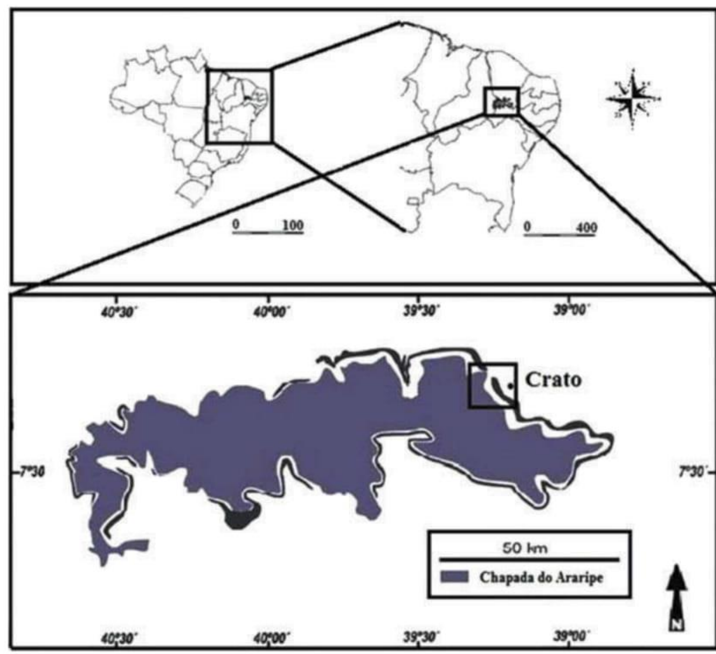

FIGURE 1 Collection area of *Psidium brownianum*.

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Biology at the Regional University of Cariri—URCA. Twigs with flowers of the species were also collected and vouchers were produced and deposited in the Herbarium Dárdano de Andrade Lima at the university under No. 10161, where the species was identified as *Psidium brownianum* DC. The collection period included January, February, March, and April, known as the "wintry block of the Cariri Ceara region." Collections were made between 8:30 and 10:30 am, and the plant material was taken to the laboratory. Leaves uncontaminated by parasites were washed and dried before being weighted and stored under refrigeration. Altogether, there were 2866 kg of leaves in perfect condition, and this quantity was divided for preparation of three types of extracts: hydroethanolic extract (70%)—EHPB, aqueous extract by decoction—AEPBD and water extract by infusion—AEPBI.

Preparation of Extracts

Aqueous extracts

Two types of aqueous extracts with natural tap water were prepared, each using 399.9 g of leaves mixed with 6 L of water (based on a proportion of 10 g/150 mL, equivalent to one cup of tea—150 mL). The decoction was made by mixing roughly cut leaves in cold water and then boiling for 15 min. Afterward, the tea was allowed to cool (4 h and 45 min), filtered and then stored under refrigeration. As for the infusion, the water was boiled without leaves, which were placed in the water after turning off the heat. The pot was covered with a lid and allowed to stand for 4 h 45 min until the tea cooled down,[23] and the preparation was then filtered and stored under refrigeration. Infusion and decoction were frozen (–60°C) and lyophilized to dryness.

The powdered extracts were stored under refrigeration for testing, using 24.9 and 23.3 g extract powder from the decoction and infusion, respectively.

EHPB

The EHPB (70%) was prepared by trituration with cold extraction, using a total of 2 kg leaves in a proportion of 5 g/mL of hydroethanol solution.[23] The leaves were cut to increase contact surface with the solvent, and the mixture was left at room temperature protected from air and light, for a period of 96 h to for maximum extraction efficiency. The mixture was then filtered and placed in a rotary evaporator (Q-344B—Quimis—Brazil) at 40 rpm and 60°C to concentrate the extract. Finally, the crude extract was frozen, lyophilized (568 g) and then stored under refrigeration.

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Chemical Analysis

Chemical, apparatus, and general procedures

All chemicals were of analytical grade. Methanol, acetic acid, gallic acid, caffeic acid, ellagic acid, and chlorogenic acid were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, rutin, kaempferol, luteolin, catechin, and coumarin were acquired from Sigma Chemical Co. (St. Louis, MO, USA). High-performance liquid chromatography–diode array detector (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A DAD, and LC solution 1.22 SP1 software.

HPLC-DAD

Reverse-phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm × 250 mm) packed with 5 μm diameter particles; the mobile phase was water containing 2% formic acid (A) and acetonitrile (B), and the composition gradient was: 17% of B until 10 min and changed to obtain 20, 30, 50, 60, 70, 20, and 10% B at 20, 30, 40, 50, 60, 70, and 80 min, respectively, following the method described[24] with slight modifications. The extracts solutions of *P. brownianum* (hidroethanolic—EHPB, infusion—AEPBI and decoction—AEPBD) were prepared at 20 mg/mL and the mobile phase were filtered through 0.45 μm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. The flow rate was 0.7 mL/min and the injection volume was 50 μL. Stock solutions of standards references were prepared in water: acetonitrile (1:1; v/v) at a concentration range of 0.025–0.250 mg/mL catechin, coumarin, quercetin, quercitrin, kaempferol, luteolin, and rutin, and 0.035–0.350 mg/mL for gallic, caffeic, ellagic, and chlorogenic acids. Quantification was carried out by integration of the peaks using the external standard method, at 270 nm for gallic acid and coumarin, 281 nm for catechin, 327 nm for chlorogenic, ellagic, and caffeic acids, and 366 for quercetin, quercitrin, luteolin, kaempferol, and rutin. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200 to 600 nm). Calibration curve for gallic acid: $Y = 13682x + 1284.5$ ($r = 0.9999$); catechin: $Y = 11956x + 1260.3$ ($r = 0.9995$); caffeic acid: $Y = 11943x + 1198.7$ ($r = 0.9998$); chlorogenic acid: $Y = 12601x + 1327.1$ ($r = 0.9994$); ellagic acid: $Y = 13075x + 1283.9$ ($r = 0.9997$); rutin: $Y = 12853x + 1186.7$ ($r = 0.9996$); quercetin: $Y = 11968x + 1273.9$ ($r = 0.9998$); quercitrin: $Y = 12658x + 1249.7$ ($r = 0.9995$), coumarin: $Y = 13159x + 1358.2$ ($r = 0.9990$), kaempferol: $Y = 11983x + 1275.8$ ($r = 0.9999$), and luteolin: $Y = 12496x + 1195.4$ ($r = 0.9997$). All chromatography operations were carried out at ambient temperature and in triplicate.

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Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves.[25] LOD and LOQ were calculated as 3.3 and 10 σ/S, respectively, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Antifungal Assay

Strains and culture media used

Standard types of strains were obtained from the Culture Collection of Oswaldo Cruz of the Brazilian Institute of Quality Control in Health (INCQS) and clinical isolates of the yeasts *Candida albicans* and *Candida tropicalis* were provided by Dr. Edeltrudes Oliveira Lima (Mycology Laboratory of Paraíba Federal University), namely CA INCQS 40006, CA LM 62, CA LM 77, CA LM 109, CA LM 111, CA LM 122, CT INCQS 40042, CT LM 18, CT LM 20, and CT LM 23. These strains were inoculated into Sabouraud dextrose agar (SDA, KASVI) and incubated for 24 h at 37°C. Afterward, small aliquots of yeast were transferred to test tubes each containing 3 mL of sterile saline (0.9%). The concentration of the inoculum was standardized by 0.5 McFarland, giving a standard yeast suspension of 1×10^5 cells/mL.[26] The inocula thus prepared were used to determine the minimum inhibitory concentration (MIC) in Sabouraud dextrose broth (SDB, HIMEDIA), double concentrated. Another culture medium was used for analysis of yeast micromorphology. The potato dextrose agar (PDA, DIFCO) was prepared by diluting it more than that recommended by the manufacturer to make it a depleted medium capable of stimulating yeast to produce hyphae. Agar was added to this diluted medium to obtain a solid medium.

Drugs, reagents, and preparation of solutions

Dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) was used for dilution of the extracts, and the antifungal fluconazole (Capsule—FLUCOMED), diluted in water, was used as the reference drug. The matrix solutions of the extracts were prepared by weighing 0.3 g of each extract and then diluting in 1 mL of DMSO. To obtain the desired concentration for testing, the extracts were further diluted in sterile distilled water so that the concentration of DMSO in the natural product did not exert any activity in the test cells.[27]

Microbiological Screening

Microbiological screening was performed to select the yeasts to be used in microbiological testing. The microdilution broth was chosen to perform this procedure, and this was done by determining the MIC.[28] The plates prepared to carry out this test would be used later in tests to find the minimum fungicidal concentration (MFC), besides facilitating the demonstration of cell viability curve and calculating the IC_{50} of the test products.

Determination of MIC

This test was performed by the broth microdilution method in 96-well plates. Each well was filled with 100 µL of SDB containing 10% fungal inoculum, and then, 100 µL of the natural product (16384 µg/mL) or fluconazole (antifungal reference) at the same concentration, were added to the first well, followed by twofold serial dilution. The concentrations in the wells ranged from 64 to 8192 µg/mL. The last well contained no extract or drug and served as the normal growth control.[28] Controls for diluent of the products (using saline instead of inoculum) and the sterile

medium were also prepared. All tests were performed in triplicate. The plates were incubated at 37° C for 24 h and afterward read in an enzyme linked immuno sorbent assay (ELISA) spectrophotometer (Thermoplate®) at a wavelength of 630 nm. The MIC was defined as "the lowest concentration of an antimicrobial agent that inhibit the visible growth of a microorganism in dilution assays."[26] The results obtained in the ELISA readout were used to construct the cell viability curve and the IC_{50} of the extracts of *P. brownianum*.

Determination of MFC

For this test, a small sterile rod was placed in each well of the MIC test plate (except for sterility control). After mixing the medium in each well, the rod was taken to a large petri dish containing SDA, streaking its surface and transferring the solution (medium + inoculum + natural product) for subculture of yeast and checking cell viability. After 24 h incubation, the plates were inspected for any formation of colonies of *Candida*[29] (with modifications). The concentration at which there was no growth of fungal colonies was considered the MFC of the natural product.

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Effect of Natural Products on Fungal Morphology

To determine if the natural product caused any change in fungal morphology, by inhibiting the development of hyphae, sterile micromorphological chamber slides were prepared for observation of yeasts. Three milliliters of PDA medium depleted by dilution were added to chambers, containing the natural product concentrations MIC/2, MIC and MIC × 2. Aliquots of the inoculi were taken from the petri dishes to make two parallel streaks on the solid medium, which were then covered with a sterile coverslip. The chambers were placed in the incubator for 24 h (37°C) and inspected under a light microscope using a 40× objective. A camera was attached to the microscope to capture images randomly at 5× zoom. A control for yeast growth (hyphae stimulated by depleting medium) was performed, as well as a control with the conventional antifungal fluconazole for comparative purposes and a control with DMSO at 100 and 0.5% (the concentration in the natural products used in the tests with some modifications).[30,31]

Statistical Analysis

The results of the tests were done in triplicate. Data obtained for each sample and concentration were checked for their normal distribution and then analyzed by one-way ANOVA by post-hoc Tukey test. EC_{50} values were obtained by non-linear regression for the purpose of interpolating values from standard curves (using the software Graphpad Prism, v. 5.0) of the percentage growth values plotted against concentration and EC_{50} values are expressed as μg/mL.

RESULTS AND DISCUSSION

HPLC was used to analyze the chemical composition of the *P. brownianum* extracts, which detected and quantified the phenolic compounds present. However, as it can be seen in Fig. 2, the HPLC profile, the HPLC profile showed other minor compounds in addition to gallic acid (retention time-t_R 9.98 min, peak 1), catechin (t_R = 14.35 min, peak 2), chlorogenic acid (t_R = 22.03 min, peak 3), caffeic acid (t_R = 25.11 min, peak 4), ellagic (t_R = 33.07 min, peak 5), rutin (t_R = 38.49 min, peak 6), quercitrin (t_R = 47.68 min, peak 7), quercetin (t_R = 49.73 min, peak 8), coumarin (t_R = 53.81 min, peak 9), kaempferol (t_R = 55.78 min, peak 10), and luteolin (t_R = 61.94 min, peak 11). The findings for each extract are shown in Fig. 2, with the

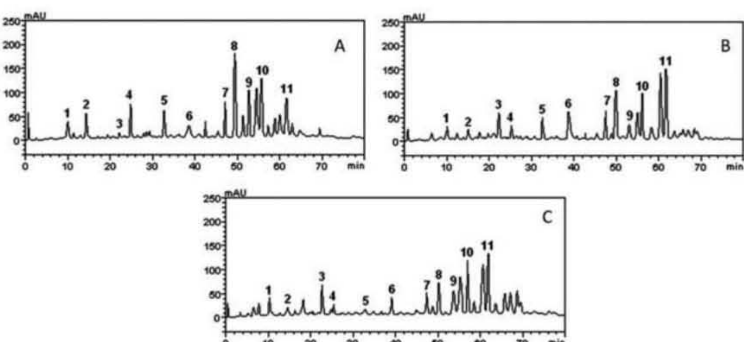

FIGURE 2 High-performance liquid chromatography phenolics and flavonoids profile of *Psidium brownianum*.

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peaks confirmed by chromatography, and the amounts for each chemical constituent are given in Table 1, demonstrating the presence of flavonoids and phenolic acids.

The different times of exposure to temperature of the aqueous (decoction and infusion) extracts resulted in the extraction of compounds in different amounts, where the decoction showed slightly higher amounts than the infusion with little significant difference. However, the decreasing order of the major constituents also varied: decoction: luteolin > quercetin > kaempferol > quercitrin > rutin; and infusion: luteolin > kaempferol > quercetin > chlorogenic acid > quercitrin acid. The results for the EHPB of the species showed the five major ones in the following order: quercetin > kaempferol > coumarin > quercitrin > luteolin. In terms of the amount of phenolic compounds extracted from 20 mg of each extract, the order was EHPB > AEPBD > AEPBI, showing that the EHPB was richer in these phytochemicals (Table 1).

The antimicrobial screening revealed that a minimum concentration of the extract able to inhibit the growth of the strains was the same (8192 μg/mL), and thus, the selection of the strains was

TABLE 1
Phenolics and flavonoids composition of *Psidium brownianum*

	P. Brownianum				
Compounds	*EHPB mg/g*	*AEPBD mg/g*	*AEPBI mg/g*	*LOD μg/mL*	*LOQ μg/mL*
Gallic acid	3.15 ± 0.02 a	1.73 ± 0.01 a	2.98 ± 0.01 a	0.019	0.062
Catechin	4.28 ± 0.01 b	1.69 ± 0.01 a	1.70 ± 0.03 b	0.008	0.025
Chlorogenic acid	0.09 ± 0.01 c	4.05 ± 0.03 b	3.95 ± 0.02 c	0.024	0.081
Caffeic acid	5.41 ± 0.03 d	1.76 ± 0.01 a	1.68 ± 0.01 b	0.035	0.116
Ellagic acid	4.35 ± 0.01 b	3.84 ± 0.02 b	1.62 ± 0.01 b	0.010	0.034
Rutin	2.67 ± 0.01 e	4.29 ± 0.01 c	2.89 ± 0.03 a	0.017	0.056
Quercitrin	5.63 ± 0.02 f	4.32 ± 0.01 c	3.11 ± 0.01 a	0.032	0.105
Quercetin	11.54 ± 0.02 g	7.08 ± 0.03 d	4.05 ± 0.02 c	0.025	0.083
Coumarin	7.18 ± 0.02 h	1.81 ± 0.02 a	3.10 ± 0.03 a	0.011	0.037
Kaempferol	8.93 ± 0.01 i	6.97 ± 0.01 d	6.92 ± 0.01 d	0.018	0.059
Luteolin	5.61 ± 0.01 f	10.34 ± 0.02 e	8.37 ± 0.01 e	0.023	0.075

Results are expressed as mean ± standard deviation of the determinations; Averages followed by different letters on each extract differ by Tukey test at $p < 0.05$; AEPBI: Aqueous extract of *P. brownianum* infusion; AEPBD: Aqueous extract of *P. bronianum* decoction; EHPB: Hidroethanolic extract of *P. brownianum*.

performed randomly. For continuity of the work, we selected the yeast strains INCQS CA 40006, CA LM 77, INCQS 400042 CT, and CT LM 23. Therefore, for comparative purposes, a standard strain and a clinical strain isolated from each species were used. Fluconazole was used as the control, which, as expected, had a lower IC50 (58.63 to 76.72 µg/mL) compared to the extracts (1056.82 to 5128.61 µg/mL; Table 2). In this context, extracts of *P. brownianum* demonstrated antifungal potential due the fact of inhibit the virulence mechanism of morphogenesis. So, these extracts can be used against the candidiasis, but not against systemic infections due the high doses required for this activity.

The IC_{50} was calculated for each test product using the ELISA readings, where the IC_{50} values differed but were generally high, ranging from 1056.82 to 5128.61 µg/mL (Table 2). A graph demonstrating the effect on cell viability of the selected yeasts is presented in Fig. 3. It is important to note that the type of solvent and extraction method did not influence the results obtained from the MIC and MFC tests. Also, the determination of the potential to inhibit the transition of yeasts was not affected, since aqueous extracts (obtained using different times of exposure to heat) and EHPBs showed the same effect at the same concentrations tested.

To determine the concentration of the test products necessary to exert a fungicidal effect, tested concentrations ranged from 64 to 8192 µg/mL and after 24 h incubation, fungal growth was observed in all cases, showing that the minimum fungicidal concentration was higher than 8192 µg/mL. DMSO (100%) was found to have an effect on the morphology of *Candida* yeasts (data not shown), but at the concentration at which the products were diluted for testing, this activity was absent, as can be seen in Fig. 4.

Fluconazole, a drug used as a control in testing, is a drug that acts on the fungal membrane, causing loss of integrity.[32] It showed an inhibitory effect on hyphae at a lower concentration compared to the products tested, as can be seen in Fig. 4. This effect was expected, since the MIC of this antifungal was well below the values expressed by the products tested. The extracts tested, at the highest concentration (16384 µg/mL), prevented the morphological transition of yeasts, with the exception of strain CA 40006, which was affected by MIC/2 (4096 µg/mL) of decoctio, with progressive inhibition at higher concentrations (8192 and 16384 µg/mL; Figs. 5 and 6). The extracts probably influenced the genetic and biochemical processes occurring in the cell wall of yeast, disrupting the formation of pseudohyphae and hyphae and thereby affecting one of the factors responsible for the virulence of the species, the ability to invade substrates. The activity of phenolic compounds against *Candida* has been reported in some studies,[33–36] also demonstrating a concentration-dependent effect.[37] The extracts evaluated here just started to

TABLE 2
IC_{50} of all products assayed (µg/mL)

Yeast strains	*EHPB*	*AEPBD*	*AEPBI*	*Fluconazole*
CA INCQS 40006	**1056.82**	**2924.15**	**4073.8**	**76.72**
CA LM 62	1840.77	3872.58	4764.31	81.35
CA LM 77	**1199.5**	**3090.3**	**4168.69**	**69.09**
CA LM 109	1396.37	3689.78	4602.57	72.49
CA LM 111	1862.09	2460.37	4677.35	103.4
CA LM 122	1076.47	3162.28	4677.35	103.4
CT INCQS 40042	**1333.52**	**3589.22**	**5011.87**	**73.98**
CT LM 18	1651.96	3890.45	4943.11	391.2
CT LM 20	1815.52	3630.78	5128.61	205.5
CT LM 23	**1883.65**	**3597.49**	**4655.86**	**58.63**

AEPBD: Aquous extract of *P. brownianum* infusion; EHPB: Aquous extract of *P. bronianum* decoction; Hidroethanolic extract of *P. brownianum*. The indicated strains in bold were utilized in the assays.

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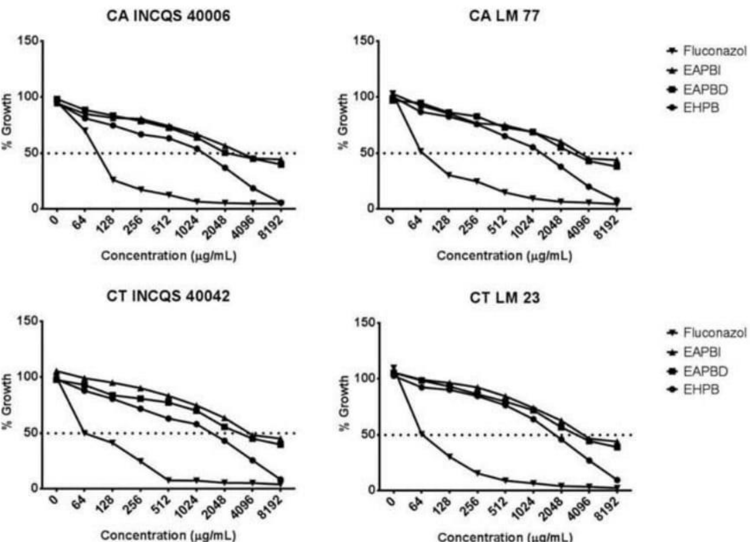

FIGURE 3 Cell viability curve *Candida* strains under the effect of *Psidium brownianum*.

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show some antifungal activity when the concentration of phenolic compounds in the extract gradually increased as demonstrated in the cell viability curves.

Micromorphological analysis could indicate a possible mechanism of action of the tested products, because it allows the observation of changes in morphology of the strains. The formation of hyphae and pseudohyphae, observable in a micromorphology assay are important virulence factors in the development of candidiasis.[38] The morphological transition between the yeast and hyphal forms of *Candida*, that is, dimorphism, is one of the pathogenicity mechanisms appearing as a fitness attribute in superficial and systemic infections, where yeast and hyphae have spreading and invasive potential, respectively.[2] Morphological changes in *Candida* are stimulated by various environmental factors, and thus, changes in pH, CO_2, temperature, depletion of nutrients, serum or presence of N-acetylglucosamine, as well as quorum sensing mechanisms, can promote the formation of hyphae.[2] This hyphal growth depends on the expression of various genes of cell wall proteins, transduction pathways, transcription factors, down regulation, activation of cyclic nucleotide-dependent protein kinase, phosphorylation reactions, and some ribosomal proteins.[39] The adhesion of the hyphae to the surface at the time of invasion, is favored by a set of proteins, adhesins.[2]

In our assays, nutrient depletion was the factor that triggered the development of hyphae. In the presence of depleted medium, the fungi responded to the stress condition by activating the genetic and biochemical processes necessary for the formation of pseudohyphae and hyphae. Figure 4 shows the growth of strains in depleted medium. A previous study found that the flavonoid extract from multifloral honey containing luteolin was able to inhibit the dimorphic conversion of *C. albicans* and that this effect could be related to the inhibition of the production of reactive oxygen species and changes in cellular levels of intracellular glutathione, both factors that significantly influence the conversion of yeasts to hyphae.[37] Also, aimed at elucidating the probable mechanism of action of the effect of this extract on yeast transition, another study found that the inhibitory activity of the

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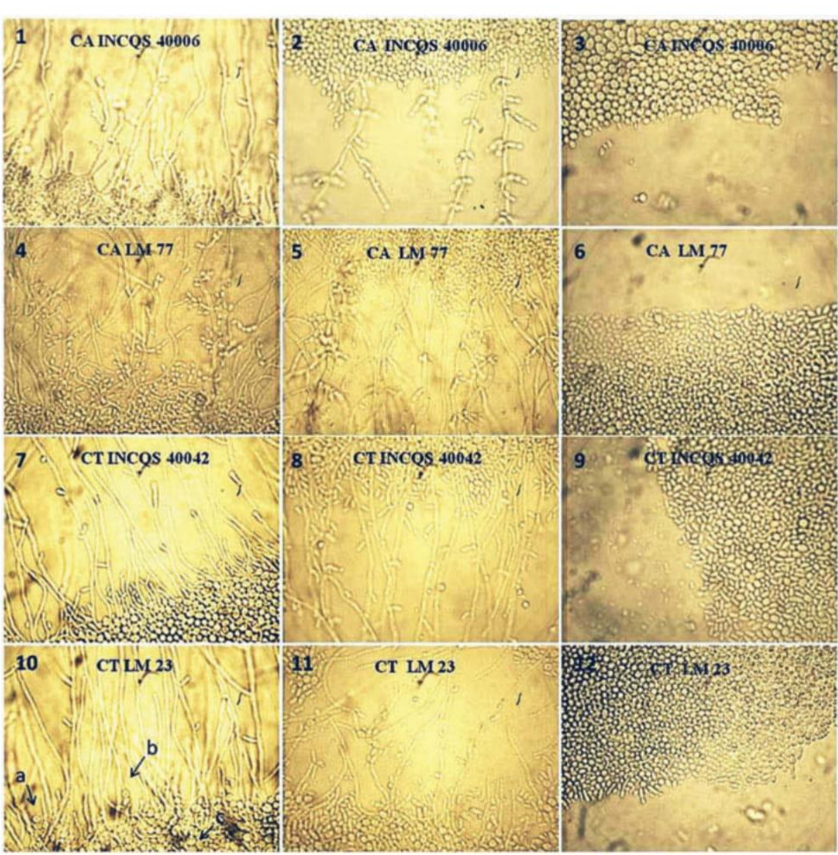

FIGURE 4 Controls used in micromorphologic assay of *Candida* yeast under the effect of the products of *Psidium brownianum*.

flavonoid extract of honey could be attributed to changes in cell cycle progression, membrane integrity, mitochondrial function, and also biogenesis.[40]

Methanolic extract of *Leiothrix spiralis* and luteolin were effective against Candida species; however, only the extract interfered with formation of hyphae, indicating that effect on inhibition of hyphae cannot be attributed to flavonoid luteolin alone, but it was probably due to phenolic compounds presents in methanolic extract.[41] Therefore, considering the potential of phenolic compounds for inhibiting the dimorphic transition process in yeast and their existence in extracts of *P. brownianum*, we can assume that the effect exhibited by extracts was due to these phytochemicals through a single contribution (except for luteolin) or in a synergistic fashion. Luteolin is the major compound in the aqueous extracts, and the fact that its presence did not interfere in the extracts' effect on yeast morphology suggests that the inhibitory effect was due to the activity of other compounds or to their synergistic action. The possibility of luteolin being involved in this possible synergistic process cannot be ruled out.

In the ether extract of Italian multifloral honey, flavonoids including luteolin, quercetin, apigenin, kaempferol and isorhamnetin were found. The extract presented a better effect than

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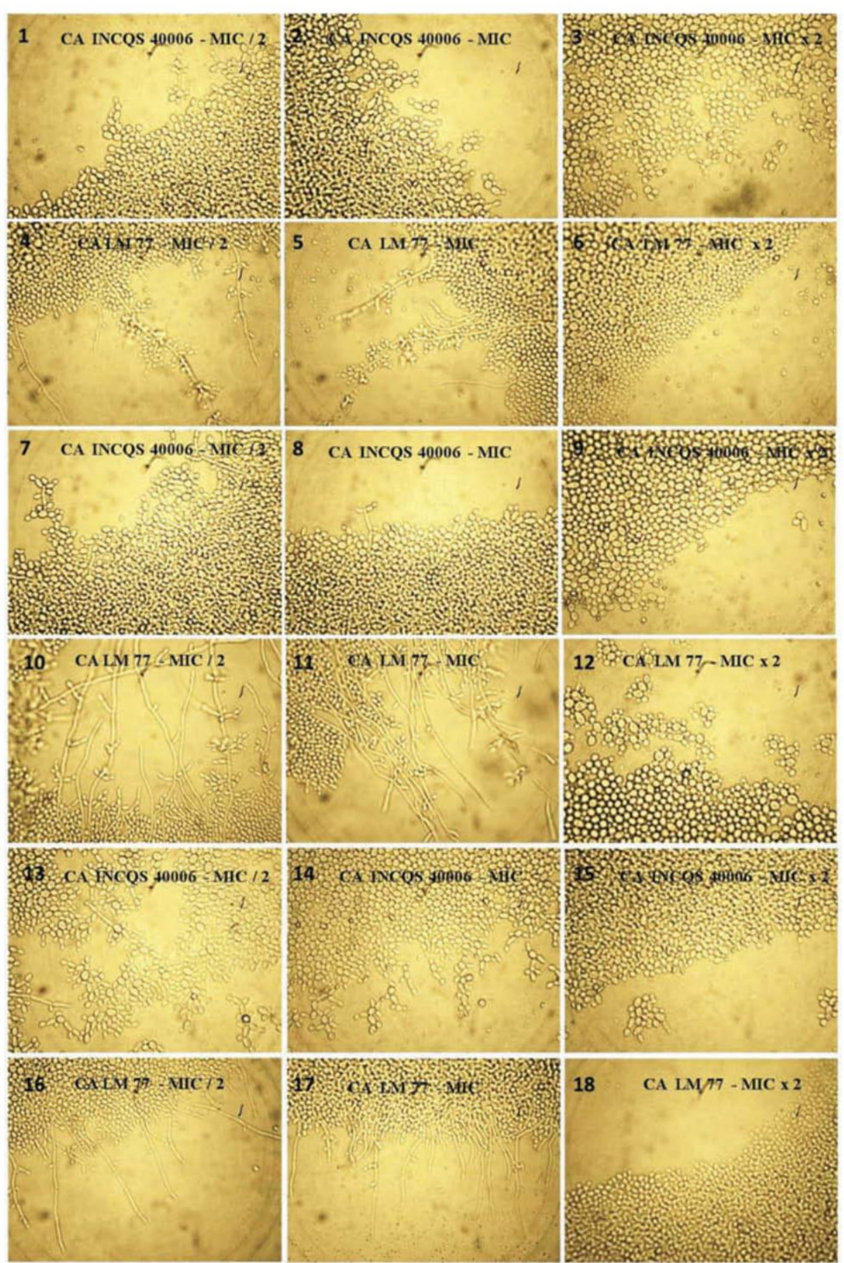

FIGURE 5 Effect of *Psidium brownianum* extracts on the morphology of *Candida albicans*.

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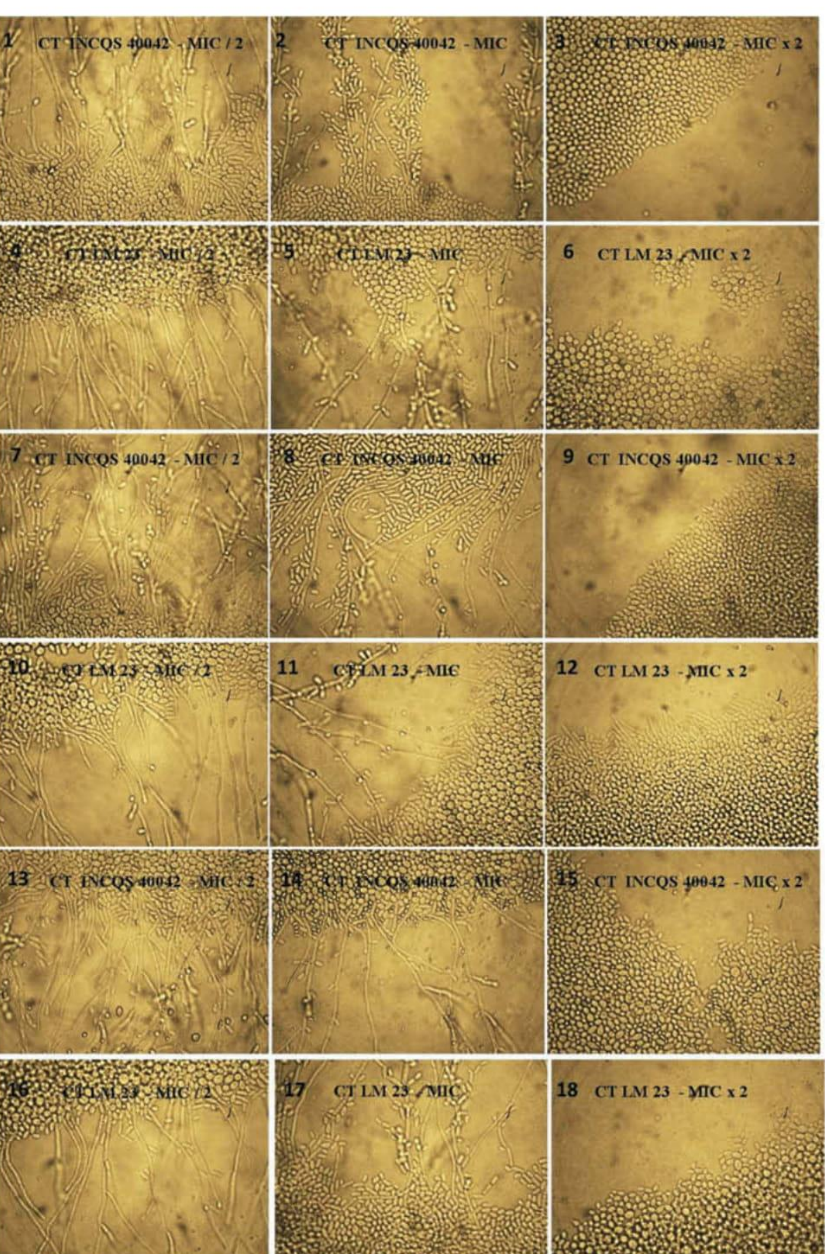

FIGURE 6 Effect of *Psidium brownianum* extracts on the morphology of *Candida tropicalis*.

components separately assayed, suggesting that synergism between phenolic compounds is an important factor in natural products exerting an antimicrobial effect.[42] Finally, given the above, this study showed low antimicrobial activity of *P. brownianum* against *Candida*. However, in relation to mucocutaneous fungal infections caused by *Candida*,[43] the topical use of this natural product can be demonstrated as a local inhibitor of fungal virulence, hindering morphogenesis and the invasion of tissues. Our results did not focus on a direct clinical indication, as many complementary tests are needed, such as toxicity tests.

CONCLUSION

In this study, it was found that the mechanism of action by which the extracts of *P. brownianum* act is by altering capacity for morphological transition in yeasts, preventing the conversion from yeast to hyphae and pseudohyphae. The complete elucidation of this mechanism needs further study. The antifungal potential observed is presumably attributed to phenolic compounds found in the extracts (flavonoids and phenolic acids), whose levels did not significantly differ between the extracts, and their possible synergistic effect. However, more specific studies are needed to reach a definitive conclusion. This is the first report of biological activity for this species, and the results reveal that *P. brownianum* is a source of phytochemicals able to inhibit the morphological transition of strains of *C. albicans* and *C. tropicalis*, neutralizing one of their major virulence factors, the ability to invade substrates and consequently tissues. This effect is potentially promising, but more studies are needed for the possible development of drugs for topical use.

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FUNDING

The authors are grateful to the Brazilian Research agencies: CNPq, CAPES, and FUNCAP for the financial and grant support.

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Microbial Pathogenesis 95 (2016) 200–207

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Microbial Pathogenesis

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Psidium guajava L. and *Psidium brownianum* Mart ex DC.: Chemical composition and anti – *Candida* effect in association with fluconazole

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A R T I C L E I N F O

Article history:
Received 7 November 2015
Received in revised form
11 April 2016
Accepted 12 April 2016
Available online 13 April 2016

Keywords:
Phytochemical characterization
Antifungal effect modulation
Psidium species
Yeasts

A B S T R A C T

The therapeutic combinations have been increasingly used against fungal resistance. Natural products have been evaluated in combination with pharmaceutical drugs in the search for new components able to work together in order to neutralize the multiple resistance mechanisms found in yeasts from the genus *Candida*. The aqueous and hydroethanolic extracts from *Psidium brownianum* Mart ex DC. and *Psidium guajava* L. species were evaluated for their potential to change the effect of commercial pharmaceutical drugs against *Candida albicans* and *Candida tropicalis* strains. The tests were performed according to the broth microdilution method. Plate readings were carried out by spectrophotometry, and the data generated the cell viability curve and IC50 of the extracts against the yeasts. A chemical analysis of all the extracts was performed for detection and characterization of the secondary metabolites. The total phenols were quantified in gallic acid eq/g of extract (GAE/g) and the phenolic composition of the extracts was determined by HPLC. Fluconazole and all extracts presented high Minimum Inhibitories Concentrations (MICs). However, when associated with the extracts at sub-inhibitory concentrations (MIC/16), fluconazole had its effect potentiated. A synergistic effect was observed in the combination of fluconazole with *Psidium brownianum* extracts against all *Candida* strains. However, for *Psidium guajava* extracts the synergistic effect was produced mainly against the *Candida albicans* LM77 and *Candida tropicalis* INCQS 400042 strains. The IC_{50} values of fluconazole ranged from 19.22 to 68.1 μg/mL when it was used alone, but from 2.2 to 45.4 μg/mL in the presence of the extracts. The qualitative chemical characterization demonstrated the presence of phenols, flavonoids and tannins among the secondary metabolites. The concentration of total phenols ranged from 49.25 to 80.77 GAE/g in the *P. brownianum* extracts and from 68.06 to 82.18 GAE/g in the *P. guajava* extracts. Our results indicated that both *P. brownianum* and *P. guajava* extracts are effective on potentiating the effect of fluconazole, and therefore, these plants have the potential for development of new effective drugs for treating fungal infections.

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1. Introduction

The human health is constantly affected by infections caused by microorganisms that are resistant to therapeutic agents. The evolution of these microorganisms, especially the fungi of the genus *Candida*, has generated resistance to a number of antifungal drugs, which has stimulated the search for new effective medications

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http://dx.doi.org/10.1016/j.micpath.2016.04.013
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[1,2].

The World Health Organization (Who) [3] draws attention to the importance of fungal infections outbreaks, especially those caused by Candida species, since they represent the most common fungal infections in humans. According to the WHO, superficial and invasive candidiasis has particularly affected vulnerable people, including those under prolonged health treatment, immunosuppressed subjects and patients in Intensive Care Units (ICU). Nevertheless, despite the clinical and epidemiological relevance of infections caused by *Candida* species, the currently available therapeutic options are limited to three main classes of antifungal drugs: azoles, echinocandins and polyenes; and, in some undeveloped countries, most people have access to only one class of these drugs, because of the lack of financial resources. Moreover, several countries have reported the occurrence of resistance to antifungal drugs, that has been increasing, especially among non-*albicans* species. Finally, most antifungal agents present serious toxic effects, justifying the importance of the search for new, safe and effective antifungal therapeutic agents.

Drug-combined therapy is a practice that has been used for many years. Combining plants through homemade formulations has generated effective therapies for treating several disease types in different locations [4,5]. On the other, many people worldwide often make the concomitant use of medicinal plants and pharmaceutical drugs without prescription, which can cause health problems due to plant-drug interactions [6]. It is also common the combination of different commercial drugs aiming to obtain the benefit of potentiating the therapeutic effectiveness in addition to the economic benefit of using previously acquired drugs [7].

In the scenario of the search for new therapeutic alternatives, the combination of natural products with commercial drugs has emerged as an important tool on drug development research [8,9]. Natural products have been tested for antimicrobial activity in the form of extracts and fractions, in association with commercial drugs, assuming that medicinal herbal formulations have been used as a complementary therapy in the management of a particular disease [10]. The investigation of these products have led to interesting results, and in the long term, i.e. after studies and applications, new therapeutic possibilities can be developed by exploring the desirable beneficial effects of drug interactions.

The *Psidium brownianum* and *Psidium guajava* species investigated in the current study were subjected to *in vitro* antimicrobial tests in order to check whether they present any potential to induce a synergistic effect when combined with the commercial antifungal drug Fluconazole against yeasts from the genus *Candida*.

2. Material and methods

2.1. The collection area

Psidium brownianum species was collected in a strict sense Cerrado area located in Barreiro Grande Farm, to the northeast of Chapada do Araripe (latitude: 07°21.685′ S and longitude: 39°28.605′ W, 907 m above sea level), in Crato County, Ceará State, Northeastern Brazil. *Psidium guajava* species was collected in a private property named Sítio Malhada, Caatinga area, in Milagres County rural area, Ceará State, Northeastern Brazil (latitude: 07° 17.119′ S and longitude: 038° 51.779′ W, 388 m above sea level; latitude: 07° 17.120′ S and longitude: 038° 51.778′ W, 389 m above sea level; latitude: 07° 17.122′ S and longitude: 038° 51.776′ W, 392 m above sea level; latitude: 07° 17.119′ S and longitude: 038° 51.779′ W, 388 m above sea level).

2.2. Plant material

Healthy young leaves of *P. guajava* and *P. brownianum* species were collected and transported to the Microbiology and Molecular Biology Laboratory of the Regional University of Cariri – URCA (Universidade Regional do Cariri). The Herbarium specimens of these species were produced and deposited in the Dárdano de Andrade Lima University Herbarium under numbers 10,161 (*P. brownianum*) and 10,935 (*P. guajava*). The samples were collected between January and April (rainy months) in the morning between 8:30 a.m. and 10:30 a.m. The plant material was weighed and stored under refrigeration after it was screened and cleaned. The total of 2866 g *P. brownianum* leaves and 2650 g *P. guajava* leaves were weighed. This amount was divided in order to prepare three extract types: the hydroethanolic extract (70%), the aqueous extract prepared by decoction and aqueous extract prepared by infusion.

2.3. Preparation of the extracts

Aqueous extracts: two aqueous extract types were prepared, using for each, 399.9 g of the leaves mixed in six liters of water (at 10 g/150 mL ratio, i.e. equivalent to a cup of tea - 150 mL). Decoction was performed by mixing coarsely chopped leaves in cold water and then boiling them for 15 min. After this time, the tea was cooled, filtered and then stored under refrigeration. As for the infusion, the water was boiled without the leaves, which were placed in the water after turning off the fire. The pan was covered and the leaves were kept there until the tea was cooled down [11]. The preparation was then filtered and stored under refrigeration. Both the infusion and the decoction were frozen and taken to a lyophilizer (at −60 °C) until complete removal of water. The powder extracts were stored under refrigeration for testing. After lyophilization, 24.9 g of the Aqueous Extract of *P. brownianum* Decoction (AEPBD), 23.3 g of the Aqueous Extract of *P. brownianum* Infusion (AEPBI), 14.46 g of the Aqueous Extract of *P. guajava* Decoction (AEPGD) and 15 g of the Aqueous Extract of *P. guajava* Infused (AEPGI) were obtained.

Hydroethanolic extract. The hydroethanolic extract (70%) was prepared by trituration followed by cold extraction. A total of 1846.5 g of the *P. guajava* leaves and 2000 g of the *P. brownianum* leaves were used at the ratio of 5 g of the leaves to each mL of the hydroethanolic solution [11]. The leaves were chopped to increase the contact surface with the extractor. The mixture was kept at room temperature and protected from air and light for 96 h in order to increase the extraction efficiency. The macerate was then filtered and transferred to a rotary evaporator (Q-344B - Quimis - Brazil - 40 rpm, 60 °C), in order to concentrate the extract. The crude extracts still containing water were frozen and then lyophilized. Fifty-point-eight (50.8) g of the Hydroethanolic Extract of *P. guajava* (HEPG) and 568 g of the Hydroethanolic Extract of *P. brownianum* (HEPB) were obtained and then they were stored under refrigeration.

2.4. Chemical analysis

2.4.1. Qualitative chemical prospecting

The chemical assays were used for the qualitative analysis of the presence of secondary metabolites. The detection tests to evaluate the presence of phenols, tannins, flavonoids and alkaloids were performed according to the method described by Matos [12]. The tests are based on the visual observation of color modifications and formation of precipitate after the addition of specific reagents.

2.4.2. Quantification of the total phenols

To perform this test, 0.01 g of the extracts were weighed and then diluted in 1 mL dimethylsulfoxide (DMSO). A stock solution (1000 μg/mL) was prepared by diluting the extracts with water. Other concentrations (500, 400, 200, 100, 50, 25 and 10 μg/mL) were prepared from the stock solution and used in the test. One hundred (100) μL of each sample were distributed in test tubes, which were added with 50 μL of Foulin-Ciocalteu; 2000 μL of calcium carbonate and 7850 μL of water. The blank contained Foulin-Ciocalteu, calcium carbonate and water. As for the calibration curve with gallic acid, different compound concentrations (200, 100, 50, 25, 10 and 5 μg/mL) were prepared. The reading was carried out by spectrophotometer at 750 nm. The test was performed in triplicate [13], with some modifications]. Calibration curve for Gallic acid: $y = 0,004500 (\pm 0,0001160)x + 0,02641 (\pm 0,005969)$.

2.4.3. Statistical analysis of the quantification of phenols

The phenol value measured by interpolation was over the linear range of the standard curve and values were expressed as g gallic acid equivalent/kg.

2.4.4. Comparative analysis of the phenol composition

The phenol composition was determined by HPLC-DAD according Kamdem et al. [14] and Silva [15], with modifications. The chromatographic data and tables are available at Morais-Braga et al. [16,17].

2.5. Antifungal test

2.5.1. Culture media and strains

The herein used standard-type strains were obtained from the Oswaldo Cruz Culture Collection at the National Institute of Quality Control in Health (INCQS - Instituto Nacional de Controle de Qualidade em Saúde). Dr. Edeltrudes Oliveira Lima (Mycology Laboratory at Federal University of Paraíba) provided clinical isolates of *Candida albicans* and *Candida tropicalis* yeasts, namely: CA INCQS 40006, CA LM 77, CT INCQS 40042 and CT LM 23. These strains were inoculated in Sabouraud Dextrose Agar (SDA - KASVI) and incubated at 37 °C, for 24 h. The inoculum concentration was standardized according to the McFarland scale, by comparing the inocula turbidity and the 0.5 standard in the scale. The prepared inocula were used in Minimum Inhibitory Concentration (MIC) tests and in the test performed to verify the potential of the extracts on modulating the antifungal activity of fluconazole. Double-concentrated Sabouraud Dextrose Broth culture medium (CSD - HIMEDIA) was used to perform the tests.

2.5.2. Drugs, reagents and preparation of solutions

Dimethylsulfoxide (DMSO - Merck, Darmstadt, Germany) was used to dilute the extracts. Fluconazole (Capsule - FLUCOMED) was diluted in water and used as reference antifungal drug in the tests. The extracts' matrix solutions were prepared by weighing 0.3 g of each extract and then diluting them in 1 mL of DMSO. In order to obtain the desired concentration for the tests, the extracts underwent new dilution in sterile distilled water so that the DMSO concentration in the natural products did affect the tested cells.

2.5.3. Determination of the minimum inhibitory concentration (MIC)

This test was performed in 96-well plates using the broth microdilution method, according to Javadpour et al. [18]. Briefly, serial dilutions were performed yielding natural products and fluconazole concentrations ranging from 64 μg/mL to 8192 μg/mL. The last well was used as microorganism growth control. Product dilutions (using saline instead of inoculum) and medium sterility controls were also achieved. All tests were performed in triplicate. The plates were incubated at 37 °C, for 24 h. After this time, the reading was carried out by ELISA spectrophotometer apparatus (Termoplate®) at 630 nm. The MIC was set as the concentration at which it was possible to observe a significant decrease in the fungal growth curve at the tested concentrations.

2.5.4. Evaluation of potential of the extracts on modulating the antifungal activity of fluconazole

The plant extracts ere used at a sub-inhibitory concentration (MIC/16) to perform this test. According to the methodology used by Coutinho et al. [19], with minor modifications, the commercial drug fluconazole was used in the serial dilution drug combination test at concentrations ranging from 1 to 1024 μg/mL. A dilution control was carried out in the drug combination test. The fluconazole MIC was also determined and it was used to perform the control dilution. A medium sterility control was also prepared. These tests were performed in triplicate and the plates were incubated at 37 °C, for 24 h. An ELISA spectrophotometer apparatus (Termoplate®) was used to perform the reading at with 630 nm and the results were used to obtain the cell viability curve.

2.5.5. Statistical analysis of the microbiological tests

The data obtained for each sample and concentration were checked for their normal distribution and then analyzed by one-way ANOVA with Tukey's post hoc test. The IC_{50} values were obtained by nonlinear regression for the purpose of interpolating values from standard curves (using the software Graphpad Prism, v. 5.0) of the *% growth* values plotted against concentration and IC_{50} values are expressed as μg/mL.

3. Results

The chemical characterization demonstrated the presence of secondary metabolites, such as phenols, flavonoids and tannins (Table 1). The chemical analysis performed to determine the total phenols revealed that *P. guajava* and *P. brownianum* extracts contained considerable amounts of these compounds (Table 2). The HPLC analysis specified some constituents, which were determined as parameters. These constituents were found in extracts which major composition [16,17] varied in concentration, according to Table 3.

In this comparative analysis, the extracts of *P. guajava* demonstrated higher concentrations of phenols. The Fig. 1 shows all compounds identified on each species. The presence of epicatechin, isoquercitrin, ellagic acid and coumarin could not be compared due

Table 1
Prospecting phytochemical extracts of *Psidium guajava* and *Psidium brownianum*.

Extracts	Metabolites													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
HEPG	+	−	+	−	−	+	+	+	+	+	+	+	+	−
HEPB	+	−	+	−	−	+	+	+	+	+	+	+	+	−
AEPGD	+	−	+	−	−	+	+	+	+	+	+	+	+	−
AEPBD	+	−	+	−	−	+	+	+	+	+	+	+	+	−
AEPGI	+	−	+	−	−	+	+	+	+	+	+	+	+	−
AEPGI	+	−	+	−	−	+	+	+	+	+	+	+	+	−

1 – Phenols; 2 – Tannin pyrogallates; 3 – Tannin Phlobaphenes; 4 – Anthocyanins; 5 – Leucoanthocyanidins; 6 – Flavones; 7 – Flavonols; 8 – Flavononols; 9 – Flavonones; 10 – Xantones; 11 – Aurones; 12 – Chalcones; 13 – Catechins; 14 – Alkaloids; (+) presence; (−) absense; HEPG: Hidroethanolic Extract of *P. guajava*; HEPB: Hidroethanolic Extract of *P. brownianum*; AEPGD: Aquous Extract of *P. guajava* Decoction; AEPBD: Aquous Extract of *P. brownianum* Decoction; AEPGI: Aquous Extract of *P. guajava* Infusion; AEPBI: Aquous Extract of *P. brownianum* Infusion.

Table 2
Quantification of total phenols in extracts of *Psidium guajava* and *Psidium brownianum*.

Species	Extracts (GAE)/g		
	Hidroetanolic	Aqueous by decoction	Aqueous by infusion
P. guajava	82.18	78.14	68.07
P. brownianum	49.25	80.77	77.74

(GAE)/g: Gallic acid equivalent per gram.

Table 3
Major phenolic compounds of *Psidium guajava* and *Psidium brownianum* (HPLC-DAD).

	Extracts					
	EHPG	EHPB	AEPGD	AEPBD	AEPGI	AEPBI
Major compounds	Quercetin	Quercetin	Quercetin	Luteolin	Quercetrin Isoquercetrin Luteolin	Luteolin

HEPG: Hidroethanolic Extract of *P. guajava;* HEPB: Hidroethanolic Extract of *P. brownianum;* AEPGD: Aqueous Extract of *P. guajava* Decoction; AEPBD: Aqueous Extract of *P. brownianum* Decoction; AEPGI: Aqueous Extract of *P. guajava* Infusion; AEPBI: Aqueous Extract of *P. brownianum* Infusion.

to maximize the effect of this drug through synergistic interactions have been made by using other commercial drugs against resistant clinical isolates. Spitzer et al. [10] systematically evaluated several combinations of pharmacological drugs and they identified 148 medications with unknown antifungal potential. These medications enhanced the fluconazole effect and surprisingly, they demonstrated strong fungicidal activity in some cases. Drug combination databases have been launched, such as the Antifungal

these compounds are not present in the composition of all extracts. According to the HPLC analysis, both extracts present similar composition, varying only in the concentration of the detected phenolic compounds.

The extract of *P. guajava* demonstrated higher levels of phenolic compounds, with significative diferences among the concentrations of chlorogenic acid, quercitrine, quercetin and luteolin (Fig. 1). In the decoction, significative diferences were observed among catequines, caffeic acid, quercetin, kaempferol and luteolin (Fig. 1). Regarding the infusions, all compounds presented quantitative differences, with the exception of luteolin, quercetin, catequines and rutine (Fig. 1).

According the results, both species of *Psidium* are chemically similar, presenting the same major compounds: quercetine and luteolin according to the chemical characterization by HPLC-DAD, the total phenols quantification and the chemical qualitative characterization.

The Minimum Inhibitory Concentration tests revealed that the extracts from both species presented an inhibitory effect on *Candida* fungal strains growth when they were used in high concentrations, with a MIC value of 8192 μg/mL, visualized by spectrophotometry. The evaluation of the potential of the extracts on modulating the antifungal activity of fluconazole against different strains revealed that combining this drug with the aqueous and hydroethanolic extracts at sub-inhibitory concentrations inhibited the microorganisms growth at concentrations lower than when it was used alone. This synergistic effect was also attested in the cell viability curve (Figs. 2 and 3). The IC50 values (Table 4) obtained from the evaluated products demonstrated that all *P. brownianum* combined extracts showed very low inhibitory concentrations in comparison to that obtained for fluconazole alone. Together, these data indicates that the extracts of both plants tested in this work potentiate the antifungal action of fluconazole against CA LM 77, CT INCQS 40042 and CT LM 23 yeast strains (Fig. 2).

4. Discussion

Fluconazole is an antifungal drug with fungistatic activity that acts by destabilizing the fungal membrane. The molecular mechanism of action of this drug consists in the inhibition of lanosterol 14α azole-demethylase (an *ERG11* gene product), which is a cytochrome P450 enzyme essentially involved in the ergosterol biosynthetic pathway [20]. Previous studies have reported the resistance of *Candida* species to fluconazole [21,22]. Thus, attempts

Synergistic Drug Combination Database (ASDCD) on which fluconazole is found in synergysm with up to 42 antifungal medications [7].

Some studies have also addressed natural products-fluconazole interactions. Nodoushan and colleagues [23] evaluated the garlic (*Allium sativum*) aqueous extract combined with fluconazole against different *Candida* clinical isolates. They showed the potentiating effect on drug's action and suggested that should be feasible to use the two antimicrobial agents topically, for therapeutic purposes. Allicin – the garlic bioactive component showing weak individual activity - was later investigated for its synergistic potential, and it presented synergism with fluconazole against 23 fluconazole-resistant strains. The study also found interesting results in *in vivo* tests [24]. A research that investigated the effect of the combination of essential oils from some medicinal plants (*Thymus broussonetii* and *Thymus maroccanus*) and commercial drugs, including fluconazole, demonstrated that they presented strong synergism. Saad and colleagues [25], based on results obtained in their studies, highlighted the benefits that might arise from the combined-agents therapy, such as reduction in the minimum effective medication dose, reduced toxic effect and, consequently, reduced side effects, and low treatment cost.

Phenolic compounds combined with fluconazole also exhibited synergistic interaction. So, it is worth mentioning the caffeic acid case. Its series of amides were investigated for their synergistic potential with fluconazole [26] and it was found that this association is able to sensitize the drug-resistant fungi by reducing the fluconazole MIC80 from 1.0 to 0.5 μg/mL to 128.0 μg/mL against *Candida albicans*. Catechins potentiated the fluconazole effect by significantly reducing its effective concentration against *C. albicans* [27]. Catechin and quercetin synergisms with fluconazole were evaluated using fluconazole-resistant *Candida tropicalis* strains. It was found that the association of these flavonoids with the drug triggered cell apoptosis, mitochondrial depolymerization, ROS (reactive oxygen species) accumulation and DNA damage [28].

Phenolic compounds such as gallic acid, catechin, luteolin and quercetin individually showed *in vitro* antifungal activity against several *Candida* species, including *C. albicans* and *C. tropicalis* [29]. Fu et al. [30] investigated the anti-candida activity of caffeic acid and its amides. Another test showed that the chlorogenic acid had effect on *Candida albicans* by disrupting its membrane lipid bilayer [31]. Bisignano et al. [32] found antifungal activity against *Candida albicans* in compounds such as gallic acid (that presented the best effect), kaempferol and rutin. Coumarins were also effective against

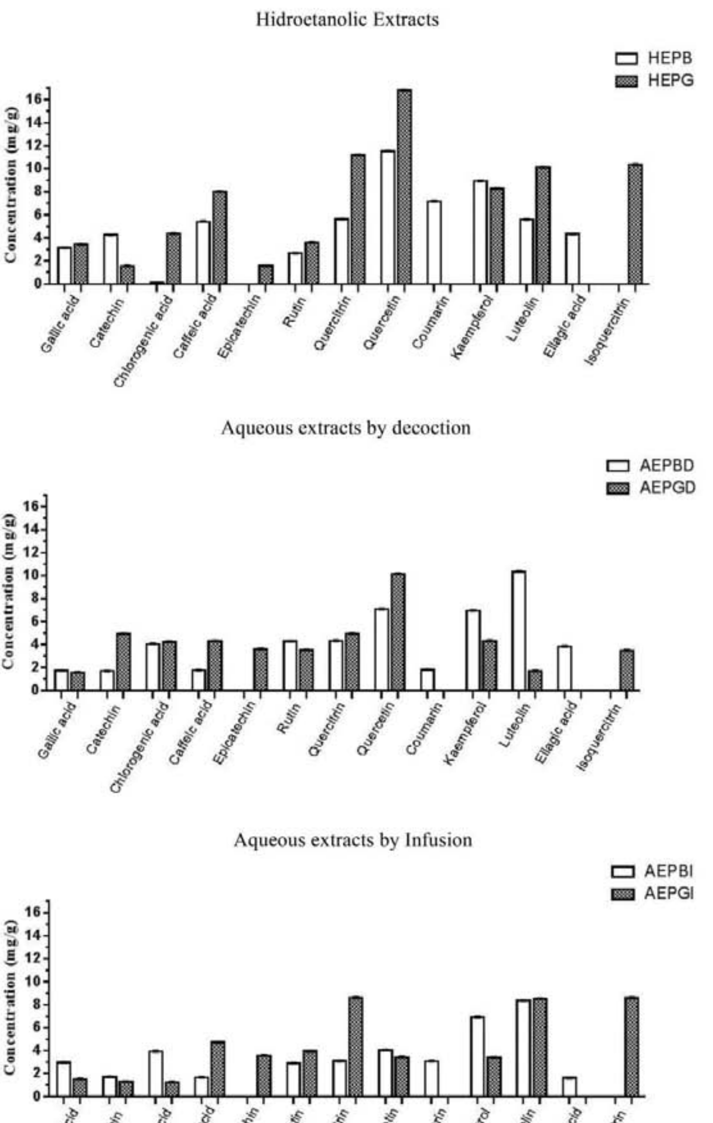

Fig. 1. Comparison of the amount of phenolic compounds found in hydroethanolic and aqueous extracts of *Psidium guajava* and *Psidium brownianum*. Investigated compounds in the extracts: gallic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, rutin, quercitrin, isoquercitrin, quercetin, kaempferol and luteolin (*P. guajava*) and gallic acid, catechin, chlorogenic acid, caffeic acid, ellagic acid, rutin, quercitrin, quercetin, coumarin, kaempferol and luteolin (*P. brownianum*).

Candida strains by affecting cellular events that, once disrupted, led to the disturbance and loss of cell membrane integrity [33] and Isoquercitrin exerts its fungicidal effect by disturbing the membrane of cells [34]. In the Table 5 is demonstrated that all compounds detected into the studied plants presented an antifungal potential.

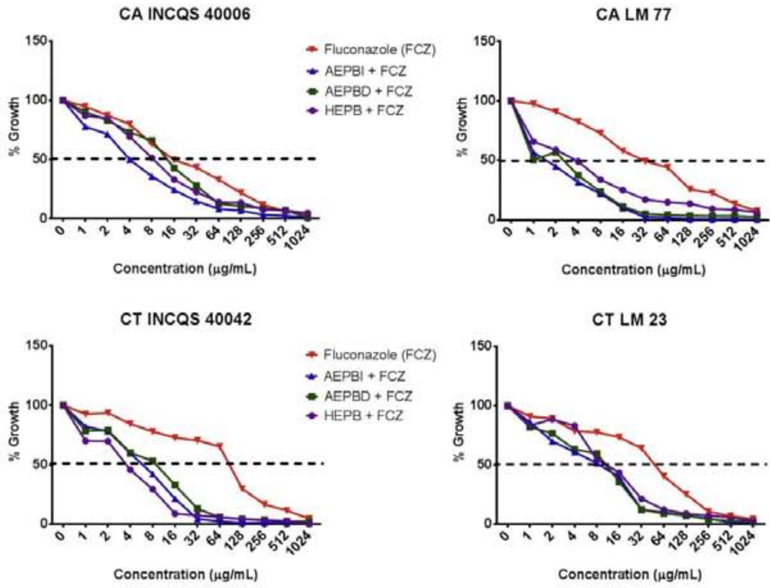

Fig. 2. Antifungal effect of fluconazole alone or in combination with the extracts of *Psidium brownianum* (MIC/16 μg/mL). FCZ: Fluconazole; CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. AEPBI: Aquous Extract of *P. brownianum* Infusion; AEPBD: Aquous Extract of *P. brownianum* Decoction; EHPB: Hidroethanolic Extract of *P. brownianum*.

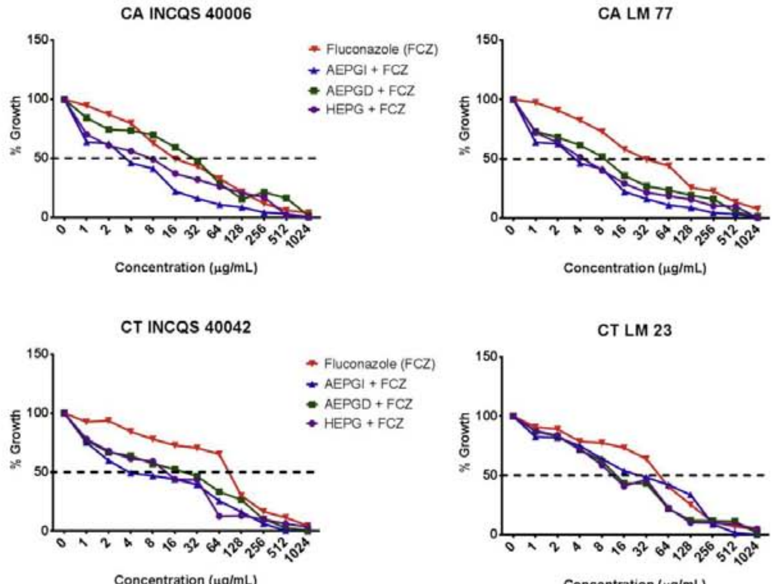

Fig. 3. Antifungal effect of fluconazole alone and in combination with the extracts of *Psidium guajava* (MIC/16 μg/mL). FCZ: Fluconazole; CA: *Candida albicans*; CT: *Candida tropicalis*; INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia. AEPBI: Aquous Extract of *P. guajava* Infusion; AEPBD: Aquous Extract of *P. guajava* Decoction; EHPB: Hidroethanolic Extract of *P. guajava*.

Table 4
IC_{50}(μg/mL) of the *Psidium guajava* and *Psidium brownianum* extracts front different *Candida* strains.

Products tested	Strains			
	CA INCQS 40006	CA LM 77	CT INCQS 40042	CT LM 23
Fluconazole (FCZ)	19.22	32.41	68.10	41.11
HEPG + FCZ	8.77	3.82	15.24	12.68
HEPB + FCZ	8.30	3.78	3.10	10.20
AEPGD + FCZ	25.89	7.88	37.52	13.66
AEPBD + FCZ	11.16	2.28	12.12	8.16
AEPGI + FCZ	3.88	3.88	6.17	45.40
AEPBI + FCZ	3.20	2.05	4.45	6.94

HEPG: Hidroethanolic Extract of *P. guajava;* HEPB: Hidroethanolic Extract of *P. brownianum*; AEPGD: Aqueous Extract of *P. guajava* Decoction; AEPBD: Aqueous Extract of *P. brownianum* Decoction; AEPGI: Aqueous Extract of *P. guajava* Infusion; AEPBI: Aqueous Extract of *P. brownianum* Infusion; CA: *Candida albicans;* CT: *Candida tropicalis;* INCQS: Instituto Nacional de Controle de Qualidade em Saúde; LM: Laboratório de Micologia.

Table 5
Phenolic compounds with activity against *Candida* spp.

Compounds	References
Gallic acid	[29–32–37–38–39–40]
Rutin	[32–37]
Chlorogenic acid	[31–39]
Ellagic acid	[40,41]
Caffeic acid	[30–39]
Catechin	[29–42]
Epicatechin	[40–42]
Quercetin	[29–38–39–43]
Quercitrin	[38–43]
Isoquercitrin	[34]
Coumarin	[33–44]
Kaempferol	[32–40–43]
Luteolin	[29–43]

This is the first study to report potential of *P. guajava* and *P. brownianum* extracts on modulating the antifungal activity of a commercialized pharmaceutical drug. In this work, we also identified constituents in the plant extracts and, regarding the drug modulator activity, it is believed that these compounds work together with fluconazole to improve its effect at lower concentrations, possibly by destabilizing the fungal membrane and facilitate the antifungal agent penetration. However, since these extracts are complex mixtures of substances, some components, which would naturally show greater fluconazole potentiating effect alone, may have masked their effect due to antagonistic interactions with the other components in the extract. Finally, although the current study has investigated the presence of some phenolic compounds in the extracts, many other constituents that have been identified in *P. guajava* [35,36], but are still unknown in *P. brownianum* may be involved in the biological activity of these plants.

5. Conclusion

In conclusion, the *P. brownianum* and *P. guajava* extracts presented significant modulator activity on potentiating the effect of Fluconazol on *C. tropicalis* and *C. albicans* strains and this effect may be associated to the presence of flavonoids and phenolic compounds in the extracts. These data suggest that these plants are important sources of bioactive compounds with the potential for antifungal drug development.

Declaration of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thanks to the Brazilian Research agencies: CNPq, CAPES and FUNCAP by the financial and grant support.

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European Journal of Integrative Medicine 8 (2016) 683–687

Contents lists available at ScienceDirect

European Journal of Integrative Medicine

journal homepage: www.elsevier.com/eujim

Research paper

Psidium guajava L. and *Psidium brownianum* Mart ex DC. potentiate the effect of antibiotics against Gram-positive and Gram-negative bacteria

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ARTICLE INFO

Article history:
Received 22 January 2016
Received in revised form 2 July 2016
Accepted 3 July 2016

Keywords:
Antibacterial activity
Psidium brownianum
Psidium guajava
Synergism
Minimum inhibitory concentration

ABSTRACT

Introduction: The survival and spreading of resistant bacterial strains has boosted research which focusses on discovering new antimicrobial agents derived from plant species. Several studies have demonstrated that substances present in plant extracts can modify the activity of antibiotics, increasing their efficacy. Species of the genus *Psidium* have been popularly used to treat bacterial infections. However, their modulatory effect on antibiotic activity remains to be elucidated. The aim of this study was to evaluate the modulatory effect of the hydroalcoholic extracts obtained from the leaves of *Psidium guajava* L. and *Psidium brownianum* Mart ex DC on bacterial proliferation, both separately and in combination with antibiotics.
Methods: The assays were performed using the microdilution method. The minimum inhibitory concentration (MIC) of the extracts and drugs were determined against standard and clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. To verify the potentiation of the antibiotic activity, the MIC of the antibiotics were determined alone and in association with the extracts.
Results: The extracts of *P. guajava* and *P. brownianum* did not present clinically significant activity against the Gram negative bacteria evaluated, with MIC values against *S. aureus* of 256 and 512 μg/mL, respectively. However, when combined at sub-inhibitory concentrations with antibiotics, both extracts presented significantly synergistic effects.
Conclusions: Our results demonstrated the effectiveness of *P. guajava* and *P. brownianum* on modulating bacterial growth, suggesting that these natural products might be used in drug development in association with antibiotics, reducing bacterial resistance and thus, improving the treatment of bacterial infections.

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1. Introduction

The development of the antibiotics in the first half of the last century has culminated in one of the greatest advances in the field of medicine, dramatically reducing human morbidity and mortality. However, the intense use of these drugs has created a strong selective pressure, which constantly results in the survival and spreading of resistant strains [1]. Infections caused by these strains considerably reduce the possibility of an effective treatment, increasing the therapy costs, as well as the risk of complications and death [2].

In this context, there has been an increasing interest on developing research aimed at discovering new antibiotics from natural products, especially from plants. In fact, plants produce several secondary metabolites, many of which presenting important biological activities, and in particular, antimicrobial activity [3]. Due to the chemical complexity of the extracts, which bear

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http://dx.doi.org/10.1016/j.eujim.2016.07.001
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several compounds with biological activities, plant extracts and essential oils, when used as antimicrobial agents, present low risk of resistance development [4–6].

Regarding the application of natural products on bacterial infections, besides the direct antimicrobial activity, plant extracts have been studied as antimicrobial drug resistance modifiers. Thus, various combinations of these extracts with synthetic antibiotics have been tested against bacterial proliferation, and many studies have demonstrated that substances present in the plant extracts can modify the activity of antibiotics, increasing their efficacy [7–10].

Psidium guajava L. is a small fruit tree found from Mexico to South America as well as in Europe, Africa and Asia [11]. This plant is very appreciated because of the excellent nutritional properties of the fruits, as well as its medicinal properties. Ethnopharmacological studies revealed that this species is used to treat diarrhea, gastroenteritis, dysentery, stomach disorders, vaginal irritation and many types of infection [12–17]. From the leaves of this plant, many secondary metabolites have been isolated, including tannins, saponins, triterpenoids and flavonoids (such as quercetin, myricetin, luteolin, and kaempferol) [18,19], and their biological activities, including: antimicrobial [20–22], hepatoprotective [23], antiproliferative [24] and anti-inflammatory [25] were previously demonstrated.

Psidium brownianum Mart ex DC., is a shrub or tree size species, reaching from 0.5 to 8m. This plant is an endemic species found in several states of the Southeast and Northeast of Brazil [26], in particular in the Chapada do Araripe, Ceará state, Northeastern Brazil. Although there are currently no reports demonstrating the pharmacological properties of this plant, it has been used in folk medicine as a therapeutic agent in the fight against influenza [27].

The objective of this study was to evaluate the antimicrobial activity of the hydroethanolic extracts obtained from the leaves of *P. guajava* and *P. brownianum* both separately and in combination with antibiotics, against bacterial strains of clinical interest.

2. Materials and methods

2.1. Plant material

Young leaves of *P. guajava* species and *P. brownianum* were collected between 9 a.m. and 10 a.m. from January to April 2014, in a property named Sítio Malhada, Milagres, Ceará, Brazil (07°17.119′S and 38°51.779′W, 388 m altitude; 07°17.120′S and 38°51.778′W, 389 m; 07°17.122′S and 38°51.776′W, 392 m; 07°17.119′S and 38°51.779′W, 388 m) and in the Araripe National Forest in Crato, Ceará (7°21, 685′S and 39°28, 605′W, 907 m altitude; 7°21, 793′S and 39°28, 605′W, 902 m; 7°21,787′S and 39°28, 558′W, 906 m), respectively. Exsiccates were prepared and deposited in the Herbarium Caririense Dárdano de Andrade Lima, under the registry numbers 10.671 (*P. brownianum*) and 10.935 (*P. guajava*).

2.2. Preparation of the hydroethanolic extracts

The plants were subjected to a screening to identify and discard those whose leaves have been attacked by herbivores and parasites. The selected leaves were cleaned and triturated before the extraction. The extract was prepared by cold maceration using as solvent ethanol 70% (v/v in water) in a proportion of 5 g of the plant material for each 1 mL of the solvent. In our work, we used 1846.5 g of *P. guajava* and 2000 g *P. brownianum* leaves. The leaves were immersed in the solvent using glass jars protected from light. After 72 h the mixture was filtered through a filter paper and taken to the rotary evaporator (40 rpm at 60 °C) and then to the bath (at 60 °C) to remove all the solvent. Then, the extracts were lyophilized, yielding 50.8 g of the hydroethanolic extract of *P. guajava* (HEPG) and 568 g of the hydroethanol extract of *P. brownianum*. The extracts were maintained under refrigeration before being used in the experiments.

2.3. Drugs, reagents, culture mediums and micro-organisms

Ethanol 70% was used in the obtaining of the extract; dimethylsulfoxide (DMSO) was used to obtain the first dilution of the extracts and the resazurin sodium was used as a colorimetric indicator in the reading of the plates. Antibiotics of the aminoglycoside (gentamicin and amikacin) and quinolone (ciprofloxacin) classes were used in drug modulation assays.

Solid heart infusion agar (HIA) medium was used in Petri dishes, for renovation of lineages and liquid brain heart infusion (BHI) medium at 10% was used in microdilution plates during the antimicrobial assays.

Determination of the Minimum Inhibitory Concentration (MIC) was performed using strains of standard bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25853 and *Staphyloccocus aureus* ATCC 25923. The modulatory activity of drugs was analyzed using multiresistant bacteria of clinical relevance, whose resistance profile is shown in Table 1.

2.4. Determination of the minimum inhibitory concentration

Initially, 0.01 g of extract was diluted into 1 mL of DMSO and then in water, reaching a concentration of 1024 μg/mL, that was used in tests. Importantly, the percentage of DMSO in the extracts at this concentration did not cause any interference in the tests. The microdilution plate was filled with 100 μL of BHI 10%, and 10% of bacterial inoculum was added in each well. The inoculum was prepared based on the MacFarland scale (0.5), equivalent to 10^5 CFU/mL. In the assay, 100 μL of each extract was added to the first well of each column of the plate (separately and in numerical order), and serially diluted in a ratio of 1:2 (512, 256, 128, 64, 32, 16 and 8 μg/mL) until the penultimate well. The last well was used as microorganism growth control. The plates were incubated at 37 °C for 24 h [28] and the readings were performed using resazurin

Table 1
Antibiotic resistance profile and source of the bacterial strains.

Bacteria	Source	Resistance profile
Escherichia coli 06	Urine culture	Cephalotin, cefalexine, cefadroxil, ceftriaxone, cefepime, ampicilin-sulbactam
Pseudomonas aeruginosa 15	Cateter tip	Cefepime, ceftazidime, imipenem, ciprofloxacin, piperacilin-tazobactam, levofloxacin, meropenem
Staphyloccocus aureus 10	Rectal swab	Cefadroxil, cefalexine, cephalotin, oxacilin, penicilin, ampicilin, amoxicilin, oxifloxacin, ciprofloxacin, levofloxacin, ampicilin-sulbactam, eritromicin, claritromicin, azitromicin, clindamicin, amoxilin/clavulanic acid

sodium as colorimetric indicator. Briefly, 20 μL of this substance was added in each well and 1 h later changes in color of the solution were checked. To attest the results, a red color indicates growth and blue indicates inhibition. The Minimum Inhibitory Concentration (MIC) was determined as the lower concentration of natural products and synthetic drugs that did not cause color changes in the well.

2.5. Evaluation of the potentiation of the antibiotic activity

To evaluate the potentiation of the antibiotic activity, the extracts were tested at sub-inhibitory concentrations (8× lower than the MIC or MIC/8). Each well of the plate received 100 μL of culture medium containing the extract (MIC/8) and the inoculum (10%). Then, 100 μL of antibiotic at 5000 μg/mL was added to the first well and serial dilutions were performed [29]. The last well was used as microorganism growth control and the readings were performed as described above. All tests were performed in triplicate.

2.6. Statistical analysis

All experiments were performed in triplicate and geometric mean, standard error of geometric mean and geometric standard deviation were calculated using the Graphpad Prism 5.0 software. The results were compared using analysis of variance (ANOVA) and the comparison between the geometric means was performed using the Bonferroni's post-test. The differences with $p > 0.05$ were considered significant [30].

3. Results

In the minimum inhibitory concentration test, we demonstrated that both extracts obtained from *P. guajava* and *P. brownianum* presented inhibitory activities against *S. aureus* ATCC 25923, with MIC values of 256 and 512 μg/mL for respectively. However, these extracts presented no antimicrobial activity at clinically relevant concentrations against *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 25853, both extracts presenting MIC ≥ 1024 μg/mL against each microorganism (Table 2).

Figs. 1–3 present the results of the activity of the extracts obtained from *P. guajava* and *P. brownianum*, demonstrating that both extracts potentiate the effect of the antibiotic against resistant bacterial strains. As shown in Figs. 1 and 2, these extracts presented significant synergic action in combination with the antibiotics against all bacteria tested. The same effect can be observed in Fig. 3, against *E. coli* and *P. aeruginosa*, except for *P. guajava* when tested with gentamicin, which presented no modulatory effect against *S. aureus*.

4. Discussion

In this study, *P. guajava* and *P. brownianum* species had their antimicrobial activity and potential for modulating the action of antibiotics evaluated. Our results demonstrated that both extracts presented significant inhibitory effects at clinically relevant

Table 2
MIC (μg/mL) of the extracts obtained from *Psidium guajava* and *P. brownianum* against standard bacteria.

Microorganisms	MIC (μg/mL)	
	Psidium guajava	*Psidium brownianum*
Escherichia coli ATCC 25922	≥1024	≥1024
Pseudomonas aeruginosa ATCC 25853	≥1024	≥1024
Staphyloccocus aureus ATCC 25923	256	512

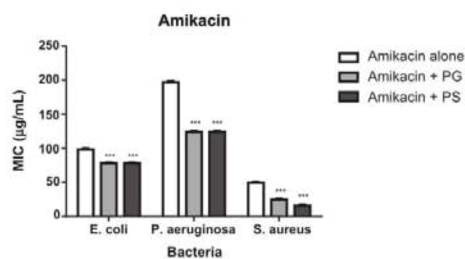

Fig. 1. Effect amikacin alone or in associations with the hydroethanolic extracts obtained from *P. guajava* and *P. brownianum*. ***—Significance with $P < 0.0001$.

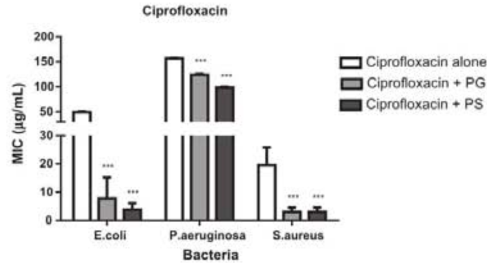

Fig. 2. Effect of ciprofloxacin alone or in associations with the hydroethanolic extracts obtained from *P. guajava* and *P. brownianum*. ***—Significance with $P < 0.0001$.

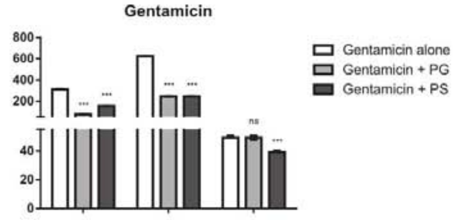

Fig. 3. Effect of gentamicin alone or in associations with the hydroethanol extract of *P. guajava* and *P. brownianum* . ***—Significance with $P < 0.0001$.

concentrations in *in vitro* tests against the Gram-positive *S. aureus* bacteria. Pereira [31] demonstrated that the essential oil obtained from *P. guajava* presented an antibacterial effect against *S. aureus*. However, when tested against *E. coli* and *P. aeruginosa*, this oil presented MIC ≥1024 μg/mL, suggesting that *P. guajava* has a better effect against gram positive bacteria.

Earlier studies which have tested at least one type of extract obtained from the leaves of *P. guajava* against *S. aureus* demonstrated that this bacterial specie is sensitive to the chemical components present in the extracts [32–34]. Accordingly, a study that tested the effect of an extract obtained from the leaves of *P. guajava* against Gram-positive and Gram-negative microorganisms, demonstrated that the extract caused higher inhibition of Gram-positive microorganisms, presenting a MIC value of 125 mg/mL for both *S. aureus* and *Bacillus subtilis* [33]. Finally, in general, it has been observed that polar extracts present higher activity against *S. aureus* [32,33,35], because they can overcome the

defense mechanisms of Gram-positive bacteria more easily, which unlike Gram-negative bacteria, do not present external protective membrane or periplasmic space [31].

Morais-Braga and colleagues [36], investigating the chemical composition of the same extracts tested in the present study, identified the presence of phenolic compounds, including: quercetin, quercitrin, luteolin, isoquercitrin, kaempferol, caffeic acid, chlorogenic acid, gallic acid, rutine, catechin and epicatechin. The antibacterial activity of some of these compounds has been previously reported, along with underlying mechanisms of action [6,33,37–39].

Therefore, the antibacterial activity of *P. guajava* can be due to the presence of these or other compounds in the extract that, when isolated, retain the antibacterial activity. In fact, some compounds, such as morin-3-*O*-lyxoside, morin-3-*O*-arabinoside, quercetin-3-*O*-arabinoside and quercetin presented pharmacological activity against strains of *E. coli*, *S. aureus* and *P.aeruginosa*, in disk diffusion tests [40]. Additionally, in another study, quercetin and derivatives quercetin-3-*O*-α-L-arabinofuranoside and quercetin-3-*O*-β-D-arabinopyranoside were isolated, identified and then tested for antibacterial activity by the above-cited method [41], and it was demonstrated that all derivatives were active against *E. coli*, although quercetin was the only compound to have presented simultaneous activity against *E. coli*, *P. aeruginosa* and *S. aureus*. From the ethanol extract and polar fractions obtained from the leaves of *P. guajava*, other compounds (identified as parts of lipid structures) were isolated and evaluated by the disk diffusion method, for their antibacterial activity against different pathogens, revealing that the aliphatic compounds 11-hydroxy-tricont-35-pentatriacontanoate, hexaeicosan-16-ol, pentatetracosan-10,25-diol, tricosan-17-ene-5-ol and nonacosan-23-ene-3-ol showed moderate activity against *S. aureus*. In addition, the compounds pentapentacont-17,31-diol, 34-octahexacontanol, heptatriacont-8-ol, heptatriacont-8-ol and nonacosan-23-ene-3-ol presented weak activity against *E. coli*. Finally, significant activity against *Pseudomonas* was achieved by the following compounds: 14,15-dimethyl(cyclopropayl)-9-ol-octadecayl-3-(4-hydroxyphenyl) propanoate and tricosan-17-ene-5-ol [42].

Because an extract is a complex chemical mixture, various compounds can act synergistically causing an antimicrobial response. Thus, besides the isolated compounds mentioned above, other phytochemical constituents of different classes of metabolites, such as tannins, may be responsible for providing this response.

Of note, at present no other study regarding the antimicrobial effect of *P. brownianum* has been reported, as well as, additional studies aimed at identifying the chemical compounds present in this plant species remain to be carried out. Importantly, this is the first study to report the antimicrobial activity and the modulatory effect of this species on potentiating the action of antibiotic.

Currently, there are poor data regarding the combination of *P. guajava* with commercial drugs. However, Pereira [31] demonstrated that when the essential oil of *P. guajava* was combined with aminoglycoside antibiotics against bacterial strains, including *S. aureus* and *E. coli*, the effects of amikacin and gentamicin against *S. aureus* were potentiated. In our previous assays, we have found that in general, the extract of a given plant present higher potentiating effect than its oil because, except for *P. guajava* in association with gentamicin, all associations have resulted in significant potentiation of the antibacterial effect of antibiotics against the multidrug-resistant bacteria tested. However, regarding the studies mentioned here, It is worthy of mention that due to the genetic specificities of the species tested and because they do not belong exactly to the same lines, no comparative pattern can be established.

The quinolones, such as ciprofloxacin, are inhibitors of the topoisomerases. These enzymes play important roles in the mechanisms involved on DNA replication, transcription and repair. The resistance mechanisms against these drugs include: modification of the target, activation of efflux pumps and reduction of the cell membrane permeability [43]. Gentamicin and the amikacin are aminoglycosides and thus, act by inhibiting the protein synthesis [44]. In this case, the bacterial resistance mechanisms involved are: enzyme degradation, cell membrane permeability alteration, activation of efflux systems and modification of the target [45].

Therefore, the synergistic effects that are usually achieved by the association of extracts with synthetic drugs can be related to an increase in drug influx, because a set of constituents present in the extracts, such as flavonoids, exert a destabilizing effect on the bacterial cell wraps, causing increased permeability to tested antibiotics (aminoglycosides and quinolones) and thus, potentiating their effect [46].

5. Conclusion

The hydroethanolic extracts obtained from *P. guajava* and *P. brownianum* demonstrated significant antibacterial activity against *S. aureus*, but not against the other strains evaluated. However, when associated with antimicrobial drugs, they caused a synergistic effect, reducing the MIC of antibiotics against both Gram-positive and Gram-negative strains. These data suggest that *P. guajava* and *P. brownianum* bear bioactive compounds capable of potentiate the effect of the commercial antibiotics, and thus, are promising natural products in the research of drug development based on combination therapies.

Conflict of interest

The authors state that they have no conflict of interest.

Funding source

FUNCAP; CAPES and CNPq.

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Journal of Ethnopharmacology xx (xxxx) xxxx–xxxx

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Review

Psidium guajava L., from ethnobiology to scientific evaluation: Elucidating bioactivity against pathogenic microorganisms

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ARTICLE INFO

Keywords:
Guava tree
Antiprotozoal
Antifungal
Antibacterial
Antiviral

ABSTRACT

Ethnopharmacological relevance: The use of popular plants has guided pharmaceutical research aimed at combating pathogenic microorganisms. *Psidium guajava* L. is a plant of great versatility and it has been used both as food and as a therapeutic agent. Root, bark, leaves, fruits, flowers and seeds are used for medicinal purposes, especially in infusions and decoctions for oral and topical use. *P. guajava* is utilized in symptomatology treatment related to organ malfunction and of diseases caused by the action of pathogenic and/or opportunistic microorganisms. Many pharmacological studies have been conducted to scientifically assess its therapeutic potential.

Aims of study: The aim of the current study is to relate the popular use of this plant and its bioscientific assessment as a therapeutic agent in the treatment of diseases and symptoms caused by the action of protozoa, fungi, bacteria and viruses, and also evaluate the safety for the usage and the interaction with drugs.

Materials and methods: A bibliographic database the ethnobiology of *Psidium guajava* (2005–2015) and the pharmacological infections and parasitic diseases (2010–2015). Searches were done in scientific disclosure databases such as PubMed, Web of Science, and Scopus.

Results: P. guajava leaf extracts were scientifically investigated for the treatment of diseases caused by protozoa (leishmaniasis, malaria, giardiasis, amoebiasis and trichomoniasis), fungi (dermatosis, systemic and mucocutaneous diseases), bacteria (respiratory, mucocutaneous and gastrointestinal infections, cholera, gastritis and stomach ulcers, oral and periodontal infections, venereal diseases and urinary infections) and viruses (herpes, influenza, rotavirus disease and AIDS). The toxicity assays indicates the safet for usage.

Conclusions: Highlight and elucidate the therapeutic potential and versatility of *P. guajava*. They also justify using ethnobiology efficiency to guide pharmacological studies. Some limitations can be observed in this kind of study, as the lack for ethnobiological informations and the absence of some controls in the assays.

1. Introduction

Psidium guajava L. (Myrtaceae, Subfamily: Myrtoideae) (Ravi and Divyashree, 2014) is found in tropical and subtropical countries. It is native to Central and Latin America and adapts well to a wide variety of soils, except for those with high clay content, low drainage capacity, or on acid and saline soils (Natale et al., 2012, 2007; Salazar et al., 2006). It can be introduced into many countries that have temperatures between 15 and 30 °C. Temperature is an important production determinant, as well as adequate water supply, which must range from 1000 to 2000 m^3/ha per year. The plant is known all over the world. It is widely present in orchards and gardens and produces a fruit known as guava (Dakappa-Shruthi et al., 2013; Gupta et al., 2011).

According to The Plant List (2016), *P. guajava* may be known by synonyms such as *Guajava pumila* (Vahl) Kuntze, *Guajava pyrifera* (L.) Kuntze, *Myrtus guajava* (L.) Kuntze, *Psidium angustifolium* Lam., *Psidium cujavillus* Burm.f., *Psidium cujavus* L., *Psidium fragrans* Macfad., *Psidium igatemyense* Barb. Rodr., *Psidium intermedium* Zipp. ex Blume, *Psidium pomiferum* L., *Psidium prostratum* O. Berg, *Psidium pumilum* Vahl, *Psidium sapidissimum* Jacq., *Psidium vulgare*

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http://dx.doi.org/10.1016/j.jep.2016.11.017
Received 28 December 2015; Received in revised form 5 November 2016; Accepted 8 November 2016
Available online xxxx
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Please cite this article as: Bezerra Morais Braga, M.F., Journal of Ethnopharmacology (2016), http://dx.doi.org/10.1016/j.jep.2016.11.017

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Rich. and *Syzygium ellipticum* K. Schum. & Lauterb. It is commonly called guava or guava tree and is a large tropical shrub or a small fruit tree (up to 7 m tall). It presents short trunk, scaly bark and open canopy; its leaves are coriaceous with prominent veins and its flowers are white. Its berry-type fruit has firm pulp and many seeds, thin to thick peel, sweet to very acidic taste, and it can be pear-shaped, round or ovoid (Rishika and Sharma, 2012; Souza et al., 2011).

The chemical constitution of this plant includes tannins, phenols, flavonoids, saponins, carbohydrates, alkaloids, sterols, terpenoids, phenolic compounds, among others (Barbalho et al., 2012; Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Thakur and Vikrant, 2014; Thenmozhi and Rajan, 2015). Compounds of known antimicrobial activity such as 1,2-Benzenedicarboxylic acid, dibut, Alpha.-bisabolol, 1,2-Benzenedicarboxylic acid, buty, hexadeca-2,6,10,14-tetraen, caryophyllene, germacrene (Thenmozhi and Rajan, 2015), quercetin, quercetin-3-O-α-L-arabinofuranoside, quercetin-3-O-β-D-arabinopyranoside (Metwally et al., 2010) morin-3-O-lyxoside, morin-3-O-arabinoside, quercetin and quercetin-3-O-arabinoside (Rattanachaikunsopon and Phumkhachorn, 2010), 11-hydroxy-35- tricont-pentatriacontanoate, hexaeicosan-16-ol, tricosan-17-ene-5-ol, nonacosan-23-ene-3-ol (Mehta et al., 2012), lupeol, betulinic acid (Ghosh et al., 2010) and antileishman activity (Nerolidol) (Thenmozhi and Rajan, 2015) have been found in the leaves of *P. guajava*.

P. guajava produces edible fruit with a high economical value, since it has wide acceptance in natura and a high industrial potential. Its high nutritional value is adaptable to any type of diet. Its dehydrated and industrialized form may also be used for human consumption through manufacturing possibilities such as sweet, jam, pasta, canned fruit, purees, soft drinks, syrups, ice cream, as well as savory and sweet and sour sauces, fermented dairy products, among others (Chauhan et al., 2015; Oliveira et al., 2012; Natale, 2009).

In addition to the use as food, parts of the guava tree, especially the leaves, have been used to treat diseases. This plant provides therapeutic components widely whether used alone or in combination with other plants of medicinal importance by local people worldwide (Gutiérrez et al., 2008; Sanda et al., 2011).

Because of the medicinal importance of *P. guajava*, various works relating to its ethnobiological history, as well as scientific investigations relating to confirmation of its general pharmacological effects, have been made. In this review we will be more specific, exclusively addressing the medicinal use for treatment of diseases and symptomologies related to the action of opportunistic/pathogenic microorganisms, whether these may be protozoa, bacteria, fungi or viruses, to summarize the scientific evaluation attesting to the potential of this species against such organisms. In a global context, where multiple populations feel the impact of poverty, pharmaceutical industry negligence and resistance of microbial pathogens to conventional antimicrobials, this study provides an ethnobiological and ethnopharmacological profile of the species *P. guajava* in the clinical microbiological scenario, pointing out interesting results on biological activities as a starting point for the deepening of scientific research in this field of pharmacology.

2. Materials and methods

The systematic search in the literature for *P. guajava* ethnobotanical reports was performed to collect publications showing the plant's medicinal importance to the treatment of signs and associated symptoms, as well as of diseases caused by microorganisms. The accepted nomenclature for the species, *Psidium guajava* L. (The Plant List), was used in the search. Synonyms were not used. The cited articles were publications made available from 2005 to 2015. Word combinations such as "*Psidium guajava*+ethnobotanical" and "*Psidium guajava* +medicinal" were used as search terms. The search for scientific reports about the theme of this review covered a period among 2010–2015. The word combinations often referred to the species "*P. guajava*+the biological activity", for example antibacterial activity, or the etiological agent+*P. guajava*, for example, *Leishmania*+*P. guajava* that we used to search. On top of the ethnobiological bibliographies of *P. guajava* and scientific studies of its biological activities, an auxiliary bibliography was used, without a delimitation period, for the description of *P. guajava* and its ecological aspects, to list and relate the main microorganisms of clinical importance and the diseases caused by these, to report the toxicity of the plant. The search was carried out in scientific publication databases, such as Pubmed, Scopus, and Web of science.

3. Results

3.1. The ethnomedicinal use of Psidium guajava

In general, ethnobiological studies covering the use of *P. guajava* were included. Parts of *P. guajava* are used for medical purposes. However, the flowers are the least used part, being commonly used as cataplasms to the treatment of ocular infections (Kumar, 2012). The leaves are commonly used to prepare infusions or decoctions and these medicinal teas are used to control diabetes, gastrointestinal and inflammatory disorders, vaginal problems, rheumatism and pain, respiratory diseases, diseases caused by microorganisms, among others (Dakappa-Shruthi et al., 2013; Gutiérrez et al., 2008; Mitchell and Ahmad, 2006; Picking et al., 2015). Young leaves are chewed to treat toothache and tincture are prepared for oral use in the treatment of oral diseases (Gutiérrez et al., 2008; Kukreja and Dodwad, 2012).

The bark, leaves and roots are used as plaster, ointment and paste to treat wounds, ulcers and skin infections. *P. guajava* sticks are chewed and used to clean the teeth. The decoction of leaves and root bark is used as gargle to treat sore throats, laryngitis, swelling in the mouth and ulcerations. These indications and forms of use have been reviewed in Dakappa-Shruthi et al. (2013), Gupta et al. (2011), Gutiérrez et al. (2008) and in Kukreja and Dodwad (2012).

The species is used as a medicinal plant in countries of the Americas, Asia, Africa and Oceania (Table 1) i.e. countries often with. Severe limitations of resources, including pharmaceutical ones, enhancing the search for alternative and/or natural therapies, as plants to be used as remedies or nutraceutics against several kinds of diseases, as observed in the specific case of *P. guajava*, that is used against a huge series of signs and symptoms observed in diseases caused by microorganisms, as may be observed in Table 2. However, the used part, the indication and form of use may vary according to the cultural context. Nevertheless, the therapeutic potential of *P. guajava* has been well recognized and disseminated in the tropical and subtropical regions of the world.

3.2. Scientific assessment of pharmacological potential based on ethno-directed studies

In the search for research evaluating the biological activity, the greatest number of articles were found for antibacterial activity, followed by antiprotozoal, antifungal and antiviral. The cited articles for bioactivity totaled 51 publications.

This section presents a review of research carried out to scientifically assess the popular use of P. guajava against microorganisms causing diseases that affect humans. Each sub-section is referring to one specific bioactivity and is composed of two parts: in the first part will report research using aqueous extracts, the main usage form. When the study reports the usage of other types of extracts, these reports are cited to compare the effects and activities. The second part demonstrates the pharmacological effects of *P. guajava*, but only citing reports using isolated metabolites and non-aqueous extracts with polar or non-polar characteristics. A summary of research that scientifically evaluating *P. guajava* extracts against pathogenic microorganisms can be seen in Table 3.

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Table 1
List of countries where *Psidium guajava* is used as a medicinal plant and its uses.

Country	Indications for *Psidium guajava*	Part (s) used	Reference (s)
Bangladesh	Diarrhea, bronchitis	Root bark, leaf, bark, fruit	Kadir et al. (2012), Khisha et al. (2012)
Bolivia	Diarrhea	Stem bark, fruit, young leaf	Hajdu and Hohmann (2012)
Brazil	Diarrhea, leukorrhea, thrush, ulcers, vaginal irritation, toothache, sore, herpes	Leaf, bark, fruit, leaf bud	Borba and Macedo (2006), Fenner et al. (2006), Ribeiro et al. (2014)
Cameroon	Malaria	fresh leaf	Betti et al. (2013)
Cambodia	Stomach ache	NI	Laval et al. (2011)
China	Diarrhea, stomachache, peptic ulcer	Leaf, root, bark, fruit	Ghorbani et al. (2011), Zheng et al. (2013)
Colombia	Nervousness	Fresh leaf	Gómez-Estrada et al. (2011)
Cuba	Catarrh, helminth	Shoot.	Volpato et al. (2009)
Ecuador	Leishmaniasis	Leaf, bark	Gachet et al. (2010)
Ethiopia	Amoeba, diabetes	Leaf	Regassa (2013)
Ghana	Malaria	Leaf	Asase et al. (2010)
Indonesia	Diarrhea, stomach pain	Wood, bark, young leaf	Mulyoutami et al. (2009)
India	Dysentery, Gastro-Intestinal Disorders, hemorrhage, diarrhea, gastroenteritis, anti cough, ulcers, bowels, cholera, ferver, pain, diabetes, inflammation	Young twigs, leaf	Beverly and Sudarsanam (2011), Das and Choudhury (2010), Dey et al. (2010)
Jamaica	Diarrhea, dysentery; tonic (strength and vitality)	NI; Root	Mitchell and Ahmad (2006), Mitchell (2011)
Kenya	Diarrhea	Leaf, fruit	Njoroge and Kibunga (2007)
Madagascar	Diarrhea, dysentery	Bark, leaf	Rakotoarivelo et al. (2015)
Malaysia	Respiratory diseases, diarrhea, stomachache	Leaf	Mohamad et al. (2011), Ong et al. (2011)
Mexico	Stomachache, vomit, herpes, diarrhea, disentery, wounds, rash, gastritis, vermifuge, toothache, cough, stomachache, fever, flu	Leaf	Alonso-Castro et al. (2012), Juárez-Vázquez et al. (2013).
Namibia	AIDS, cough, diarrhea, tuberculosis	Leaf, bark	Chinsembu, et al. (2015)
Nepal	Gastritis, flatulence, blood pressure	Leaf	Singh et al. (2011), Thapa (2012)
Nigeria	Hemorrhoids, fever, dysentery, diarrhea, malaria, stomach ache	Stem bark, root, leaf	Ariwaodo et al. (2012)
Pakistan	Diarrhea, cough, stomachache and dysentery, toothaches, indigestion, constipation	Leaf, fruit, root	Khan et al. (2013)
Papua New Guinea	Chicken pox, measles, alcohol intoxication; rhinitis	Young leaf	Waruruai et al. (2011)
Peru	Diarrhea, dysentery	Bark	Sanz-Biset et al. (2009)
Philippines	Diarrhea, Cuts, Wounds	Leaf, fruit	Abe and Ohtani (2013), Tantiado (2012)
South Africa	Vaginal candidiasis, AIDS, Gonorrhoea, non-specified venereal diseases,	Leaf bulb, leaf, root	Otang et al. (2012), Van-Vuuren and Naidoo (2010), York

(continued on next page)

Table 1 *(continued)*

Country	Indications for *Psidium guajava*	Part (s) used	Reference (s)
	respiratory infections		et al. (2011)
Tanzania	Tuberculosis, Chronic diarrhea	Leaf	Kisangau et al. (2007)
Thailand	Diarrhea	Leaf	Srithi et al. (2009)
Togo	Diabetes	Leaf, root	Holaly et al. (2015)
Uganda	AIDS, infection, tuberculosis	Root bark, leaf, root	Asiimwe et al. (2013), Lamorde et al. (2010), Kamatenesi-Mugisha et al. (2008)
Vanuatu	Diarrhea, dysentery	Leaf	Bradacs et al. (2011)
Zimbabwe	Cough, flu and fever	Leaf	Maroyi (2011)

NI: no information

3.2.1. *Scientific assessment of pharmacological potential from* Psidium guajava

3.2.1.1. *Antiprotozoal activity*

3.2.1.1.1. Aqueous extracts highlighted. Ethnobotanical studies have recorded the use of *P. guajava* via infusion and decoction of leaves, roots and seeds (Ariwaodo et al., 2012) to treat malaria. The plant has also caught the attention of ethnopharmacologists who have investigated both its *in vitro* and *in vivo* potential and found interesting results.

The individual and combined evaluation of extracts with different polarities of *P. guajava* (petroleum ether, dichloromethane, ethyl acetate, methanol and water) and of other species (*Citrus limon* (L.) Osbeck *Carica papaya* L., *Cymbopogon citratus* (DC.) Stapf and *Vernonia amygdalina* Delile) was investigated based on ethnomedicinal use in Nigerian communities. *Plasmodium falciparum* strains sensitive (D10) and resistant (Dd2) to chloroquine were used, and the antiplasmodial effect of the species was observed both individually and in combination (in doubles, with fixed concentrations of a species – 50% and variations of the other – 50%, 25% and 5%). The standard drug used in this study was cloroquine, that demonstrated an antiplasmodial activity with IC_{50} of 8.55 ± 2.81 ng/mL (D10) and 98.5 ± 26.1 ng/mL (Dd2). The best effect verified for *P. guajava* was from the dichloromethane (leaf) extract with an IC_{50} of 6 µg/mL. The aqueous extract demonstrated an Inhibitory concentration $IC_{50} > 50$ µg/mL. However, the latter case indicated that the antiparasitic effect was potentiated by the phytochemical combination of more than one species, with the best indices of fractional concentration (IFC) being observed for *C. limon+P. guajava* (1.99), *C. papaya+P. guajava* (2.39) and *C. citratus+P. guajava* (2.58) against D10. It probably reached more than one target; thus, it could be an alternative to combat parasitic resistance (Melariri et al., 2012).

Nefang, which is a herbal medicine composed of species such as *Mangifera indica* L. (bark and leaves), *P. guajava*, *C. papaya*, *C. citratus*, *Citrus sinensis* (L.) Osbeck and *Ocimum gratissimum* L. (leaves) in boiled water, is used orally in Cameroon to treat malaria caused by *P. falciparum* (Tarkang et al., 2012). An *in vitro* evaluation of the antiplasmodial activity was conducted using the 3D7 and Dd2 strains of the parasite. The species were tested individually and in combination. *P. guajava* showed antiparasitic potential for aqueous and ethanol leaf extracts, with effective concentration EC_{50} lower than 48 µg/mL. *P. guajava* leaf aqueous extract showed synergistic relation (Combination Index CI < 0.7) with both leaf and bark *M. indica* extracts in combination, whereas the ethanol extract showed additivity with all other extracts, except for the ethanol extract of *M. indica* bark (Tarkang et al., 2014). This study used as control combinations between cloroquine: cloroquine and cloroquine: artemisin, both with antiplasmodial activities in nM.

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Table 2
Use of *Psidium guajava* in the treatment of signs and symptoms related to infectious and parasitic diseases.

Etiological agent	*P. guajava* indication in folk medicine	Use of forms	Part (s) used	Reference (s)
• ***Protozoa*** • Amebiasis • Giardiasis • Leishmaniasis • Malaria • Trichomoniasis	• Stomach pain, flatulence, fever, diarrhea. • Stomach pain, diarrhea. • Sore, fever, pain, respiratory problems, diarrhea, bleeding. • Fever, pain. • Vaginal discharge, itching.	• Infusion, decoction • Infusion, decoction • Infusion, decoction • Infusion, decoction • Infusion	• Leaves, roots and stem bark. • Leaves, roots and stem bark. • Leaves, roots and stem bark. • Leaves, roots and seeds. • Leaves, roots.	• Semenya and Maroyi (2012), Alonso-Castro et al. (2012), Rishika and Sharma (2012), Juárez-Vázquez et al. (2013). • Semenya and Maroyi (2012), Alonso-Castro et al. (2012), Rishika and Sharma (2012), Juárez-Vázquez et al. (2013), Neiva et al. (2014). • Andrade-Cetto (2009), Semenya and Maroyi (2012), Alonso-Castro et al. (2012), Rishika and Sharma (2012). • Ariwaodo et al. (2012), Juárez-Vázquez et al. (2013). • Van-Vuuren and Naidoo (2010).
• ***Fungi***	• Cough, rhinitis. • Diarrhea, digestive problems, pain. • Skin diseases, itching, sores, mycosis, thrush. • Vaginal discharge, unspecified venereal disease.	• Infusion, decoction • Infusion, decoction, gargle • Infusion, decoction, gargle • Infusion.	• Leaves. • Leaves, roots, stem bark • Leaves, roots. • Leaves.	• York and Van Vuuren (2011), Juárez-Vázquez et al. (2013). • York and Van Vuuren (2011), Semenya and Maroyi (2012), Alonso-Castro et al. (2012), Rishika and Sharma (2012). • Fenner et al. (2006), Van-Vuuren and Naidoo (2010), Andrade-Cetto (2009), Oliveira et al. (2010). • Van-Vuuren and Naidoo (2010).
• ***Bacteria***	• Tuberculosis. • Cholera. • Gonorrhea, unspecified venereal diseases, vaginal discharge. • Urinary problems. • Lung problems, respiratory disorders, cough, rhinitis, catarrh, fever, sore throat. • Toothache. • Diarrhea, dysentery, digestive problems, constipation. • Stomach pain, gastritis, ulcer. • Boils, skin wounds, infections.	• Decoction, macerated. • Decoction. • Infusion. • Infusion. • Infusion, decoction, gargle. • Infusion, decoction. • Infusion, decoction. • Infusion, decoction. • Infusion, decoction, cataplasm.	• Leaves, bark. • Leaves. • Leaves and roots. • Leaves and roots. • Leaves, roots, stem bark. • Leaves. • Leaves, roots, stem bark, fruit. • Leaves, Roots and bark of the stem. • Leaves.	• Ogbole e Ajaiyeoba (2010), Asiimwe et al. (2013). • Sahu et al. 2014. • Van-Vuuren and Naidoo (2010). • Van-Vuuren and Naidoo (2010). • York and Van Vuuren (2011), Alonso-Castro et al. (2012). • Alonso-Castro et al. (2012), Khan et al. (2013). • Alonso-Castro et al. (2012), Semenya and Maroyi (2012), Rishika and Sharma (2012). • Andrade-Cetto (2009), Alonso-Castro et al. (2012), Rishika and Sharma (2012), Khan et al. (2013). • Alonso-Castro et al. (2012), Otang et al. (2012).
• ***Virus***	• Diarrhea, pain. • Flu, rhinitis, colds, cough, fever. • Herpes • AIDS	• Infusion, decoction. • Infusion, decoction, gargle. • Infusion • Decoction	• Leaves, roots and bark of the stem. • Leaves. • Leaves. • Leaves and bark root.	• Semenya and Maroyi (2012), Rishika and Sharma (2012), Juárez-Vázquez et al. (2013). • Andrade-Cetto (2009),Alonso-Castro et al. (2012), York and Van Vuuren (2011). • Alonso-Castro et al. (2012). • Lamorde et al. (2010), Chinsembu et al. (2015).

Rajendran et al. (2014) performed *in vivo* tests using aqueous extracts of fresh *P. guajava* leaves and unripe fruits against the NK65-chloroquine-resistant strain of *Plasmodium berghei*. Doses were orally administered to infected rats for 7 days. It was observed that the parasitaemia reduced as the volume of extracts increased, with suppression percentage of 85.8% (leaf) and 62.0% (fruits), when both extracts were used at a concentration of 1000 mg/kg, the leaf extract being more active. The control of this assay was performed using 50 mg/kg of cloroquine (0%).

The tea of *P. guajava* leaves made by decoction and the fruit bud have been used to treat giardiasis (Birdi et al., 2011; Neiva et al., 2014). The leaf decoction was investigated in a previous study (Birdi et al., 2011), and decoction was done as local people do it. The viability of *G. lamblia* trophozoites was affected at 5% (1.350 ± 0.063 mg/mL) and 10% dilutions (2.7 ± 0.125 mg/mL) and the antigiardial potential of *P. guajava* leaf tea was proven, besides the effect observed was lower (% of viable trophozoites ranging between 20–40%) than that observed using the standard drug, metronidazole at 10 µg/mL (% viable trophozoites lower than 20%) (Birdi et al., 2011).

Ethnomedicinal formulations are used to treat intestinal infections. People living in the Yucatan peninsula use fresh maceration and decoction to prepare mixtures of different plants, and *P. guajava* is the therapeutic component used in at least three homemade medicinal preparations (Vera-Ku et al., 2010). As a result, the species was tested to determine its *in vitro* pharmacological effect on parasites that cause infections, such as *G. lamblia* and *Entamoeba histolytica*. The preparation by decoction (2 leaves of *Teucrium cubense* Jacq, *P. guajava, Guazuma ulmifolia* Lam. anChavand *Senna atomaria* (L.) H.S.Irwin & Barneby each and a pinch of baking soda) showed IC_{50} of 14.33 µg/mL against *E. histolytica* and IC_{50} of 50.05 µg/mL against *G. lamblia*. The metronidazole was used as standard drug, with IC_{50} =0.22 µg/mL on both assays (amebicidal activity and giardicidal activity). Products with $IC_{50} \leq 20$ µg/mL were considered active against the parasites. The other preparations containing *P. guajava* did not exert significant effects on the parasites (Vera-Ku et al., 2010).

The effect of *P. guajava* on *Trichomonas vaginalis* was evaluated because the plant is indicated to treat non-specific venereal diseases (Van-Vuuren and Naidoo, 2010). The medication is prepared through infusions of leaves and roots. The ciprofloxacin was used as the standard drug to control the growth of *Trichomonas* at a concentration of 0.12 µg/mL. The dichloromethane: methanol and aqueous extracts of leaves were active against the protozoa at the concentrations of 1 and 4 mg/mL, respectively (Van-Vuuren and Naidoo, 2010).

3.2.1.1.2. Other extracts. Parts of *P. guajava* have been used to treat leishmaniasis (Gachet et al., 2010). The bark was pulverized and leaves macerated, these were then topically applied (in cold) to the injury. Gachet et al. (2010) investigated the *in vitro* potential of *P. guajava* bark extracts (dichloromethane, methanol) to find out whether the plant has any effect on the *Leishmania donovani* protozoan. However, *P. guajava* bark extracts (4.8 and 0.8 µg/mL) showed lower leishmanicidal activity (< 50%) on amastigote forms (found within phlebotomines) than other plants evaluated and mentioned by the communities. In this assay, the comparison drug miltefosine was used (IC_{50} of 0.104 µg/mL). A study conducted by Gawad et al. (2015) used an ethanolic extract of *P. guajava* leaves (80 µg/mL) and found that the extract exerts 90.3% inhibition against

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Table 3
Scientific validation of *Psidium guajava* effect against pathogenic microorganisms.

Microbial group	Part (s) used	Extract/Fraction/Compound (s)	Microorganism (s)	Scientific validation
Protozoa	Bark, leaf, root, flower and fruit	Aqueous extract (decoction and infusion), methanol extract (80%), hydroethanolic extract (maceration and percolation), ethanolic extract, dichloromethane extract, cyclohexane extract e cyclohexane extract + chloroquine, petroleum ether extract, ethyl acetate extract, methanolic extract	*Entamoeba histolytica, Giardia lamblia, Leishmania donovani, Plasmodium falciparum, Plasmodium berghei, Trichomonas vaginalis*	Birdi et al. (2011), Chenniappan e Kadarkarai (2010), Chinchilla-Carmona et al. (2011), Chinchilla et al. (2012), Gachet et al. (2010), Gawad et al. (2015), Inyang-Etoh and Ohanu, (2015), Kaushik et al. (2015), Melariri et al., (2012), Neiva et al. (2014), Rajendran et al. (2014), Tarkang et al. (2014), Van-Vuuren and Naidoo (2010), Vera-Ku et al. (2010)
Fungi	Leaf	Hexane extract, methanol and acetone extracts, aqueous extract (infusion and decoction), methanol extract and ethyl acetate fractions, hydrometanolic extract (70%), ethyl acetate extract, hexane extract, petroleum ether extract, toluene extract, ethanol extract (30%)	*Cryptococcus neoformans, Microsporum canis, Microsporum gypseum, Trichophyton tonsurans, Trichophyton rubrum, Sporothrix schenckii, Saccharomyces cerevisiae,Trichophyton mentagrophytes, Epidermophyton floccosum, Aspergillus niger, Penicillium* spp, *Microsporum canis, Candida albicans, Candida parapsilosis, Candida tropicalis, Candida krusei, Candida neoformans, Candida epicola*	Chanda and Kaneria (2011), Chanu et al. (2011), Egharevba et al. (2010), Ferreira et al. (2013), **Fonseca e Botelho (2011)**,Ghosh et al. (2010), Mailoa et al. (2014), Morais-Braga et al. (2015), Padrón-Márquez et al. (2012), Perera et al. (2014), Suwanmanee et al. (2014)
Bacteria	Leaf, bark, fruit	Methanol extract, petroleum ether extract, toluene extract, ethyl acetate extract, acetone extract, aqueous extract (decoction), ethanol extract, dichloromethane-methanol extract, hydromethanolic extract, chloroform extract. Compounds: 11-hydroxy-35- tricont-pentatriacontanoate, hexaeicosan-16-ol, tricosan-17-ene-5-ol, nonacosan-23-ene-3-ol, morin-3-O-lyxoside, morin-3-O-arabinoside, quercetin and quercetin-3-O-arabinoside	*Staphylococcus aureus, Klebsiella pneumonia, Streptococcus pneumonia, Pseudomonas aeruginosa, Moraxella catarrhalis, Mycobacterium tuberculosis, Mycobacterium bovis, Escherichia coli, Salmonella enteric, Vibrio cholera, Salmonella typhi, Shigella sp, Lactobacillus sp., Streptococcus pyogenes, Pseudomonas fluorescence, Enterobacter sp, Citrobacter sp., Xanthomonas campestris, Xanthomonas citri, Klebsiella sp., Shigella flexneri, Helicobacter pylori, Streptococcus sanguinis, Streptococcus mitis, Actinomyces* sp., *Streptococcus mutans, Streptococcus salivarius, Streptococcus mitior, Streptococcus milleri, Oligella ureolytica, Ureaplasma urealyticum, Neisseria gonorrhoeae, Gardnerella vaginalis*	Adesina et al. (2015), Alo et al. (2012), Bai et al. (2014), Birdi et al. (2010), Chanda and Kaneria (2011), Chavan et al. (2015), Fathilah (2011), Garode e Waghode (2014), Ghosh et al. (2010), Ibekwe et al. (2014), Ifeanyichukwu et al. (2015), John et al. (2013), Madduluri et al. (2013), Mehta et al., (2012), Philip et al. (2015), Rahim et al. (2010), Rajan et al. (2015), Rattanachaikunsopon and Phumkhachorn (2010), Uyub et al. (2010), Van-Vuuren and Naidoo (2010), Vieira et al. (2014), York et al. (2012)
Virus	Leaf	Hydroethanolic extract (70%), aqueous extract, ethanol extract, saponins	Herpes simplex virus type 1 (HSV-1) H1N1 virus (A/Narita/1/2009, A/Yamaguchi/20/06 and A/Kitakyushu/10/06 - resistant to Oseltamivir) AS-11 simian rotavirus HIV	Birdi et al. (2011), Faral-Tello et al. (2012) Lamorde et al. (2010);Mao et al., (2010); Sriwilaijaroen et al. (2012)

the promastigote forms of *Leishmania donovani*, which parasitizes mammalian hosts, including humans. Amphotericin B was used as the standard drug, demonstrating an inhibition of 98.7%.

Gachet et al. (2010) tested the dichloromethane extract against *Plasmodium falciparum* K1 and found that the bark extract was able to inhibit the protozoan at percentages above 80%, and with IC_{50} of 2.7 μg/mL. The results were compared using chloroquine (0.07 μg/mL). Chenniappan and Kadarkarai (2010) tested the *in vitro* cyclohexane and methylene chloride extracts (10 μg/mL) of the species against a chloroquine-resistant *P. falciparum* strain. They found that the leaf extracts showed moderate activity (69% and 58%, respectively). Chinchilla and collaborators (2011 and 2012) investigated the antimalarial potential of 25 plants from Costa Rica. Among these plants, they tested hydroethanolic extracts from bark, roots, young and mature leaves, as well as flowers and ripe or unripe fruits of *P. guajava*. Their experiments were carried out against the *Plasmodium berghei* ATCC NK 65. Regarding the *in vitro* test using the extracts, the plant was considered to be very active, with the lowest IC_{50} recorded for the dried flower extract (1 μg/mL). When compared, the dry extracts had a better effect than the fresh ones. However, they showed low activity in tests performed in rats, with percentages ranging from 30% to 50%, and the lowest IC_{50} (70 mg/kg) was attributed to the dry extract of the ripe fruit.

Antiplasmodial activity was also observed by testing the methanolic and ethyl acetate leaf extracts of *P. guajava*. The IC_{50} of 15 and 12.5 μg/mL were obtained from the respective extracts when they were used against (3D7) chloroquine-sensitive strains of *P. falciparum;* and IC_{50} of 9 and 18 μg/mL were obtained against (INDO) chloroquine-resistant strains. The IC_{50} of the standard drugs used in this work (chloroquine and artemisinin) was found to be respectively 0.0021 and 0.0045 μg/mL. The antiplasmodial activity was considered dose-dependent and of significant importance, thus it scientifically validates the plant's popular use and guides researches geared toward the production of medications against malaria (Kaushik et al., 2015).

Neiva and colleagues (2014) tested the hydroethanolic extract of *P. guajava* leaves prepared through maceration and percolation against *Giardia lamblia* trophozoites to scientifically assess the plant's effect on the etiological agent. The results showed moderate activity for both extracts (IC_{50} ≤500 μg/mL), but the drugs used as positive controls demonstrated an IC_{50} of 0.22 μg/mL.

An *in vivo* test using male and female albino Wistar rats was conducted to investigate the antiamoebic potential of *P. guajava*. The results showed that the methanol extract (80%) of fresh *P. guajava* leaves used at the concentration of 400 mg/kg of body weight is as effective in reducing the number of parasites (*E. histolytica*) as the reference medication - metronidazole – used at the concentration of 125 mg/kg (Inyang-Etoh and Ohanu, 2015).

3.2.1.2. Antifungal activity

3.2.1.2.1. Aqueous extracts highlighted. A survey conducted to find plants used to treat signs and symptoms related to fungal infections in Northeastern Brazil indicated that *P. guajava* is one of the plants showing antifungal potential according to popular indications (Fenner et al., 2006). *P. guajava* has been used to treat symptoms such as leukorrhea, aphthae, ulcers, vaginal irritation and skin problems through the preparation of cataplasms, decoctions and infusions using different parts from the plant such as fruits, bark, flowers and leaves (Fenner et al., 2006; Gutiérrez et al., 2008). Fungal infections have generated great impact on public health due to the increasing number of immunocompromised patients. Several genera may be involved in infectious processes such as *Cryptococcus, Candida, Aspergillus, Pneumocystis* and *Coccidioidomycosis* (Brown et al., 2012), among others.

Suwanmanee et al. (2014) tested the effect of *P. guajava* aqueous extract (leaf) against fungi that are clinically important to humans and animals. These fungi were categorized in three groups: yeasts (*C. albicans* and *Saccharomyces cerevisiae*), dermatophytes (*Trichophyton mentagrophytes, T. rubrum, T. tonsurans, Epidermophyton floccosum, M. canis* and *M. gypseum*) and filamentous fungi (*Aspergillus niger* and *Penicillium* spp). The aqueous extract showed activity against all the strains, with Minimum Inhibitory Concentration (MIC) between 2.67 and 16 mg/mL and inhibition zones ranging from 7 to 15 mm. The fluconazole (1 mg/mL) demonstrated a MIC ranging between 2 - > 128 μg/mL and inhibition zones ranging between 2 and 17 mm. Against *A. niger*, the extract of *P. guajava* demonstrated a better effect than the fluconazole (with inhibition zones ranging between 9 and 11 mm and between 2 and 3 mm, respectively).

The methanol extract of *P. guajava* leaves and fractions also showed antifungal activity when they were tested against dermatophyte strains (*T. mentagrophytes, T. rubrum, Microsporum canis, M. gypseum* and *E. floccosum*). The methanol and the aqueous extracts and ethyl acetate fractions were bioactive against all strains. The methanol extract was the most effective against *E. floccosum* (22.5 mm), the acetate fraction demonstrated a better effect against *M. canis* (27.13 mm) and the aqueous extract was more effective against *T. rubrum* (26 mm). The MIC was determined only for the ethyl-acetate fraction (6.25–25 mg/mL) and demonstrated a better activity against *M. canis* (MIC 6.25 mg/mL). In this assay, the standard drugs was the econazole (10 μg/mL), with inhibition zones higher than 30 mm against all microorganisms (Perera et al., 2014).

Chanu et al. (2011) tested the anti-Candida activity of *P. guajava* aqueous and ethanolic extracts at concentrations of 1%, 3%, 5%, 7% and 9% by diffusion, verifying the dose-dependent effect with the greatest verified inhibition zones of 30 mm when assayed using the aqueous extract and 20 mm when assayed using the ethanol extract. Beside the good results reported by the author, no standard drug was used in this work.

On the other hand, the aqueous, ethanol and acetone extracts (leaves) showed effect on *C. albicans, C. krusei, C. glabrata* and *C. dubliniensis* yeasts. The extracts demonstrated antifungal effect with MICs ranging between 62.50 and 250 μg/mL (aqueous), 15.62 and 62.50 μg/mL (ethanol) and 15.62 and 31.25 μg/mL (acetone). When assayed with the fluconazole, the MIC ranged between 1 and 64 μg/mL. In the same work, the Minimum Fungicide Concentration (MFC) was determined against *C. dublinensis, C. glabrata* and *C. krusei*. The best antifungal effect was observed against *C. dublinensis*, with MFC=500 μg/mL (Ferreira et al., 2013). Chanda and Kaneria (2011) verified that the extracts of petroleum ether, toluene, ethyl acetate, acetone and water of *P. guajava* leaves at a concentration of 2 mg/well presented an inhibition zone with values above 7 mm and below 15 mm against *C. albicans* and *Candida neoformans* and only the three most polar extracts presented activity against *Candida epicola*. The aqueous extract demonstrated an inhibition zone ranging between 7 and 10 mm. However, in this work, no antifungal drug was used as a standard.

Morais-Braga et al. (2015), observed that the hydroethanolic (70%) and aqueous (infusion and decoction) extracts of *P. guajava* leaves, prepared according to popular indication (150 mL of H_2O/10 g of leaves), inhibited both the fungal growth (MIC 8192 μg/mL – aqueous and hydroethanol extracts), as well as one of the important virulence factors of *C. albicans* and *C. tropicalis*, the cellular dimorphism. The inhibition of the morphological transition was concentration dependent, with maximal effect of all extracts at 16,384 μg/mL. Fluconazole was used as comparative standard and according with the authors, in 150 mL (similar to a cup of tea) of the extracts we can find concentrations 4–25 times higher than the inhibitory concentrations and 2–12 times the inhibitory concentration of the dimorphism.

3.2.1.2.2. Other extracts. A study conducted by Padrón-Márquez et al. (2012) reports the action of *P. guajava* leaf extracts (50 mg/mL) against dermatophytes. The hexane extract showed effect against all strains (*Candida albicans, Candida parapsilosis, Cryptococcus neoformans, Microsporum canis, Microsporum gypseum, Trichophyton tonsurans, Trichophyton rubrum*, and *Sporothrix schenckii*), whereas the methanol and acetone extracts were active against 70% of them. The inhibition zones ranged between 10 and 19 mm for the extracts and between 25 and 50 mm to the ketoconazole (250 μg).

Two triterpenoids were isolated from the toluene extract from *P. guajava* leaves, these being betulinic acid and lupeol. Through assessing these compounds against fungi causing superficial and deep infections, *Fusarium equisitae, Curvularia eragrostidis*, and *Alterneria alternata* (Ghosh et al., 2010), *the authors reported MICs of 2.5, 10 and 5* μg/mL for the betulinic acid and 5, 10 and 5 μg/mL for the lupeol against the fungal strains. These results were compared to streptomycin, with MIC of 2.5, < 2.5 and 2.5 μg/mL.

Egharevba et al. (2010) evaluated the hydromethanolic, methanolic, ethyl acetate and hexane extracts from *P. guajava* leaves against *Candida albicans* ATCC and clinical isolates. In the diffusion test, using the 10 mg/mL and 20 mg/mL concentrations (hexane) the formation of halos varying between 15 and 30 mm were found while in the broth dilution test the MIC varied between 1.25 and10 mg/mL, especially with the methanol extract. In this test, growth inhibition of *M. gypseum* and *T. rubrum* were also verified by methanolic and ethyl acetate extracts with halos of 14–17 mm and MIC of 5 mg/mL. In the diffusion disk method, the standard drug used was fluconazole (0.5 mg/mL) with inhibition zones ranging between 22 and 27 mm.

Using the hydroethanolic extract from leaves (70%), Fonseca and Botelho (2011) observed inhibited growth of *C. albicans, C. tropicalis* and *C. krusei* strains, with inhibition zones of 14, 11 and 10 mm, respectively. Fluconazole demonstrated an inhibition zone of 13 mm to *C. albicans* and *C. krusei*, but no effect was observed against *C. tropicalis*. The ethanolic extract (30%) of *P. guajava* leaves containing 2.351 mg/g of tannins presented inhibition zones of 9 mm against the fungi *Aspergillus niger* and *C. albicans* (Mailoa et al., 2014).

3.2.1.3. Antibacterial activity

3.2.1.3.1. Aqueous extracts highlighted. Psidium guajava has been used to treat several diseases that suggest bacterial activity, such as respiratory infections, gastrointestinal problems, genitourinary and related to oral health, and diseases such as tuberculosis and cholera (Gutiérrez et al., 2008). The macerated plant bark has been used to treat tuberculosis (Ogbole and Ajaiyeoba, 2010). The decoction of leaves (tea) taken three times a day in order to treat respiratory infections (York et al., 2011). Furuncles and ulcers are orally treated with the infusion of the leaves (Otang et al., 2012.). Sexually

transmitted infectious diseases are treated with the infusion of leaves and roots (Vuuren and Naidoo, 2010). Decoctions of bark and leaves are used against cholera (Sahu et al., 2014). The decoction of the bark is administered from 3 to 4 times a day to treat intestinal infections. The infusion of young leaves is used in association with other plants to treat diarrhea (Kadir et al., 2012). The frequent popular use of guava to treat infections has led to research aimed at proving its antibacterial effect.

Pneumonia is presently among the most present acute respiratory infections in the population and it can be caused by viruses, bacteria or fungi. *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Mycobacterium tuberculosis*, *Moraxella catarrhalis*, and others can be found among the bacteria causing the infection (Bjarnason et al., 2015; Souza, 2010).

Chanda and Kaneria (2011) investigated the potential microbial activity of Indian nutraceutical plants. *P. guajava* was among these plants and it was evaluated against 10 pathogenic bacteria species, including *S. aureus* and *K. pneumoniae*. Different types of extracts obtained from leaves (petroleum ether, toluene, ethyl acetate, acetone and water) were evaluated and showed antibacterial effect in the *in vitro* assay. The microbial activity was evaluated at a concentration of 2 mg/well. *P. guajava* stood out as the species showing the best antimicrobial activity among the plants evaluated with inhibition zones between 7 and 25 mm. The antibacterial effect of all extracts was evaluated against Gram positive and Gram negatives strains, the better effect being observed when the aqueous extract was assayed against Gram negative bacteria. In this work, no standard drug was used as reference.

Microorganisms such as *S. pneumoniae*, *K. pneumoniae*, *P. aeruginosa*, among others were tested *in vitro* against ethanolic and aqueous extracts of *P. guajava* leaves and fruits (10 µg/disc). The leaves showed better effect than the fruits, with inhibition zones ranging from 10 to 19 mm. *K. pneumoniae* was the most extract-sensitive bactéria. The extracts exerted activity ranging from low to intermediate in comparison to the reference antibiotic (tetracycline 36 µg), with inhibition zone ranging between 20 and 36 mm. In general, the ethanol extract was more effective (Philip et al., 2015).

P.guajava is used to treat respiratory infections in South Africa (York et al., 2012). Thus, it was evaluated in the form of organic dichloromethane-methanol and aqueous leaf extracts against different bacterial strains (ATCC standard) and it proved to be effective against *M. catarrhalis*, *K. pneumonie* and *S. aureus*, with the MIC of the extracts ranging from 0.5 µg/mL at 1 mg/mL (organic) and 0.5 µg/mL at 4 mg/mL (aqueous). The aqueous extract was more effective against *K. pneumonie* and *M. catarrhalis* and the results were comparable with ciprofloxacin (0.08–0.42 µg/mL) (York et al., 2012).

P. guajava is mostly indicated to treat gastrointestinal infections. Several studies have reported the use of guava leaves to treat diarrhea and dysentery, cholera and stomach problems (Dakappa-Shruthi et al., 2013; Gómez-Estrada et al., 2011; Gutiérrez et al., 2008). Intestinal infections may be caused by viruses, bacteria and parasites. The following bacterial species causing intestinal pathogens stand out: *Shigella species*, *Salmonella species*, *Yersinia enterocolitica*, *Vibrio species*, *Escherichia coli*, *Clostridium difficile* (Bresee et al., 2012; Njume and Goduka, 2012).

Garode and Waghode (2014) highlighted the antimicrobial potential of chloroform, ethanol, petroleum ether and water leaf extracts (50 µL/disc) of *P. guajava* against *Salmonella typhi* bacteria, with inhibition zones of 12, 18, 13 and 16 mm, respectively. None standard drug was used as reference. Another study also showed that *P. guajava* leaf extracts have growth inhibitory potential over five *S. typhi* isolates with a concentration dependent effect, where the best results were observed at the concentration of 0.6 g/mL with inhibition zones varying from 10 to 15 mm and from 12 to 17 mm for the cold water and ethanolic extracts, respectively. The results were compared with cloramphenicol (2.5 µg/disc) (Alo et al., 2012).

Based on ethnobotanical studies and searching for naturally occurring molecules with antimicrobial activity, six plants were investigated in India against 14 clinical isolates of enteropathogenic strains responsible for gastrointestinal tract infections in humans, namely: *E. coli*, *Shigella sp.*, *S. typhi*, *Lactobacillus sp.*, *Streptococcus pyogenes*, *Pseudomonas fluorescence*, *Enterobacter sp*, *Citrobacter sp.*, *Xanthomonas campestris*, *Xanthomonas citri*, *P. aeruginosa*, *Klebsiella sp.*, *Streptococcus faecalis* and *S. aureus*. Disk diffusion tests were performed and the results were confirmed by MIC determination. The aqueous and ethanol extracts of *P. guajava* (800 µg/disc) were described as active, with inhibition halos of 10.5–24.5 mm and 12.5–24.6 mm, respectively. The smallest and the largest effects were seen on *Enterobacter* sp. and on *E. coli*, respectively. The results were compared with tetracycline, with inhibition zones ranging between 0–18 mm. The MIC for the other plants ranged between 41.6 and 383.3 µg/mL for the aqueous extract and 41.6–216.6 µg/mL for the ethanol extract, thus revealing that *P. guajava* overall presented the best results among the assessed plant species (Rajan et al., 2015).

Based on the popular use, the aqueous extract of *P. guajava* leaves prepared by decoction has been tested against *E. coli*, *V. cholerae* and *Shigella flexneri* bacteria, which cause diarrhea. The extract was assayed in the follow concentrations: 0.1% (1:1000 dilution), 1% (1:100 dilution), 5% (1:20 dilution), and 10% (1:10 dilution) (v/v), inhibiting the growth of *S. flexneri* (EC_{50} of 0.98 ± 0.2%) and of *V. cholerae* (EC_{50} of 2.88 ± 0.36%) and it decreased the production of the *E. coli* labile toxin as well as the cholera toxin. The effect was compared with the Ofloxacin (1 µg/mL), that inhibited the growth of all microrganisms. In addition, it inhibited both the enteropathogenic adhesion of *E. coli* and the invasion by *S. flexneri* and enteroinvasive *E. coli*. (Birdi et al., 2010). Rahim et al. (2010) found antibacterial activity against the causative agent of cholera. Their studies showed that the crude aqueous extract of associated leaf and bark and the methanol extract of leaves showed bioactivity against *V. cholerae* MDR at the minimal inhibitory concentrations of 1,250 µg/mL and 850 µg/mL, respectively. No standard drugs were used by the authors of this work.

Psidium guajava, among other plants have been used in Malaysian traditional medicine for gastrointestinal disorders and wounds. For this reason, Uyub et al. (2010) searched 32 plant species indicated by the population, including *P. guajava*. Different types of *P. guajava* extracts were tested against *H. pylori* bacteria and they found inhibitory activity with 8.5 mm (petroleum ether), 10 mm (Chloroform), 33 mm (methanol) and 7 mm (water) inhibition zones. Among the 32 plants species screened, only 4 species demonstrated any effect associated with the extract, *P. guajava* being among them. None standard drug was used as reference.

The use of *P. guajava* against oral infections has also stimulated pharmacological research. The *P. guajava* aqueous extracts were tested against cariogenic and dental plaque causative microorganisms (*Streptococcus sanguinis*, *Streptococcus mitis* and *Actinomyces* sp.) and the MIC was determined, varying between 2.61 and 4.69 mg/mL. The assays were performed using the microdilution method and the MIC was determined by the turbidity of the medium, indicating the bacterial growth. In this research, the anti-biofilm activity of the *P. guajava* leaf extract was evaluated, but the effect observed was lower (25–45% reduction in adhesion) than the control using CHX-containing mouthrinse at 0.12 µg/mL (reduction in adhesion >90%) (Fathilah, 2011). The aqueous, ethanolic and hydroethanolic extracts of young *P. guajava* leaves (hot and cold extraction) exhibited an inhibition zone varying from 11.8 to 25 mm. The extracts were obtained using two methods: warm or cold extraction. The better effect among the warm extracts was observed in the ethanol extract (25 mm), however, using the cold extracts, the better result was demonstrated by the aqueous extract (21 mm). The control used demonstrated an inhibition zone of 25 mm. The extracts also demonstrated an anti-

adherent and antiplatelet activity in the consortium test in which the *Streptococcus mutans, S. mitis, S. salivarius, S. mitior, S. sanguinis* and *S. milleri* bacteria were used, some with the capacity to form biofilms (John et al., 2013). The aqueous extract of *P. guajava* branch was also active against *S. mutans*, with a 15 mm inhibition zone. The inhibition zone of ethanol extract was 14.33 mm. Besides, the extract showed an inhibitory effect, inhibiting partially thee development of biofilm, but the control using ethanol inhibited completely the biofilm of *S. mutans* (Chavan et al., 2014).

P. guajava is used to treat gonorrhea and non-specific venereal diseases in South Africa through the infusion of leaves and roots. Dichloromethane-methanol and aqueous extracts of *P. guajava* leaves were tested against bacteria such as *Oligella ureolytica, Ureaplasma urealyticum, Neisseria gonorrhoeae* and *Gardnerella vaginalis*. The results showed that *P. guajava* had an inhibitory effect at concentrations ranging from 0.8 µg/mL to 1.5 mg/mL when it came to the dichloromethane-methanol extract The aqueous extract was bioactive at higher concentrations (from 6 to 16 mg/mL) against *O. ureolytica, U. urealyticum* and *G. vaginalis*. The results were compared using ciprofloxacin that was active at concentrations ranging between 0.04 and 0.63 µg/mL (Van-Vuuren and Naidoo, 2010).

Bacterial urinary tract infections have been treated by medications that may have urease inhibition as the action mechanism. In the search for alternative therapies, medicinal plants used to treat this type of infection, including *P.guajava*, were tested to check their anti-urease potential. Methanol, aqueous and cow-urine extracts were tested against urease-positive bacteria and showed MICs ranging from 125 to 500 µg/mL for *S. aureus* and *P. aeruginosa*. The better result was observed with the methanol extract. No standard drug was used in this work (Bai et al., 2014).

3.2.1.3.2. Other extracts. The pathogenic bacteria *E. coli, K. pneumonia* and *S. aureus* can be the causal agents of gastroenteritis and urinary infections, pneumonia and wound infections, respectively. *P. guajava* methanol and ethanol leaf extract (100 µg/mL) were assayed against these microorganisms. The ethanol and methanol extracts demonstrated inhibitions zones ranging between 11 and 15 mm and between 9.2 and 10.2 mm. The inhibition zone observed with the ciprofloxacin (10 µg/mL) ranged between 17 and 23 mm. The better effect of the extracts was observed against *S. aureus* and the MICs of the extract are higher than observed with the ciprofloxacin (Madduluri et al., 2013).

A preliminary study conducted by Ifeanyichukwu et al. (2015) used ethanol and methanol extracts of leaves and bark of *P. guajava*. Their results showed that these extracts (100 mg/mL) inhibited the growth of bacteria *S. aureus, K. pneumoniae, Pseudomonas aeruginosa, E. coli* and *S. pneumoniae* with inhibition zones evidenced through the disk diffusion test. For methanol and ethanol extracts of leaves the zone of inhibition ranged from 13 to 22 mm, while for the bark extracts the variation was 13–20 mm. Chloramphenicol (10 mg) was the standard drug used in this work with inhibition zone ranging between 18 and 28 mm. The bulk extracts were more efefctive than the chloranphenicol against strains of *E. coli* and *P. aeruginosa*.

Chemical constituents such as 11-hydroxy-35- tricont-pentatria-contanoate, hexaeicosan-16-ol, tricosan-17-ene-5-ol and nonacosan-23-ene-3-ol found in the *P. guajava* ethanol extract (leaf) were isolated and subsequently tested through disk diffusion against bacteria. The results showed that the compounds had moderate activity against *S. aureus* (12–14 mm), however, no standard drug was used as reference in this work (Mehta et al., 2012). This microorganism is involved in the etiology of respiratory infections and it is also responsible for skin infections, wounds and furuncles for which *P. guajava* is also cited as a therapeutic agent (Dakappa-Shruthi et al., 2013; Joseph and Priya, 2011; Tong et al., 2015).

Ghosh et al. (2010) tested two isolated compounds of the toluene extract from *P. guajava* leaves and verified that betulinic acid inhibited the growth of *E. coli* and *S. aureus*, with MICs of 150 and 100 µg/mL respectively, while lupeol presented MICs of 200 µg/mL against both bacteria. The results were compared with the ampicillin, with a MIC ranging between 64 and 128 µg/mL.

P. guajava is medicinally used against tuberculosis in Uganda in the form of a decoction twice a day (Asiimwe et al., 2013). The *P. guajava* leaf hydromethanolic extract is used in traditional medicine in Malaysia. Ibekwe et al. (2014) found that the extract showed high activity (< 500 µg/mL) against bacteria such as *Mycobacterium bovis* BCG and *M. tuberculosis* $H_{37}Rv$, with MIC of 466 µg/mL and 154 µg/mL, respectively. The standard drug used in this study was Isoniazid.

Rattanachaikunsopon and Phumkhachorn (2010) evaluated the effect of flavonoids isolated from fresh *P. guajava* leaves (morin-3-O-lyxoside, morin-3-O-arabinoside, quercetin and quercetin-3-O-arabinoside) and found that these compounds exert bacteriostatic effect on enteropathogenic bacteria such as *E. coli, Salmonella enteric, Listeria monocytogenes* and *Vibrio cholerae* (MICs ranging between 40 and 114 µg/mL). The compounds were effective against *S. aureus* (MIC of 80 and 82 µg/mL). The oxytetracycline demonstrated similar values, with MICs ranging between 56 and 112 µg/mL.

Several plants used by folk medicine in Jordan were evaluated against *Helicobacter pylori*, which is the major cause of gastric disease. Among them, *P. guajava* ethanol extract (100,000 µg/mL) was tested against clinical isolates in *in vitro* assays and the results showed that the species presented moderate activity against *H. pylori* (isolates 1 and 2) with inhibition zones 33 and 28 mm, respectively. Amoxicillin (10 mg/disc), metronidazole (5 mg/disc), clarithromycin (20 mg/disc) and tetracycline (30 mg/disc) were used as standard drugs and shown inhibition zones ranging between 17 and 75 mm. In this work, one microrganism resistant to metronidazole and clarithromycin was inhibited by the *P. guajava* leaf extract (Masadeh et al., 2014).

Due the possible usage against oral diseases, the hydroethanolic extract of *P. guajava* leaves at 100 mg/mL was tested and showed 19 mm inhibition zone over *S. mutans*. The researchers demonstrated a concentration-dependent antibacterial effect when compared with the Chlorohexamine at 0.12% (20 mm) (Vieira et al., 2014).

Adesina et al. (2015) used the methanol extract of the leaves to assess the plant's inhibitory effect on biofilm formation of *E. coli* in urinary catheters. Upon covering the catheters with the extract, inhibition at the concentrations of 5, 10 and 20 mg/mL were verified, with bacterial counts varying between 4.3×10^5 and 1.9×10^{3} after 120 h and 7.7×10^5 and 3.8×10^{5} for 128 h. They found that the extract presence delayed biofilm formation in a week and inhibited bacterial proliferation.

3.2.1.4. Antiviral activity

3.2.1.4.1. Aqueous extracts highlighted. The use of *P. guajava* for the treatment of viral infections and associated symptoms has been reported in the literature. In this sense, *P. guajava* leaves are used together with the *Citrus lemon* fruit and *Eucalyptus* sp. leaves in the preparation of a beverage used for the treatment of flu by populations in Zimbabwe (Maroyi, 2011). Respiratory infections are treated with the decoction of *P. guajava* leaves administered three times daily in the South Africa (York et al., 2011). People living with HIV/AIDS are susceptible to infections caused by opportunistic microorganisms like the herpes virus, rotavirus, among others. Therefore, due to the fragility of the immune system, a series of symptoms are observed such as diarrhea, blisters and sores in the skin and mucosa (Bosco et al., 2014; Faral-Tello et al., 2012; Kisangau et al., 2007). The infusion and decoction of the white guava peel, the sap and the leaves are used in Brazil for tea preparation for ingestion or mouthwash to treat herpes and sores. Meanwhile, tea from the leaves and peel of the red guava (decoction and infusion) are taken for diarrhea (Borba and Macedo, 2006). In Mexico however, the infusion of leaves is made and taken for the treatment of herpes, diarrhea, sores and skin rashes

(Alonso-Castro et al., 2012).

Influenza, which is a respiratory virus infection, has been treated in different locations with tea from guava leaves. The guava leaf tea prepared by infusion was tested by Sriwilaijaroen et al. (2012) against clinical isolates of the H1N1 virus in a screening of different types of teas used as antiviral medication alternative in the folk medicine of oriental countries. The guava leaf tea at 4% showed strong inhibitory activity, with IC_{50} =0.05 ± 0.06, 0.42 ± 0.14 and 0.24 ± 0.05 to the viruses A/Narita/1/2009, A/Yamaguchi/20/06 and A/Kitakyushu/10/06 - resistant to Oseltamivir, respectively, affecting both the entry of the influenza virus and its propagation. Comparatively, the oseltamivir carboxylate demonstrated a IC_{50} =3.83 ± 1.64 nM, 11.57 ± 2.08 nM and 15.97 ± 7.76 nM against the respective strains. Its action mechanism was found on surface glycoproteins, such as the viral hemagglutinin, which prevents the virus from binding to the cell membrane in the initial stage of infection, and neuraminidase, which prevents the formation of new viruses. According to the researchers, the multiple chemical constituents of the tea may act on multiple targets, thus resulting in a synergistic effect.

Rotavirus is one of the most responsible agent for the onset of diarrhea among children. One of the main uses of *P. guajava* in popular medicine lies on the treatment of diarrhea and gastrointestinal disorders (Cecílio et al., 2012). Birdi et al. (2011) demonstrated an anti-rotaviral activity using the decoction of dry leaves of *P. guajava*. MA-104 cll lines were infected with the simian rotavirus SA-11, that causes cell death. The aqueous extract lowered the death of the cell lines at concentrations of 5% (1.35 ± 0.063 mg/mL) and 10% (2.7 ± 0.125 mg/mL), demonstrating that tea from the leaves of guava has effect against these viruses.

3.2.1.4.2. Other extracts. The infusion of guava leaves has been used to treat herpes, which is caused by Herpes simplex virus type 1 (HSV-1) (Faral-Tello et al., 2012). Assays using plant extracts commonly used to treat this viral infection were performed by Faral-Tello et al. (2012). *P. guajava* was one of the assessed species and its hydroethanolic extract demonstrated EC_{50}=118 µg/mL, which led to *in vitro* inhibition of HSV-1 replication in monkey kidney cells (ATCC CCL-81). In comparision, the acyclovir demonstrated an EC_{50}=45 µg/mL.

The use of *P. guajava* to treat symptoms of HIV patients has been reported by Lamorde et al. (2010) in Uganda and by Chinsembu et al. (2015) who report its use to concomitantly treat AIDS and tuberculosis in Namibia. A study using total saponins of *P. guajava* leaves (TSGL) showed that this concentrate was able to inhibit the HIV env polyprotein with an IC_{50} of 7.33 µg/mL. In addition, it had an effect on the virus-membrane-cell fusion, with inhibitory activity of 95.93% at 25 µg/mL, by blocking the viral transmembrane glycoprotein (gp41) (Mao et al., 2010).

4. *Psidium guajava* toxicity

The toxicity of *P. guajava* has been assessed in some pharmacological tests. The cytotoxicity of the aqueous infusion of *P. guajava* leaves was tested in peritoneal macrophages of mice. This assay use immunological cell, as macrophages, to *in vitro* cytotoxicity methods. The infusion was prepared using 100 g of fresh leaves in 1 L of warm water. Almeida et al. (2006) tested the infusion both immediately after preparation, as well as some hours after it. Three different control were used in the assays: a negative control with no treatement; a control using the tea no-stored and another using the tea under storage. The recently prepared infusion exhibited an initial lethality index of 10%, which increased to 31.82% (4 °C, 48 h) and 76.18% after 48 h, within 60 min of exposure, revealing that the infusions in these time and stocking conditions, present an immunotoxic effect. This effect is due the presence in the tea of oxidated compounds due the free radical production from aromatic and termolabile substances. According the authors, these substances can not be found in the no-stored tea.

In the work of Suwanmanee et al. (2014), the aqueous extract of leaves demonstrated a Lethal Dose LD_{50}=3.5 mg/mL, indicating a low toxicity, with an inhibition of Human skin fibroblast (HSF) cells ≤7%. The fluconazole, the standard drug used in the treatment of fungal infections, with a LD_{50} =1 mg/mL and inhibition of fibroblasts ≤14%, demonstrating that the extract demonstrated lower toxicity than the fluconazole.

The acute toxicity of the aqueous extract of *P. guajava* was tested using concentrations of 50, 100 and 800 mg/kg body weight and in a second phase of the experiment, higher concentrations were administrated (1000, 2000 and 5000 mg/kg). No sign of toxicity and mortality were observed after the analysis, indicating a low toxicity of the extract (Shekins and Dorathy, 2014). The work of Etuk and Francis (2003) aimed at evaluating the acute toxicity using males and females rats (200 a 250 g). Aqueous extracts from leaves of *P. guajava* (5 g/500 mL) were administrated in concentrations ranging between 10 and 50 mg/100 g. After 72 hs of observations, no death or unusual behavior were observed in the groups assayed with 40 and 50 mg/100 g, demonstrating low toxicity again.

Other works reported cytotoxic effects when higher concentrations were assayed. Tea from fresh leaves by infusion was investigated regarding its cytotoxic potential. In the assay of acute toxicity, the LD_{50} of the extract was lower than 20 g/kg. In the assay of chronic toxicity, were administrated daily 0.2, 2.0 and 20.0 g/Kg/day of extract in different groups (equivalent at 1, 10 and 100 times of usual therapeutic dose for the treatmente of diarrhea). An untreated group was used as control. Symptoms of hepatotoxicity and renal problems were observed, such as hydronephrosis (males), nephrocalcinosis and pyelonephritis (females) after six months of observations (Attawish et al., 2013).

The aqueous extract of dry leaves of *P. guajava* was also evaluated to evaluate blood and hepatic damages. The aqueous extract in the form of a gel was administered orally during 30 days in rats (200 mg/kg). The serum level of aspartate aminotransferase (AST), alanine aminotransaminase (ALT) and alkaline phosphatase (ALP) and total proteins and albumine were evaluated. No effect was observed in the hepatic functions of the animals. However, the concentration of red cells was enhanced (Uboh et al., 2010).

Some studies demonstrated that the aqueous extract of *P. guajava* have not affected fertility. Ekaluo et al. (2013) evaluated the effect of this extract against the fertility and production of reproductive hormones. During 70 days, doses ranging 100–300 mg/kg were administered. Enhanced levels of luteinizing hormone/interstitial cell stimulating hormone (LH/ICSH), follicle stimulating hormone (FSH) and testosterone were observed. Also an enhancement in the number of births was observed. Other study demonstrated previously that the aqueous extract of the leaves of *P. guajava* (250 mg) protected the toxic effect of caffein against rat spermatozoids (Ekaluo et al., 2016).

Infusions of *P. guajava* were evaluated *in vitro* and *in vivo* for their effect against chromossomes and cell cycle. Using the plant model of *Allium cepa* L., the extract of *P. guajava* (2.62 and 26.2 mg/mL) reduced the number of mitosis in the higher concentration assayed. However, the effect was considered cytostatic. *In vivo* assays using medular cell from rats demonstrated that doses with 1 mL/100 g b.w. (2.62 e 26.2 mg/100 g. b. w.) did not affected the chromossomes, neither the cell cycle. *In vitro* assays using human limphocytes (0.262 and 2.62 µg/mL culture medium) determined no mutagenic effect when compared with the non-treated control (Teixeira et al., 2003).

The toxicity studies about this plant indicated that the usage of P. guajava can be safe if the tea can be consumed immediately. However, more *in vivo* research is necessary to determine the correct time and dose to be used in the human therapy.

Studies about the cytotoxic effect of metabolites isolated from *P. guajava* were performed. Betulinic acid demonstrated effect against tumor cells of different kinds of câncer due the induction of apoptosis and mitochondrial damages, caunsing few effect in normal cells (Mullauer et al., 2010). Lupeol is also considered an important anti-cancer agent with low systemic toxicity (> 30 mg/Kg) (Chaturvedi et al., 2008; Siddique and Saleem, 2011). Antitumor and chemopreventive activities were demonstrated by the morin and toxic effects were observed only when assayed in high doses (> 300 mg/Kg/day) (Caselli et al., 2016). The quercetine presents a paradoxal toxic effect: some works demonstrated a cytoprotective activity, however, other works indicated a genotoxic and carcinogenic potential (Harwood et al., 2007).

All these works indicated a low toxicity of this plant. The project "Farmácias Vivas" in the Brazil indicates the use of leaves from *P. guajava* in this form: the preparation of a cup of tea (150 mL) use 8–10 g of fresh leaves or 4–5 g of dry leaves (Matos, 2002). Based on these amounts, it can be affirmed that the use of this plant recipe is safe. However, ever must be informed that the inadequate use of *P. guajava* instead of promoting well-being, may lead to undesired effects to the human organism. Therefore, care must be taken with the method of preparation, the time of stocking, and concentration used in the preparation of the medicinal plant and with its interactions with other plants and medications. However, currently not enough is known about the variability of the plant material and the impact of the various modes of preparation and, therefore, only limited conclusions in terms of the drugs' safety can be drawn.

5. *Psidium guajava*-drug interaction

Few works demonstrated the effect of combinations between the extracts of *P. guajava* with drugs against infectious agents. These works combining plants extracts with drugs have the aim to potentiate the effect of the drug, affecting multiple targets in the microbial cell and reducing the risk of drug resistance development (Matias et al., 2013; Morais-Braga et al., 2016).

Chenniappan and Kadarkarai (2010) evaluated the *in vitro* effect of the combination between the cyclohexane (CH) and methylene chloride (MC) guava leaf extracts (5 µg/mL) with the chloroquine against chloroquine-resistant *P. falciparum* strain. When evaluated alone, the extracts inhibited 69% and 58% of the parasite population and demonstrated an IC_{50} of 6.78 µg/mL and 7.91 µg/mL, respectively. When combined with the chloroquine (0.02 µm/mL), a synergistic effect was observed, leading to a reduction of the IC_{50} to 5.30 µg/mL and 7.91 µg/mL and enhanced the antiplasmodial activity of 80% and 64% for the CH and MC, respectively.

The effect of the association between the aqueous (infusion and decoction) and hydroethanol extracts (70%) with the antifungal fluconazole was verified by Morais-Braga et al. (2016) against different strains of *Candida* spp. The fluconazole alone demonstrated IC_{50} values ranging between19.22 and 45.40 µg/mL, but when associated with the infusion and decoction, the values were reduced (3.88–41.11 µg/mL and 7.88–37.52 µg/mL, respectively), indicating that all extracts potentiated the antifungal effect of fluconazole.

Betoni et al. (2006) evaluated the interaction of the methanol extract from the leaves of *P. guajava* with antibiotics against 32 different strains of *S. aureus*. The assays were performed using disk diffusion method and the results were analysed as synergism or non-synergism. Associations between the extract (one-fourth the MIC 90%) with tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), vancomycin (30 µg), oxacillin (1 µg), cephalothin (30 µg), ampicillin (10 µg), cefoxitin (30 µg) and cotrimoxazole (25 µg) were indicated as synergism, uma vez que houve potencialização de efeito. However the molecular basis of the interactions was not elucidated.

Ushimaru et al. (2012), using a similar methodology, evaluated the interaction among the methanol extract of *P. guajava* leaves with antibiotics against 15 strains of *E. coli*. The interactions with ampicillin (10 g), amoxicillin+clavulanic acid (30 g), cephalothin (30 g), cefoxitin (30 g) and tetracycline (30 g) was indifferent. Antagonism was detected when the extract was associated with ciprofloxacin (5 g), gentamicin (10 g), polymyxin (300 UI) and sulfamethoxazole+trimethoprim (25 g). No synergism was observed.

In another work, different extracts from dry leaves of *P. guajava* were assayed alone and in association with tetracycline against strains of *E. coli* and *Salmonella* spp. Synergism was observed between the extracts and the drug. The better effect was observed in the association of aqueous extract and tetracycline (Dalee et al., 2015).

The hydroethanol extract of fresh leaves from *P. guajava* was evaluated alone and in association with antibiotics against standard and multidrug resistant clinical strains of *S. aureus, E. coli* and *P. aeruginosa*. Sub-inhibitory concentrations of the extract were combined with the antibiotics. The hydroethanol extract potentiated the effect of all antibiotics, with exception of gentamicin against *S. aureus* (Morais-Braga et al., 2016).

These researches are important contributions to the study of ethnopharmacology because drugs can modify the effect of a cup of guava tea. However, due the lack of *in vivo* studies about these combinations, is impossible to presume the effect in humans. The consumption of *P. guajava* teas and drugs in the treatment of infectious and parasitic diseases is a very common practice for several populations and these effects require more studies by pharmacists and ethnobiologists, for the simultaneous usage of drugs and plants. These evaluations of side effects of plants products a great challenge for the health system of many countries (Shetti et al., 2011). However, due the possible simultaneous usage of plant products and drugs, the interaction among then and the safe usage must be investigated, being necessary more studies to evaluate the good and side effect of these combinations.

6. Considerations about the stady and conclusions

The main indication is for the treatment of gastrointestinal disorders, and it has also been used for the treatment of several microbial diseases caused by protozoans, fungi, bacteria and viruses.

Many ethnobiological works leave considerable gaps as they lack detailed information over methods of use, type of preparation, administration, time and nature of consumption, if used only for the disease (medicinal) or as a tea (nutraceutical), the condition of the natural product used and even if the plant is utilized in conjunction with a commercial drug or with other species. We believe that the interviews must be more explicative, collecting more informations about the plant, its usage and including the possible interactions with drugs.

Some interactions have been demonstrated but only a few studies used the correct methodology to clarify the type of interactions and there are no works demonstrating the *in vivo* effect in the organism.

In studies for scientific validation of the medicinal use of *P. guajava* the leaf is the most evaluated, followed by the stem bark. However, it is important to highlight the necessity to research active compounds in dry and fresh extracts, both from every part of the plant. The truth is that the lack of more detailed investigations can lead to a waste of pharmacological potential of a species. Besides it is necessary determine a standard form to prepare and use by the populations, being used as na internal control reference drugs to compare the effect.

In the extract preparations, solvents like water, water-ethanol, ethanol and methanol, that is, polar solvents are the most utilized. It is important to note that in many cases the aqueous extract was more active than the other extracts. This is interesting as water is thought to take out less phytochemicals than the other solvents, yet it was more active. When compared, only in a few works were the extracts more effective than the standard drug, but is important to note that many works have not used these standard drugs, seriously limiting their usefulness. However, *P. guajava* isolated compounds are still under-

explored, if we consider the versatility of the plant and its ethnomedicinal importance.

In the bioactive test, *P. guajava* presents the best effect against protozoa (*Leishmania* and *Plasmodium*) and bacteria (*in vitro*). For fungi and viruses, the plant is active, however, more elevated concentrations are needed for these microorganisms. In this review, it was observed that *P. guajava* was effective against 6 different species of protozoans, 19 different species of fungi, 34 different species of bacteria and 4 different types of viruses.

Another relevant observation is the difference in results testing for the same bioactivity. The employment of different methodologies can generate different results, as is the case of microbiological tests using different methodologies like disc diffusion and microdilution. Diffusion tests usually reveal an activity of a product, even weak ones, once the tested concentrations are in milligrams, whereas in most microdilution tests the concentration used are in micrograms. Microdilution tests are more indicated in pharmacological searches, precisely because of the low concentrations used in *in vitro* systems. If a plant, exhibits activity (*in vitro*) only at high concentrations, certainly the possibility of it being directed for pharmacological research will be discarded, since very high concentrations of a determined compound would be needed for the constitution of a drug in order that it may act in a complex *in vivo* system where, normally, in such concentrations it would also exert toxic effects.

For the activities addressed there is still a lack of *in vivo* studies, maybe due to some *in vitro* results needing high concentrations, or even due to *P. guajava* having shown toxicity in some studies. In some cases, where the effect is obtained at low concentrations, studies on the mechanism of action that may elucidate the means by which extracts, fractions or compounds of a plant cause death or interfere in the cellular mechanisms of microorganisms, for example, are missing, impeding their applicability. Maybe due to the fact that guava's effects may be related to its polyphenols, which are reactive species and hard to study.

Although tests with extracts and fractions point to the species' pharmacological potential, more studies are needed in order to isolate the relevant compounds and in many cases studies need to be repeated using lower, more realistic concentrations and with a lower toxicity against human cells.

More studies are necessary to isolate and determine the potential bioactivities of the bioactive metabolites from this species and evaluate its acute and chronic toxicity. The review highlights the therapeutic potential and versatility of *P. guajava* and also justifies the the use of ethnobiological studies in guiding pharmacological studies.

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