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ATIVIDADES BIOLÓGICAS, ISOLAMENTO DE COMPOSTOS ATIVOS E CARACTERIZAÇÃO DE EXTRATOS OBTIDOS DE *Scutia buxifolia* Reissek

TESE DE DOUTORADO

Aline Augusti Boligon

Santa Maria, RS, Brasil.
2014

ATIVIDADES BIOLÓGICAS, ISOLAMENTO DE COMPOSTOS ATIVOS E CARACTERIZAÇÃO DE EXTRATOS OBTIDOS DE *Scutia buxifolia* Reissek

Aline Augusti Boligon

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Doutor em Ciências Farmacêuticas.

Orientadora: Profª. Drª. Margareth Linde Athayde

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elaborada por
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RESUMO

Tese de Doutorado

Programa de Pós-Graduação em Ciências Farmacêuticas

Universidade Federal de Santa Maria

ATIVIDADES BIOLÓGICAS, ISOLAMENTO DE COMPOSTOS ATIVOS E CARACTERIZAÇÃO DE EXTRATOS OBTIDOS DE *SCUTIA BUXIFOLIA* REISSEK.

AUTORA: ALINE AUGUSTI BOLIGON

ORIENTADORA: Profª. Drª. MARGARETH LINDE ATHAYDE

Local e Data da Defesa: Santa Maria, 20 de março de 2014.

A espécie *Scutia buxifolia* Reissek pertence à família Rhamnaceae e é conhecida na medicina popular como coronilha. A infusão das cascas do caule da planta é utilizada popularmente como cardiotônica, hipotensora e diurética. O presente trabalho objetivou determinar a capacidade antimicrobiana, antiviral, antiúlcerogênica e a toxicidade de *S. buxifolia* frente a adultos de *Apis mellifera,* bem como, extrair e identificar os componentes do óleo essencial, caracterizar os extratos e frações utilizados e isolar constituintes químicos da espécie. As cascas do caule e as folhas de *S. buxifolia* foram coletadas em outubro de 2007 no município de Dom Pedrito-RS. O material está depositado no herbário do Departamento de Biologia da UFSM catalogado sob o número de registro SMBD 10919. O material vegetal foi seco, moído e macerado utilizando como solvente etanol:água (70:30, v/v). Fez-se fracionamento do extrato bruto com solventes orgânicos de polaridades crescentes (diclorometano, acetato de etila e n-butanol). O extrato bruto de *S. buxifolia* (100, 200 e 400 mg/Kg) reverteu os danos gástricos causados pelo etanol em estômago de ratos, de maneira dose-dependente. Além disso, a espécie não apresentou toxicidade frente a *Apis mellifera* após 15 dias de observação. Os principais componentes identificados no óleo essencial foram espatulenol, β-cubebene, germacreno D e carvacrol. Ácidos fenólicos (ácido gálico, ácido clorogênico e ácido cafeico) e flavonoides (rutina, quercetina, isoquercitrina, quercitrina e canferol) foram os principais compostos identificados e quantificados no extrato bruto e frações de *S. buxifolia*. Finalizando nosso estudo, sitosterol-3-*O*-β-D-glucosídeo, β-sitosterol e ácido ursólico foram isolados da fração diclorometano das cascas do caule da espécie. Nossos resultados indicam que *S. buxifolia* apresenta um papel benéfico no tratamento e prevenção de algumas enfermidades, e os compostos químicos presentes na espécie contribuem para as propriedades encontradas.

Palavras – chave: *Scutia buxifolia*; Rhamnaceae; antimicrobiana; antiviral; antiúlcera; CLAE.

ABSTRACT

Doctoral Thesis

Postgraduate Program in Pharmaceutical Sciences

Federal University of Santa Maria

ATIVIDADES BIOLÓGICAS, ISOLAMENTO DE COMPOSTOS ATIVOS E CARACTERIZAÇÃO DE EXTRATOS OBTIDOS DE *SCUTIA BUXIFOLIA* REISSEK.

AUTHOR: ALINE AUGUSTI BOLIGON

ADVISER: Profª. Drª. MARGARETH LINDE ATHAYDE

Place and Date of Defense: Santa Maria, March 20, 2014.

Scutia buxifolia Reissek belongs to the Rhamnaceae family and is popularly known as coronilha. The stem bark infusion is popularly used as cardiotonic, antihypertensive and diuretic. This work aims to determine the antimicrobial and antiviral activities and antiulcer capacity and the toxicity of *S. buxifolia* against *Apis mellifera* adults, as well as identify and extract the essential oil components, characterize the utilized extracts and fractions and isolate chemicals constituints. Stem bark and leaves of *S. buxifolia* were collected in the city of October 2007 in Dom Pedrito-RS (coordinates 30º 59'09"S and 54º27'44"W). The material is deposited in the herbarium of the Department of Biology UFSM cataloged under the registration number SMBD 10919. The dried plant material was ground and macerated using ethanol: water (70:30, v/v) as solvent. The crude extract was fractionated with solvents of increasing polarity (dichloromethane, ethyl acetate, n-butanol). Ethyl acetate and n-butanol fractions showed the best antimicrobial and antiviral activities. The crude extract of *S. buxifolia* (100, 200 and 400 mg/kg) reversed the gastric damage caused by ethanol in the stomach of rats in a dose-dependent manner. Moreover, the species showed no toxicity against *Apis mellifera* after 15 days of observation. The main components identified in the essential oil were spathulenol, β-cubebene, germacrene D and carvacrol. Phenolic acids (gallic, chlorogenic and caffeic acids) and flavonoids (rutin, quercetin, isoquercitrin, quercitrin and kaempferol) were the main compounds identified and quantified in the crude extract and fractions of *S. buxifolia*. Finally, sitosterol-3-*O*-β-D-glucoside, β-sitosterol and ursolic acid were isolated from the dichloromethane fraction of the species. Our results indicated that *S. buxifolia* have a beneficial role in the treatment and prevention of certain diseases, and the chemical compounds present in the species contribute to the properties found.

Key words: *Scutia buxifolia*; Rhamnaceae; antimicrobial; antiviral; antiulcer; HPLC.

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1 INTRODUÇÃO

O uso das plantas para o tratamento, cura e prevenção de doenças é uma das práticas medicinais mais antigas da humanidade. As civilizações primitivas perceberam que ao lado das plantas que usavam como alimentos, existiam outras que ao serem experimentadas no combate às suas enfermidades, revelavam grande potencial curativo. Todas as informações sobre essas plantas medicinais foram sendo transmitidas pelas populações ao longo de várias gerações, para depois, serem registradas em monografias (CUNHA, SILVA, ROQUE, 2003; NEWMAN, CRAGG, SNADER, 2000).

A fitoterapia se caracteriza pelo tratamento com o uso de plantas medicinais em suas diferentes formas farmacêuticas sem a utilização de princípios ativos isolados (ARAÚJO et al., 2007). Nas últimas décadas, o interesse pelas terapias naturais tem aumentado significativamente nos países industrializados e encontra-se em expansão o uso de plantas medicinais e de fitoterápicos. Apesar da medicina moderna estar bem desenvolvida na maior parte do mundo, a Organização Mundial da Saúde (OMS) considera que, aproximadamente 80% da população mundial depende principalmente da medicina tradicional para seus cuidados primários de saúde, bem como do uso de extratos de plantas ou de seus princípios ativos (BRAZ FILHO, 2010; FARNSWORTH et al., 1985; KAUR, 2005).

A estratégia utilizada para o uso das plantas medicinais é a abordagem etnofarmacológica, que consiste em combinar informações adquiridas junto a comunidades locais, que fazem uso da flora medicinal, com estudos químico-farmacológicos. Desse modo, é possível formular hipóteses quanto às atividades farmacológicas e os ativos responsáveis pelas ações terapêuticas relatadas pelos usuários, além disso, estudos também podem descrever possível toxidade das plantas (ELISABETSKY, SETZER, 1985; ELISABETSKY, 1987). As potencialidades de uso das plantas medicinais encontram-se longe de estarem esgotadas. O Brasil, por possuir a maior biodiversidade do planeta (15-20%), associada a uma rica diversidade étnica e cultural, além de conhecimentos valiosos sobre o manejo e uso de plantas na medicina popular, detém grande potencial para o desenvolvimento de

pesquisas com plantas medicinais (AGRA, FREITAS, BARBOSA-FILHO, 2007; BRASIL, 2006).

As plantas medicinais são importantes para a pesquisa farmacológica e para o desenvolvimento de fármacos quando seus constituintes são usados diretamente como agentes terapêuticos (fitoterápicos) ou como matérias-primas isoladas (fitofármacos). A importância das substâncias naturais derivadas de plantas é reconhecida no desenvolvimento de modernos fármacos devido à sua função biológica e sua ampla diversidade molecular (CALIXTO, 1997; GUERRA, NODARI, 2007; SIMÕES et al., 2004; SIMÕES et al., 2010). O avanço da química orgânica permitiu a obtenção de compostos puros e ainda possibilitou as modificações estruturais, visando produzir fármacos potencialmente mais ativos, seguros e com menos efeitos colaterais. Alguns exemplos de fármacos importantes obtidos de plantas são digoxina (*Digitalis* spp.), atropina (*Atropa belladona*), morfina e codeína (*Papaver somniferum*), reserpina (*Rauvolfia serpentina*), quinina e quinidina (*Cinchona* spp.), vincristrina e vinblastina (*Catharanthus roseus*) e paclitaxel (*Taxus brevifolia*) (BUSS, COX, WAIGH, 2003; GUERRA, NODARI, 2007; NEWMAN, CRAGG, SNADER, 2000; RATES, 2001).

Aproximadamente 40% dos medicamentos atualmente disponíveis foram desenvolvidos direta ou indiretamente a partir de fontes naturais, assim subdivididos: 25% de plantas, 12% de microorganismos e 3% de animais (CALIXTO et al., 2001). Dos 252 fármacos considerados essenciais pela OMS, 11% são originários de plantas e um número significativo destes são sintetizados a partir de precursores naturais (RATES, 2001). No Brasil, de 1999 para 2000, o crescimento do mercado de medicamentos fitoterápicos aumentou 15%, enquanto o crescimento do mercado de medicamentos sintéticos foi de apenas 3 a 4% (ABIFITO, 2001).

Em 2006, o governo brasileiro aprovou a Política Nacional de Plantas Medicinais e Fitoterápicos, que incluem no Sistema Único de Saúde (SUS) o uso de plantas medicinais e de fitoterápicos, e que possui como objetivo geral, a garantia de acesso seguro e racional dessa terapia alternativa, promovendo o uso sustentável da biodiversidade e desenvolvimento da cadeia produtiva e da indústria nacional. Essa política reforça a importância de produtos naturais na saúde pública, estimulando novos estudos que garantam e promovam a segurança, a eficácia e a qualidade na utilização de plantas medicinais e fitoterápicos (BRASIL, 2006).

A OMS estima que no ano de 2020 a população mundial chegará a 7,5 bilhões de pessoas e que destas, 75% viverão em paises em desenvolvimento, os quais consomem hoje menos de 15% do mercado total de medicamentos. Essas observações indicam que, no futuro, esta população dependerá ainda mais das plantas medicinais. Visando diminuir o número de excluídos dos sistemas governamentais de saúde, a OMS recomenda aos órgãos responsáveis pela saúde pública de cada país, que realizem levantamentos regionais das plantas utilizadas na medicina popular tradicional estimulando o uso daquelas que tiverem comprovado sua eficácia e segurança terapêutica (LORENZI, MATOS, 2002).

O estudo aqui apresentado tem como perspectiva contribuir para caracterização, isolamento de compostos ativos e avaliação de atividades biológicas de extratos obtidos a partir das cacas do caule e folhas *Scutia buxifolia* Reissek. Essa espécie, pertencente à família Rhamnaceae, é conhecida popularmente como coronilha e na medicina tradicional suas cascas são utilizadas sob forma de infusão devido a suas propriedades cardiotônica, hipotensora e diurética (WASICKY, WASICKY, JOACHIMOVITS, 1964). A espécie *S. buxifolia*, apesar de ser distribuída no Sul da América do Sul, ainda é uma planta pouco conhecida dos pontos de vista químico e farmacológico, tornando-se importante a realização de um estudo aprofundado sobre esta espécie e assim contribuir para o conhecimento da flora brasileira e também na busca por novos compostos bioativos de interesse.

2 OBJETIVOS

2.1 Objetivo geral

Investigar atividades biológicas, isolar compostos bioativos e caracterizar os extratos obtidos de *Scutia buxifolia* Reissek.

2.2 Objetivos específicos

- Caracterizar o extrato bruto (EB) e as frações diclorometano (DCM), acetato de etila (AcOEt) e *n*-butanol (BUT) das cascas do caule e folhas de *S. buxifolia* por Cromatografia Líquida de Alta Eficiência (CLAE);
- Isolar e identificar os compostos ativos;
- Extrair e caracterizar o óleo essencial das folhas de *S. buxifolia*;
- Avaliar atividades biológicas: Atividade antimicrobiana, atividade antiviral (HSV-1), atividade antiulcerogênica e toxicidade frente à *Apis mellifera*.

3 REVISÃO DA LITERATURA

3.1 Descrição da planta

3.1.1 A família Rhamnaceae

Rhamnaceae é uma família de plantas angiospérmicas que abrange plantas com os mais variados hábitos, desde erva até árvores, ocorrendo em florestas tropicais ou subtropicais de todo o mundo (LIMA, 2000). Esta família abrange 58 gêneros com aproximadamente 900 espécies (HEYWOOD, 1993). As folhas são simples, alternadas e normalmente estipuladas. As flores são actinomorficas, bissexual e pentâmeras. O androceu é formado por cinco estames e pétalas que revestem o receptáculo floral. O gineceu é composto por pistilo de usualmente 2-4 carpelos e os frutos são drupas. No noroeste da Europa é descrita a ocorrência de dois gêneros com quatro espécies. Todos são arbustos dos quais dois são comuns nessa área, *Frangula alnus e Rhamnus catharticus*; *R. alaternus e R. saxatilis* ocorrem, geralmente, no sul e centro da Europa (ERDTMAN et al., 1961).

As plantas da família Rhamnaceae apresentam uma variada classe de constituintes químicos, como alcaloides ciclopeptídicos (MOREL et al., 1998), flavonoides, antocianinas, taninos, esteroides e triterpenos (SHAH et al., 1985). Os alcaloides ciclopeptídicos são bastante difundidos na medicina popular no tratamento de várias moléstias, tais como, disenteria, hipertensão arterial (KLEIN, RAPOPORT, 1968) e vários tipos de infecções (SHAH et al., 1985).

3.1.2 O gênero Scutia

Scutia é um gênero de arbustos que apresenta algumas dificuldades para sua identificação. É um dos três gêneros da família Rhamnaceae em que cada fruto maduro tem dois ou três pedras uniovulado, as espécies pertencentes a este gênero são: *Scutia buxifolia, Scutia myrtina, Scutia colombiana, Scutia spicata* e *Scutia arenicola* (JOHNSTON, 1974).

3.1.3 A espécie Scutia buxifolia Reissek

A espécie *Scutia buxifolia* (Figura 1), conhecida popularmente como coronilha, canela-de-espinho ou espinho-de-touro, é uma planta nativa da América do Sul, ocorrendo principalmente no Rio Grande do Sul, Argentina e Uruguai (BOLIGON et al., 2009; WASICKY et al., 1964). No Rio Grande do Sul, encontra-se em florestas com araucárias, florestas ribeirinhas na Serra do Sudeste e no Litoral Sul (SOBRAL et al., 2006). Ocorre também ocasionalmente na Depressão Central e é frequente em certas regiões elevadas da Serra do Sudeste.

É uma pequena árvore ou arbusto de até seis metros de altura, sua copa é esférica generalizada e possui espinhos. Sua folhagem persistente varia de cor em função da sua exposição ao sol, a partes expostas ao sol apresentam cor verde claro e a sombra verde escuro, as folhas são opostas até alternas, inteiras ou com poucos dentes e lustrosas, variam de 2-4 cm de comprimento e 1-2 cm de largura. Inflorescências em fascículos axilares com flores pequenas e verdes, que podem ser vistas na primavera (floresce entre outubro e janeiro). O fruto tem de 3 a 5 mm de diâmetro, drupa globosa, e apresenta uma cor escura quando atinge a maturidade (a espécie frutifica no mês de março). *Scutia buxifolia*, pertencente à família Rhamnaceae, é usada popularmente como cardiotônica, hipotensora e diurética através da infusão em água da casca do caule e folhas (WASICKY et al., 1964).

3.1.3.1 Compostos isolados de Scutia buxifolia

Os primeiros relatos sobre a presença de alcaloides na espécie *S. buxifolia* ocorreram em 1964, quando Wasicky e colaboradores observaram a presença de alcaloides ciclopeptídicos nesta planta. Porém, um estudo mais aprofundado com esta espécie foi realizado em 1967 por um grupo da Universidade de Bonn (TSCHESCHE et al., 1970), que, trabalhando com a raiz da planta coletada em Santa Catarina, isolou um alcalóide peptídico, scutianina A (Figura 2). Além desse alcalóide detectaram a presença de outras bases, o que serviu de incentivo para novas tentativas de isolamento. Mais tarde, pesquisadores da Universidade de

Buenos Aires (SIERRA et al., 1974), determinaram todos os centros quirais da scutianina A.

Figura 1 - *Scutia buxifolia* Reissek (Coronilha) – Aspectos da planta.

Disponível em: <http://www.brazilian-plants.com/brsearch.cfm>. Acesso em 10/08/2012.

Tschesche e colaboradores (1974) isolaram da raiz da mesma planta três alcaloides denominados como: scutianina C, scutianinas D e E. Morel e colaboradores, em 1979, isolaram do extrato metanólico da casca da raiz da espécie

S. buxifolia os alcaloides scutianinas B, C, D e E, todos conhecidos, e dois alcaloides novos, scutianinas H e I (Figura 2).

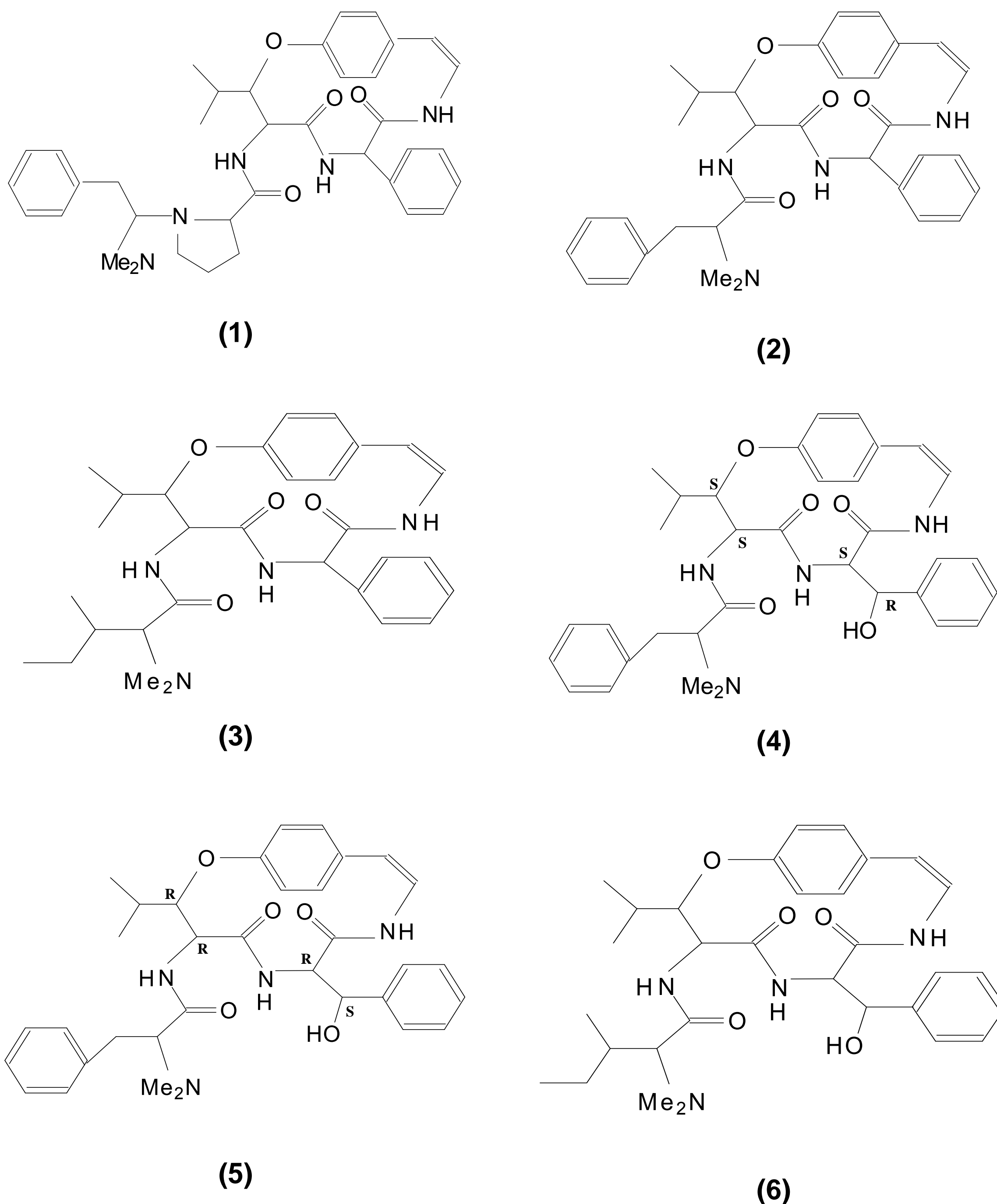

Figura 2 – Estruturas químicas de alcaloides isolados de *S. buxifolia*: (1) scutianina A, (2) scutianina B, (3) scutianina C, (4) scutianina D, (5) scutianina E e (6) scutianina H.

Em 1977, Tschesche e colaboradores isolaram dois novos alcaloides da mesma planta: scutianina G e F. A casca do caule de *S. buxifolia* também contém substâncias que pertencem ao grupo dos alcaloides peptídicos. Menezes e colaboradores (1995) isolaram do extrato metabólico das cascas de *S. buxifolia*, coletada em Santana do Livramento em março de 1993, as scutianinas B, C, D, E, H e um novo alcalóide, a scutianina J. Em continuação ao estudo de Menezes e colaboradores (1995), foram isolados dois novos alcaloides ciclopeptídicos: scutianina K e L (MOREL et al., 1998). Em 2005, Morel e colaboradores isolaram um novo alcaloide, a scutianina M (Figura 3).

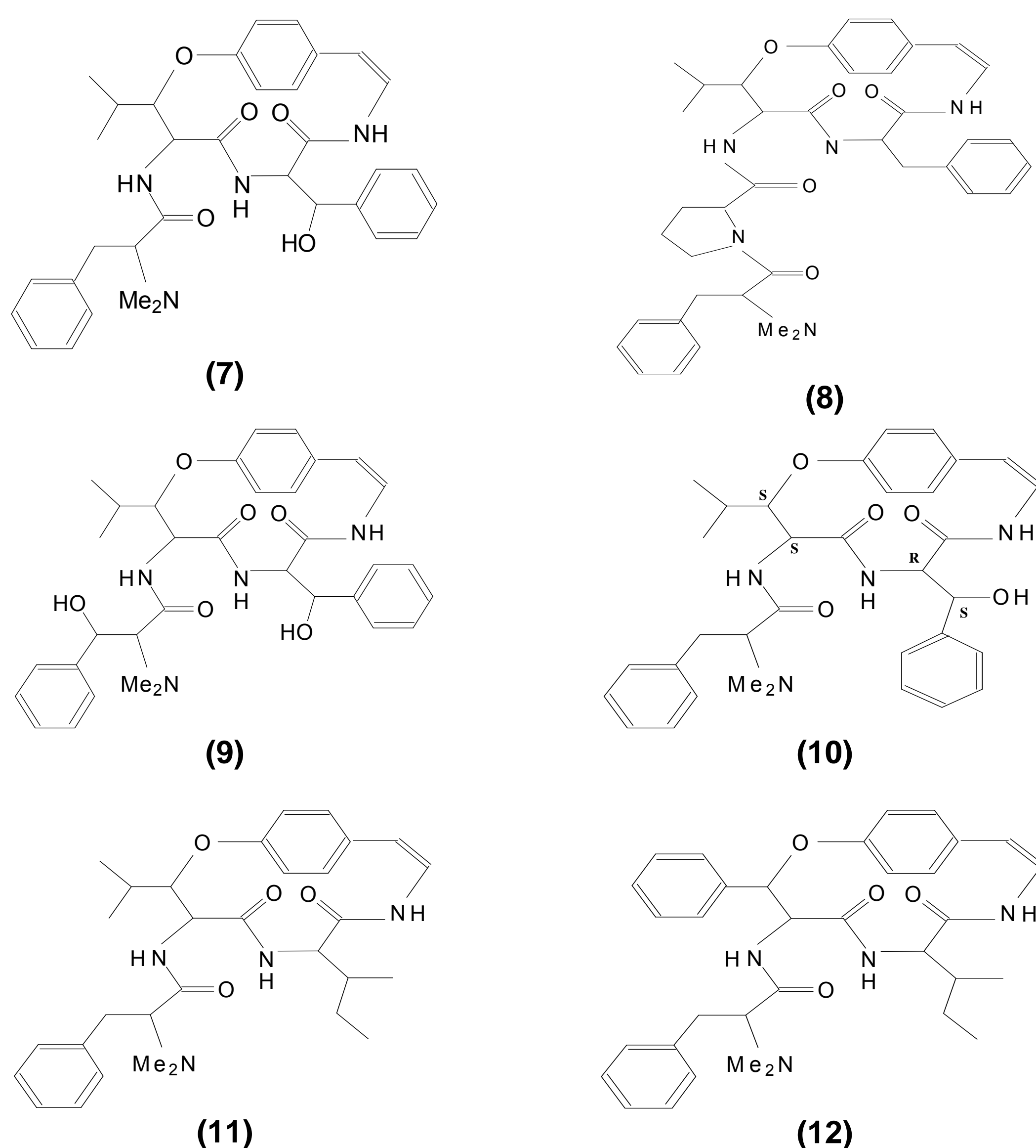

Figura 3 - Estruturas químicas de alcaloides isolados de *S. buxifolia*: (7) scutianina I, (8) scutianina F, (9) scutianina J, (10) scutianina K, (11) scutianina L e (12) scutianina M.

A presença de flavonoides em *S. buxifolia* também já foi descrita. Quercetina, quercitrina, isoquercitrina e rutina foram isoladas e quantificadas na fração acetato de etila de *S. buxifolia* por Boligon e colaboradores (2009). Além dos flavonoides citados, foram isolados, identificados e quantificados alguns compostos triterpenóides (β-sitosterol, estigmasterol e lupeol) presentes na fração diclorometano da espécie (BOLIGON et al., 2010).

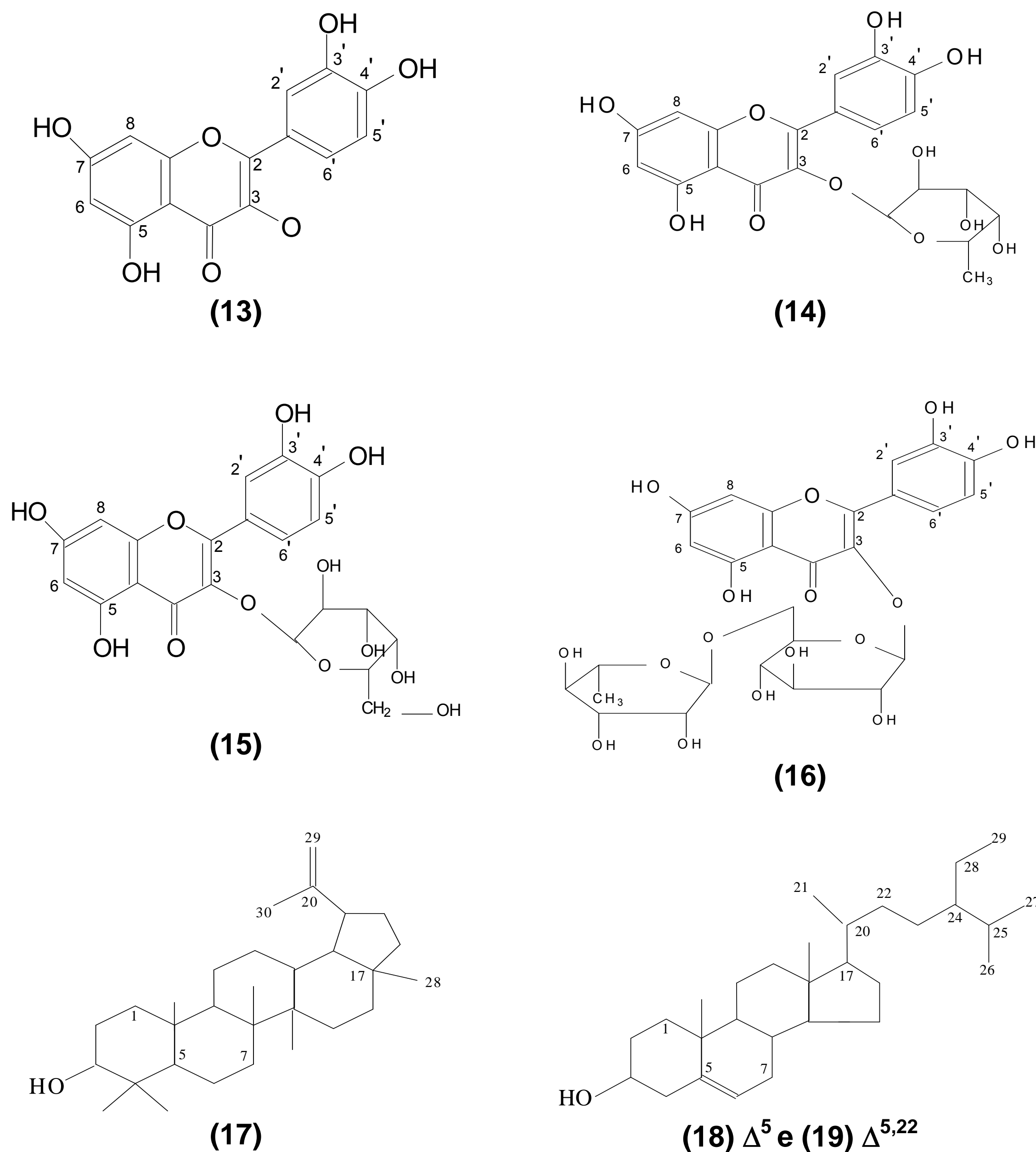

Figura 4 – Estruturas químicas de flavonoides e triterpenos isolados de *S. buxifolia*: (13) quercetina, (14) quercitrina, (15) isoquercitrina, (16) rutina, (17) lupeol, (18) β-sitosterol e (19) estigmasterol.

3.1.3.2 Atividades descritas para S. buxifolia

A atividade antimicrobiana de alguns alcaloides ciclopeptídicos isolados da casca da raiz de *S. buxifolia* foi relatada por Morel et al. (2005), utilizando o método de bioautografia. O composto com o maior espectro de atividade foi a scutianina E, sendo efetiva contra *Staphylococcus aureus* ATCC 6538; *Staphylococcus epidermidis* ATCC 12228; *Micrococcus luteus* ATCC 9341; *Escherichia coli* ATCC 25792 e *Klebsiella pneumoniae* ATCC 10031. Scutianina D apresentou modesta atividade antibacteriana contra *M. luteus*; *S. epidermidis* e *E. coli*, enquanto a scutianina B só foi ativa contra a *E. coli* e a scutianina M não apresentou atividade frente às cepas testadas. Além disso, as frações (diclorometânica, acetato de etila e butanólica) das folhas, ramos e cascas do caule de *Scutia buxifolia* mostraram efeitos antimicrobianos frente a *Saccharomyces cerevisiae* (concentração inibitória mínima; CIM = 62,5 – 250 μg/mL), *Pseudomonas aeruginosa*, *Staphylococcus aureus* (CIM = 125 – 500 μg/mL) e *Bacillus subtilis* (CIM = 250 – 500 μg/mL) (BOLIGON et al., 2012).

Trevisan et al. (2009) investigaram o potencial analgésico das scutianinas B, C e D através do teste de retirada da cauda (*tail-flick*), as scutianinas foram administradas via intratecal em ratos. Scutianina B (10 nmol) induziu efeito antinociceptivo em 15 e 60 minutos após a sua administração, a scutianina C (10 nmol) produziu hiperalgesia em 15 e 60 minutos após a sua administração e a scutianina D não apresentou alterações.

Extratos e frações de *S. buxifolia* apresentaram eficaz inibição da produção de espécies reativas ao ácido tiobarbitúrico (TBARS), além disso, demostraram atividade captadora de radicais livres, a presença de compostos polifenólicos em concentrações elevadas na espécie contribuem para a capacidade antioxidante descrita. A presença de flavonoides indica que *S. buxifolia* possui compostos promissores para o tratamento de doenças resultantes do stress oxidativo. A avaliação fitoquímica de *Scutia buxifolia* revelou a presença de cumarinas, ácidos orgânicos, heterosídeos flavônicos, heterosídeos cianogênicos, fenóis e alcaloides (BOLIGON et al., 2009).

Recentemente, efeitos citotóxicos de extratos das folhas, galhos e cascas do caule da planta foram avaliados pelo ensaio de *Artemia salina.* Todos os extratos

testados foram considerados tóxicos para este microcrustáceo (concentração letal 50%, CL_{50} = 82,23 ± 0,34 – 50,00 ± 0,22 μg/mL) (BOLIGON et al., 2012). Entretanto, Freitas et al. (2012) descreveram que o extrato aquoso liofilizado das cascas do caule de *S. buxifolia* não apresentou toxicidade aguda nas concentrações testadas (100, 200 e 400 mg/kg de peso corporal do animal). Os autores especulam que a dose letal de extrato aquoso liofilizado seja superior a 400 mg/kg, porque as doses testadas não causaram mortalidade em ratos.

Além disso, o efeito hepatotóxico também foi investigado (animais foram tratados sub-cronicamente nas doses de 100, 200 e 400 mg/kg de peso corporal, com o extrato aquoso liofilizado das cascas do caule de *S. buxifolia*). Observou-se que o extrato aquoso de *S. buxifolia* não causou alterações nas defesas antioxidantes enzimáticas analisadas (catalase e superóxido dismutase), não elevou o conteúdo de malondialdeido no tecido hepático e não houve alterações na atividade das enzimas alanina aminotransferase (ALT) e aspartato aminotransferase (AST). Não foram observadas características negativas (inflamação, cirrose, necrose, congestão sinusoidal e ruptura das veias centrais) na análise histopatológica do fígado, desta forma, concluiu-se que *S. buxifolia* não causou hepatotoxicidade (FREITAS et al., 2013).

Scutia buxifolia é usada na medicina tradicional como um agente anti-hipertensivo, a fim de avaliar esta atividade, Da Silva et al. (2012) investigaram os efeitos vasculares provocados por *S. buxifolia* em aorta de ratos. Os resultados demonstraram que as frações da planta induziram o relaxamento em anéis isolados da aorta, sendo o relaxamento vascular completamente relacionado com a ativação do sistema NO/GMPc (óxido nítrico/guanosina monofosfato cíclico) e canais de K^+.

3.2 Atividades relacionadas ao potencial antioxidante

3.2.1 Ação antimicrobiana e antiviral

As plantas contêm uma ampla variedade de metabólitos secundários com capacidade antioxidante, ou seja, estrutura química favorável à captação e neutralização de radicais livres. Polifenóis, flavonoides, taninos, carotenoides, terpenoides, quinonas, cumarinas, antocianinas e vitaminas possuem propriedades

antioxidantes (CHOI et al., 2002; REEDY, ODHAV, BHOOLA, 2003). Estudos têm demonstrado que muitas destas substâncias antioxidantes também possuem atividade antimicrobiana, antiviral, antitumoral, anti-inflamatória e antiúlcera (BHOURI et al., 2010; HARBONE, WILLIANS, 2000; SHAHAT, 2002).

Apesar da evolução das pesquisas farmacêuticas para a produção de novos antibióticos, a resistência microbiana a estes fármacos se tornou um problema de saúde pública. Em geral, bactérias, fungos e vírus têm a habilidade genética de adquirir e de transmitir resistência aos fármacos utilizados como agentes terapêuticos (COHEN, 1992; MEHRGANA et al., 2008). Assim, existe a necessidade de pesquisas que visem o descobrimento de novas substâncias com atividade antiviral e antimicrobiana. O sistema de medicina tradicional baseada em produtos naturais continua a desempenhar um papel importante no tratamento de muitas doenças, especialmente das doenças infecciosas (RAUHA et al., 2000). Conforme a estimativa da OMS, cerca de 80% da população mundial depende principalmente da medicina tradicional para sua saúde primária. O desenvolvimento de novos produtos obtidos a partir de plantas medicinais é vital para controlar as ameaças representadas pelos microrganismos patogênicos (JASSIM, NAJI, 2003). Devido a este fato, ultimamente muitos trabalhos têm sido realizados em busca de novas plantas com atividade antimicrobiana. Sabe-se que esta atividade tem sido atribuída a diferentes compostos bioativos, como flavonoides, fenóis, terpenos e taninos (COS et al., 2006; MAHLKE et al., 2009). Estas observações levam à busca de novos compostos antimicrobianos e antivirais derivados de plantas, que surgem como uma promissora alternativa terapêutica para várias doenças.

A Loque Americana ou *American foulbrood* (AFB) é uma doença bacteriana produzida pelo bacilo *Paenibacillus larvae* um microrganismo móvel com flagelos, que possui a forma de um bastão. Uma característica fundamental de *P. larvae* é a formação de endósporos, extremamente resistentes ao calor, aos desinfetantes químicos, ao cloro, à radiação UV e água quente (GONZÁLEZ; MARIOLLI, 2010). A AFB é uma das doenças bacterianas mais graves que afetam abelhas (*Apis mellifera*), está distribuída em todo o mundo e causa uma diminuição na população de abelhas e na produção de mel (GENERSCH et al., 2010; GONZÁLEZ; MARIOLLI, 2010). Apicultores têm dificuldade em tratar AFB porque o agente etiológico produz esporos ambientalmente estáveis, virulentos e resistentes (THOMPSON et al., 2007), por isso, esta doença é considerada uma ameaça global para a apicultura

(GENERSCH, 2010). Alguns produtos naturais, tais como: a própolis, óleos essenciais e extratos de *Myrtus communis, Eucalyptus gunnii, Rosmarinus officinalis* e *Zingiber officinale* foram previamente investigados e apresentaram efeito frente *P. larvae*, sendo uma alternativa natural para a prevenção e/ou controle da AFB (FLESAR et al., 2010; MIHAI et al., 2012; SANTOS et al., 2012).

3.2.2 Ação antiulcerogênica

As principais patologias do estômago são as úlceras gástricas e gastrites. A etiologia da úlcera gástrica é influenciada por fatores agressivos (secreção de ácido gástrico) e defensivos (integridade da mucosa gástrica) (LAINE, TAKEUCHI, TARNAWSKI, 2008; MOTA et al., 2011). A incidência de úlcera gástrica é aumentada devido ao estresse, tabagismo, *Helicobacter pylori,* ácidos, analgésicos, anti-inflamatórios não-esteroidais e etanol (VONKEMAN et al., 2007; INEU et al., 2008).

O excesso de espécies reativas de oxigênio (EROs), tais como radical hidroxila ($OH^{\bullet}$), ânion radical superóxido ($O_2^{\bullet}$), hidroperoxila ($ROO^{\bullet}$), peróxido de hidrogênio (H_2O_2) e oxigênio singleto ($^1O_2$) causa a oxidação de biomoléculas como o DNA, lipídios e proteínas levando eventualmente às doenças crônicas decorrentes do estresse oxidativo, como câncer, arterosclerose, doenças degenerativas, doenças cardiovasculares e pulmonares (CAI et al., 2004; SOUSA et al., 2007). Além disso, falhas nos mecanismos que controlam a produção exacerbada das EROs provocam danos à mucosa gástrica (HAHN et al., 1997; SMITH et al., 1996). Esses danos são facilmente produzidos pela formação de radicais livres endógenos e exógenos, os quais são extremamente reativos. O etanol aumenta os níveis do ânion superóxido, radical hidroxil e peroxidação lipídica no tecido estomacal (BAGCHI et al., 1998). Por isso, substâncias antioxidantes oferecem uma significativa proteção contra os efeitos do etanol na mucosa gástrica (SATHISH, VYAWAHARE, NATARAJAN, 2011). Os antioxidantes são capazes de interceptar os radicais livres gerados pelo metabolismo celular ou por fontes exógenas, impedindo o ataque sobre os lipídeos, os aminoácidos das proteínas, a dupla ligação dos ácidos graxos poliinsaturados e as bases do DNA, evitando a formação de lesões e perda da integridade celular (GILL et al., 2012).

A exposição crônica ao etanol está envolvida na manifestação de diversas patologias do estômago, tais como irritação e inflamação da mucosa gástrica, gastrite, úlcera gástrica e péptica. Embora o fígado seja a principal via de metabolização do álcool, este também pode ser oxidado pela enzima álcool desidrogenase e pelo citocromo P450, localizada no estômago (LAINE, TAKEUCHI, TARNAWSKI, 2008). Na literatura encontra-se uma gama de estudos sobre os efeitos benéficos dos antioxidantes de origem natural que são usados no tratamento do dano oxidativo em modelos de úlcera gástrica. Os compostos mais investigados são os flavonoides, ácidos fenólicos, taninos e esteroides (GILL et al., 2012; MOTA et al., 2011, SATHISH et al., 2011).

3.3 Generalidades sobre óleos essenciais

Óleos essenciais são misturas de constituintes voláteis à temperatura ambiente, originados, na maioria das vezes, do metabolismo secundário, sendo produzidos e armazenados em estruturas secretoras próprias formadas nas folhas, flores, ramos, caules ou raízes de diversas espécies (KAMATOU et al., 2007). Apresentam-se na forma de líquidos oleosos voláteis, dotados de aroma forte geralmente agradável. São extraídos de plantas por processo específico, sendo o mais frequente a destilação por arraste com vapor d’água (CRAVEIRO et al., 1981; EDRIS, 2007). Praticamente todos os óleos essenciais constituem-se de misturas químicas complexas formadas por: hidrocarbonetos, álccois, cetonas, aldeídos, éteres, óxidos, ésteres, entre outros (EDRIS, 2007). Essas misturas heterogêneas podem conter dezenas de compostos, em diferentes concentrações. Cada óleo essencial é caracterizado por um ou mais componentes principais que podem atingir níveis elevados, em comparação com outros compostos presentes em quantidades vestigiais (ZOUARI, 2013).

Segundo Paduch et al. (2007) os constituintes químicos dos óleos essenciais podem ser divididos em duas classes baseadas na origem de sua biossíntese: derivados de terpenos (fomados pela via acetato-ácido mevalônico) e compostos aromáticos (formados pela via do ácido fenilpropanóico). No entanto, para uma dada espécie, fatores naturais podem interferir na variabilidade da composição química e

na qualidade dos óleos essenciais. Estes fatores podem ser intrínsecos (relacionados com a planta) ou extrínseco (ambiente em que a planta se desenvolveu), o que faz com que ocorra vários quimiotipos dentro da mesma espécies (ZOUARI, 2013).

Estudos têm demonstrado várias propriedades farmacológias benéficas atribuídas aos óleos essenciais, como: propriedade antisséptica (frente a bactérias e fungos diversos), antiviral, antioxidante, anti-inflamatória, expectorante, diurética, sedativa, anestesica local, entre outras (EDRIS, 2007; PASSOS et al, 2007; SHIMIZU, 1990;).

APRESENTAÇÃO

Os resultados desta tese estão apresentados na forma de publicações científicas, as quais constituem os capítulos 1 ao 6, sendo formatada de acordo com os periódicos onde os artigos foram publicados.

Além disso, esta tese contempla um item de considerações finais, no qual os resultados são interpretados em conjunto, das conclusões e das referências bibliográficas, as quais são relativas às seções introdução, revisão da literatura e considerações finais.

Na seção dos anexos são apresentados os espectros de ressonância magnética nuclear dos compostos isolados.

4 PUBLICAÇÕES CIENTÍFICAS

4.1 Capítulo 1

Boligon, A.A.; Kubiça, T.K.; Mario, D.N.; Brum, T.F.; Piana, M.; Weiblen, R.; Lovato, L.; Alves, S.H.; Santos, R.C.V.; Alves, C.F.S.; Athayde, M.L. Antimicrobial and antiviral activity-guided fractionation from *Scutia buxifolia* Reissek extracts. Acta Physiol Plant. DOI 10.1007/s11738-013-1259-0

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ORIGINAL PAPER

Antimicrobial and antiviral activity-guided fractionation from *Scutia buxifolia* Reissek extracts

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Abstract Antimicrobial and antiviral activities of the fractions from *Scutia buxifolia* stem bark and leaves were evaluated. Best antimicrobial results occurred with the ethyl acetate (EA) and n-butanolic (NB) fractions from the leaves against *Micrococcus* sp. (minimal inhibitory concentration—MIC = 62.5 μg/ml), and NB fraction from stem bark and leaves against *Klebsiella pneumoniae* and *Enterococcus faecalis* (MIC = 62.5 μg/ml). The most active fractions were selected and fractioned into silica column to perform an in vitro antibiofilm assay, which evidenced subfractions EA2 and EA3 as the more active against *Candida albicans* (biofilm inhibitory concentration—BIC = 582 ± 0.01 μg/ml) and *Staphylococcus aureus* (BIC = 360 ± 0.007 μg/ml), respectively. The NB (selectivity index—SI = 25.78) and the EA (SI = 15.97) fractions from the stem bark, and the EA (SI = 14.13) fraction from the leaves exhibited a potential antiviral activity towards Herpes Simplex Virus type 1 whereas EA2 and EA3 subfractions from leaves (SI = 12.59 and 10.06, respectively), and NB2 subfraction from stem bark (SI = 12.34) maintained this good activity. Phenolic acids and flavonoids (gallic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, quercitrin and quercetin) were identified by HPLC and may be partially responsible for the antimicrobial and antiherpes activities observed. The results obtained in this study showed that *Scutia buxifolia* has antibiofilm and anti-herpetic activities and that these properties are reported for the first time for this species.

Keywords Scutia buxifolia · Antimicrobial activity · Biofilm · HSV-1 · HPLC

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Introduction

Associated with the extensive use of antibiotics the problem of drug resistance has become widespread. Therefore, intensive search for new antibiotics is needed on a worldwide basis and numerous efforts have been made to find new antibacterial compounds from various sources, such as microorganisms, soils, animals and plants. The use of medicinal plants for the treatment of several diseases, including fungal and bacterial infections is typical in Brazil (Fontenelle et al. 2008), and worldwide many research groups screen plant extracts to detect secondary metabolites with antimicrobial and antiviral properties (Freitas et al. 2009; Rangasamy et al. 2007; Soberón et al. 2007) in an attempt to find new antibacterial, antifungal and antiviral compounds. Essential oils, alkaloids, phenolic acids, quinones, flavones, flavonols, flavonoids, tannins and

coumarins represent the principal groups of compounds extracted from plants with antimicrobial properties described in the literature (Duarte et al. 2007; Esquenazi et al. 2002; Morel et al. 2005; Hatano et al. 2005; Peng et al. 2008).

Biofilms have important roles in many diseases. Prosthetic device colonization, dental plaque formation, infection of the cystic fibrosis, food spoilage, and unusual resistance to antibiotics are some important conditions originating from biofilms, which can compromise over 70 % of all nosocomial infections (Merritt et al. 2005; Kite et al. 2004; Ji-Dong et al. 2001). *Candida albicans* and *Staphylococcus aureus* are among the leading pathogens causing bloodstream infections able to form biofilms on host tissue and indwelling medical devices (Harriott and Noverr 2009). Infections caused for these agents are becoming more difficult to treat because of increasing resistance to antibiotics. In a biofilm environment particularly, microbes exhibit enhanced resistance to antimicrobial agents (Jabra-Rizk et al. 2006).

Herpes Simplex Virus type I (HSV-1) is a member of the subfamily *Alphaherpesvirinae*, family *Herpesviridae*. HSV-1 is an enveloped, double-stranded DNA virus that causes oral and genital vesicular lesions, keratoconjunctivitis, and encephalitis among a variety of clinical symptoms (Khan et al. 2005; Pellet and Roizman 2007). Moreover, HSV infections may cause severe illnesses in immunocompromised patients and neonates (Khan et al. 2005). The virus establishes latency in neural ganglia after the acute infection and recurrent episodes of reactivation are common (Pellet and Roizman 2007). Acyclovir and other nucleoside analogues are the drugs commonly used to treat HSV infections although the emergence of drug-resistant virus strains has increased mainly among immunocompromised individuals (Gilbert et al. 2002; Khan et al. 2005). Therefore, the development of new antiherpetic agents and complementary therapy with currently available drugs are still needed.

Scutia buxifolia Reiss belongs to the Rhamnaceae family and is popularly known as “coronilha”. It is a native plant from South America, with a dispersion that includes Rio Grande do Sul state in Brazil, Argentina and Uruguay, where it is popularly used as cardiotonic, antihypertensive and diuretic (Wasicky et al. 1964). This plant has shown to contain four classes of biologically active compounds, namely phenolic compounds (gallic, caffeic and chlorogenic acids), flavonoids (quercetin, quercitrin, isoquercitrin and rutin), alkaloids (scutianines A-M) and triterpenes (β-sitosterol, stigmasterol and lupeol) (Boligon et al. 2009, 2010, 2012a; Maldaner et al. 2011; Morel et al. 2005). Among the few studies that were conducted, alkaloids isolated from *Scutia buxifolia* displayed in vitro antimicrobial activity demonstrated by the bioauthograpy method and in vivo antinociceptive/analgesic effects (Morel et al. 2005; Trevisan et al. 2009), while polyphenols and flavonoids were associated with antioxidant, antimicrobial, and antimycobacterial activities (Boligon et al. 2012b, c). Taking into consideration these preliminary findings on *S. buxifolia's* antimicrobial activities, the present work reports a detailed study including an extended panel of fungal and bacterial together with an inhibition of biofilm formation assay in microtiter plate against *S. aureus* and *C. albicans*, which is described for the first time for this plant. In addition, the presence of caffeic and chlorogenic acids, whose antiviral properties are described in the literature, led us to deeply evaluate for the first time the antiviral activity of the plant fractions and subfractions against HSV-1 by a bioguided assay coupled with a High-Performance Liquid Chromatography–Diode Array (HPLC–DAD) analysis.

Materials and methods

Chemicals apparatus and general procedures

Methanol and acetonitrile were of HPLC grade and acquired from Merck (Darmstadt, Germany). All extraction reagents, such as dichloromethane, ethyl acetate (EA), and n-butanol were of reagent grade. Reagents for cell culture, such as fetal bovine serum (FBS), trypsin–EDTA and antibiotics were purchased from Gibco BRL (Gaithersburg, MD, USA). Culture medium (M199), dimethyl sulfoxide (DMSO), 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), crystal violet solution, acyclovir, penicillin G, streptomycin, ampicillin, cefoperazone, imipenem, fluconazole, amphotericin B, gallic acid, caffeic acid, chlorogenic acid, rutin and quercetin were purchased from Sigma (Sigma Chemical Co., St. Louis, USA). Sabouraud dextrose agar medium, Muller Hinton (MH) agar medium and Tripticase soy broth (TSB) were from Difco (Detroit, MI, USA). Silica Gel Merck 70-230 mesh was used for column chromatography and silica gel Merck GF_{254} nm was used for thin layer chromatography. 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.

Plant collection

Stem bark and leaves of *Scutia buxifolia* Reissek (Rhamnaceae) were collected from Dom Pedrito, Rio Grande do Sul State, Brazil (30°59′09″S, 54° 27′44″ W), in October 2007. Exsiccate was archived as voucher specimen

in the herbarium of Department of Biology at Federal University of Santa Maria by register number SMBD 10919 for future references.

Bioassay-guided fractionation

The aerial parts of the plant were separately dried at room temperature and powdered in a knife mill (0.86 µm). Leaves (372.34 g) and stem bark (651.52 g) were macerated with 70 % ethanol for 7 days based on the traditional methods of tincture preparation. After filtration, a portion of 100 ml of the hydroalcoholic extracts were reserved and evaporated to dryness under reduced pressure (±40°C) to obtain the crude extracts (CE from leaves and stem bark). After that, the remainder hydroalcoholic extracts were partitioned with dichloromethane (DCM), EA and n-butanol (NB), successively; the yield of the crude extract and fractions was calculated using the following formula: Yield (%) = [(Mass of the extract/mass of material) × 100]. The bioguided chromatographic fractionation process began with the evaluation of antimicrobial, cytotoxicity and antiviral screening of these samples. NB fraction of the stem bark was the most active and as active fraction that was submitted to chromatographic separation processes. NB fraction (3.5 g) was submitted to a column chromatography on silica gel column (225 g), eluted with EA/EtOH (1:0–0:1 v/v). Seventy subfractions (±50 ml each) were collected and their composition was monitored by thin-layer chromatography (TLC) and so grouped on the basis of similarity chromatographic profile to furnish four new subfractions (NB1 to NB4): NB1 (subfractions 1–9, 28 mg), NB2 (subfractions 10–33, 47 mg), NB3 (subfractions 34–50, 106 mg) and NB4 (subfractions 51–70, 80 mg).

Furthermore, EA fraction of the leaves (5 g) was chromatographed on silica gel column (225 g), eluted with CH_2Cl_2/EtOH (1:0–0:1 v/v). Ninety-six subfractions (±50 ml each) were collected, their composition monitored by TLC and grouped on the basis of similarity profile to obtain four new subfractions (AE1 to AE4). AE1 (subfractions 1–12, 50 mg), AE2 (subfractions 13–51, 168 mg), AE3 (subfractions 52–80, 184 mg) and AE4 (subfractions 81–96, 122 mg).

From AE2 and EA3 subfractions, the compounds quercetin, quercitrin (quercetin 3-O-rhamnoside), isoquercitrin (quercetin-3-O-β-D-glucopyranoside) and rutin were isolated. Detailed isolation of quercetin, quercitrin, isoquercitrin and rutin was published elsewhere (Boligon et al. 2009).

HPLC analysis

The subfractions NB1 to NB4 from the stem bark and EA1 to EA4 from the leaves were analyzed by HPLC–DAD. Reverse-phase chromatographic analyses were carried out in isocratic conditions using C-18 column (4.6 mm × 250 mm) packed with 5-µm diameter particles; the mobile phase was methanol–acetonitrile-water (40:15:45, v/v/v) containing 1.0 % acetic acid. The mobile phase was filtered through a 0.45-µm membrane filter and then degassed by an ultrasonic bath prior to use. Stock solution of gallic acid, caffeic acid, chlorogenic acid, quercetin and rutin standard reference were prepared in the HPLC mobile phase at a concentration range of 0.018–0.280 mg/ml (Artani et al. 2006). The subfractions EA1–EA4 and NB1–NB4 were also dissolved in the mobile phase. Quantification was carried out by the integration of the peak using external standard method. The flow rate was 1.0 ml/min, injection volume was 40 µl and detection was done at 257 nm for gallic acid, 325 nm for caffeic and chlorogenic acids and 365 nm for quercetin and rutin. The chromatographic peaks were confirmed by comparing their retention time and UV spectra with those of the reference standards and by spiking the isolated compounds in the plant sample. The calibration curve for gallic acid was $Y = 13{,}985x + 1{,}020.6$ ($r = 0.9998$), caffeic acid: $Y = 15{,}972x + 1{,}281.4$ ($r = 0.9985$), chlorogenic acid: $Y = 14{,}752x + 1{,}175.0$ ($r = 0.9991$), quercetin: $Y = 15{,}083x + 4{,}627.1$ ($r = 0.9997$) and rutin: $Y = 16{,}499x + 5{,}356.2$ ($r = 0.9994$). All chromatographic operations were performed at room temperature and in triplicate. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by ICH (2005). 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.

Antimicrobial assay

The crude extract and fractions were individually evaluated against *Micrococcus* sp. ATCC 7468, *Aeromonas* sp. (clinical isolate), *Klebsiella pneumoniae* ATCC 700603, *Proteus mirabilis* ATCC 7002, *Enterococcus faecalis* ATCC 51299, *Bacillus subtilis* ATCC 6633, *Candida tropicalis* (clinical isolate), *Candida parapsilosis* (ATCC 90018), *Candida dubliniensis* (clinical isolate), *Cryptococcus neoformans* ATCC 2857, *Cryptococcus gattii* ATCC 56990, *Malassezia pachydermatis*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Fusarium solani* (clinical isolates). The minimal inhibitory concentration (MIC) of each fraction against the test microorganisms was determined by the broth microdilution method M27-A2 (NCCLS 2002). The experiments were repeated twice and the results were determined as an average value. Six different dilutions of each fraction (1000, 500, 250, 125, 62.5 and 31.25 µg/ml) were prepared in DMSO. Bacterial

strains were cultured overnight at 37 °C in Mueller–Hinton agar. Yeasts were cultured overnight at 30 °C in Potato dextrose agar. The first column of the plate was reserved for negative control wells (without inoculants) and the last column, for the positive growth control wells (without antimicrobial agents). The MIC was considered as the lowest concentration of the extract or fraction inhibiting the total growth of microorganisms. MIC was detected by lack of visual turbidity (matching the negative growth control).

Determination of percent activity (A%) and bacterial susceptible index (BSI)

These parameters were determined according the equations listed below (Ellof 2004; Rangasamy et al. 2007).

$$A\% = \frac{100 \times \text{number of susceptible strains to a specific extract or fraction}}{\text{Total of tested strains}}$$

$$BSI = \frac{100 \times \text{number of extracts of fractions effective against each strain}}{\text{Total samples tested}}$$

Microtiter plate biofilm assay

Biofilm study was performed by the method published by Merritt et al. (2005). Briefly, bacterial strains were individually inoculated in 2–5 ml of Tripticase soy broth (TSB) and grow up to stationary phase, respectively. Cultures diluted to 1:100 in TSB, and 100 µl of each dilution pipetted to four wells in a sterile flat bottom microtiter plate. After incubation for 24 h in 37 °C, planktonic bacteria removed from all of the wells and the extracts were added in the wells and serially diluted in TSB. After incubation for 24 h in 37 °C, planktonic bacteria removed from all of the wells and washed with distilled water for three times. 125 µl of 0.1 % crystal violet solution (Sigma Chemical Co) added to each well, and then washed with distilled water. Microplates were inverted and vigorously tap on paper towels to remove any excess liquid and air dried. 200 µl of 95 % ethanol poured in *Candida albicans* ATCC 90028 and *Staphylococcus aureus* ATCC 25923 wells, respectively. Biofilm stains solubilized at room temperature. After shaking and pipetting of wells, 125 µl of the solution from each well transferred to a new microtiter plate and relative biofilm formation was assayed by measuring the absorbance of the crystal violet solution at 600 nm (optical density—OD_{600}). Negative control (only culture media), positive control for *C. albicans* or *S. aureus* (without extracts) and Chlorhexidine (culture media plus *C. albicans* or *S. aureus* plus Chlorhexidine) were used as controls.

Cells and viruses

HEp-2 cells were grown in minimum essential medium (MEM—GIBCO Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS—GIBCO Invitrogen Corporation, Grand Island, NY, USA) and penicilin, streptomycin and amphotericin B, at the concentrations 100 U/ml, 100 and 2.5 µg/ml, respectively. The cells were prepared in 96-well plates for both cytotoxicity and antiviral assays and maintained at 37 °C in a 5 % CO_2 incubator. The strain KOS of HSV-1 was kindly provided by Dr Paulo Roehe from the Universidade Federal do Rio Grande do Sul (UFRGS). The viruses stocks were prepared as described in Simões et al. (1999) and kept at −70 °C. After three cycles of freezing/thawing, the fluids were titrated on the basis of cytopathogenicity and expressed as 50 % tissue culture infectious dose (TCID50)/mL (Reed and Muench 1938).

Cytotoxicity evaluation

The cytotoxicity and antiviral tests were performed through the colorimetric assay MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] according to Mosmann (1983) with modifications. Shortly, minimum essential medium (MEM) with 10 % bovine fetal serum and increasing concentrations of the samples from 1.95 to 250 µg/ml were added to the HEp-2 cells (2×10^4 cells/well), in 96 well plates, in a total of six repetitions for each concentration. After 72 h in 5 % CO_2 incubator at 37 °C, the compound MTT (1 mg/ml) was added to the cells. The reagent was removed after 4 h of incubation and 100 µl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The supernatant was transferred to a new plate and readings were performed in an ELISA Spectra Count reader at the wavelength of 540 nm. The viable cell percentage for each compound was calculated by the following formula: absorbance of the compound/absorbance of the cell control $\times 100$ %. The CC_{50} (50 % cytotoxic concentration) was obtained from the concentration-effect curves after linear regression as described in Freitas et al. (2009). The maximum non-toxic concentration (MNTC) of the essential oil or compounds that did not cause cytotoxicity was used for the antiviral tests.

Antiviral evaluation

HEp-2 cells monolayers were prepared in 96-well plates 24 h before performing the tests. The maintenance media on the preformed monolayer was then removed and replaced by a new media containing 100 µl/well of the virus suspension containing 10^4 $TCID_{50}$/ml. The control

cell media was replaced by 200 µl/well of MEM media, without virus. After that, 100 µl/well of the MNTC of the samples was added to the wells. The plates were then incubated for 72 h at 37 °C in a 5 % CO_2 incubator. The readings were performed as described above, for the cytotoxicity assays. Acyclovir (10 µg/ml) was used as positive control for the HSV-1 inhibition. The viral inhibition for each compound was calculated according to the following formula: (absorbance of the compound—absorbance of the viral control)/(absorbance of the cell control—absorbance of the viral control) × 100 %. The 50 % inhibitory concentration (IC_{50}) was obtained from the concentration-effect curves after linear regression as described by Freitas et al. (2009). The selectivity index (SI) was calculated from the formula CC_{50}/IC_{50}.

Data analysis

CC_{50} and IC_{50} values were obtained from linear regression analysis of concentration-effect curves. Results of the HPLC–DAD quantification were considered statistically significant when $p < 0.05$ by Tukey test. Data of biofilms assay were analyzed using one-way analysis of variance (ANOVA) followed by the Duncan's multiple range test when appropriate. Results were expressed as the mean ± standard deviation (SD) and differences were considered statistically significant when $p < 0.0001$.

Results

The yields of crude extract, dichloromethane, EA, and *n*-butanol-soluble fractions obtained from the leaves and stem bark are given in Table 1. The highest yield was obtained with crude extracts (CE) and EA fraction obtained from the leaves.

Antimicrobial potential

Antimicrobial activities of CE extracts, DCM, EA and NB fractions of the *S. buxifolia* were tested; the overall view is given in Table 2. Regarding the stem bark, strong antimicrobial activity were obtained against *K. pneumoniae* and *E. faecalis* for EA and NB fractions, the last one showing the minimal inhibitory concentration (MIC = 62.5 µg/ml). Considering the leaves, NB fraction was effective against

Table 1 Yield of the extracts and fractions of the *S. buxifolia*

Extracts and fractions	Stem bark	Leaves
CE	65.15 g (10.1 %)	26.07 g (7.0 %)
DCM	1.30 g (0.2 %)	1.12 g (0.30 %)
EA	23.5 g (3.6 %)	20.13 g (5.41 %)
NB	28.67 g (4.4 %)	13.85 g (3.72 %)

CE crude extract, *DCM* dichloromethane fraction, *EA* ethyl acetate fraction, *NB* n-butanolic, fraction

Table 2 Minimum Inhibitory Concentration (MIC) values for crude extracts and fractions of *Scutia buxifolia* leaves and stem bark

Microorganisms	*S. buxifolia*							
	Stem bark				Leaves			
	CE	DCM	EA	NB	CE	DCM	EA	NB
Micrococcus sp	1000	500	250	125	1000	500	62.5	62.5
Aeromonas sp.	>1000	1000	250	250	1000	1000	125	250
K. pneumoniae	1000	500	125	62.5	1000	500	250	62.5
P. mirabilis	>1000	>1000	>1000	500	>1000	>1000	250	500
E. faecalis	>1000	>1000	125	62.5	>1000	>1000	250	62.5
B. subtilis	1000	500	500	250	1000	1000	500	250
C. tropicalis	>1000	>1000	1000	500	>1000	>1000	500	250
C. parapsilosis	>1000	>1000	500	500	>1000	>1000	>1000	250
C. dubliniensis	>1000	>1000	500	250	>1000	>1000	250	125
C. neoformans	>1000	>1000	>1000	500	>1000	1000	250	500
C. gattii	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
M. pachydermatis	>1000	1000	500	250	>1000	500	250	250
A. flavus	1000	1000	1000	500	1000	500	500	500
A. fumigatus	>1000	1000	1000	500	>1000	1000	250	125
F. solani	>1000	1000	500	250	>1000	1000	1000	500

Values are expressed in µg/ml

Controls: ampicillin (8 µg/ml), cefoperazone (16 µg/ml), imipenem (0.6 µg/ml), fluconazole (16 µg/ml), amphotericin B (0.5 µg/ml)

CE crude extract, *DCM* dichloromethane fraction, *EA* ethyl acetate fraction, *NB* n-butanolic fraction

Micrococcus sp., *K. pneumoniae* and *E. faecalis* (MIC = 62.5 μg/ml), in addition to *C. dubliniensis* and *A. fumigatus* (MIC = 125 μg/ml). The *Micrococcus* sp. and *Aeromonas* sp. were vulnerable for EA leaves (MIC = 62.5 and 125 μg/ml, respectively). The set of data indicated that EA and NB fractions of the stem bark and leaves were the most effective ones in the antimicrobial assay.

Biofilm activity

The extracts NB2 (stem bark), EA2 and EA3 (leaves) were active against *C. albicans* biofilm (biofilm inhibitory concentration—BIC of 810 ± 0.030, 582 ± 0.011, 622 ± 0.018 μg/ml, respectively). The extracts EA, NB and NB2 (stem bark) and EA, NB and EA3 (leaves) were active against *S. aureus* (BIC of 810 ± 0.009; 615 ± 0.011; 933 ± 0.012; 855 ± 0.005; 855 ± 0.010 and 360 ± 0.007 μg/ml, respectively) (Fig. 1).

Antiviral activity

In this study, CE extract, DCM, EA and NB fractions of the *S. buxifolia* leaves and stem bark were also tested for cytotoxicity and antiviral activity by MTT assay. The data were used to calculate the selectivity index ($SI = CC_{50}/IC_{50}$) (Table 3). The NB and the EA fractions of the stem bark and the EA fraction of the leaves exhibited a potential antiviral activity with SI values of 25.78, 15.97 and 14.13, respectively. Subfractions also were examined for its cytotoxicity and anti-HSV activity, EA2 ($IC_{50} = 6.50 \pm 1.94$ μg/ml; SI = 12.59) and EA3 ($IC_{50} = 11.27 \pm 2.97$ μg/ml; SI = 10.06) were the most active of the EA subfractions towards HSV-1. The flavonoids quercetin, quercitrin, isoquercitrin and rutin were previously isolated from subfractions EA2 and EA3 (Boligon et al. 2009). Among the isolated flavonoids, only rutin (SI = 6.74) showed moderate activity against HSV-1 (Table 3). In addition, three phenolic acids (gallic, caffeic and

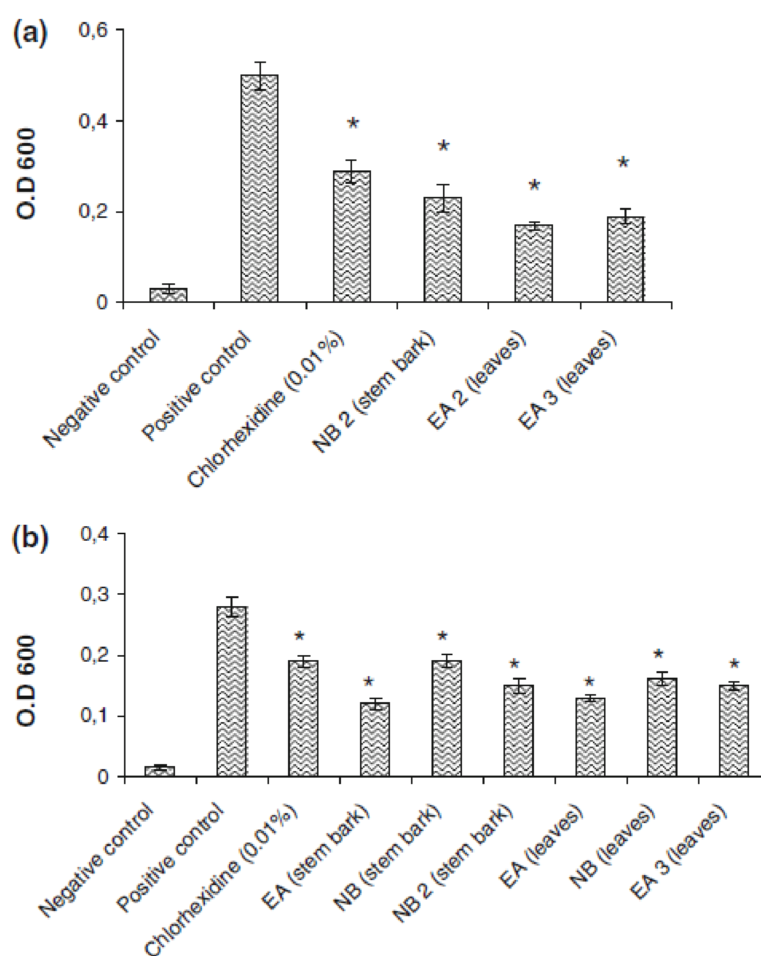

Fig. 1 Inhibition of biofilm formation of *C. albicans* (a) and *S. aureus* (b) from the active extract and fractions of *S. buxifolia* (360–933 μg/mL). *Error bars* are shown as one-standard deviation and differences were considered statistically significant when $p < 0.01$. Each experiment was carried out three times yielding similar results

chlorogenic acids) were quantified in these subfractions, which also may have contributed to the antiviral activity (Fig. 1; Table 4). On the other hand, the subfraction NB2 (IC_{50} = 1.12 ± 0.91 μg/ml; SI = 12.34) was the most effective when compared with other subfractions obtained from the stem bark (Table 3).

Phytochemical analysis

The chromatographic process of separation yielded four subfractions of the EA (EA1 to EA4) and NB (NB1 to NB4) fractions. HPLC profile of EA1 to EA4 active subfractions leaves (Fig. 2) and NB1 to NB4 subfractions stem bark (Fig. 3) of the *S. buxifolia* were obtained and showed an elution diagram where the peaks were grouped into three regions based on the UV absorption profile. These regions showed typical patterns of UV absorption, supporting the presence of gallic acid with retention time (t_R) of 12.5 min (peak 1), chlorogenic acid with t_R = 22.3 min (peak 2), caffeic acid with t_R = 27.8 min (peak 3), rutin with t_R = 38.1 min (peak 4), isoquercitrin with t_R = 39.8 min (peak 5), quercitrin with t_R = 41.5 min (peak 6) and quercetin with t_R = 48.0 min (peak 7). Subfractions with the highest quantities of compounds (flavonoids and phenolic acids) were EA3 (53.8 %), EA2 (49.8 %), NB4 (39.1 %) and NB2 (36.2 %); results n are shown in Table 4.

Table 3 Cytotoxicity and anti-HSV-1 activity of *Scutia buxifolia*

Sample	Cytotoxicity	Anti-HSV-1 activity	
	CC_{50}	IC_{50}	SI
Stem bark			
CE	171.08 ± 8.76	34.95 ± 5.35	4.89
DCM	147.09 ± 812	12.85 ± 3.63	11.44
EA	101.59 ± 7.21	6.36 ± 1.67	15.97
NB	119.37 ± 9.59	4.63 ± 1.25	25.78
Leaves			
CE	165.84 ± 11.31	22.40 ± 5.17	7.40
DCM	123.76 ± 7.19	21.26 ± 3.24	5.82
EA	91.03 ± 7.97	6.44 ± 2.13	14.13
NB	138.20 ± 8.42	18.47 ± 4.24	7.48
NB subfractions (stem bark)			
NB 1	1.95 ± 1.51	1.19 ± 0.52	1.64
NB 2	13.82 ± 5.39	1.12 ± 0.91	12.34
NB 3	2.92 ± 1.02	0.58 ± 0.02	5.03
NB 4	4.56 ± 1.31	0.48 ± 0.07	9.5
EA subfractions (leaves)			
EA 1	67.85 ± 5.65	28.45 ± 3.65	2.38
EA 2	81.84 ± 7.54	6.50 ± 1.94	12.59
EA 3	113.35 ± 6.35	11.27 ± 2.97	10.06
EA 4	268.07 ± 9.42	101.33 ± 8.11	2.64
EA isolated (leaves)			
Quercetin	96.46 ± 8.32	NI	–
Quercitrin	161.66 ± 9.12	NI	–
Isoquercitrin	160.09 ± 9.41	NI	–
Rutin	882.74 ± 15.32	130.87 ± 8.32	6.74
Acyclovir (10 μg/mL)	>280.00	1.50 ± 0.30	>186.66

CE crude extract, *DCM* dichloromethane fraction, *EA* ethyl acetate fraction, *NB* n-butanolic fraction *NB1-4* n-butanolic subfractions, *EA1-4* ethyl acetate subfractions, CC_{50} 50 % cytotoxic concentration (μg/ml), IC_{50} 50 % inhibitory concentration (μg/ml), *SI* selectivity index (= CC_{50}/IC_{50}), *NI* no inhibitory activity

Discussion

Besides the determination of MIC, other methods could be used to express the antimicrobial effectiveness of the fractions. The percent activity (A%) and the bacterial susceptibility index (BSI) may help in choosing the appropriate plant antimicrobial or its fraction in natural antimicrobial applications. Percent activity indicates EA and NB as the effective fractions, whereas BSI indicated *Micrococcus* sp., *K. pneumoniae*, *B. subtilis* and *A. flavus* as the most sensitive microorganisms (BSI = 100 %), showing sensitivity for the CE, DCM, EA and principally, to NB fraction, followed by *Aeromonas* sp., *M. pachidermatis*, *A., fumigatus and F. solani* (BSI = 75.0 %); *E. faecalis*, *C. dubliniensis* and *C. neoformans* (BSI = 50.0 %); *P. mirabilis*, *C. tropicalis* and *C. parapsilosis* (BSI = 37.5 %), with intermediate sensibility; and *C. gattii* as the resistant micrarganism (BSI = 0 %). In fact, EA and NB fractions extracted the more polar compounds, such as phenolics, flavonoids and tannins; these compounds could explain the good antimicrobial activities achieved in this assay. Phenolics, especially flavonoids, have the ability to inhibit spore germination and have therefore antifungal activity (Harborne and Williams 2000). This fact could explain, at least in part, the antifungal activities displayed against *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. neoformans*, *M. pachydermatis*, *A. flavus*, *A. fumigatus* and *F. solani* in this study. This class of secondary metabolites also possesses antibacterial activity (Pereira et al. 2006; Oliveira et al. 2007). They may act as antibacterial agents by means of at least three or more mechanisms: inhibition of nucleic acid synthesis, disruption of bacterial membranes and/or inhibition of energy metabolism (Cowan 1999). The tested fractions here were effective against *K. pneumoniae* and *Micrococcus* sp. In a preliminary study with *S. buxifolia*, Boligon et al. (2012b) showed that AE and NB were active against *S. aureus* (MIC = 125–500 μg/ml); our in vitro studies revealed that *S. buxifolia* extracts could easily release biofilm layer from

Table 4 Subfraction composition of *S. buxifolia* (%), LOD and LOQ variations for compounds

Components in *S. buxifolia*	Gallic acid	Chlorogenic acid	Caffeic acid	Rutin	Isoquercitrin*	Quercitrin*	Quercetin
Stem bark							
NB 1	0.41 ± 0.3[a]	9.26 ± 0.5[a]	4.81 ± 0.1[a]	4.38 ± 0.6[a]	0.71 ± 0.4[a]	1.35 ± 0.3[a]	0.69 ± 0.1[a]
NB 2	0.59 ± 0.5[a]	4.70 ± 0.2[b]	8.84 ± 0.1[b]	16.50 ± 0.3[b]	4.81 ± 0.1[b]	0.51 ± 0.2[b]	0.42 ± 0.1[b]
NB 3	–	5.19 ± 0.2[b]	4.33 ± 0.3[a]	6.42 ± 0.4[c]	–	1.14 ± 0.5[c]	–
NB 4	–	4.53 ± 0.3[b]	6.11 ± 0.1[c]	19.31 ± 0.9[d]	8.00 ± 0.2[c]	1.23 ± 0.1[ac]	–
Leaves							
EA 1	0.99 ± 0.2[b]	0.96 ± 0.2[c]	1.58 ± 0.6[d]	4.92 ± 0.3[a]	–	–	1.15 ± 0.4[c]
EA 2	20.7 ± 0.1[c]	1.65 ± 0.1[d]	12.82 ± 0.1[e]	1.59 ± 0.1[e]	–	4.91 ± 0.1[d]	8.34 ± 0.1[d]
EA 3	11.3 ± 0.1[d]	0.64 ± 0.3[e]	5.98 ± 0.5[c]	9.27 ± 0.1[f]	1.19 ± 0.2[d]	8.53 ± 0.2[e]	17.11 ± 0.3[e]
EA 4	1.75 ± 0.4[e]	–	1.40 ± 0.1[d]	5.03 ± 0.1[a]	–	1.37 ± 0.1[c]	1.59 ± 0.1[f]
LOD	0.025	0.043	0.017	0.023	–	–	0.035
LOQ	0.075	0.130	0.051	0.070	–	–	0.106

Results are expressed as means ± standard deviations (% ± SD) of three determinations. [a–f]Means with the different letters in each column are significantly different ($p < 0.05$), by analysis of variance (One-way ANOVA) ($n = 3$)

CE crude extract, *DCM* dichloromethane fraction, *EA* ethyl acetate fraction, *NB* n-butanolic fraction, *NB1-4* n-butanolic subfractions *EA1-4* ethyl acetate subfractions. *LOD* limit of detection (μg/ml), *LOQ* limit of quantification (μg/ml)

* Quantified as quercetin

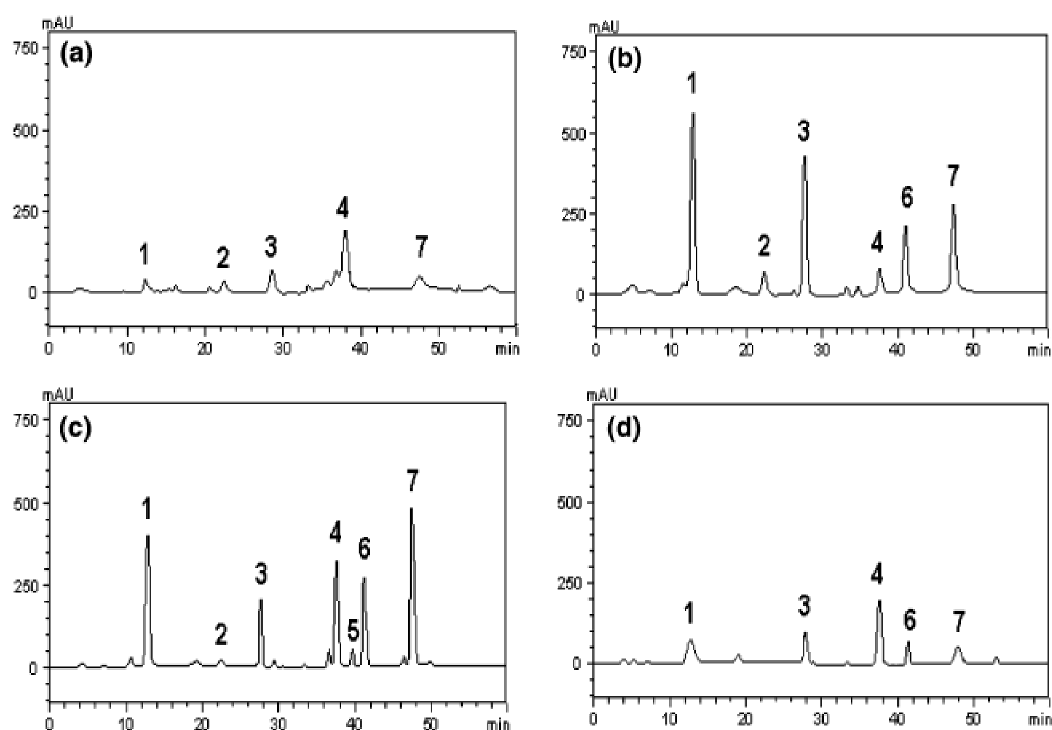

Fig. 2 High-performance liquid chromatography (HPLC) profile of ethyl acetate subfractions from the *S. buxifolia* leaves: EA 1 (**a**), EA 2 (**b**), EA 3 (**c**) and EA 4 (**d**). Gallic acid (1), chlorogenic acid (2), caffeic acid (3), rutin (4), isoquercitrin (5), quercitrin (6) and quercetin (7). Chromatographic conditions described in the experimental section

microtiter wells and its effect is greater on *S. aureus* than on *C. albicans*. Disrupting the multicellular structure of bacterial biofilm was proposed as one of the most potential strategies for increasing the sensitivity of pathogens in biofilm to antibiotics and host immune systems (Stewart and Costerton 2001).

The antiviral activity observed for the fractions of *S. buxifolia* could be partly attributed to the presence of flavonoids in the fractions examined. Hence, the NB fraction of the stem bark and the EA fraction of the leaves were selected for the activity-guided study against HSV-1.

The anti-HSV-1 activity of phenolic compounds such as caffeic and chlorogenic acids and flavonoids has already been described in the literature (Bourne et al. 1999; Chiang et al. 2002; Khan et al. 2005; Suárez et al. 2010; Gescher et al. 2011; Lückemeyer et al. 2012). The actions of these polyphenol are partly attributed to its ability to complex with protein (Haslam 1996), and earlier studies have shown that polyphenols bind to the virus or the cell receptor avoiding virus absorption (Vanden Berghe et al. 1986). Such concept has been confirmed by Gescher et al. (2011) showing that aqueous extract of *Rhododendron ferrugineum* complexed with the gD protein of the HSV envelope. Therefore, the potent antiviral activity of the EA2 (Table 3; Fig. 2b), EA3 (Table 3; Fig. 2c), and NB2 (Table 3; Fig. 3b) may be explained by the high concentrations of the phenolic compounds present in these subfractions. On the other hand, gallic acid was found to be very effective against the HSV-1 both in HEp-2 or Vero cells by inhibiting the virus multiplication at early stages of replication or inactivating the virus directly (Uozaki et al. 2007). The inhibition of the HSV attachment to and its penetrations into cells by gallic acid was also described by Kratz et al. (2008).

Previous reports have also described a potential antiviral activity of quercetin (Chiang et al. 2003; Suárez et al. 2010; Gravina et al. 2011). Quercetin was also the main compound showing antiviral activity against HSV when the plant *Caesalpinia pulcherrima* and its related flavonoids were tested towards this virus (Chiang et al. 2003). Besides, quercetin showed virucidal action against EHV-1 (Gravina et al. 2011), another *Alphaherpesvirus* in the family *Herpesviridae*. In our conditions, quercetin did not show any activity on HSV-1 while rutin was able to inhibit the virus moderately (Table 3). The results on the rutin antiherpetic activity have varied in different studies. In the study of Chiang et al. (2003), rutin had no activity against HSV-1. However, the presence of rutin and its synergistic action with other components such as caffeic and chlorogenic acids were responsible for anti HSV-1 and 2 activity of *Ilex paraguariensis* leaves extract (Lückemeyer et al. 2012).

Conclusion

This study shows, for the first time, the antibiofilm activity of *S. buxifolia*, being effective against *S. aureus* and

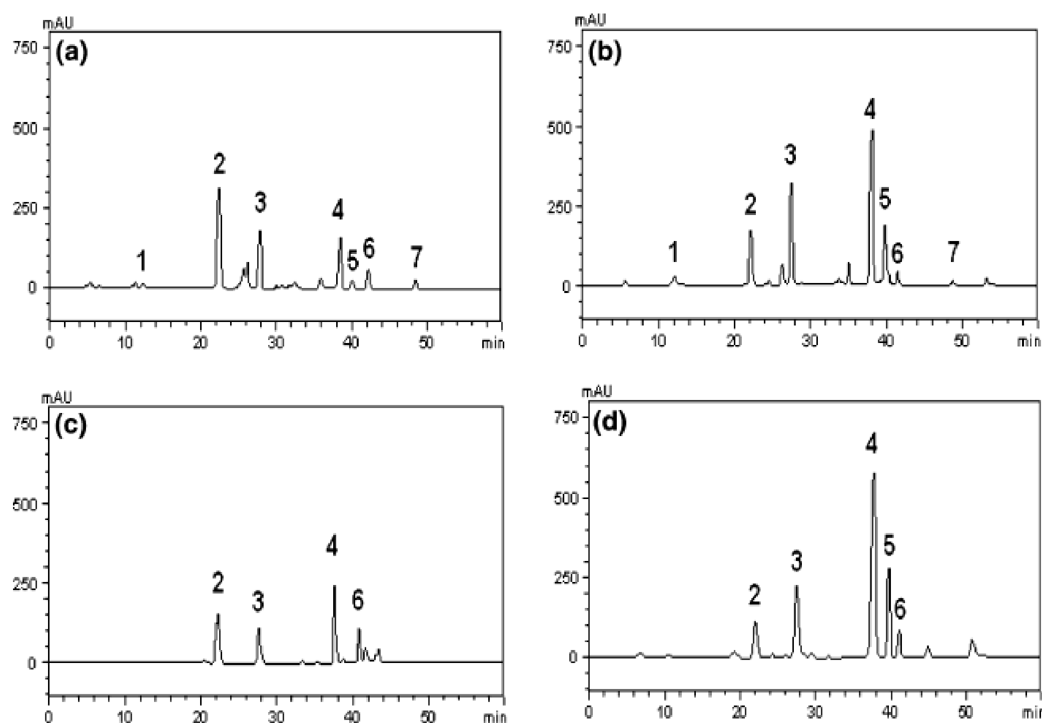

Fig. 3 High-performance liquid chromatography (HPLC) profile of *n*-butanolic subfractions from the *S. buxifolia* stem bark: NB 1 (a), NB 2 (b), NB 3 (c) and NB 4 (d). Gallic acid (1), chlorogenic acid (2), caffeic acid (3), rutin (4), isoquercitrin (5), quercitrin (6) and quercetin (7). Chromatographic conditions described in the experimental section

C.albicans. Additionally, the antibacterial and antifungal activity of this plant extracts was demonstrated here. The bioguided assay evidenced some fractions and subfractions from the leaves and stem bark of the *S. buxifolia* which showed strong antiviral activity against HSV-1 in vitro. The high contents of phenolic and flavonoid compounds (gallic acid, chlorogenic acid, caffeic acid, rutin, isoquercitrin, quercitrin and quercetin) in the fractions suggest that these components are responsible for the antiviral activity and antimicrobial found. The results obtained in the present study highlight the importance of the research of the natural products as a source for new therapies.

Author contribution A.A. Boligon, T.F. Brum, M. Piana and M.L. Athayde performed the extraction, purification, HPLC analyses, results interpretation and contributed to the preparing of final manuscript version. T.F. Kubiça, R. Weiblen and L. Lovato were responsible for experimental antiviral study, data analysis and results interpretation as well as paper preparing. D.N. Mario and S.H. Alves, R.C.V. Santos and C.F.S. Alves contributed to the experimental process related antimicrobial assay and biofilm formation and also were responsible for the statistical treatment.

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Conflict of interest The authors have no conflict of interest.

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4.2 Capítulo 2

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Antimycobacterial activity of the fractions and compounds from *Scutia buxifolia*

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Article

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Abstract: The antimycobacterial activity of *Scutia buxifolia* Reissek, Rhamnaceae, leaves extracts and fractions were evaluated for the first time. Four compounds were identified, flavonoids (quercetin and quercitrin) and phenolic acids (gallic and caffeic acids) and quantified by HPLC-DAD. Promising anti-*Mycobacterium smegmatis* activity was observed with ethyl acetate extract (MIC 312.50 µg/mL) and their fractions (MIC values ranging from 78.12 to above 312.50 µg/mL). The fractions III and VI of *S. buxifolia* leaves showed a high level of activity against *M. smegmatis* (MIC 78.12 and 156.25 µg/mL, respectively), *M. tuberculosis* (MIC 156.25 µg/mL) and *M. avium* (MIC 312.50 µg/mL), whereas to the other fractions the values varied from 312.50 to 1250.00 µg/mL against these strains. The better MIC result was associated with two fractions that contain bigger amounts of quercetin, quercitrin, gallic and caffeic acids. The results provided evidence that the studied plants fractions might be potential sources of new antimicrobial drug.

Keywords:
antimycobacterial
flavonoids
HPLC
phenolics
Scutia buxifolia

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Introduction

More and more people in developing countries utilize traditional medicine for their major primary health care needs (Houghton, 1995). The use of medicinal plants for the treatment of several diseases, including fungal and bacterial infections is typical in Brazil (Carvalho et al., 2002; Pereira et al., 2007; Schubert et al., 2007) and worldwide many research groups screen plant extracts to detect secondary metabolites with antimicrobial properties in an attempt to find new antimycobacterial or antifungal compounds (Cos et al., 2006; Soberón et al., 2007; Rangasamy et al., 2007). Plant-derived compounds are a potential source for investigation of alternative lead chemical structures for drug development (McGaw et al., 2008). Essential oils, alkaloids, terpenoids, phenolics acids, peptides, flavonoids, tannins and coumarins represent the principal groups of compounds extracted from plants with antimycobacterial properties described in the literature (Esquenazi et al., 2002; Morel et al., 2005; Duarte et al., 2007; Peng et al., 2008; Lechner et al., 2008; McGaw et al., 2008; Honda et al., 2010).

The great increase of the resistance of pathogenic microorganisms to multiples drugs occurs owing to the indiscriminate use of antimicrobial, hence there is worry for a search of the new alternative therapeutics (Novais et al., 2003; Antunes et al., 2006; Oliveira et al., 2006; Oliveira et al., 2007; Edgar et al., 2009). Medicinal plants are of great interest scientific, because there is the possibility of use like to phytopharmacos, or big chance to obtain up molecules prototypes due its diversity of constituents (Grayer & Harborner, 1994; Nascimento et al., 2000; Mallavarapu, 2001; Lima et al., 2006). Over 350 natural products, mainly plant species, which have been used in traditional medicine, have been assessed for their antimycobacterial activities (Newton et al., 2000; Copp, 2003). A number have been shown to demonstrate significant *in vitro* antimycobacterial activities and active plant-derived compounds belonging to various chemical classes have been isolated. These findings have therefore stimulated further research towards the isolation of new

antimycobacterial agents from natural products (Newton et al., 2002).

Mycobacterial infections including *Mycobacterium tuberculosis* as well as fast-growing strains are increasing globally; the tubeculosis caused 8 million new cases and 1.8 million fatalities per annum worldwide (Dye, 2006). The mycobacterial are bacilli aerobic no mobile alcohol acid resistant, its surface is rich in mycolic acid. The *M. smegmatis* is a mycobacterium associate to infections as lung disease, despite not being the causative agent of tuberculosis; *M. smegmatis* has been used successfully in many studies due to the characteristic of rapid growth and ease of handling (Pauli et al., 2005). *Mycobacterium avium* is usually opportunistic pathogen, non-pathogenic in healthy individuals, infections caused by *M. avium* is prevalent in patients with AIDS, affecting as many as 40% of patients with advanced disease, which is resistant to most existing antimicrobials (Ramos et al., 2000; Collins & Franzblau, 1997). The additional prevalence of multidrug-resistant strains and extensively drug-resistant tuberculosis (De Rossi et al., 2006) stimulates an urgent need for the development of new drugs for the treatment of mycobacterial infections.

Scutia buxifolia Reissek belongs to the Rhamnaceae family and is popularly known as "coronilha". It is native tree from South America, with a dispersion area that comprises Rio Grande do Sul State in Brazil, and the countries Argentina and Uruguay. The root bark infusion is popularly used as cardiotonic, antihypertensive and diuretic (Wasicky et al., 1964). Antimicrobial activities of some cyclopeptide alkaloids isolated from the root bark of *S. buxifolia* were reported by Morel et al. (2005), using the bioautography method. The objectives of the present work were obtain the profile by HPLC of fractions obtained from the leaves of *S. buxifolia* and evaluate the antimycobacterial activity of the same fractions, using the broth microdilution method (NCCLS, 2003) which is a very sensitive method to screen plant extracts to prospect antimicrobial.

Material and Methods

Chemicals apparatus and general procedures

Methanol and acetonitrile were of HPLC grade and acquired from Sigma Chemical Co. (St. Louis, MO, USA). Solvents for the extractions, dichloromethane, ethyl acetate, ethanol, methanol, n-butanol and dimethylsulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Quercetin, quercitrin, gallic acid and caffeic acid, Alamar Blue and Tetrazolium bromide [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromode] (MTT) were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Lowenstein-Jensen medium, Middlebrook 7H9 broth, OADC (oleic acid-albumin-dextrose-catalase) were purchased from Difco Laboratories (Detroit, Mich). Silica Gel Merck 70-230 mesh was used for column chromatography and silica gel Merck GF254nm was used for thin layer chromatography. High performance liquid chromatography (HPLC-DAD) was performed with the HPLC system (Shimadzu, Kyoto, Japan), Prominence Auto-Sampler (SIL-20A), equipped with Shimadzu LC-20 AT reciprocating pumps connected to the degasser DGU 20A5 with integrator CBM 20A, UV-VIS detector DAD SPD-M20A and Software LC solution 1.22 SP1.

Plant collection

Leaves of *Scutia buxifolia* Reissek, Rhamnaceae, were collected on October of 2007 in the first district of the council of Dom Pedrito, in the Rio Grande do Sul State, Brazil (coordinates 30º59'09" S and 54º27'44" W). Voucher specimen was archived in the herbarium of Department of Biology at Federal University of Santa Maria, register number SMBD 10919.

Extraction

The leaves (372.34 g) of the plant were dried at room temperature, powdered in a knife mill (0.86 µm) and macerated with 2 L of ethanol 70% during a week. After filtration, the extracts were evaporated under reduced pressure to remove the ethanol. The remaining aqueous extract was partitioned with dichloromethane, ethyl acetate and *n*-butanol (3x 100 mL for each solvent). At the end of all extractions procedures, furnished three extracts with different polarities and properties. These extracts were dried under reduced pressure using a rotatory evaporator at 40 °C.

Fractionation

The ethyl acetate extract of the leaves (4 g) was submitted to a column chromatography on silica gel 60 (225 g), eluted with CH_2Cl_2/EtOH (1:0-0:1). Ninety-six fractions (±50 mL each fraction) were collected and their composition monitored by thin-layer chromatography (TLC), so grouped on the basis of similarity chromatographic profile to obtain seven fractions (I-VII). Fraction I (fractions 1-8, 15 mg), fraction II (fractions 9-51, 24 mg), Fraction III (fractions 52-68, 92 mg), Fraction IV (fractions 69-80, 67 mg), Fraction V (fractions 81-82, 51 mg), Fraction VI (fractions 83-89, 17 mg), VII (fractions 90-96, 18 mg). Detailed isolation of quercitrin and quercetin is published elsewhere (Boligon et al., 2009a).

Quantification of phenolics compounds by HPLC

Reverse phase chromatographic analyses were carried out in isocratic conditions using C-18 column (4.6 mm x 250 mm) packed with 5 µm diameter particles, the mobile phase was methanol-acetonitrile-water (40:15:45, v/v/v) containing 1.0% acetic acid. The mobile phase was filtered through a 0.45 µm membrane filter and then degassed by an ultrasonic bath prior to use. Stock solution of quercetin, gallic acid and caffeic acid standard reference was prepared in the HPLC mobile phase at a concentration range of 0.018 to 0.280 mg/mL (Artani et al., 2006). The fractions I, II, III, IV, V, VI and VII from the leaves were also dissolved in the mobile phase. Quantification was carried out by the integration of the peak using external standard method. The flow rate was 1.0 mL/min, injection volume was 20 µL and detection were done at 257 nm for gallic acid, 325 nm for caffeic acid and 365 nm for quercetin and quercitrin. The chromatographic peaks were confirmed by comparing their retention time and DAD-UV spectra with those of the reference standards and by spiking the isolated compounds in the plant sample. The calibration curve for gallic acid was: Y=53985x-1020.6 (r=0.998), the curve of caffeic acid was: Y=15972x-7081.4 (r=0.995), and the curve of quercetin was: Y=15083x-4627.1 (r=0.989). All chromatographic operations were performed at room temperature and in triplicate.

Antimicrobial assay on Mycobacterium

Antimycobacterial activity was tested against *Mycobacterium smegmatis* mc^2 155 (ATCC 700084), *M. tuberculosis* H37Rv (ATCC 25618) and *M. avium* LR541CDC. The stored mycobacterias were seeded onto Löwenstein-Jensen medium and incubated during 3-5 days. From this culture a portion was removed and placed into Middlebrook 7H9 broth, supplemented with 10% OADC and 0.2% glycerol (MD7H9) and then homogenized in ultrasonic bath for one minute. The concentration of bacteria in this medium was determined by optical density on spectrophotometer (0.08 to 0.1 of absorbance at 625 nm) of 0.5 McFarland scale and then diluted with MD7H9 up to 10^5 CFU/mL for *M. smegmatis,* to reach the inoculum. The *M. tuberculosis* and *M. avium* were used at a concentration of 0.5 McFarland (not diluted) because they are slow-growing. Plant extracts, fractions and isolated were dissolved in DMSO, at a concentration of 50.00 mg/mL and then diluted in MD7H9 until the desired concentrations, beginning the series with 2500.00 µg/mL.

The activity test were performed using the broth microdilution method (NCCLS, 2003), which presented as a result the minimum inhibitory concentrations (MIC) of each compound. 100 µL of mycobacterial culture were placed in each well of a microtitre plate, as well as the extracts and fractions at correspondent concentrations. Analysis were carried out in triplicate, controls were made to the medium and to the mycobacterium, and one blank for each concentration of samples. The plates were incubated during 48 h at 37 °C. The Alamar Blue (Franzblau et al., 1998) or MTT (Sankar et al., 2008) dye were used to check the growth of microorganisms. The Alamar Blue was diluted in a ratio of 1/10 with Tween 80, diluted to 1/9 with sterile water, supplemented with 0.025 mL in each well, considering the MIC into the pit where there was no change in color from purple to pink. MTT solution at 0.5 mg/mL were prepared by the dilution with absolute ethanol up to 1 mg/mL, and then by dilution half to half with a solution of 10% Tween 80. 25 µL of the final solution were added to each plate well, and the well where the drug prevented the color change from yellow to purple was considered the MIC.

Results and Discussion

The yields of dichloromethane, ethyl acetate and *n*-butanol-soluble fractions obtained after the partition of the remaining aqueous extract are given in Table 1. The highest yield was obtained with ethyl acetate solvent.

Table 1. Yielding expressed in grams and in percentage for all extracts.

Extracts of the leaves	Yielding of the extracts
Dichloromethane	1.134 g (0.30%)
Ethyl acetate	20.183 g (5.41%)
Butanolic	13.490 g (3.62%)

Extracts from the leaves of *S. buxifolia* were tested against *M. smegmatis* to determine the promissory extract to continue the experiment. The dichloromethane and ethyl acetate extracts of the leaves showed the good MIC (312.50 µg/mL), due to the higher yield, the ethyl acetate extract was prioritized. The results for the dichloromethane extract is probably related to the presence of triterpenes and sterol reported for this extract (Boligon et al., 2010) which have antimycobacterial activity demonstrated (Cantrell et al., 2001). However, the values found for the ethyl acetate extract are related to the presence of flavonoids contents (Boligon et al., 2009a; Boligon et al., 2009b).

The fractions I, II, III, IV, V, VI and VII of *S. buxifolia* leaves were analyzed by HPLC. The result indicates that the *S. buxifolia* contains several compounds including gallic acid with retention time (t_R) of 3.3 min (peak 1), caffeic acid with t_R=5.0 min (peak 2), quercitrin with t_R=6.4 min (peak 3) and quercetin with t_R=12.1 min (peak 4), (Figure 1).

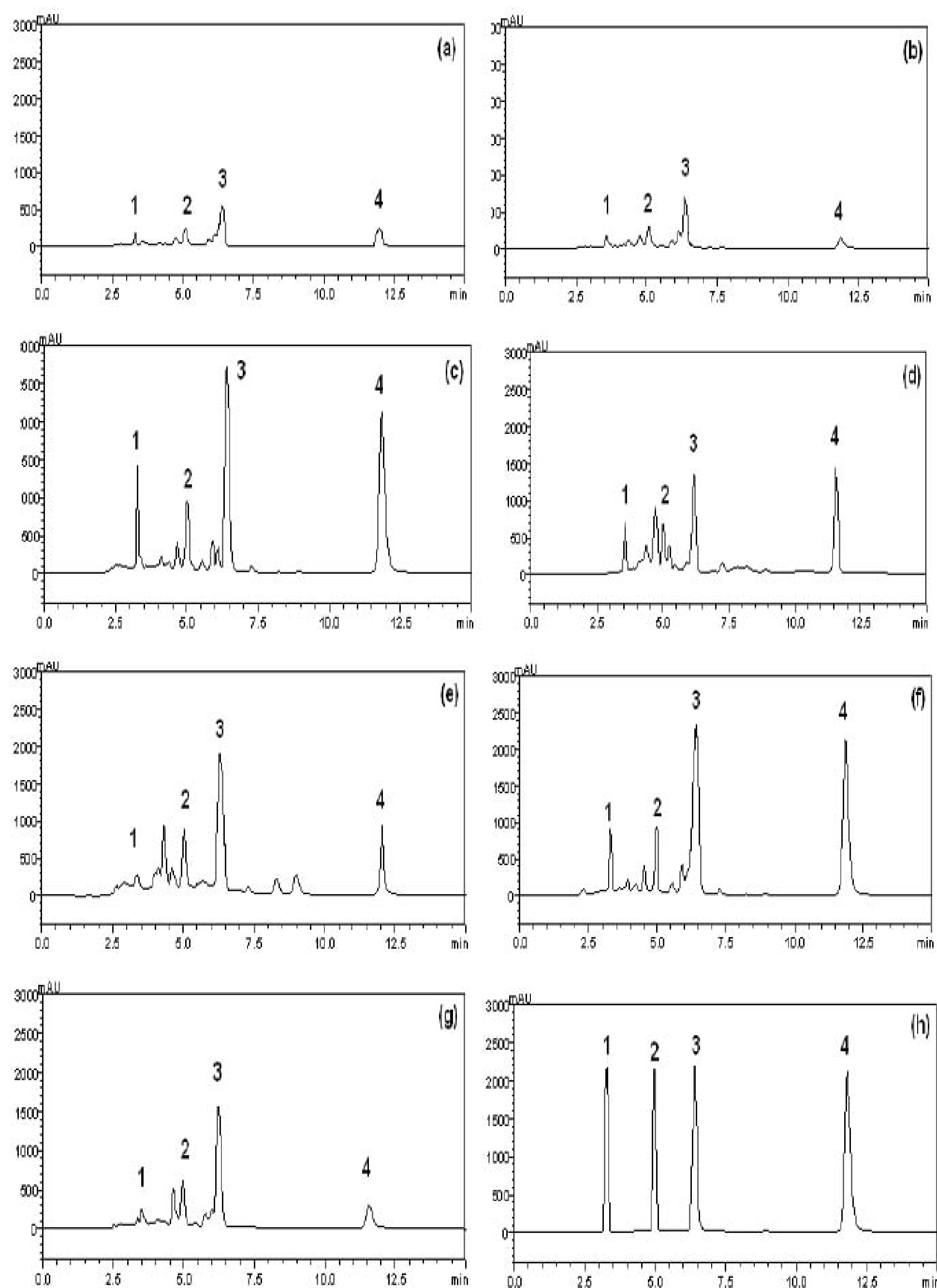

Figure 1. High performance liquid chromatography phenolic profile of ethyl acetate fraction from the leaves: fraction I (a), fraction II (b), fraction III (c), fraction IV (d), fraction V (e), fraction VI (f), fraction VII (g) and standards (h). Gallic acid (1), caffeic acid (2), quercitrin (3) and quercetin (4). Chromatographic conditions described in the experimental section.

The HPLC profile of seven active fractions was also acquired, as well the quantification of quercetin, quercitrin, gallic and caffeic acids by HPLC-DAD based in the reference standards (Table 2). Quercetin (27.1±0.03 mg/g) and quercitrin (183.2±0.24 mg/g) were also quantified by Boligon et al. (2009b) in the ethyl acetate extract of *S. buxifolia*, using the same chromatographic conditions.

In vitro screening techniques for detecting antimycobacterial in plant extracts and isolated compounds are varied; the Broth dilution techniques offer benefits such as ease of operation, no expensive equipment needs and, in a microplate format, low sample volumes. Many researchers have made use of the 96-well microplate format to screen test substances for antimycobacterial activity against *M. tuberculosis*, for example the Microplate Alamar Blue Assay or MABA. Microplate assay that use Alamar Blue or tetrazolium-type compounds have the potential of becoming the methods of choice for drug susceptibility testing of *M. tuberculosis*. Similar considerations would apply for any of the slow-growing pathogenic mycobacteria including *M. avium* and *M. bovis* (McGaw et al., 2008; Collins & Franzblau, 1997).

Several researchers have utilized non-pathogenic, fast-growing *Mycobacterium* species in rapid and easy screens for antimycobacterial activity in plant extract and pure plant-derived compounds (Brown et al., 2007; Kuete et al., 2008). In one study realized by McGaw et al. (2008) was concluded that *M. smegmatis* was better predictor of activity against pathogenic *M. tuberculosis*. However, more research is needed to validate the use of non-pathogenic species such as *M. smegmatis* as models for detecting activity of plant-derived extracts against pathogenic *M. tuberculosis*.

The antimycobacterial activity against *M. smegmatis, M. tuberculosis* and *M. avium* is a new addition to the activities reported for this plant, the results of the antimycobacterial assays have been reported in Table 3. The all tested samples of the leaves

Table 2. Fractions composition of *Scutia buxifolia* leaves.

Fractions of the leaves	Gallic acid (mg/g of dry fraction*)	Caffeic acid (mg/g of dry fraction*)	Quercitrin** (mg/g of dry fraction*)	Quercetin (mg/g of dry fraction*)
Fraction I	21.03±0.07	29.12±0.09	83.56±0.04	51.64±0.34
Fraction II	18.55±0.15	31.05±0.06	89.43±0.12	45.89±0.02
Fraction III	132.36±0.18	109.29±0.37	418.28±0.33	312.86±0.56
Fraction IV	79.13±0.19	67.22±0.11	191.9±0.67	170.02±0.19
Fraction V	31.04±0.24	97.08±0.05	248.8±0.16	96.43±0.43
Fraction VI	99.11±0.07	103.92±0.23	318.17±0.02	277.34±0.08
Fraction VII	36.22±0.41	74.06±0.09	226.6±0.09	59.27±0.56

*Results are expressed as mean±SE of three determinations; **Quantified as quercetin.

Table 3. Minimal inhibition concentration (µg/mL) of fractions and isolated of the leaves of *Scutia buxifolia* against *M. smegmatis*, *M. tuberculosis* and *M. avium*.

Extract and fractions	*M. smegmatis*	*M. tuberculosis*	*M. avium*
		Leaves	
Crude extract	>2500.00	ND	ND
Dichloromethane	312.50	ND	ND
Ethyl acetate	312.50	ND	ND
n-Butanolic	>2500.00	ND	ND
Fraction I	1250.00	312.50	1250.00
Fraction II	1250.00	625.00	625.00
Fraction III	78.12	156.25	312.50
Fraction IV	312.50	312.50	625.00
Fraction V	312.50	312.50	625.00
Fraction VI	156.25	156.25	312.50
Fraction VII	312.50	625.00	625.00
Quercetin	625.00	312.50	2500.00
Quercitrin	312.50	625.00	1250.00
Caffeic acid	>2500.00	ND	ND
Gallic acid	>2500.00	ND	ND

ND: Not Determined.

(except crude extract and *n*-butanolic fraction) were found to be active on *M. smegmatis* and the MIC ranged from 78.12 to 1250.00 µg/mL. The lowest MIC of the 78.12 µg/mL against *M. smegmatis* was noted for the fraction III, this fraction together with the fraction VI showed the best activity against *M. tuberculosis* and *M. avium* exhibiting an MIC of 156.25 µg/mL and 312.50 µg/mL, respectively. Tosun et al. (2004) considered inactive the plant extracts that could not prevent growth of mycobacterium up to concentration of 200.00 µg/mL. In this sense, we considered a promising result the MIC of 78.12 and 156.25 µg/mL found in fraction III and VI.

The fraction III (Figure 1c) and VI (Figure 1f) have the highest amount of flavonoids and phenolics acids when compared to other fractions tested (Table 2). This suggests that the interaction between compounds presents from these fractions might be important for the activity against *M. tuberculosis, M. smegmatis* and *M. avium.*

Quercetin and quercitrin were predominantly present in the fraction III and VI, flavonoids are well known for their antimicrobial potencies (Cowan, 1999). Several studies have demonstrated activity of flavonoids against mycobacterium, such as luteolin (MIC 78.12 µg/mL for *M. tuberculosis* and *M. smegmatis*), genistein (MIC 19.53 µg/mL for *M. tuberculosis* and *M. smegmatis*) and alpinum isoflavone (MIC 19.53 µg/mL for *M. smegmatis*) isolated from *Ficus chlamydocarpa*, licoisoflavanone isolated from the medicinal plant *Glycyrrhiza glabra* and quercetin 3'7-di-*O*-methyl-3 sulphate and kaempferol 7-*O*-methyl 3 sulphate isolated of *Argyreia speciosa* presented MIC of 25.00 µg/mL against *M. tuberculosis* and 4'-hydroxy-5,7,3'-trimethoxyflavan-3-ol isolated from *Cinnamomum kotoense* (MIC 150.60 µg/mL) (Copp, 2003; Chen et al., 2005; Kuete et al., 2008).

In a recent study by Askun et al. (2009) reports the antimycobacterial activity (*M. tuberculosis*) of the methanolic extracts of *Thymbra spicata* var. *spicata* and *Origanum minutiflorum*, which presented MIC of 196.00 and 392.00 µg/mL respectively, among the components of these extracts was identified flavonoid that screened in this study, like quercetin. Brown et al (2007) investigated the antimycobacterial activity of the flavonoid butein (MIC 43.00 µg/mL) and isoliquiritigenin (MIC 50.00 µg/mL) and concluded that its effects may be related to their inhibitory effect on fatty acid and mycolic acid biosynthesis.

Lechner et al. (2008) tested flavonoids with different substitution patterns, using different mycobacterial strains, *M. smegmatis* (ATCC 14468), *M. smegmatis* mc^2 155 (ATCC 700084) and *M. smegmatis* mc^2 2700. The results using the same strain *M. smegmatis* mc^2 155 (ATCC 700084) used in our study were epicatechin (MIC>128.00 µg/mL), isorhamnetin (MIC>256.00 µg/mL), kaempferol (MIC>256.00 µg/mL), luteolin (MIC=128-256.00 µg/mL), myricetin (MIC 32.00 µg/mL), quercetin (MIC>256.00 µg/mL), rutin and taxifolin (MIC>128.00 µg/mL). Generally, simple flavonols and its glycosides like quercetin, quercitrin and rutin possess a moderated antimycobacterial activity against both, fast (*M. smegmatis*) and slow (*M. tuberculosis*) growing mycobacteria. Flavones and flavanones have been exhibiting better results, like those obtained by Kuete et al. (2008) and Lechner et al. (2008), where genistein, alpinum isoflavone, laburnetin presented low MIC against *M. tuberculosis.*

Flavonoids can inhibit enzymes involved in the fatty acid and mycolic acid biosynthesis *in vivo*. Mycolic acids are one of the most distinctive features of the mycobacteria cell wall, essential for its survival. Considering the structure of flavonoids, the ketone group emulates the carbonyl group of a fatty acid substrate, and they all possess a 2,3 double bond consistent with a product mimic. Furthermore, the most potent inhibitors of FAS-II (fatty acid synthase II) do not support the oxygen containing cycle possessed by quercetin. The activity of these compounds against *Mycobacterium* certainly represents an important potential target for future drug development studies (Brown et al., 2007).

The data obtained from this study demonstrated that flavonoids are among the chemical classes responsible for the antimycobacterial activity of the plant reported here. Several authors have documented the antibacterial and antifungal potency of flavonoids (Cowan, 1999; Koysomboon et at., 2006; Kuete et al., 2007a; Kuete et al., 2007b; Kuete et al., 2008). This activity may be due to its ability to complex with bacterial cell wall (Cowan, 1999) and thus inhibiting microbial growth. Though the plant mixture is used traditionally, the results this study showed that the fractions from *S. buxifolia* leaves could be used alone with good efficiency.

Gallic and caffeic acids standards showed no antimycobacterial (both MIC over 2500.00 µg/mL against *M. smegmatis*). Rauha et al. (2000) demonstrated that caffeic acid does not even have an inhibitory effect against Gram-positive and negative bacteria. This way, do not contribute to the promising activities found for the fractions of *S. buxifolia.*

To the best of our knowledge the antimycobacterial activity from *S. buxifolia* and its fractions is being reported for the first time. Fraction III and VI of plant were active against *M. tuberculosis* and *M. smegmatis*. The present study provides an important basis for the use of fraction from *S. buxifolia* for the treatment of infections associated with the studied microorganisms. The fractions could be useful for the development of new antimicrobial drugs. However, pharmacological and toxicity studies are currently going on in our laboratory and will be necessary to confirm this hypothesis.

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4.3 Capítulo 3

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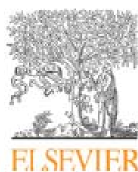

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Antimicrobial activity of *Scutia buxifolia* against the honeybee pathogen *Paenibacillus larvae*

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ABSTRACT

The honeybee disease American foulbrood (AFB) is a serious problem since its causative agent (*Paenibacillus larvae*) has become increasingly resistant to conventional antibiotics. One of the feasible alternative treatments being used for control of this disease are plants extracts. The aim of the present work was to evaluate the effect of crude extract and fractions of *Scutia buxifolia* against six *Paenibacillus* species, including *P. larvae*, and its potential use for the control of AFB. *In vitro* activity of *S. buxifolia* samples against *Paenibacillus* species were evaluated by the disk diffusion and microdilution methods, and the minimal inhibitory concentration (MIC) were also determined. All *Paenibacillus* species were sensitive to crude extract and fractions of *S. buxifolia*. The dichloromethane (DC) fraction showed the better MIC (1.56 mg/mL), followed by ethyl acetate (EtAc) (6.25 mg/mL), *n*-butanol (BuOH) (25 mg/mL) and Crude extract (CE) (50 mg/mL). Toxic effect of *S. buxifolia* crude extracts and fractions against bees were also evaluated by the spraying application method of the same concentrations of MICs. The samples tested showed no toxic effects for the bees after 15 days of observation. These results are first time described for this species and showed that *S. buxifolia* presented a important activity against *Paenibacillus* species and proved to be a natural alternative for the prevention/control of AFB.

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1. Introduction

One of the most severe bacterial diseases that affecting honeybee (*Apis mellifera*) is American foulbrood (AFB) (Genersch et al., 2005). AFB is among the most deleterious bee diseases and is produced by Gram positive and sporeforming bacterium *Paenibacillus larvae*, which is distributed worldwide and causes a decrease in bee population and honey production (Genersch et al., 2006; González and Marioli, 2010). Beekeepers have difficulty in treating AFB, because the etiologic agent produces environmentally stable spores which are very virulent, as resistant to heat, to desiccation and to disinfectants (Thompson et al., 2007). Hence, this disease is considered as a global threat to apiculture (Genersch, 2010).

Plants extracts, herbs, spices, essential oils and isolated compounds are known to retard or inhibit the growth of bacteria, yeast and moulds (Hayouni et al., 2008). In this context, some natural products, such as, propolis (Antunez et al., 2008; Bastos et al., 2008; Mihai et al., 2012); essential oils (González and Marioli, 2010; Santos et al., 2012); *Myrtus communis, Eucalyptus gunnii, Rosmarinus officinalis, Zingiber officinale*, among other species (Flesar et al., 2010) were previously investigated and exhibited growth-inhibitory effect against AFB.

Scutia buxifolia Reissek belongs to the Rhamnaceae family and is popularly known as "coronilha". It is a native plant from South America, with a dispersion that include Rio Grande do Sul state in Brazil, Argentina and Uruguay, where is popularly used as cardiotonic, antihypertensive and diuretic (Wasicky et al., 1964; Da Silva et al., 2012). This plant has shown to contain four classes of biologically active compounds, namely phenolic compounds (gallic, caffeic and chlorogenic acids), flavonoids (quercetin, quercitrin, isoquercitrin and rutin), alkaloids (scutianines A-M), and triterpenes (β-sitosterol, stigmasterol and lupeol) (Boligon et al., 2009a, 2010, 2012a; Maldaner et al., 2011). Crude extract and fractions of *S. buxifolia* showed *in vitro* antioxidant, antimicrobial and antimycobacterial activities (Boligon et al., 2012b, 2012c), where polyphenols, flavonoids and alkaloids present in this fractions were associated with this effects (Trevisan et al., 2009; Boligon et al., 2012b).

The aim of the present work was to evaluate, for the first time, the use of *S. buxifolia* crude extract and fractions against *Paenibacillus* species. Furthermore, toxicity against honey bees *Apis mellifera* was also investigated.

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2. Materials and methods

2.1. Plant collection and extraction

Stem bark of *S. buxifolia* Reissek (Rhamnaceae) was collected in Dom Pedrito, Rio Grande do Sul State, Brazil (30°59′09″S, 54°27′44″W), in October of 2007. Exsiccate was archived as voucher specimen in the herbarium of Department of Biology at Federal University of Santa Maria by register number SMBD 10919, for future references. The stem bark were dried at room temperature and powdered in a knife mill (0.86 μm), resulting in a mass of 651.52 g. The crude extract was obtained by maceration in hydroalcoholic solution (EtOH:H_2O 7:3, v/v), using a container covered with the solvent. The mash was daily subjected to manual agitation for seven days. To remove ethanol, at this time the content was filtered through cotton and concentrated using a rotating evaporator under reduced pressure at a low temperature. Following evaporation of ethanol, the crude extract (CE) of stem bark was partitioned by sequential extraction using solvents with increasing polarity: dichloromethane (DCM), ethyl acetate (EtAc), and *n*-butanol (BuOH). Detailed description of preparation and characterization of crude extract and fractions, as well as phytochemical screening of the fractions have been previously published (Boligon et al., 2009a, 2010, 2012a).

2.2. Microorganisms tested

In this study, six isolates of *Paenibacillus* species from the collection of Ministry of Agriculture (LANAGRO/RS) Brazil were used. The test organisms included isolates of *P. alginolyticus, P. pabuli, P. azotofixans, P. borealis, P. validus* and *P. larvae* (ATCC 9545). The microorganisms were grown in Mueller–Hinton broth (Difco, Sparks, Maryland, USA) at 37 °C for 24 h and maintained on slopes of nutrient agar (Difco).

2.3. Determination of the minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of crude extract and fractions of *S. buxifolia* were determined by microdilution techniques in Mueller–Hinton broth (Difco) for *Paenibacillus* species (CLSI, 2008). The assay was carried out in 96-well microtitre plates. Each sample was mixed with an inoculum prepared in the same medium at a density adjusted per tube to 0.5 of the McFarland scale (1.5×10^8 CFU/mL) and diluted 1:10 for the broth microdilution procedure. Microtitre trays were incubated at 37 °C and the MICs were recorded after 24 h of incubation. The MIC was defined as the lowest concentration of compounds that inhibits bacterial growth. This test was performed in triplicate on separate occasions. The 2,3,5-triphenyltetrazolium chloride was used as an indicator of bacterial growth.

2.4. Toxicity assay

The crude extract and fractions of *S. buxifolia* were dissolved in DMSO to reach the final concentrations of 50, 25, 6.25 and 1.56 mg/mL for CE, BuOH, EtAc and DCM, respectively. These concentrations used in the toxicity test were determined from the determination of MIC values (Table 1). The spraying application method was performed according to Santos et al. (2012). Petri dishes (150 × 15 mm) padded with absorbent filter paper on the inner bottom and with an extra lid of plastic mesh were used. Six adult worker bees were placed in every modified Petri dish. Then, 1 mL of each concentration (crude extract and fractions) was individually sprayed on the bees throughout the plastic lid using a hand sprayer. A device with candy and water was placed inside each unit

Table 1
MICs of *S. buxifolia* crude extract and fractions on *Paenibacillus* species.

Microorganisms	*S. buxifolia* (mg/mL)			
	CE	DCM	EtAc	BuOH
P. larvae (ATCC 9545)	50.00	1.56	6.25	25.00
P. borealis	6.25	1.50	6.25	6.25
P. validus	1.56	1.56	1.56	3.12
P. pabuli	1.56	1.56	1.56	3.12
P. alginolyticus	1.56	1.56	1.56	3.12
P. azotofixans	1.56	1.56	1.56	3.12

Crude extract (CE), dichloromethane (DCM), ethyl acetate (EtAc) and *n*-butanol (BuOH).

as food for the bees. Six bees in a modified Petri dish sprayed with DMSO were included as negative control, and six bees in a modified Petri dish sprayed with 0.07% Deltamethrin (DTT) (Pirisa-Piretro Industrial Ltda, Brazil) were included as positive death control. Four replicates for each experimental group were run. Bioassay dishes were placed in incubators at 28 ± 1 °C and 60% relative humidity. Mortality of bees was evaluated by visual inspection daily up to 15 days.

2.5. Statistical analysis

Differences in survival after 15 days of observation were assessed by Kaplan–Meier analysis followed by the Logrank test. A p value < 0.05 was considered statistically significant. All statistical analyses were performed with the software package GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Antimicrobial susceptibility test and determination of MIC

All *Paenibacillus* species were susceptible to the assessed CE, DCM, EtAc and BuOH of the *S. buxifolia*. The MICs of these samples ranged 1.56–50.00 mg/mL (Table 1).

3.2. Lethal concentration on bees

Toxicity analysis for honeybees, evaluated by spraying application method of CE, DCM, EtAc and BuOH, demonstrated that *S. buxifolia* samples are not toxic during 15 days of treatment (Fig. 1). Bee mortality was evident only in treatment with DTT (positive death control group).

4. Discussion

The present work reports the first study about the use of the crude extract and fractions of *S. buxifolia* for the treatment of *P. larvae*-affected beehives. Results indicate that *S. buxifolia*

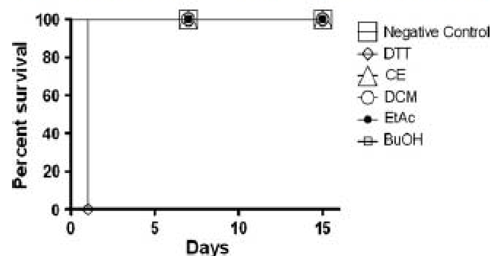

Fig. 1. Effects of *S. buxifolia* spraying applications on bees. For further details, see Section 2.

samples showed *in vitro* antibacterial activity against *Paenibacillus* species included the *P. larvae* and that low concentrations of *S. buxifolia* are required to inhibit its growth. In this research, the DCM and EtAc fractions showed better results for *P. larvae* with MIC of 1.56 and 6.25 mg/mL, respectively (Table 1). The antibacterial activity these fractions could be related to chemical composition of *S. buxifolia* that is rich in steroids, triterpenes, phenolics, flavonoids and alkaloids (Maldaner et al., 2011; Boligon et al., 2009a, 2010, 2012a). It has been proposed that the antimicrobial activity could be due to the synergism between its different components, since it was observed that not even a single component has shown an activity higher than the crude extract and fractions (Flesar et al., 2010). Mihai et al. (2012) also attribute the inhibitory effect of propolis extract again *P. larvae* to synergism between flavonoids and phenols.

These effects are in accordance with previous works that reported the antibacterial activity of *S. buxifolia* against diverse pathogens as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis* and *Mycobacterium avium*, between others (Boligon et al., 2012b, 2012c).

Plant extracts comprises a complex unique mixture of different secondary metabolites and are considered as important sources of bioactive molecules, some of them exhibiting antibacterial effect with future prospects in pharmaco-chemistry or food control (Flesar et al., 2010). The chemical nature of these constituents may vary considerably between species (Paul et al., 2009). Flesar et al. (2010) described that 13 natural compounds and 16 crude extracts exhibited an antimicrobial effect gainst *P. larvae* with MICs values ranging from 2 to 256 μg/mL, using broth microdilution method. In case of extracts, *Humulus lupulus* and *Myrtus communis* methanolic-dichloromethane extracts exhibited the highest growth-inhibitory effect. Propolis extract also was described with as a natural alternative for the control of AFB (Antunez et al., 2008; Bastos et al., 2008).

In this research, the CE, DCM, EtAc, BuOH of *S. buxifolia* were sprayed on *A. mellifera* adults to verify the possible toxic effects during 15 days. All samples tested presented similar results to the control group, causing not toxic effects or animal death at tested concentration (same concentration that inhibits the growth of *P. larvae*), showing that *S. buxifolia* CE and fractions can be used for the treatment of AFB. Santos et al. (2012) tested the toxicity of Amazonian oils against *A. mellifera*, copaiba oil showed no toxic effects after 10 days of observation, using the same method described here. The use of perspective non-toxic compounds could represent a natural alternative to synthetic antibiotics in the control of AFB, because of their significant growth-inhibitory action on *P. larvae* and their not toxicity to bees (Flesar et al., 2010), the *Scutia buxifolia* is a potentially useful alternative in suppressing bacterial diseases that affecting honeybee.

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4.4 Capítulo 4

Boligon, A.A.; Schwanz, T.G.; Brum, T.F.; Frohlich, J.K.; Nunes, L.; Mario, N.D.; Alves, S.H.; Athayde, M.L. Chemical composition, antioxidant and antimicrobial activities of the essential oil of *Scutia buxifolia* Reissek leaves. Pharmaceut Anal Acta 3: 199, 2012. Doi:10.4172/2153-2435.1000199

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Research Article Open Access

Chemical Composition, Antioxidant and Antimicrobial Activities of the Essential Oil of *Scutia buxifolia* Reissek Leaves

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Abstract

The chemical composition, antioxidant and antibacterial activities of essential oil isolated by hydrodistillation from the leaves parts of *Scutia buxifolia* were evaluated. The chemical composition was analyzed by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Twenty-five substances were identified, consisting of a complex mixture of sesquiterpenes (73.69%) and monoterpenes (18.74%). The main components in the oil were spathulenol (27.09%), β-cubebene (11.26%), germacrene D (9.81%), carvacrol (7.01%), globulol (5.36%), α-copaene (4.17%), γ-eudesmol (3.59%), thymol (3.27%), 1,8-cineol (3.08%), *p*-cymene (2.56%), α-eudesmol (2.34%), β-elemene (2.04%), butylated hydroxytoluene (2.00%) along with eugenol acetate, n-hexanol, α-pinene, α-humulene, eugenol, humulene epoxide, phytol as minor constituents. The antioxidant property of the oil was assessed by free radical scavenging (DPPH) assay. *S. buxifolia* essential oil presented interesting radical scavenging activity (IC_{50}=13.62 ± 0.17 µg/mL). The antibacterial properties of the oils also was tested by broth microdilution method and was effective only against *S. aureus* and *E. coli* (MIC=500 and 750 µg/mL, respectively). To the best of our knowledge, this is the first study of the composition, antioxidant and antimicrobial activities of essential oil from the *S. buxifolia* collected from Brazil.

Keywords: *Scutia buxifolia;* Essential oil; Antioxidant; Antimicrobial

Introduction

Essential oils in plant are complex volatile mixtures exist at low concentrations and are commonly found in aromatic plants [1,2]. Studies have demonstrated beneficial properties of essential oils in the prevention and treatment of cancer, cardiovascular diseases including atherosclerosis and thrombosis, as well as their bioactivity as antibacterial, antiviral, antioxidants, antidiabetic, anti-inflammatory agents, local anaesthetic and immunomodulatory [1,3-5].

Biologically, the essential oils perform the function of adaptation of the plant to the environment, acting in the defense against the attack of predators, attraction of pollination agents, protection against water loss and temperature increase and as inhibitors of germination [6,7]. Economically, they are employed in food, cosmetic and cleaning products industries, as well as in alternative medicine due to their many therapeutic properties.

Scutia buxifolia Reissek belong to the Rhamnaceae family and is popularly known as "coronilha". It is native tree from South America, with a dispersion area that comprises Rio Grande do Sul State in Brazil, and the countries Argentina and Uruguay. The root bark infusion is popularly used as cardiotonic, antihypertensive and diuretic [8]. Antimicrobial activities of some cyclopeptide alkaloids isolated from the root bark of this species were reported by Morel et al. [9] using the bioautography method. Cytotoxicity of extracts from leaves, twigs and stem bark of the plant was evaluated by the *Artemia salina* assay, as well as the antimicrobial activity against a panel of microorganism strains [10]. Extracts from the leaves and stem bark of *S. buxifolia* were effective inhibitors of TBARS production and also presented DPPH scavenger activity [11].

The literature search did not reveal any report on the essential oil composition of *S. buxifolia.* The aim of the present work was to determine the chemical composition and evaluate the antioxidant and antimicrobial activity of the essential oil from leaves of *S. buxifolia*, accessed by gas chromatography (GC-FID) and gas chromatography-mass spectrometry (GC-MS) analysis, 1,1-diphenil-2-picrylhydrazil (DPPH) method and microdilution assay.

Materials and Methods

Plant material

Scutia buxifolia (Rhamnaceae) leaves were collected in Dom Pedrito, State of Rio Grande do Sul, Brazil, on June of 2011 (coordinates 30°59'09"S and 54°27'44"W). It was identified and archived as voucher specimens in the herbarium of Department of Biology at Federal University of Santa Maria by register number SMBD 10919.

Extraction of the essential oil

The fresh material (250 g) of the plant leaves was extracted using a hydrodistillation process in a Clevenger apparatus for 4 hours. Oil was dried over anhydrous sodium sulphate and, after filtration, stored at -4°C until test and analysis. The yield in terms of percentage of the fresh weight of the leaves was determined.

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Gas chromatography (GC-FID)

The gas chromatography (GC) analyses were carried out using an Agilent. Technologies 6890N GC-FID system, equipped with DB-5 capillary column (30 m×0.25 mm; film thickness 0.25 mm) and connected to an FID detector. The injector and detector temperatures were set to 280°C. The carrier gas was helium, at a flow rate of 1.3 mL/min. The thermal programmer was 50-300°C at a rate of 5°C/min. Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors. The injection volume of the oil was 1 µL [4,12].

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed on a Agilent Technologies AutoSystem XL GC-MS system operating in the EI mode at 70 eV, equipped with a split/splitless injector (250°C). The transfer line temperature was 280°C. Helium was used as carrier gas (1.3 mL/min) and the capillary columns used were an HP 5MS (30 m×0.25 mm; film thickness 0.25 mm) and an HP Innowax (30 m×0.32 mm i.d., film thickness 0.50 mm). The temperature programmer was the same as that used for the GC analyses. The injected volume was 1 µL of the essential oil.

Identification of the components

Identification of the constituents was performed on the basis of retention index (RI), determined with reference of the homologous series of n-alkanes, C_7-C_{30}, under identical experimental conditions, comparing with the mass spectra library search (NIST and Wiley), and with the mass spectra literature date Adams [13]. The relative amounts of individual components were calculated based on the CG peak area (FID response).

Qualitative analysis of antioxidant activity

Ten microlitres of 1:50 dilution of the essential oil in methanol was applied to TLC plates (silica gel 60 GF_{254}), quercetin and ascorbic acid (Sigma-Aldrich, ≥ 98% HPLC) standards also were used. The TLC plate was sprayed with a 0.2% 1,1-diphenyl-2-picrylhydrazyl (DPPH) solution in methanol and left at room temperature for 30 minutes. Active compounds appear as yellow spots against a purple background, indicating possible antioxidant activity [14].

Quantitative analysis of antioxidant activity

The antioxidant activity of the essential oil was evaluated by monitoring their ability in quenching the stable free radical DPPH, according to a slightly modified method previously described by Mensor et al. [14]. Spectrophotometric analysis was used to measure the free radical-scavenging capacity and to determine the scavenging concentration or inhibitory concentration (IC_{50}). The DPPH quenching ability was expressed as IC_{50} (the essential oil concentration (µg/mL) required to inhibit 50% of the DPPH in the assay medium).

Six different ethanol dilutions of essential oil at 250, 125, 62.5, 31.25, 15.62 and 7.81 µg/mL were mixed with 1.0 mL of DPPH 0.3 mM in ethanol solution. After 30 min, absorption was measured at 518 nm, where the radical DPPH shows maximum absorption. A solution of DPPH (1 mL; 0.3 mM) in ethanol (2.5 mL) was used as a negative control and ascorbic acid in the same concentrations used for the essential oil provided the positive control. Ethanol was used to calibrate the spectrophotometer. The test was performed in triplicate and the calculation of the antioxidant activity followed the equation: % Inhibition=$[(A_0-A_1)/A_0]\times100$, where A_0 was the absorbance of the control sample (without essential oil) and A_1 was the absorbance in the presence of the sample [11].

Antimicrobial assay determination

The essential oil was evaluated against *Candida albicans* ATCC 28967, *Cryptococcus neoformans* ATCC 2857, *K. pneumoniernaae* ATCC 700603, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 51299, *P. mirabilis* ATCC 7002, *S. aureus* ATCC 29213, *Malassezia sp*., *Aspergillus sp*., *Aeromonas sp*., *S. aureus* and *E. coli* (clinical isolates). The minimal inhibitory concentration (MIC) of the oil against the test microorganisms were determined by the broth microdilution method M27-A2 [15]. The experiments were repeated twice and the results were determined as an average value. Six different dilutions (1000, 750, 500, 250, 125, and 62.5 µg/mL) were prepared in DMSO. Bacterial strains were cultured overnight at 37°C in Mueller-Hinton agar. Yeasts were cultured overnight at 30°C in Potato dextrose agar. The first column of the plate was reserved for negative control wells (without inoculants) and the last column, for the positive growth control wells (without antimicrobial agents). The MIC was considered as the lowest concentration of the essential oil inhibiting the total growth of microorganisms. MIC was detected by lack of visual turbidity (matching the negative growth control).

Statistical analysis

The obtained antioxidant and antimicrobial results were stated in mean ± standard deviation of three replicates.

Results and Discussion

The pale yellowish essential oil of the fresh leaves of *S. buxifolia* was obtained by hydrodistillation in the yield of 0.4%. Essential oil was analyzed by GC-FID and GC-MS systems and the oil components were identified both quantitatively and qualitatively. Twenty-five components, representing 98.38% of the total composition, were identified, of which 73.69% are sesquiterpenes and 18.74% are monoterpenes (Table 1).

The main components in the oil were spathulenol (27.09%), β-cubebene (11.26%), germacrene D (9.81%), carvacrol (7.01%), globulol (5.36%), α-copaene (4.17%), γ-eudesmol (3.59%), thymol (3.27%), 1,8-cineol (3.08%), *p*-cymene (2.56%), α-eudesmol (2.34%), β-elemene (2.04%), butylated hydroxytoluene (2.00%) along with eugenol acetate, n-hexanol, α-pinene, α-humulene, eugenol, humulene epoxide, phytol as minor constituents. In addition, *p*-cymene showed a maximum when carvacrol was at its minimum, which is in agreement with the literature, reporting that *p*-cymene is the precursor of carvacrol [16].

Spathulenol, the most abundant component of this oil, has also been reported in the oil of other species such as *Baccharis uncinella* (16.41%), *Stevia rebaudiana* (15.41%), *Origanum vulgare* (11.67%) and *Baccharis dracunculifolia* (9.54%) [17-19]. The second major component of the oil, β-cubebene, has also been found in the oils of *Dendropanax morbifera* [20] and *Cinnamomum osmophloeum* [21] in about 4.19% and 59.4%, respectively. Germacrene D, the third major compound present, has also been found in the oil of *Artemisia annua* (15.64%), *Baccharis uncinella* (14.87%), *Campomanesia adamantium* (11.82%), *Tagetes minuta* (10.00%) and *Origanum vulgare* (8.11%) [19,22].

Many *in vitro* studies have addressed the antioxidant and radical-scavenging properties of essential oils [1,23]. In particular, DPPH

Citation: Boligon AA, Schwanz TG, de Brum TF, Frohlich JK, Nunes L, et al. (2012) Chemical Composition, Antioxidant and Antimicrobial Activities of the Essential Oil of *Scutia buxifolia* Reissek Leaves. Pharmaceut Anal Acta 3: 199. doi:10.4172/2153-2435.1000199

Rt (min)	Compounds	(%)	RI[a]	RI[b]	Mol. Formula
	Monoterpenes				
3.261	α-Pinene	1.27	939	939	$C_{10}H_{16}$
3.880	β-Pinene	0.42	981	980	$C_{10}H_{16}$
10.625	*p*-Cymene	2.56	1026	1026	$C_{10}H_{14}$
11.381	1,8-Cineol	3.08	1029	1033	$C_{10}H_{18}O$
16.153	Thymol	3.27	1288	1290	$C_{10}H_{14}O$
16.947	Carvacrol	7.01	1298	1298	$C_{10}H_{14}O$
18.042	Eugenol	1.13	1357	1356	$C_{10}H_{12}O_2$
	Sesquiterpenes				
19.511	Thymol acetate	0.92	1357	1355	$C_{12}H_{16}O_2$
23.760	α-Copaene	4.17	1378	1376	$C_{15}H_{24}$
25.934	β-Cubebene	11.26	1400	1390	$C_{15}H_{24}$
28.486	β-Elemene	2.04	1390	1391	$C_{15}H_{24}$
28.627	Methyl eugenol	0.49	1400	1401	$C_{11}H_{14}O_2$
29.002	α-Humulene	1.21	1454	1454	$C_{15}H_{24}$
30.165	Germacrene D	9.81	1477	1480	$C_{15}H_{24}$
31.931	Butylated hydroxytoluene	2.00	1509	1512	$C_{15}H_{24}O$
32.471	Eugenol acetate	1.55	1537	1536	$C_{12}H_{16}O_3$
33.502	Spathulenol	27.09	1577	1576	$C_{15}H_{24}O$
35.099	Globulol	5.36	1583	1583	$C_{15}H_{26}O$
35.710	Humulene epoxide	1.08	1598	1606	$C_{15}H_{24}O$
36.420	γ-Eudesmol	3.59	1630	1630	$C_{15}H_{26}O$
40.015	Cubenol	0.78	1643	1642	$C_{15}H_{26}O$
43.127	α-Eudesmol	2.34	1651	1652	$C_{15}H_{26}O$
	Other				
5.618	n-Hexanol	4.43	867	867	$C_6H_{14}O$
34.701	Hexadecanol	0.32	1871	1879	$C_{16}H_{34}O$
47.095	Phytol	1.00	1957	1949	$C_{20}H_{40}O$
54.32	α-tocoferol	0.24	2960	-	$C_{29}H_{50}O_2$
Total identified (%)		98.38			

Relative proportions of the essential oil constituents were expressed as percentages. Rt=Retention time according their order on MS. [a]Retention indices experimental (based on homologous series of *n*-alkane C_7-C_{30}). [b]Retention indices from literature (Adams, 1995)

Table 1: Chemical compounds present in *Scutia buxifolia* essential oil.

radical is widely used for quickly assessing the ability of antioxidants to transfer labile H atoms to radicals [24]. Following a similar line of thought, the essential oil was subjected to a preliminary test in order to verify the antioxidant activity using the DPPH free radical scavenging assay. Therefore, the anti-scavenging ability of the essential oil applied on silica gel TLC plate was performed. One sample yellow spot could be observed immediately after spraying DPPH reagent on the TLC plate, suggesting some antioxidant activity for this oil, with intensity and color similar to quercetin and ascorbic acid used as standards. However, in order to get relevant data, a single method for testing antioxidant activities of essential oils is not recommended due to their complex composition. So, this test was the first step in the screening of the potential activity of this essential oil and DPPH test quantitative also was performed.

In the DPPH assay quantitative, antioxidants are typically characterized by their IC_{50} value, concentration necessary to reduce 50% of DPPH radical. The efficiency of the essential oil of *S. buxifolia* and ascorbic acid standard were evaluated for this method, and presented IC_{50} values of 13.62 ± 0.17 and 15.98 ± 1.30 μg/mL, respectively; compared to *Thymbra capitatus* (IC_{50}=19.27 μg/mL), *Pistacia atlantica* (IC_{50}=18.95 μg/mL), *Stevia rebaudiana* (IC_{50}=19.26 μg/mL), *Acacia Senegal* (IC_{50}=17.89 μg/mL), *M. peregrinum* (IC_{50}=13.48 μg/mL) [16,18,23], these results proved that the essential oil from *S. buxifolia* leaf possess significant antioxidant properties.

For this oil, the medicinal benefit derived from their use may include prevention of oxidative damage and subsequent disease progression. Essential oils are complex mixtures and the determination of the component(s) responsible of the activity is difficult. Antioxidant activity of essential oils has often been attributed to the presence of phenolic constituents, especially spathulenol, carvacrol and thymol [16,18,25,26]. This association was confirmed in our study, but other compounds also seem to play an important role such as eugenol (IC_{50}=1.26 μg/mL by DPPH method), β-cubebene (IC_{50}=19.3 μg/mL), butylated hydroxytoluene (BHT) and *p*-cymene [21,27-29]; these compounds are also present in the essential oil of *S. buxifolia*, and may account, in part, the good antioxidant potential reported here. The results presented here may contribute to the knowledge of the antioxidant potential of the essential oil and provide some information for its uses.

The essential oil of *S. buxifolia* leaves was tested also against 11 microorganisms; the antimicrobial screening is summarized in table 2. The essential oil showed only moderately activity against *S. aureus* and *E. coli* (MIC=500 and 750 μg/mL, repectively), previous study describes the activity of *S. buxifolia* leaves against *S. aureus* [11]. Sesquiterpenoids spathulenol, β-cubebene, germacrene D and carvacrol were the main components identified in this essential oil and may be responsible, in part, for the antimicrobial activity described, since that spathulenol [30] and carvacrol [16,31] have been reported to present notable antimicrobial activity against bacterial infections. Spathulenol showed also a decrease in the proliferation of lymphocytes demonstrating immunomodulatory effects [5].

The antimicrobial activity of thymol (3.27% in the essential oil of *S. buxifolia*) has been confirmed on bacteria such as *E. coli* [32]. Thymol has been shown to cause disruption of the cellular membrane, inhibition of ATPase activity, and release of intracellular ATP and other constituents [26,33]. The spathulenol, major compound described in the essential oil of *S. buxifolia* leaves (27.09%), evidenced a high activity against the fungi strains dermatophytes as *T. mentagrophytes* and *M. gypseum* with MIC and MFC values ranging from 32 to 64 μg/ml. Furthermore, the MIC value against C. *lactis-condensi* and the MIC and MFC values against *P. purpurogenum* for the spathulenol were 32 μg/ml [17]. However, in our work that was not observed, since the essential oil of the *S. buxifolia* showed no activity against strains of fungi.

In conclusion, the analysis of the chemical composition of the essential oil of this plant and the preliminary evaluation of its

Microorganisms	Essential oil (μg/mL)
C. albicans	>1000.00
C. neoformans	>1000.00
K. pneumoniernaae	>1000.00
P. aeruginosa	>1000.00
E. faecalis	>1000.00
P. mirabilis	>1000.00
Malassezia sp.	>1000.00
Aspergillus sp.	>1000.00
Aeromonas sp.	>1000.00
S. aureus	500.00
E. coli	750.00

Table 2: Minimal inhibitory concentrations (MIC) of essential oil of the *S. buxifolia* leaves.

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antioxidant and antimicrobial activity is the first work described in the literature for this species, and, taken together, the data obtained here inspire more studies supporting the possibility of linking the chemical contents with particular biological properties.

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4.5 Capítulo 5

Boligon, A.A.; Brum, T.F.; Piana, M.; Jesus, R.S.; Freitas, R.B.; Froeder, A.L.F.; Athayde, M.L. Triterpenes isolated of *Scutia buxifolia* Reissek. Anais da Academia Brasileira de Ciências.

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Classifications:	Ciências da Saúde (Health Sciences)

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Triterpenes isolated of *Scutia buxifolia* Reissek

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ABSTRACT

Rhamnaceae family includes 58 genera and approximately 900 species that occur in tropical and subtropical areas around the world. This work, describe the phytochemical investigation of the dichloromethane fraction of *S. buxifolia* stem bark, popularly known as coronilha, which led to the isolation of three triterpenes compounds, β-sitosterol (1), sitosterol-3-*O*-β-D-glucoside (2) and ursolic acid (3). The structures of the isolated compounds were elucidated by spectroscopic (NMR) techniques as well as literature data comparisons. β-sitosterol (25.39%), sitosterol-3-*O*-β-D-glucoside (13.08%) and ursolic acid (19.74%) were quantified in dichloromethane fraction by high performance liquid chromatography (HPLC/DAD), being the major compound in this fraction the β-sitosterol.

Keywords: Steroids, Rhamnaceae, *Scutia buxifolia*.

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INTRODUCTION

The terpenes or terpenoids belong to a diverse class of natural substances, are formed by five-carbon isoprene units $(C_5H_8)n$. In plants, terpenoids exert communicative and defensive responses, serving as attractants to pollinators, plant toxins, or plant antibiotics to inhibit the spread of plant pathogens (MacGarvey and Croteau. 1995; Wagner and Elmadfa. 2003). This is a group of compounds that exhibit a diverse biological relevance and have attracted more attention in recent years (Wagner and Elmadfa. 2003). Terpenoid compounds have been reported to be beneficial to the human body for anti-inflammatory, anti-oxidant, anti-cancer and anti-biotic effects (Ku and Lin. 2013).

Rhamnaceae family includes 58 genera and approximately 900 species occurring in tropical and subtropical areas around the world (Boligon et al. 2009a). *Scutia buxifolia* Reissek belongs to the Rhamnaceae family and is popularly known as ''coronilha''. It is a native plant from South America, with a dispersion that includes Rio Grande do Sul state in Brazil, Argentina and Uruguay, where it is popularly used as cardiotonic, antihypertensive and diuretic (Da Silva et al. 2012). This plant has shown to contain four classes of biologically active compounds, namely phenolic compounds, flavonoids, alkaloids and triterpenes (Boligon et al. 2009a, 2009b, 2010, 2012a; Maldaner et al. 2011).

In this study, we describe the isolation and structural elucidation of three new triterpenes compounds from the dichloromethane fraction of *S. buxifolia* stem bark, as well their quantification by High-Performance Liquid Chromatography-Diode Array (HPLC–DAD).

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MATERIALS AND METHODS

Chemicals, apparatus and general procedures

All chemicals were of analytical grade. Silica Gel 60 and Silica Gel 60 F254 coated plates were purchased from Merk (Darmstadt, Germany and Rio de Janeiro, Brazil). The solvents for the extractions and analytical procedures as dichloromethane, ethyl acetate, ethanol, methanol and n-butanol were obtained from Vetec and Proquimios. β-sitosterol and ursolic acid were acquired from Sigma Chemical Co. (St. Louis, MO, USA). NMR spectra were carried out on a Bruker AMX 400 spectrometer equipped with a broadband 5-mm probe, using a spectral width of 10 ppm (parts per million). Chemical shifts were expressed as ppm relative to the TMS. Deuterated dimethyl sulfoxide (DMSO) and chloroform were used as solvent for the samples. 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.

Plant collection and extraction

Stem bark of *Scutia buxifolia* were collected in Dom Pedrito (Rio Grande do Sul State) in October of 2007 (coordinates 30°59'09''S and 54°27'44'' W). Exsiccate was archived as voucher specimen in the herbarium of Department of Biology at Federal University of Santa Maria by register number SMBD 10919. Plant material was dried at room temperature and powdered in a knife mill, resulting in a mass of 651.52 g, which was submitted to maceration at room temperature with ethanol 70% for a week with daily shake. After filtration, the extract was evaporated under reduced pressure to remove the ethanol and after this step, the aqueous

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extract was partitioned successively with dichloromethane, ethyl acetate and *n*-butanol (3 x 200 mL for each solvent).

Isolation and purification

The dichloromethane fraction from stem bark (3.5 g) was submitted to column chromatography on silica gel 60 (240 g) using initially Hex/CH_2Cl_2 (9:1 v/v, 300mL); followed by Hex/CH_2Cl_2 (8:2 v/v, 300mL); Hex/CH_2Cl_2 (7:3 v/v, 300mL); Hex/CH_2Cl_2 (5:5 v/v, 300mL); Hex/CH_2Cl_2 (3:7 v/v, 300mL); Hex/CH_2Cl_2 (1:9 v/v, 300mL); CH_2Cl_2 (500mL), CH_2Cl_2:EtOH (9:1 v/v, 500 mL), CH_2Cl_2:EtOH (7:3 v/v, 500 mL) and CH_2Cl_2:EtOH (5:5 v/v, 600 mL). The procedure describe above fifty-six (56) fractions of ± 100 mL each, which were analyzed by TLC and pooled together on the basis of similarities in their chromatographic profile (solvent system: hexane: dichloromethane, 5:5, v/v). The separated fractions were observed on UV light and detection was performed with sulfuric anisaldehyde/100 °C for ten minutes. Authentic samples of β-sitosterol and ursolic acid were used as reference standards in order to guide the fractions pool process. Fractions 10-34 furnished a subfraction (0.37 g), which was further chromatographed under silica gel 60 and eluted with Hex:CH_2Cl_2 (8:2, v/v), Hex:CH_2Cl_2 (6:4, v/v), Hex:CH_2Cl_2 (5:5, v/v) and Hex:CH_2Cl_2 (2:8, v/v) to give, after precipitation with methanol, isolated compounds **1** (28 mg) and **2** (19 mg). Compounds **3** (54 mg) was obtained from the sub-fraction 39-51 (0.48 g) after additional column chromatographic procedures, Hex:CH_2Cl_2 (1:9, v/v), CH_2Cl_2 and gradient from CH_2Cl_2:EtOH 9:1 to pure EtOH).

Preparation of standard and sample solutions for HPLC quantification

Standard stock solutions of β-sitosterol and ursolic acid were prepared in mobile phase, at a concentration range of 0.030 to 0.400 mg/ml for β-sitosterol and 0.025 to 0.500 mg/ml for ursolic acid. The dichloromethane fraction was dissolved in the mobile phase. All

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solution were filtered through a filter paper and a 0.45 μm membrane filter (Millipore). Triplicate injections were made for each level, and a linear regression was generated.

Chromatographic conditions

Chromatographic analyses were carried out in isocratic conditions using RP-C8 column (4.6 mm x 150 mm, packed with 5μm diameter particles) eluted at a rate of 0.5 mL/min with the mobile phase acetonitrile: water (86:14, v/v) containing 1.25% H_3PO_4, with a detection wavelength set at 206 nm and 40 μL injection (Chen et al. 2003; Silva et al., 2008). The chromatographic peaks were confirmed by comparing their retention time and UV spectra with those of the reference standards. Quantification was carried out by the integration of each peak using the external standard method. All chromatographic operations were carried out at ambient temperature. The curves obtained were: $Y = 12567x + 1509.3$ ($r = 0.9997$) and $Y = 11846x + 1058.5$ ($r = 0.9992$) for ursolic acid and β-sitosterol, respectively.

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 Boligon et al. (2012). 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.

RESULTS AND DISCUSSION

Column chromatography of the dichloromethane fraction of *S. buxifolia* stem bark led to the isolation of the β-sitosterol (**1**), sitosterol-3-*O*- β-D-glucoside (2) and ursolic acid (3) (Figure 1). The structure of β-sitosterol (1) was identified based on the ^{13}C-NMR data and by

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comparing with the literature data (Boligon et al. 2010; Janovik et al. 2012; Salem et al. 2013). The ^{13}C-NMR assignments (400 MHz/ppm (parts per million) of β-sitosterol in deuterated $CDCl_3$ solvent are as follows: δ_C 140.95 (C-5); 121.71 (C-6); 71.83 (C-3); 56.79 (C-14); 56.18 (C-17); 50.17 (C-9); 45.92 (C-4); 42.31 (C-13); 39.79 (C-1); 39.20 (C-10); 38.91 (C-20); 37.27 (C-12); 36.18 (C-22); 34.03 (C-2); 32.41 (C-7), 31,93 (C-8); 31.67 (C-25); 30.34 (C-16); 29.31 (C-23); 28.28 (C-15); 26.28 (C-28); 24.30 (C-11); 23.11 (C-26); 21.10 (C-27); 19.39 (C-19); 19.05 (C-21); 18.79 (C-18); 18.71 (C-29). The presence of the β-sitosterol in this species was been described recently by Boligon et al. (2010), this authors isolated the lupeol and mixture of β-sitosterol and stigmasterol.

The presence of sitosterol-3-*O*-β-D-glucoside (2) was first reported for this species. The structures were identified based on the ^{13}C-NMR data and by comparing with the literature data (Agrawal 1992; Boligon et al. 2010; Janovik et al. 2012; Frohlich et al. 2013). The signals at δ 140.53 and 121.29 ppm indicate the presence of steroids with a double bond at C5 and C6 (De-Eknankul and Potduang 2003), whereas the anomeric carbon signals at 100.67 ppm indicate the presence of O-glycosides. The appearance of anomeric resonances in chemical shifts at a range of 90-112 ppm helps greatly in determining the number of O-glycosides, since none of the carbon atoms of the aglycone are absorbed in this region and anomeric resonances are usually non-equivalents and do not superimpose on one another (Agrawal 1992). The presence of glucosyl residue at C-3 of the aglycone of sitosterol was attributed to the carbon with a chemical shift at 79.01 ppm. The glucosyl residue at C-3 of aglycone increases the shift of this carbon in 6–7 ppm to a low-field region compared to the shift of this carbon without glucosyl residue (Chaurasia and Wichtl 1987; Frohlich et al. 2013). The glucosyl residue was identified as β-D-glucose by comparing with the ^{13}C-NMR spectrum of the literature (Agrawal 1992).

The ^{13}C-NMR assignments (400 MHz/ppm) of compounds sitosterol-3-*O*-β-D-glucoside (2) in solvent deuterated dimethyl sulphoxide (DMSO) are as follows: δ C 140,53

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(C-5), 121,29 (C-6), 100,67 (C-1'), 79,01 (C-3), 76,91 (C-3'), 76,83 (C-2'), 73,47 (C-5'), 70,17 (C-4'), 61,04 (C-6'), 56,35 (C-14), 55,19 (C-17), 49,55 (C-9), 45,26 (C-24), 41,83 (C-4 e C-13), 39,57 (C-12), 37,86 (C-1), 36,20 (C-10), 35,59 (C-20), 33,41 (C-22), 31,45 (C-8), 31,34 (C-7 e C-2), 28,72 (C-25), 27,80 (C-16), 25,49 (C-23), 24,19 (C-15), 22,56 (C-28), 20,63 (C-11), 19,75 (C-26), 19,19 (C-19), 18,28 (C-21 e C-27), 11,36 (C-29) e 11,27 (C-18).

The compound 3, appeared as a white amorphous powder, was identified as ursolic acid (3β-hydroxy-urs-12-en-28-oic acid). ^{13}C NMR (DMSO-d_6) assignments (400MHz/ppm) spectrum indicate the presence of 29 carbon atoms, the signal at: δ 178.13 was attributed to a carbonyl carbon placed at C-28, the other signals were: 137.29 (C-13); 124.52 (C-12); 76.80 (C-3); 55.49 (C-5); 40.13 (C-18); 39.93 (C-9); 39.71 (C-16); 39.50 (C-14); 39.29 (C-8); 39.07 (C-20); 38.66 (C-19); 38.32 (C-4); 38.25 (C-1); 36.51 (C-10); 36.28 (C-22); 32.59 (C-15); 30.81 (C-7); 28.23 (C-23); 27.52 (C-21); 26.78 (C-2); 23.88 (C-11); 23.24 (C-27); 22.80 (C-16); 21.16 (C-30); 17.94 (C-6); 16.97 (C-29); 16.90 (C-24); 16.03 (C-25); 15.08 (C-26). The spectral data were compatible with those of ursolic acid (Mahato and Kandu 1994; Silva et al. 2008; Huang et al. 2012).

Ursolic acid is a naturally occurring ursane-type pentacyclic triterpene widely distributed in dietary and medicinal herbs (Sheng and Sun 2011; Huang et al. 2012). It shows a wide spectrum of biological activities, including antibacterial, antiviral, antitumor, anti-mutagenic, cardiotonic, antidysrhythmic, hepatoprotective, antioxidative and immunomodulatory (Somava et al. 2004; Ma et al. 2005; Saravanan et al. 2006; Fonranay et al. 2008; Ramachandran and Prasad 2008; Jang et al. 2009). Extracts and fractions of *S. buxifolia* possesses several properties, such as, antioxidant, antiviral, antimicrobial and cardiotonic (Da Silva et al. 2012; Boligon et al. 2009b, 2012a, 2012b, 2013) which may be related to the presence of ursolic acid described herein.

Dichloromethane fraction of *S. buxifolia* was analysed by HPLC (Figure 2, Table 1). The fraction contains other minor compounds in addition to ursolic acid (retention time-t_R =

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12.56 min; peak 1; 19.74%), sitosterol-3-*O*- β-D-glucoside (t_R = 18.73 min; peak 2; 13.08%) and β-sitosterol (t_R = 26.18 min; peak 3; 25.39%). These compounds are found widely in the plant kingdom and presenting several pharmacological properties (Frohlich et al. 2013; Ku and Lin 2013), confirming that *S. buxifolia* is a promising species.

CONCLUSION

The ursolic acid and sitosterol-3-*O*- β-D-glucoside compounds were first described for *S. buxifolia*. Quantification by HPLC showed that β-sitosterol is the major component, followed by ursolic acid and sitosterol-3-*O*-β-D-glucoside, respectively, in the dichloromethane fraction of this species. However, the chemical potential of *S. buxifolia* deserves to be further investigated and their effectiveness biological evaluated.

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RESUMO

A família Rhamnaceae inclui 58 gêneros e aproximadamente 900 espécies que ocorrem em áreas tropicais e subtropicais de todo o mundo. Este trabalho, descreve a investigação fitoquímica da fração diclorometano das cascas do caule de *S. buxifolia* (coronilha), o que conduziu ao isolamento de três compostos triterpênicos, β-sitosterol (1), sitosterol-3-O-β-D-glicosídeo (2) e ácido ursólico (3). As estruturas dos compostos isolados foram elucidadas por métodos espectroscópicos (RMN), bem como em comparação com dados da literatura. β-sitosterol (25,39%), o sitosterol-3-O-β-D-glicosídeo (13,08%) e ácido ursólico (19,74%) foram quantificados na fração de diclorometano por meio de cromatografia líquida de alta eficiência (CLAE), sendo o composto principal nesta fracção do β-sitosterol.

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Figure 1 - Chemical structures of terpenoids isolated from *Scutia buxifolia*. β-sitosterol (**1**), sitosterol-3-*O*- β-D-glucoside (**2**) and ursolic acid (**3**).

Figure 2 - High performance liquid chromatography profile of *S. buxifolia*. Ursolic acid (peak 1), sitosterol-3-*O*- β-D-glucoside (peak 2) and β-sitosterol (peak 3).

Table 1 – Quantification of steroids in dichloromethane fraction of *S. buxifolia* stem bark. LOD and LOQ variations for compounds.

Compounds	*S. buxifolia*		LOD	LOQ
	mg/g of fraction	%	µg/mL	µg/mL
Ursolic acid	197.49 ± 0.012	19.74	0.023	0.075
Sitosterol-3-*O*- β-D-glucoside*	130.86 ± 0.09	13.08	0.009	0.029
β-sitosterol	253.94 ± 0.017	25.39	0.014	0.047

*Quantified was β-sitosterol. LOD limit of detection (µg/ml), LOQ limit of quantification (µg/ml).
Results are expressed as mean ± standard deviations (SD) of three determinations.

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4.6 Capítulo 6

Boligon, A.A.; Freitas, R.B.; Brum, T.F.; Waczuk, E.P.; Klimaczewski, C.V.; Ávila, D.S.; Athayde, M.L.; Bauermann, L.F. Antiulcerogenic activity of *Scutia buxifolia* on gastric ulcers induced by ethanol in rats. Acta Pharmaceutica Sinica B

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Short title: *S. buxifolia* protection on gastric ulcer

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Antiulcerogenic activity of *Scutia buxifolia* on gastric ulcers induced by ethanol in rats

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ABSTRACT

Gastric ulcers affect many people around the world and their development is a result of the imbalance between aggressive and protective factors in the gastric mucosa. In this study, we evaluated the crude extract of *Scutia buxifolia* (ceSb); this plant, more commonly known as coronilha, has attracted the interest of the scientific community due to its pharmacological properties and its potential therapeutic applications. Here, we tested the preventive effects of the ceSb against gastric ulcer induced by 70% ethanol in male Wistar rats. In addition, we aimed to clarify the composition of ceSb by High-performance liquid chromatography (HPLC). *S. buxifolia* extract (100, 200 and 400 mg/Kg body weight) attenuated oxidative and histopathological features induced by EtOH. Moreover, all evaluated doses of ceSb caused a significant ($p < 0.001$ and $p < 0.0001$) and dose dependent increase in NPSH levels, CAT and SOD activities. Furthermore, the administration of ceSb reversed the increase in lipid peroxidation produced by ethanol. The protective effect of the extract could be attributed to antioxidant compounds present in the ceSb, such as flavonoids and phenolic acids, which were quantified by HPLC. Thus, an antioxidant effect of the extract leads to a protection on gastric tissue. These results indicate that *S. buxifolia* could have a beneficial role against EtOH toxicity by preventing oxidative stress and gastric tissue injury.

Keywords: *Scutia buxifolia*; antioxidant, gastric ulcer, HPLC.

1. Introduction

Gastric ulcer is one of the major gastrointestinal disorders, which occurs due to an imbalance between the offensive (gastric acid secretion) and defensive (gastric mucosal integrity) factors.[1,2] The incidence of peptic ulcer is increased due to stress, smoking, alcohol, *Helicobacter pylori* and ingestion of non-steroidal anti-inflammatory drugs (NSAID).[3-5] It has been suggested that reactive oxygen species (ROS), primarily super-oxide anions, hydroxyl radicals, and lipid peroxides, are the harmful species known to cause the gastric ulcer development.[6] To scavenge ROS, gastric cell have several enzymatic and non-enzymatic antioxidants including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), endogenous glutathione (GSH), and sulfhydryl groups (NPSH), but excessive generation of ROS enhance lipid peroxidation and depletes these antioxidants enzymes. [7-9]

There are many different experimental models of gastric ulcer induction, including ethanol and acetic acid.[2] Using such animal models, researchers simulate conditions to which humans may be exposed and, as a result, develop gastric ulcers. Ethanol is known as a cause of gastric damage by altering protective factors, including decreasing mucus production and blood circulation within the mucosa.[4,10] In addition, the gastric damage caused by ethanol may be due to the generation of reactive species, decreased cell proliferation, and an exacerbated inflammatory response. [10-12]

The prevention or cure of peptic ulcers is one of the most important challenges confronting medicine nowadays, as it is certainly a major human illness affecting nearly 8 to 10 % of the global population, and of these 5% suffer from gastric ulcers.[13] Gastric ulcer therapy faces nowadays a major drawback because most of the drugs currently available in the market show limited efficacy against gastric diseases and are often associated with severe side effects. [14,15]

Controlling the formation of reactive species and secretion of gastric acid are essential for the treatment of these pathologies. In this context, medicinal plants containing a wide variety of antioxidants such us phenolic acids, flavonoid, coumarins, tannins and terpenoids compounds, are some of the most attractive sources of new drugs and have been shown to produce promising results in the treatment of gastric ulcers.[16-19]

Scutia buxifolia Reissek (Rhamnaceae), popularly known in Brazil as "coronilha", is native tree from South America, with a dispersion area that comprises Rio Grande do Sul State in Brazil, and the countries Argentina and Uruguay. In these regions, an aqueous infusion prepared with stem bark of *S. buxifolia* has been described and widely used in folk

medicine for cardiotonic, diuretic and antihypertensive properties.[20,21] Phytochemical screening of *S. buxifolia* fractions revealed the presence of cyclopeptide alkaloids, steroids, polyphenols and flavonoids.[22-25] Among the studies that were conducted, alkaloids isolated from *S. buxifolia* displayed *in vitro* antimicrobial activity.[22,26] Cytotoxicity effects of extracts from leaves, twigs and stem bark of the plant was evaluated by the *Artemia salina* assay, as well as the antimicrobial, antimycobacterial and antiviral activities.[23,27,28] Furthermore, Freitas et al.[29] showed that the lyophilized aqueous extract of the stem bark of *S. buxifolia* not cause hepatotoxicity. Extracts from the leaves and stem bark of *S. buxifolia* were effective inhibitors of TBARS production and also presented DPPH scavenger activity, while polyphenols and flavonoids were associated with this properties indicating that this plant have promising compounds to be tested as potential drugs for the treatment of diseases resulting from oxidative stress.[25]

The aim of the present study was to evaluate the protective effect of *S. buxifolia* crude extract against toxicity of EtOH on gastric mucosal by evaluating oxidative stress markers, antioxidant defense along with morphological and histopathological damage. In order to clarify the properties of the crude extract of *S. buxifolia* (ceSb), we also evaluated the extract composition by High-performance liquid chromatography (HPLC/DAD).

2. Experimental

2.1. Chemicals, apparatus and general procedures

Methanol, ethanol, acetic acid, gallic acid, chlorogenic acid and caffeic acid were purchased from Merck (Darmstadt, Germany). Quercetin, rutin, kaempferol and omeprazole were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Milli-Q ultra-purified water was used in preparing the samples. 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.

2.2.Plant collection and extract preparation

Stem bark of *Scutia buxifolia* were collected on October of 2007 in the first district of the council of Dom Pedrito, in the Rio Grande do Sul State, Brazil (coordinates 30º59'09" S and 54º27'44" W). Voucher specimen was archived in the herbarium of Department of Biology at Federal University of Santa Maria, register number SMBD 10919. The stem

bark were dried at room temperature and powdered in a knife mill (0.86 μm), resulting in a mass of 651.52 grams of plant material, which was submitted to maceration at room temperature with ethanol 70% for a week with daily shake. After filtration, the extract was evaporated under reduced pressure to remove the ethanol. Then, the extract was stored and subjected to a slow evaporation of the water fraction of the solvent in an oven, for future use of the remaining solids (ceSb).

2.3. Determination of total phenolics contents

The determination of total phenolic contents in ceSb was determined by the Folin-Ciocalteu method with slightly modifications.[30] The samples were read at 730 nm in spectrophotometer. Gallic acid in the range of 0.005 - 0.030 mg/mL was used as a standard phenol, giving the calibration equation: $Y = 11.969x - 0.0454$ ($r = 0.9987$). Test was carried out in triplicate and the result was expressed in milligrams equivalents of gallic acid (GAE) per gram of crude extract.

2.4. Determination of total flavonoids content

The flavonoid content in ceSb was determined based on the formation of flavonoid-aluminium complex.[31] One milliliter of sample was mixed with 1mL of 2% aluminium chloride solution. After incubation for 15 min at room temperature, the absorbance of the reaction mixture was measured at 420 nm. A standard curve was first plotted using quercetin (0.012 - 0.200 mg/mL) as a standard, giving the calibration equation: $Y = 0.0045x - 0.014$ ($r = 0.9992$). The amount of flavonoids was expressed as quercetin mg/g dry crude extract and all tests were carried out in triplicate.

2.5. Determination of total tannins content

The tannins content in ceSb was performed using the method described by Morrison et al.[32] Samples in concentrations of 0.25 mg/mL, 5mL of solution A (1 g vanillin in 100 mL of methanol) and solution B (8 mL HCl in 100 mL of methanol) were used to experiment. The samples were read at 500 nm in spectrophotometer. The total tannins content was expressed in milligrams equivalents of catechin per gram of each fraction. The equation obtained for the calibration curve of catechin in the range of 0.001 - 0.025 mg/mL was $Y = 0.00015x + 0.005$ ($r = 0.9979$). The experiments were conducted in triplicate.

2.6. *Determination of total alkaloids content*

The alkaloids content in ceSb (20 mg/mL) was determined using the method described by Sreevidja and Mehrotra[33], where Dragendorff's reagent precipitates alkaloids in plants materials. It is based on the formation of yellow bismuth complex in nitric acid medium with thiourea. Mixture of thiourea and nitric acid were used as a blank. The samples were read at 435 nm in spectrophotometer. The equation obtained for the calibration curve of bismuth nitrate pentahydrate solution in the range of 0.01 - 0.09 mg/mL was Y = 2.2783x + 0.0361 (r = 0.9997). The experiments were conducted in triplicate.

2.7. *HPLC/DAD analyses of Scutia buxifolia extract composition*

Reverse phase chromatographic analyses were carried out under gradient conditions using C_{18} column (4.6 mm x 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 Amaral et al.[12] with slight modifications. The ceSb and mobile phase were filtered through 0.45 µm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use, the extract was analyzed dissolved in methanol at a concentration of 8 mg/mL. Stock solutions of standards references were prepared in methanol at a concentration range of 0.031 – 0.250 mg/mL for kaempferol, quercetin and rutin, and 0.006 – 0.250 mg/mL for gallic, chlorogenic and caffeic acids. Quantification was carried out by integration of the peaks using the external standard method, at 254 nm for gallic acid, 325 nm for caffeic and chlorogenic acids, and 365 nm for quercetin, rutin and kaempferol. The flow rate was 0.8 mL/min and the injection volume was 40 µL. The chromatography peaks were confirmed by comparing their retention time and Diode-Array-UV spectra with those of the reference standards. All chromatography operations were carried out at ambient temperature and in triplicate. The respective standard solutions calibrations curves were: Y = 53985x + 1020.6 (r = 0.9859) for gallic acid; Y = 52548x + 1082.3 (r = 0.9850) for chlorogenic acid; Y = 87846x + 1093.0 (r = 0.9938) for caffeic acid; Y = 103861x – 1235.8 (r = 0.9921) for rutin; Y = 150833x – 4741.7 (r =0.9949) for quercetin and Y = 130745x – 1897.9 (r = 0.9928) for kaempferol.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Sabir et al.[34] 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.

2.8. Animals

Male Wistar rats (200-250 g), obtained from the General Animal House of the Federal University of Santa Maria, were kept on a separate animal room, in a 12-hour light/dark cycle at room temperature and were fasted 16 hours with free access to water before the experiment. All animals were used according to the guidelines of the Committee on Care and Use of Experimental Animal Resources from Federal University of Santa Maria, Brazil (013/2012).

2.8.1 The experimental protocol and ethanol-induced gastric lesions method

The animals were randomly divided into ten groups (A-J), with six animals each. Five groups of animals received distillated water as vehicle (0.5 mL/100 g body weight) and the other five groups received a 70% aqueous solution (v/v) of EtOH by oral gavage (0.5 mL/100 g body weight). After 1 hour of EtOH or vehicle administration, the animals received ceSb intragastrically at doses of 0 g/kg (vehicle), 100 mg/kg, 200 mg/Kg and 400 mg/kg body weight, the group positive control received omeprazole 30 mg/Kg body weight. The chosen model of gastric damage induced by EtOH has already been described.[35] The *Scutia buxifolia* extract doses used were adapted from a previous study.[36]

The treatment groups and experimental protocol are detailed below:

Group A - Control group: Received only distilled water (0.5 mL/100 g body weight).

Group B - EtOH group: Received only EtOH 70% (0.5 mL/100 g body weight).

Group C – Omep. control group: Received distilled water (0.5 mL/100 g body weight), 1 h after omeprazole (30 mg/kg body weight).

Group D – Omep. + EtOH group: Received EtOH (0.5 mL/100 g body weight), 1h after omeprazole (30 mg/kg body weight).

Group E – 100 ceSb control group: Received distilled water (0.5 mL/100 g body weight), 1h after ceSb (100 mg/kg body weight).

Group F – 100 ceSb + EtOH group: Received EtOH (0.5 mL/100 g body weight), 1 h after ceSb (100 mg/kg body weight).

Group G – 200 ceSb control group: Received distilled water (0.5 mL/Kg body weight), 1 h after ceSb at (200 mg/kg body weight).

Group H - 200 ceSb + EtOH group: Received EtOH (0.5 mL/100 g body weight), 1 h after ceSb (200 mg/kg body weight).

Group I – 400 ceSb control group: Received distilled water (0.5 mL/100 g body weight), 1 h after ceSb (400 mg/kg body weight).

Group J - 400 ceSb + EtOH group: Received EtOH (0.5 mL/100 g body weight), 1 h after ceSb (400 mg/kg body weight).

One hour after ceSb or omeprazole administration, the animals were euthanized by deep anesthesia induced by thiopental at 100 mg/kg body weight, administered intraperitoneally. The stomachs were immediately removed, washed with saline solution (NaCl 0.9%) and the glandular portion was separated for macroscopic evaluation (gastric lesion index). Afterwards, a portion of gastric tissue was collected for histopathological analysis and the remained tissue was homogenized in 9 volumes of potassium phosphate buffer 0.1 M, pH 7.4 using a Polytron mixer (Kinematica AG, Switzerland). The homogenate was centrifuged at 3000g at 4°C for 10 min to yield a low-speed supernatant that was used to measure the biochemical parameters.

2.8.2. Macroscopic evaluation

The stomachs were opened along the greater curvature and washed with 0.9% NaCl and examined by a blinded pathologist for macroscopic lesions in the glandular part under a dissecting microscope. The severity of macroscopic lesions was estimated using an index as previously reported,[37,38] where normal stomachs received score 0 and discolored stomachs received score 1. For hemorrhage, petechiae, edema and mucus, stomachs with no injuries received score 0, stomachs with minor injury received score +1, those with moderate and severe injuries were given a score of +2 and +3, respectively.[38]

2.8.3. Histopathological examinations

For microscopic analysis, a portion of stomach from each experimental group was fixed in 10% formalin and immersed in paraffin. Sections of 5 mm were obtained with a standard microtome and were stained with hematoxylin and eosin.[39] The sections were examined by a pathologist without knowledge of the experimental groups for presence of any negative features, such as edema, erosion, ulceration and necrosis. The severity of histopathological changes was quantified according to an arbitrary scale as described before, with some modifications.[40] Gastric tissue with no negative features was given a

score of 0. Gastric tissue with mild histopathological damage was given a score of +1. Those with moderate and severe negative features were given a score of +2 and +3, respectively. Results were expressed as a histopathological score.

2.8.4. Thiobarbituric acid reactive substances (TBARS)

Stomach tissue lipoperoxidation (LPO) estimation was performed using the TBARS assay as previously described, where the colorimetric reaction of the LPO product malondialdehyde (MDA) with thiobarbituric acid (TBA) is quantified. The concentration of thiobarbituric acid reactive substances was measured at 532 nm using a standard curve of malondialdehyde, and the results were expressed as nmol MDA/mg protein.[41]

2.8.5. Non-Enzimatic Antioxidant Defense

Tissue sulfhydryl groups (NPSH) were quantified after mixing the homogenate with 10% trichloroacetic acid (1:1, v/v), followed by centrifugation, as described by Ellman.[42] Cysteine was used for preparation of a standard curve.

2.8.6. Catalase activity (CAT) assay

Catalase (CAT) activity was determined by measuring the decrease in hydrogen peroxide (H_2O_2) absorption at 32 °C. The method is based on the consumption of H_2O_2 by CAT and loss of absorbance at 240 nm.[43]

2.8.7. Superoxide dismutase activity (SOD) assay

Superoxide dismutase is an enzyme which catalyzes the dismutation of superoxide radical to form hydrogen peroxide and oxygen. The assay for determination of indirect SOD-activity is based in the inhibition of reaction between superoxide radical with adrenaline.[44]

2.8.8. Protein quantification

The amounts of LPO were normalized to the amount of stomach protein content. The quantification of the protein was performed following Lowry method, where the maximum absorbance for the solution of Folin-Ciocalteu due to its interaction to bovine serum albumin (BSA) protein, occurs at 625 nm.[45]

2.9. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical comparisons were performed by one-way analysis of variance followed Tukey's *post-hoc* test. The data were analyzed by using Statistical Package for the Social Sciences (SPSS, version 18.0). A *p*-value less than 0.001 and 0.0001 were considered to be significant different.

3. Results

3.1. Total phenols, flavonoids, tannins and alkaloids contents

The quantitative phytochemical results showed the presence of phenolics (141.09 ± 0.71 mg GAE/g of extract), flavonoids (100.37 ± 0.56 mg quercetin/g of extract), tannins (66.67 ± 0.17 mg catechin/g extract) and alkaloids (1.59 ± 0.08 mg alkaloids/g extract) (Table 1). The large amount of total phenolics and flavonoids contents detected in ceSb can be attributed to the antioxidant potential formerly described for this species (Boligon et al., 2009).

3.2. HPLC/DAD analysis

HPLC fingerprinting of ceSb revealed the presence of the gallic acid (retention time-t_R 12.4 min; peak 1; 4.13%), chlorogenic acid (t_R = 23.1 min; peak 2; 1.92%), caffeic acid (t_R = 28.6 min; peak 3; 7.75%), rutin (t_R = 37.5 min; peak 4; 0.89%), quercetin (t_R = 47.6 min; peak 5; 9.03%) and kaempferol (t_R = 54.9 min; peak 6; 0.54%) (Figure 1, Table 1). The HPLC analysis revealed that flavonoids (quercetin, rutin and kaempferol) and phenolics acids (gallic, chlorogenic, caffeic acids) are the major components of the extract. All found substances are well-know antioxidants.

3.3. Macroscopic analysis

The assay revealed a significant effect of EtOH group on gastric tissue ($p< 0.001$; Figure 2 and 3). The animals that received 70% ethanol developed a consistent macroscopic damage which were evidenced by presence of ulceration hemorrhagic (Figure 2B), it is attenuated by the administration of omeprazole (30 mg/kg b.wt.) with a few fields of hyperemia (Figure 2D). In addition, the ceSb did not show any macroscopic toxicity, preserving the morphological integrity of the gastric mucosa (Figure 2E, G and I) when compared to non-treated control group (Figure 2 A). Furthermore, the animals treated with ceSb at 200 and 400 mg/kg b.wt. weight was able to reversed the damage

induced by ethanol (Figure 2 H and J, respectively), with very similar aspect to the control group (Figure 2 A).

Post-hoc comparisons demonstrated that 1 hour exposure to EtOH is able to cause injury to gastric tissue characterized by macroscopic features such as discoloration ($p < 0.001$; Figure 3A), petechiaes ($p < 0.001$; Figure 3B), edema ($p < 0.001$; Figure 3C), hemorrhage ($p < 0.001$; Figure 3D) and mucus loss ($p < 0.001$; Figure 3E). Although ceSb at dose of 200 and 400 mg/kg b.wt. totally reversed all macroscopic lesions induced by EtOH ($p < 0.001$; Figures 2 and 3), the dose of 100 mg/kg b.wt. completely restored just edema and mucus loss occurrence ($p < 0.001$; Figure 3C and E). Moreover, ceSb at dose of 100 mg/kg b.wt. partially ameliorated color, petec, and hemorrhage ($p < 0.001$; Figure 3A, B and D). The omeprazole completely reversed the color and edema induced by EtOH ($p < 0.001$; Figure 3A and C).

3.4. Histopathology

Acute exposure of rats to EtOH caused mucosal necrosis, edema and congestion along with inflammatory process characterized by neutrofils infiltration, as demonstrated by the histopathological score ($p < 0.001$; Figure 4B and L). These results confirm that EtOH causes gastric damage also at a microscopic level. Post-treatment with *S. buxifolia* extract (100, 200 and 400 mg/kg b.wt.) ameliorated injuries caused by EtOH ($p < 0.0001$; Figure 4F, H and J, respectively, and Figure 4L) and did not induce any damage to gastric tissue per se (Figure 4E, G and I).

3.5. Effect on lipid peroxidation

The EtOH group showed significant change on oxidative markers with an increase on lipid peroxidation when compared to control group (MDA = 4.02 ± 0.42 and 0.94 ± 0.3 nmol/mg protein, respectively, $p < 0.0001$). However, the animals which received omeprazole at 30 mg/Kg b.wt., ceSb at 100, 200 and 400 mg/kg b.wt. completely attenuated the damage induced by ethanol (MDA = 1.83 ± 0.36; 2.25 ± 0.47; 1.14 ± 0.26 and 1.01 ± 0.09 nmol/mg protein, respectively). Furthermore, the ceSb, at all doses tested, was able to significantly prevent the increase on lipid peroxidation in relation to respective control animals ($p < 0.0001$ and $p < 0.001$) (Figure 5A).

3.6. Effect on Tissue Sulfhydryl Groups (NPSH)

EtOH group caused a decreased NPSH content when compared to control group (25.56 ± 8.4 and 72.45 ± 9.1 nmol/mg protein; respectively, $p < 0.001$; Figure 5B). These results confirm the ability of EtOH in depleting antioxidant defenses. In addition, omeprazole (30 mg/Kg b.wt.) and ceSb extract (100, 200 and 400 mg/Kg b.wt.) restored NPSH levels (56.14 ± 7.3; 39.05 ± 9.11; 89.26 ± 10.58 and 95.87 ± 2.94 nmol/ mg protein; $p < 0.0001$). However, the level of NPSH in stomach tissue was not affected by the treatment only with ceSb at different dosages (100, 200 and 400 ceSb control group), maintaining similar levels to the respectively control group ($p < 0.0001$; Figure 5B).

3.7. Enzymatic antioxidant defense

Statistical analysis revealed a significant decrease in catalase (CAT) and superoxide dismutase (SOD) activities in gastric tissue after EtOH administration (1.65 ± 0.35 nmol/mg protein and 1.07 ± 0.20 nmol/mg protein, respectively, $p < 0.0001$), when compared to control group. In addition, the omeprazole at 30 mg/Kg b.wt. group, ceSb, at all doses tested, were able to significantly reversed, dose-dependent manner, the decrease on CAT and SOD activities induced by EtOH in relation to respective control groups animals (Figure 5C and D).

4. Discussion

Phytochemical screening of the crude extract of *S. buxifolia* stem bark (ceSb) showed the presence of acids phenolics, flavonoids, tannins and alkaloids (Table 1). In addition, HPLC analysis revealed that gallic acid, chlorogenic acid, caffeic acid, quercetin, rutin and kaempferol are the main compounds present in ceSb (Figure 1 and Table 1). These compounds scavenge the free radical and play important role in the prevention and therapy of diseases. Gallic acid, caffeic acid, rutin and quercetin are strong natural antioxidant, decrease the peroxidation and have anti-ulcerogenic, anti-mutagenic and anti-cancerogenic properties. [14,17,46,47]

Acute exposure of the gastric mucosa of rats to EtOH can result in gastric lesions similar to those occurring in gastric ulcer; hence, ethanol-induced gastric ulcers have been widely used for the evaluation of gastroprotective activity.[4,10] Accordingly, was observed that EtOH administration to rats caused macroscopic lesions to gastric tissue, such as loss of normal color and mucus along with presence of petechiae, hemorrhage and edema (Figure 2B and 3). These lesions are most likely related to mucus depletion and a constrictive effect on veins and arteries of the gastric mucosal, producing congestion,

inflammation and tissue injury.[8] The reduction of gastric mucosal blood flow can result in hemorrhage and necrosis in damaged tissue.[5,14]

In order to confirm the results of antiulcer experiment, the stomachs were also evaluated by histopathological examination (Figure 4). In histological observation, the stomach of control animals showed no damage (Figure 4A). However, rats 1 hour after of exposure to EtOH presented damage to gastric tissue at a microscopic level. Histopathological injury caused by EtOH administration is characterized by edema and congestion of mucosal, as well as, inflammatory process characterized by neutrofils infiltration (Figure 4B). However, ceSb was able to reverse the damage caused by ethanol, probably exert potent antiinflammatory effect in gastric mucosa. This activity can be confirmed by microscopic evidence obtained in our analysis, decreasing the infiltration of inflammatory cells (neutrophils) (Figure 4F, H and J) in relation to samples from stomachs of rats that received ethanol-only (Figure 4B). Furthermore, the ceSb at 200 and 400 mg/kg b.wt. was able to protect the histological structure of the gastric mucosa, preventing swelling (Figure 4H and J, respectively) and preventing the infiltration of inflammatory cells (neutrophils) at 100, 200 and 400 mg/kg b.wt. (Figure 4F, H and J, respectively).

The abnormal elevation of reactive species corresponds to one of the main aggressive mechanisms of ethanol, which can cause gastric cell damage and death.[4,12] In this study, EtOH induced depletion of non-enzymatic defenses (NPSH groups) and inhibition of the antioxidant enzyme CAT and SOD. In fact, depletion of glutathione (the major non protein thiol), CAT and SOD inhibition after EtOH exposure has already been described [5,8,47] is directly involved in increased of lipid peroxidation observed in EtOH-treated rats. Besides, lipid peroxidation in gastric tissue plays a significant role in the pathogenesis of EtOH-induced gastric lesions.[10,11,48] Previous reports confirm that EtOH increases superoxide anion and hydroxyl radical production by neutrophils and these reactive oxygen species (ROS) cause LPO in the gastric mucosa and tissue damage.[1]

Scutia buxifolia extract restored, in a dose dependent manner, the gastric mucosal damage and oxidative stress induced by EtOH administration (Figure 5A-D). The broad antioxidant properties of ceSb were demonstrated by decreased levels of MDA and increase of antioxidant defenses (NPSH, SOD and CAT). These protective effects described for the crude extract of *S. buxifolia* can be associated with the presence of phenolic acids, mainly gallic, chlorogenic and caffeic acids, besides, the flavonoids quercetin, rutin and kaempferol in ceSb. The free radical scavenging activity of the ceSb[25] might be considered as one of the possible mechanisms of its gastroprotective effect

observed. Because oxygen derived radicals and agents with antioxidant properties have been implicated in the pathogenesis of ethanol-induced gastric ulcers.[49] In agreement with our findings, high levels of flavonoids also have already been found in the ethyl acetate fraction of *S. buxifolia* by Boligon et al.[25] Similar results were also obtained in related to antioxidant enzyme activities by Alimi et al.[48] and Liu et al.[9]

Several studies have associated the protection of gastric ulcer to the presence of phenolic acid and flavonoids in plant extracts.[7,8,12,16 48] Hussain et al.[17] describe the significant gastroprotective effect of rutin by scavenging the reactive oxygen species produced by gastric damage. Furthermore, quercetin and kaempferol also showed protective effects in ethanol-induced gastric ulcer by decreasing oxidative stress and increasing antioxidant enzyme activity.[15,47] Flavonoids are antioxidant compounds that efficiently remove superoxide anion, hydroxyl, peroxyl and alcoxyl radicals,[8] while the removal of these same ROS along with peroxynitrite radicals has been described also for chlorogenic and caffeic acids.[12] Since superoxide anion and hydroxyl radical are the ROS involved in oxidative stress caused by EtOH and peroxyl and alcoxyl radicals are the major products of the LPO process,[1,14] the scavenging of these species explains the protective effects of *S. buxifolia* against gastric injury induced by EtOH. In addition, some flavonoids also interfere in inflammation process and increase mucus content in gastric mucosal, resulting in cytoprotective effects.[2,12]

In conclusion, we demonstrated that ceSb (100, 200 and 400 mg/Kg b.wt.) protect gastric mucosal against oxidative injuries caused by EtOH administration and this protection is most likely due to antioxidant properties of *S. buxifolia*. In addition, the presence of phenolic acids and flavonoides in ceSb certainly contribute to the antiulcerogenic activity described here.

Conflict of interest

The authors declare that there is no conflict of interest.

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Figure Caption

Figure 1. High performance liquid chromatography (HPLC) chromatogram of *Scutia buxifolia* crude extract. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), rutin (peak 4), quercetin (peak 5) and kaempferol (peak 6). Detection UV was at 325 nm; other chromatographic conditions are described in HPLC analysis in the Material and Methods section.

Figure 2. Demonstrative images of stomachs from all experimental groups. Observe images from control group (A), EtOH group (B), omep. control group (C), Omep. + EtOH group (D), 100 ceSb control group (E), 100ceSb + EtOH group (F), 200 ceSb control group (G), 200ceSb + EtOH group (H), 400 ceSb control group (I) and 400ceSb + EtOH group (J).

Figure 3. Color (A), petechiae (B), edema (C), hemorrhage (D) and mucus loss (E) indexes (magnification of x 10) of stomach from rats treated with EtOH and/or omeprazole or *Scutia buxifolia* extract. Data are means + SD (n = 6). *Different from all control groups (p < 0.0001). #Different from EtOH control group (p < 0.0001). [X]Different from omep. + EtOH group (p < 0.0001). ‡Different from 100ceSb + EtOH group (p < 0.0001). [δ]Different from 200ceSb + EtOH group (p < 0.001). †Different from 400ceSb + EtOH group (p < 0.0001).

Figure 4. Representative histology (magnification of x 100; A-J) and histopathological damage score of gastric tissue from animals treated with EtOH and/or *Scutia buxifolia* extracts (L). Control group (A), EtOH group (B), omep. control group (C), Omep. + EtOH group (D), 100 ceSb control group (E), 100ceSb + EtOH group (F), 200 ceSb control group (G), 200ceSb + EtOH group (H), 400 ceSb control group (I) and 400ceSb + EtOH group (J). In panel L, the score 0 indicates absence of negative features (edema, erosion, ulceration and necrosis) while score 3 indicates severe negative features. Data are means ± SD (n = 5-6). *Different from all control groups (p < 0.001). #Different from *Scutia buxifolia* 0-EtOH group (p < 0.0001). Observe the preserved architecture of mucosal cells (A). In the EtOH-treated animals (B), there is edema and congestion of mucosal, as well as, inflammatory process characterized by neutrofils infiltration. In all other groups (C-J), there is absence of any of the negative features described above.

Figure 5. Thiobarbituric acid reactive substances – TBARS (A), sulfhydryl Groups – NPSH (B), CAT activity (C) and SOD activity (D) levels of gastric tissue from rats treated with EtOH and/or omeprazole or *Scutia buxifolia* extract. Data are means ± SEM (n = 5-9). *Different from all control groups (p < 0.0001). #Different from EtOH control group (p < 0.0001). [X]Different from omep. + EtOH group (p < 0.0001). ‡Different from 100ceSb + EtOH group (p < 0.0001). [δ]Different from 200ceSb + EtOH group (p < 0.001). †Different from 400ceSb + EtOH group (p < 0.0001).

Table Caption

Table 1. Content of phenolics, flavonoids, tannins and alkaloids in crude extract of *S. buxifolia*; LOD and LOQ variations for antioxidant compounds.

Expressed as (*) gallic acid equivalent, (#) quercetin equivalent, (‡) catechin equivalent. LOD: limit of detection; LOQ: limit of quantification. Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$.

Table 1

S. buxifolia **compounds**	**Quantities** (mg/g)	(%)	**LOD** (µg/mL)	**LOQ** (µg/mL)
Total phenolics (*)	141.09 ± 0.71	-	-	-
Total flavonoids (#)	100.37 ± 0.56	-	-	-
Total tannins (‡)	66.67 ± 0.17	-	-	-
Total alkaloids	1.59 ± 0.08	-	-	-
Gallic acid	41.3 ± 0.22	4.13 a	0.017	0.056
Chlorogenic acid	19.2 ± 0.19	1.92 b	0.008	0.025
Caffeic acid	77.5 ± 0.03	7.75 c	0.023	0.075
Rutin	8.9 ± 0.34	0.89 d	0.010	0.032
Quercetin	90.3 ± 0.05	9.03 e	0.009	0.029
Kaempferol	5.4 ± 0.15	0.54 d	0.032	0.104

Expressed as (*) gallic acid equivalent, (#) quercetin equivalent, (‡) catechin equivalent. LOD: limit of detection; LOQ: limit of quantification. Results are expressed as mean ± standard deviations (SD) of three determinations. Averages followed by different letters differ by Tukey test at $p < 0.05$.

Figure 1

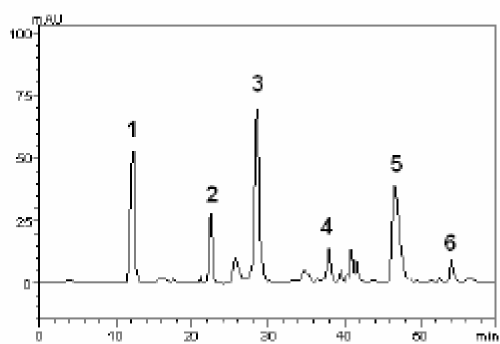


Figure 2

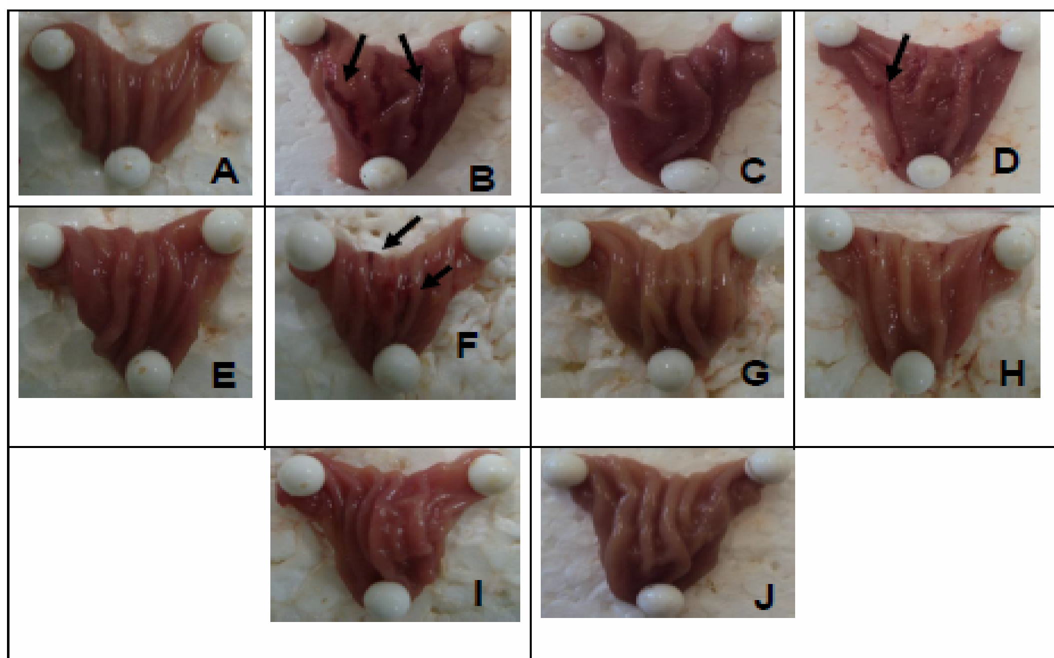


Figure 3

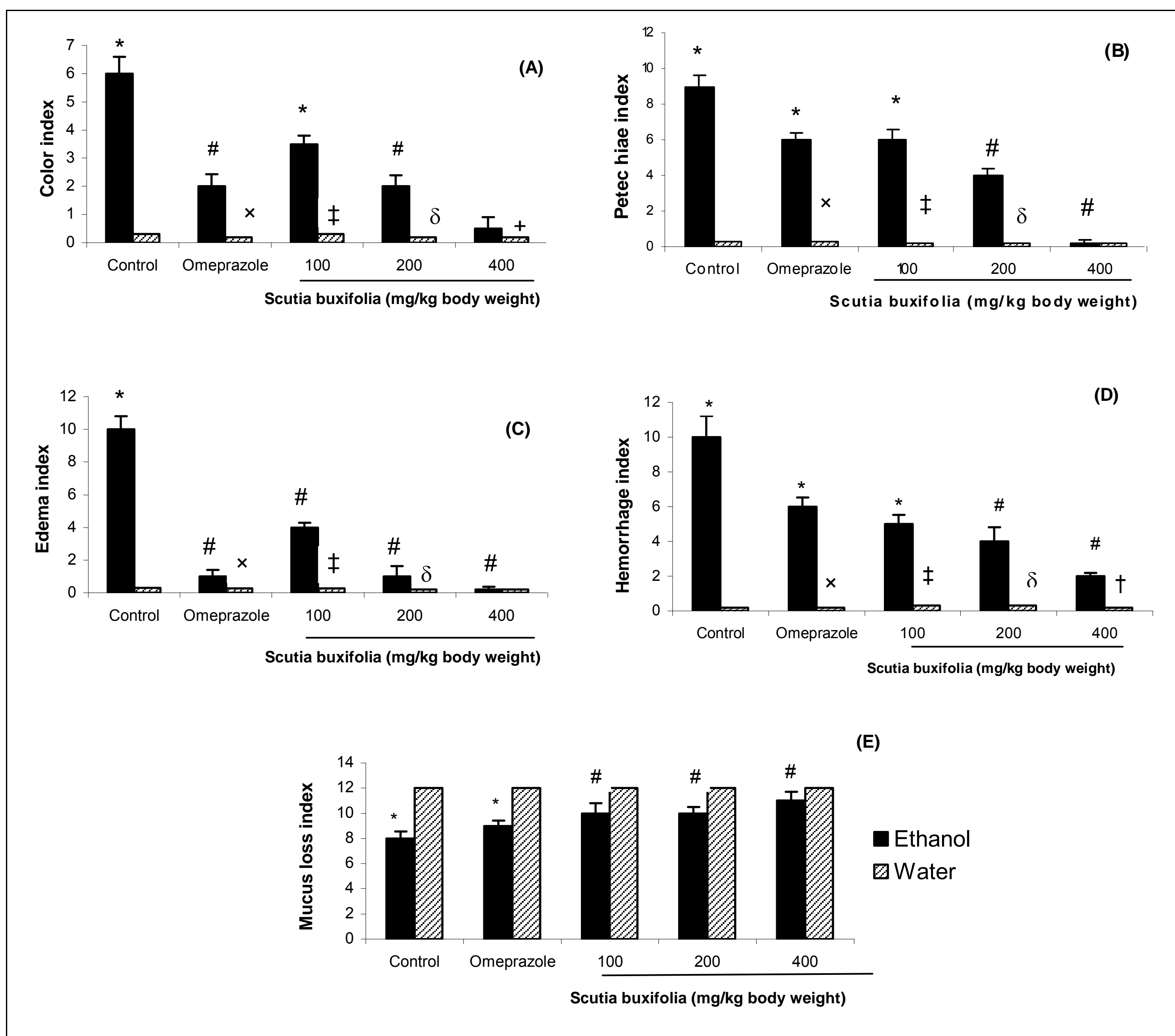


Figure 4

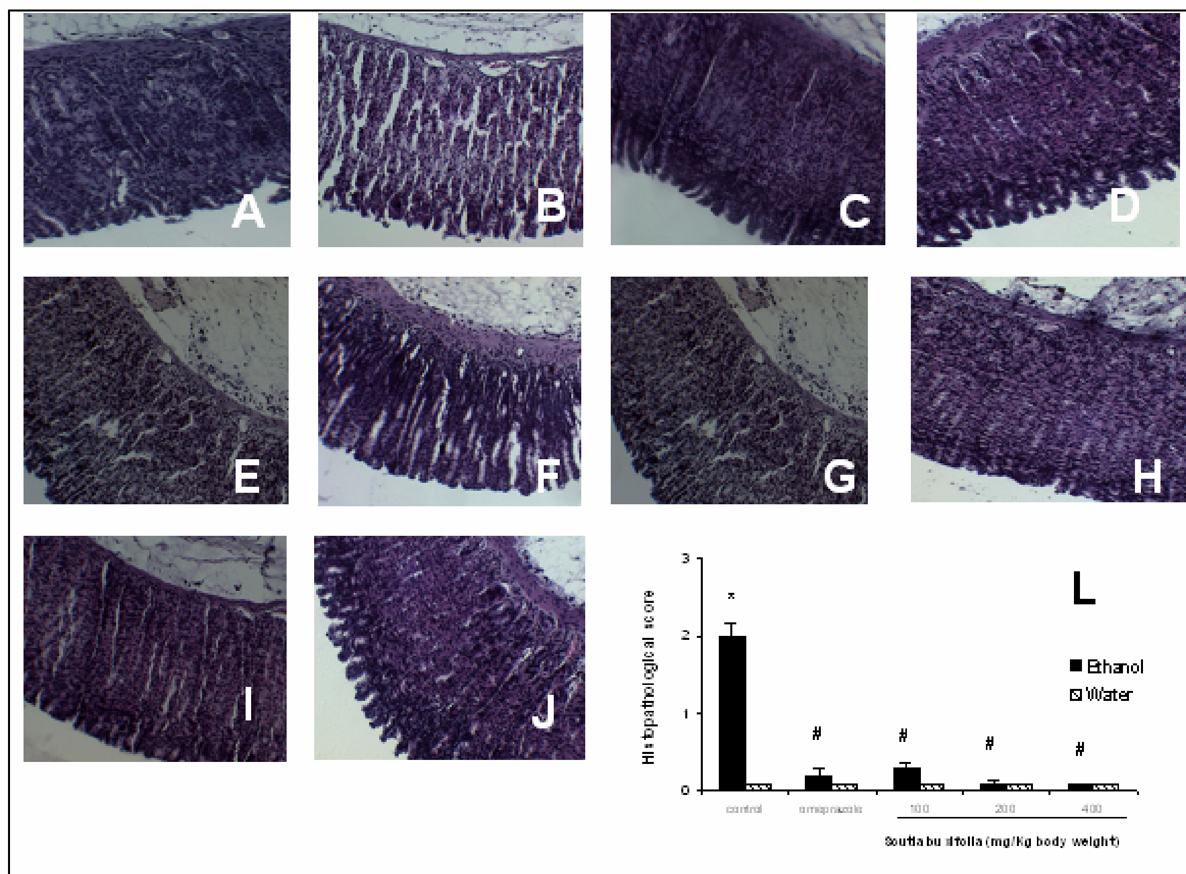
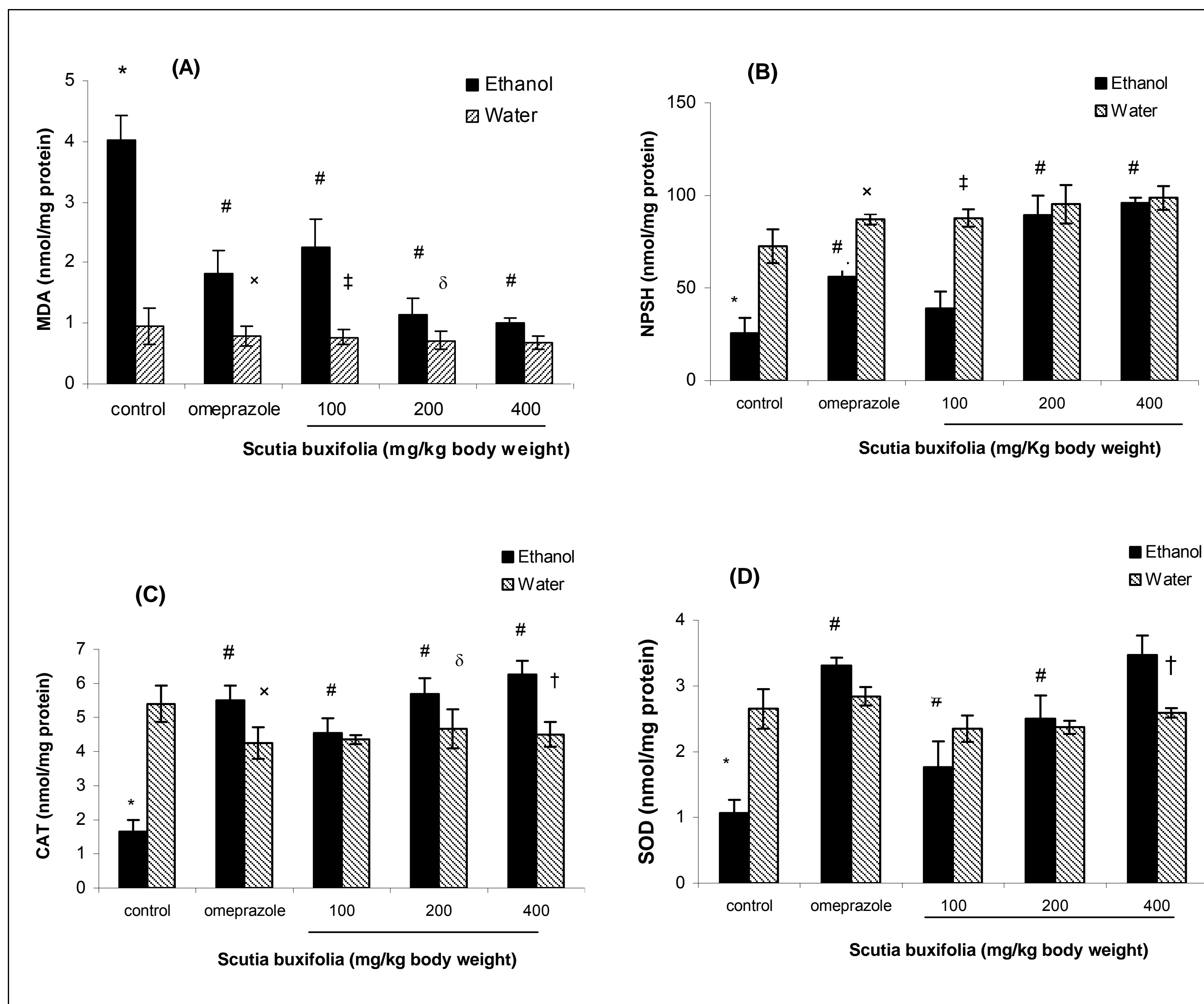


Figure 5



5 CONSIDERAÇÕES FINAIS

A utilização de plantas com fins medicinais, para tratamento, cura e prevenção de doenças, é uma das mais antigas formas de prática medicinal da humanidade. No início da década de 1990, a Organização Mundial de Saúde (OMS) divulgou que 65-80% da população dos países em desenvolvimento dependiam das plantas medicinais como única forma de acesso aos cuidados básicos de saúde (AKERELE, 1993). Apesar da grande evolução da medicina a partir da segunda metade do século XX, ainda existem obstáculos básicos na sua utilização pelas populações carentes, que vão desde o acesso aos centros de atendimento hospitalares, à obtenção de exames e medicamentos. Estes motivos, associados com a fácil obtenção e a grande tradição do uso de plantas medicinais, contribuem para sua utilização pelas populações dos países em desenvolvimento (VEIGA JUNIOR et al., 2005)

No Brasil, as plantas da flora nativa são consumidas com pouca ou nenhuma comprovação de suas propriedades farmacológicas, as quais são propagadas por usuários ou comerciantes. No entanto, o uso popular não é suficiente para validar a utilização das plantas medicinais como medicamentos eficazes e seguros. Nesse sentido, essas espécies vegetais não se diferenciam de qualquer outro xenobiótico sintético, e a preconização ou a autorização oficial do seu uso medicamentoso deve ser fundamentada em evidências experimentais comprobatórias (BRASIL, 1995; SIMÕES et al., 2010).

O conhecimento do uso popular das plantas medicinais contribui de forma relevante para a divulgação das virtudes terapêuticas destes vegetais. A cultura das práticas alternativas faz despertar o interesse de pesquisadores que buscam novas biomoléculas (NODARI, 2007). As plantas medicinais constituem uma fonte importante de pesquisa para a descoberta de novos agentes terapêuticos, uma vez que são produtoras de uma grande quantidade de metabólitos secundários com estruturas químicas diversificadas. Estes metabólitos estão sendo cada vez mais conhecidos através do isolamento, elucidação estrutural, testes farmacológicos e ensaios de toxicidade, fatos que tem impulsionado as indústrias farmacêuticas na busca por novos fármacos (RATES, 2001; SIMÕES et al., 2004).

Scutia buxifolia Reissek (Rhamnaceae) é uma espécie pertencente à flora gaúcha, sendo popularmente utilizada devido a suas propriedades diuréticas, hipotensoras e cardiotônicas (WASICKY, WASICKY, 1964). Alguns estudos anteriores descrevem que *S. buxifolia* contém três classes principais de compostos biologicamente ativos, ou seja, flavonoides (quercetina, quercitrina, isoquercitrina e rutina), alcaloides (scutianinas A-M) e triterpenos (β-sitosterol, estigmasterol e lupeol) (BOLIGON et al., 2009, 2010; MALDANER et al., 2011; MOREL et al., 1979, 1998). Entre as atividades observadas para a espécie, alcaloides isolados a partir *S. buxifolia* apresentam atividade antimicrobiana (MOREL et al., 2005). Além disso, os mesmos compostos demostraram efeitos antinociceptivos e analgésicos (TREVISAN et al., 2009).

Extratos e frações de *S. buxifolia* apresentaram excelentes propriedades antioxidantes (BOLIGON et al., 2009). Pesquisas têm demostrado que a presença de compostos com atividade antioxidante em espécies vegetais está diretamente relacionada a várias atividades biológicas, como, atividade antimicrobiana, antiviral, antitumoral, anti-inflamatória e antiúlcera (BHOURI et al., 2010; HARBONE, WILLIANS, 2000; SHAHAT, 2002).

Propriedades antimicrobianas do extrato bruto (EB) e das frações diclorometano (DCM), acetato de etila (AcOEt) e *n*-butanol (BUT) das cascas do caule e folhas de *S. buxifolia* foram invetigadas. Em relação a casca do caule, a fração BUT apresentou forte atividade antimicrobiana frente a *Klebsiella pneumoniae* e *Enterococcus faecalis* (Concentração Inibitória Mínima - CIM = 62,5 μg/mL). Considerando-se as folhas, as frações mais ativas foram AcOEt e BUT, sendo eficazes contra *Micrococcus sp.*, *Klebsiella pneumoniae* e *Enterococcus faecalis* (CIM = 62,5 μg/mL). As frações AcOEt e BUT contém os compostos mais polares, tais como, ácidos fenólicos, flavonoides e taninos, a presença destes compostos pode justificar a boa atividade encontrada para estas frações, uma vez que podem atuar como agentes antibacterianos, por meio de, pelo menos, três mecanismos: inibição da síntese de ácido nucleico, rompimento de membranas bacterianas e inibição do metabolismo energético (COWAN, 1999; OLIVEIRA et al., 2007). Além disso, as frações AcOEt e BUT também mostraram boas atividades frente aos fungos *Candida tropicalis*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida neoformans*, *Malassezia pachydermatis*, *Aspergillus flavus*, *Aspergillus fumigatus* e

Fusarium solani (MIC = 125 a 1000 μg/mL). Compostos fenólicos, especialmente flavonoides, possuem a capacidade de inibir a germinação dos esporos e têm, por conseguinte, atividade antifúngica (HARBORNE, WILLIAMS, 2000). A presença de quatro flavonoides (quercetina, quercitrina, isoquercitrina e rutina), isolados por Boligon e colaboradores (2009) da espécie *S. buxifolia*, pode estar diretamente relacionada a atividade antifúngica encontrada.

Vírus Herpes Simplex tipo 1 (HSV-1), membro da família Herpesviridae, é um vírus de DNA envelopado, de cadeia dupla que causa lesões vesiculares orais e genitais, conjuntivite e encefalite (KHAN et al., 2005; PELLET, ROIZMAN, 2007). O Aciclovir e outros análogos de nucleosídeos são fármacos comumente usados para o tratamento de infecções causadas pelo HSV, entretanto, o aparecimento de estirpes de vírus resistentes a esses fármacos tem aumentado principalmente entre os indivíduos imunocomprometidos (GILBERT et al., 2002; KHAN et al., 2005). Portanto, o desenvolvimento de novos agentes anti-HSV que complementem a terapia com farmacos atualmente disponíveis ainda são necessários. Neste sentido, um ensaio antiviral (citotoxicidade e atividade antiviral) frente Herpes Vírus Simples Tipo 1 (HSV-1) também foi realizado utilizando o EB, DCM, AcEOt, BUT das cascas do caule e folhas de *S. buxifolia.* As frações BUT e AcOEt das cascas do caule a e fração AcOEt das folhas apresentam os melhores potenciais de atividade antiviral (Índice de Seletividade – SI = 25.78, 15.97 e 14.13, respectivamente). O índice de seletividade (IS), parâmetro importante para avaliar a atividade antiviral, é calculado a partir da CC_{50}/CI_{50}, onde, a concentração citotóxica 50% (CC_{50}) é definida como a concentração da amostra, que reduz a viabilidade celular em 50% quando comparados aos controles não tratados. A concentração inibitória 50% (CI_{50}) é definida como a concentração que inibe 50% da replicação viral quando comparados aos controles de vírus (FREITAS et al., 2009).

Devido a promissora atividade encontrada, a fração BUT das cascas do caule e a fração AcOEt das folhas de *S. buxifolia* foram submetidas ao fracionamento cromatográfico, resultando em quatro subfrações das cascas do caule (BUT I-IV) e quatro subfrações das folhas (AcOEt I-IV) as quais também foram testadas frente ao HVS-1. As subfrações AcOEt II (CI_{50} = 6,50 ± 1,94 μg/mL; IS = 12,59), AcOEt III (CI_{50} = 11,27 ± 2,97 μg/mL; IS = 10,06) e BUT II (CI_{50} = 1,12 ± 0,91 μg/mL; IS = 12,34) foram as mais ativas. Estas subfrações possuem flavonoides (quercetina,

quercitrina, isoquercitrina e rutina) e ácidos fenólicos (ácido gálico, ácido cafeico e ácido clorogênico) em sua composição. A presença destes compostos na espécie *S. buxifolia* foi descrita anteriormente (BOLIGON et al., 2009; FREITAS et al., 2012, 2013).

Atividade anti-HSV-1 de compostos fenólicos tais como, ácido cafeico, ácido clorogênico e flavonoides é bem descrita na literatura (CHIANG et al., 2002, 2003; LUCKEMEYER et al., 2012; SUÁREZ et al., 2010). As ações destes polifenóis são parcialmente atribuídas a sua capacidade de formar complexo com proteínas (HASLAM, 1996). Tal conceito foi confirmado por Gescher et al. (2011) através da formação de complexo do extrato aquoso de *Rhododendron ferrugineum* com a proteína gD do envelope do HSV. Outros estudos demostram que os polifenóis se ligam ao vírus ou ao receptor da célula evitando a penetração viral (VANDEN BERGHE et al., 1986). Além disso, Uozaki e colaboradores (2007) descreveram que o ácido gálico apresentou eficaz atividade contra o HSV-1. Este composto inibiu a multiplicação do vírus em estágios iniciais de replicação e/ou inativou diretamente do vírus.

Estudos descrevem um bom potencial anti-HSV para a quercetina (CHIANG et al., 2003; GRAVINA et al., 2011; SUÁREZ et al., 2010). Quercetina apresentou ação viricida contra HSV-1 e outras espécies de vírus da família Herpesviridae (GRAVINA et al. 2011). Porém, em nossas condições, a quercetina não apresentou qualquer atividade frente a HSV-1, enquanto rutina foi capaz de inibir o vírus moderadamente (CI_{50} = 130,87 ± 8,32 μg/mL; IS = 6.74). Os resultados sobre a atividade anti-herpética da rutina variam em diferentes estudos. Chiang e colaboradores (2003) descrevem que a rutina não apresentou atividade contra HSV-1. No entanto, a presença de rutina e a sua ação sinérgica com outros componentes, tais como ácido cafeico e ácido clorogênico, foram responsáveis pela ação anti-HSV-1 e 2 dos extratos das folhas de *Ilex paraguariensis* (LUCKEMEYER et al., 2012).

Vários pesquisadores têm utilizado micobactérias não-patogênicas, de fácil e rápido crescimento para investigar a atividade antimicobacteriana de extratos e compostos derivados de plantas (BROWN et al., 2007; KUETE et al., 2008). Em um estudo realizado por McGaw e colaboradores (2008), concluiu-se que *Mycobacterium smegmatis* foi melhor indicador da atividade contra o patógeno *Mycobacterium tuberculose*. No entanto, mais pesquisas são necessárias para

validar o uso de espécies não-patogênicas, como *M. smegmatis,* como modelo para detectar a atividade de extratos de plantas contra a *M. tuberculosis*. A atividade antimicobacteriana do EB, frações e subfrações das folhas de *S. buxifolia* frente *M. smegmatis*, *M. tuberculosis* e *M. avium* foi investigada pela primeira vez. Em relação as frações, somente DCM e AcOEt mostraram atividade frente a *M. smegmatis* (CIM = 312,50 μg/mL). A ação antimicobacteriana descrita para a fração DCM, provavelmente está relacionada com a presença de triterpenos e esteroides (BOLIGON et al., 2010), os quais possuem propriedade antimicobacteriana (CANTRELL et al., 2001), enquanto que a atividade da fração AcOEt pode ser atribuída a presença dos flavonoides (BOLIGON et al., 2009).

Considerando as subfrações (I-VII) das folhas de *S. buxifolia*, a menor CIM frente a *M. smegmatis* foi observada para a subfração III (78,12 μg/mL). Esta subfração juntamente com a subfração VI apresentaram a melhor atividade contra *M. tuberculosis* e *M. avium* (MIC = 156,25 μg/mL e 312,50 μg/mL, respectivamente). Tosun e colaboradores (2004) consideram inativos os extratos de plantas que não impedem o crescimento de micobactérias a uma concentração superior a 200,00 μg/mL. Neste sentido, consideramos como resultados promissores as MICs de 78,12 e 156,25 μg/mL encontradas para as subfrações III e VI. Estas subfrações são compostas principalmente por flavonoides (quercetina e quercitrina) e ácidos fenólicos (ácido gálico e ácido cafeico). A interação entre estes compostos, provavelmente, seja responsável pela importante atividade encontrada contra *M. tuberculosis*, *M. smegmatis* e *M. avium* (COWAN, 1999).

Askun e colaboradores (2009) atribuiram a boa atividade antimicobacteriana (*M. tuberculosis*) do extrato metanólico de *Timbra spicata* e *Origanum minutiflorum* à presença de quercetina. Seus efeitos podem estar relacionados com a inibição da biossíntese de ácidos graxos e ácidos micólicos. A presença de ácido micólico é uma das características mais distintivas da parede celular de micobactéria, sendo fundamental para a sua sobrevivência. Considerando a estrutura dos flavonoides, o grupo cetona simula o grupo carbonilo de um substrato de ácido graxo, e todos eles possuem uma ligação dupla 2-3 consistente com um produto similar. Além disso, os inibidores mais potentes da ácido graxo sintase II (FAS-II) não suportam o oxigênio presente na quercetina. A atividade destes compostos contra *Mycobacterium*

certamente representa um importante alvo potencial para estudos futuros e desenvolvimento de medicamentos (BROWN et al., 2007).

Lechner e colaboradores (2008) testaram flavonoides com diferentes padrões de substituição, utilizando diferentes cepas de micobactérias, os resultados encontrados utilizando a mesma cepa usada em nosso estudo (*M. smegmatis* mc^2 155 - ATCC 700084) foram: epicatequina (CIM > 128,00 μg/mL), isoramnetina (CIM > 256,00 μg/mL), campferol (CIM > 256,00 μg/mL), luteolina (CIM = 128-256,00 μg/mL), miricetina (CIM = 32,00 μg/mL), quercetina (MIC > 256,00 μg/mL), rutina e taxifolina (CIM > 128.00 μg/mL). Geralmente, flavonóis simples e seus glicosídeos como quercetina, quercitrina e rutina possuem uma atividade antimicobacteriana moderada contra micobactérias de crescimento rápido (*M. smegmatis*) e lento (*M. tuberculosis*) (KUETE et al., 2008; LECHNER et al., 2008).

A Loque Americana (AFB) é uma das doenças bacterianas mais graves que afetam as abelhas (*Apis mellifera*) (GENERSCH et al., 2005; GENERSCH, 2010). O agente causador da AFB é a bactéria *Paenibacillus larvae*, um bacilo formador de esporos que é extremamente resistente a antibióticos, ao calor e aos desinfetantes clássicos (THOMPSON et al., 2007). Extratos de plantas, especiarias, óleos essenciais e substâncias isoladas são conhecidos por retardar ou inibir o crescimento de bactérias, leveduras e fungos (HAYOUNI et al., 2008). Neste contexto, o EB e as frações das cascas do caule de *S. buxifolia* foram testadas frente a *Paenibacillus* espécies. As frações DCM e AcOEt apresentaram os melhores resultados contra *P. larvae* (CIM = 1,56 e 6,25 mg/mL, respectivamente). A atividade antibacteriana destas frações está relacionada com a composição química de *S. buxifolia* que é rica em esteroides, triterpenos, compostos fenólicos, flavonoides e alcaloides (BOLIGON et al., 2009, 2010, 2012; MALDANER et al., 2011). Provavelmente ocorra um sinergismo entre os diferentes componentes, uma vez que foi observado que nenhum desses compostos apresentou atividade quando testado isoladamente (FLESAR et al., 2010). Mihai e colaboradores (2012) também atribuem a o efeito inibitório do extrato de própolis frente a *P. larvae* ao sinergismo entre flavonoides e ácidos fenólicos.

Além disso, o EB e as frações DCM, AcOEt, BUT de *S. buxifolia* foram pulverizados sobre abelhas adultas, durante 15 dias, para verificar os possíveis efeitos tóxicos. Nenhuma das amostras apresentou toxicidade para as abelhas nas

concentrações testadas (mesma concentração que inibiu o crescimento da *P. larvae*), mostrando que o EB e as frações de *S. buxifolia* CE podem ser usados para o controle da AFB. Santos e colaboradores (2012) testaram a toxicidade de óleos de plantas amazônicas contra a *A. mellifera*. O óleo de copaíba não apresentou efeito tóxico, após 10 dias de observação. A utilização de compostos que inibem o crescimento da *P. larvae* e que não apresentem toxicidade representa uma alternativa natural aos antibióticos sintéticos utilizados atualmente para o controle da AFB (FLESAR et al., 2010).

Para finalizarmos o estudo do potencial antimicrobiano de *S. buxifolia*, o óleo essencial das folhas da espécie foi extraído, caracterizado e suas atividades antimicrobiana e antioxidante foram testadas. Obteve-se um rendimento de 0,4% e vinte e cinco compostos, representando 98,38% do total da composição, foram identificados, dos quais 73,69% são sesquiterpenos e 18,74% monoterpenos. Os principais componentes do óleo essencial são: espatulenol (27,09%), β-cubebeno (11,26%), germacreno D (9,81%), carvacrol (7,01%), globulol (5,36%), α-copaeno (4,17%), γ-eudesmol (3,59%), timol (3,27%), 1,8-cineol (3,08%), p-cimeno (2,56%), α-eudesmol (2,34%), β-elemeno (2,04%), juntamente com acetato de eugenol, hexanol, α-pineno, α-humuleno, eugenol e fitol, presentes em menor concentração. O espatulenol, componente mais abundante no óleo essencial de *S. buxifolia*, foi descrito em outras espécies, tais como, *Baccharis uncinella* (16,41%), *Stevia rebaudiana* (15,41%), *Origanum vulgare* (11,67%) e *Baccharis dracunculifolia* (9,54%) (MUANDA et al., 2011; XAVIER et al., 2011).

Muitos estudos *in vitro* têm demostrado a atividade antioxidante de óleos essenciais (EDRIS, 2007; GOURINE et al., 2010). Em particular, o radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) é amplamente utilizado para avaliar rapidamente a capacidade de antioxidante de extratos de plantas medicinais (BRAND-WILLIAMS et al., 1995; BOLIGON et al., 2009). O óleo essencial de *S. buxifolia* foi submetido a uma avalição qualitativa. O surgimento de uma mancha amarela foi observada imediatamente após a nebulização de DPPH sobre a placa de cromatografia em camada delgada (CCD), sugerindo a presença de compostos antioxidantes na amostra. A intensidade da cor foi semelhante aos padrões de quercetina e ácido ascórbico. Além disso, um ensaio quantitativo com o DPPH também foi realizado, e os resultados foram expressos através do valor de CI_{50}, concentração necessária para reduzir de 50% de DPPH radical. O óleo essencial de *S. buxifolia* apresentou

boa propriedade antioxidante (CI_{50} = 13,62 ± 0,17 μg/mL). A capacidade antioxidante desse óleo essencial pode ser atribuída à presença de constituintes fenólicos, especialmente carvacrol e timol, os quais já possuem esta atividade descrita na literatura (BOUNATIROU et al., 2007; MUANDA et al., 2011: VIUDA-MARTOS et al., 2011). Além disso, outros compostos também parecem desempenhar um papel importante, tais como o eugenol (CI_{50} = 1,26 μg/mL pelo método de DPPH), β-cubebeno (CI_{50} = 19,3 μg/mL); e p-cimeno (JIROVETZ et al., 2006; YANISHLIEVA et al., 1999).

O óleo essencial das folhas de *S. buxifolia* foi também testado frente a microrganismos, mostrando apenas atividade moderada contra *Staphylococcus aureus* e *Escherichia coli* (CIM = 500 e 750 μg/mL, respectivamente). Um estudo anterior descreve que a espécie *S. buxifolia* possui atividade contra *S. aureus* (BOLIGON et al., 2012). Espatulenol, β-cubebeno, germacreno D e carvacrol foram os principais componentes identificados neste óleo essencial os quais podem ser responsávies, em parte, pela atividade antimicrobiana descrita. Compostos como espatulenol e carvacrol possuem notável atividade antimicrobiana contra infecções bacterianas (BOUNATIROU et al., 2007; BURT, 2004; CHINOU et al., 2004), o potencial antimicrobiano do timol (3,27% no óleo essencial de *S. buxifolia*) também já foi descrito para *E. coli* (RIVAS et al., 2010). Sua ação ocorre devido a ruptura da membrana celular bacteriana, inibindo a atividade da ATPase, e liberação de ATP intracelular (VIUDA-MARTOS et al., 2011). A análise da composição química do óleo essencial de *S. buxifolia* e a avaliação preliminar das atividades antioxidante e antimicrobiana é o primeiro trabalho descrito na literatura para o óleo essencial dessa espécie, e, em conjunto, os dados obtidos servem de apoio para a investigação de novas propriedades biológicas particulares.

Seguindo os estudos referentes às atividades biológicas da espécie *S. buxifolia*, foi investigado o efeito protetor do EB das cascas do caule da planta frente a lesão gástrica causada pelo etanol (70%, v/v) em ratos. Além disso, a composição química deste extrato também foi determinada. A exposição aguda da mucosa gástrica ao etanol pode resultar em lesões gástricas pela alteração dos fatores de proteção, incluindo a diminuição na produção de muco e na circulação de sangue nesse tecido (CHOI et al., 2009; INEU et al., 2008). Além disso, a lesão gástrica provocada pelo etanol pode ser devido à geração de espécies reativas e uma

resposta inflamatória exacerbada (AMARAL et al., 2013; CHOI et al., 2009; KAHARAMAN et al., 2003). Deste modo, em nosso estudo, foi observado que após 1 hora da administração do etanol por gavagem em ratos ocorreram alterações macroscópicas no tecido gástrico, tal como a perda de cor normal e muco, juntamente com o surgimento de petéquias, hemorragia e edema. Estas lesões são provavelmente relacionadas à depleção de muco e um efeito constritivo em veias e artérias da mucosa gastrica, produzindo, congestão, inflamação e lesão tecidual (MOTA et al., 2011). Além das lesões macroscópicas, a exposição ao etanol causou danos ao tecido gástrico em nível microscópico. As injúrias histopatologicas foram caracterizadas pelo edema e congestão das mucosas, bem como pelo processo inflamatório (infiltração de neutrófilos). A administração do EB de *S. buxifolia* nas concentrações de 100, 200 e 400 mg/Kg de peso corporal reverteu o dano da mucosa gástrica causado pelo etanol, impedindo o inchaço e a infiltração de neutrófilos.

Além disso, o EB de *S. buxifolia* restaurou, de maneira dose dependente, os parâmetros bioquímicos relacionados ao estresse oxidativo induzido pela administração do etanol. As propriedades antioxidantes do EB foram demonstradas pela diminuição dos níveis de malondialdeído MDA (marcador de peroxidação lipídica) e aumento das defesas antioxidantes: não-enzimáticas, grupos sulfidrilo (NPSH), e enzimáticas, superóxido dismutase (SOD) e catalase (CAT). Os efeitos protetores do EB de *S. buxifolia* estão associados com a presença de ácidos fenólicos e flavonoides, principalmente ácido gálico, ácido clorogênico, ácido cafeico, quercetina, rutina e canferol. A presença destes compostos antioxidantes em extratos e frações de *S. buxifolia* já foram descritos anteriormente por Boligon e colaboradoes (2009, 2012).

Vários estudos têm demostrado que ácidos fenólicos e flavonoides presentes em extratos de plantas previnem ou revertem os danos causados pela úlcera gástrica (ALIMI et al, 2010; AMARAL et al., 2013; CADIRCI et al., 2007; MOTA et al., 2011; SATHISH et al., 2011). Hussain e colaboradores (2009) descreveram o efeito gastroprotetor de rutina pela eliminação de espécies reativas de oxigênio na mucosa gástrica. Além disso, a quercetina e o canferol também mostraram efeitos protetores frente a úlcera gástrica induzida por etanol, diminuindo o estresse oxidativo e aumentando a atividade das enzimas antioxidantes (BARROS et al., 2008; MOTA et al., 2009). Neste contexto, o EB de *S. buxifolia* pode ser visto como uma promissora

e acessível droga gastroprotetora, uma vez que reverteu os danos da mucosa gástrica e não apresentou toxicidade hepática em estudo realizado por Freitas et al (2013).

As plantas medicinais têm sido utilizadas para a obtenção de moléculas protótipo, as quais podem ser exploradas terapeuticamente. Neste contexto, a fração DCM das cascas do caule de *S. buxifolia*, fração rica em compostos apolares como os terpenos, foi selecionada para a investigação de compostos ativos. Da fração DCM foram isolados o β-sitosterol (já descrito para esta espécie por Boligon et al., 2010), sitosterol-3-*O*-β-D-glucosídeo e ácido ursólico, ainda não relatados em *S. buxifolia*. A estrutura de β-sitosterol foi identificada com base nos dados de ^{13}C-RMN e por comparação com os dados da literatura (BOLIGON et al., 2010; JANOVIK et al., 2012; SALEM et al., 2013). Recentemente foi descrito que *S. buxifolia* possui lupeol e a mistura dos esteroides, β-sitosterol e estigmasterol em sua composição química (BOLIGON et al., 2010).

A presença de sitosterol-3-*O*-β-D-glucosídeo foi relatado pela primeira vez para esta espécie. As estruturas foram identificados com base nos dados de ^{13}C-RMN e por comparação com os dados da literatura (AGRAWAL, 1992; BOLIGON et al., 2010; FROHLICH et al., 2013; JANOVIK et al., 2012). Os sinais em δ 140,53 e 121,29 ppm indicam a presença de esteroides com uma ligação dupla em C5 e C6 (DE-EKNANKUL, POTDUANG, 2003), enquanto que os sinais de carbono anomérico de 100,67 ppm indicam a presença de *O*-glicosídeos. O aparecimento de ressonâncias anoméricas de desvios químicos em um intervalo de 90-112 ppm, contribui grandemente para a determinação do número de *O*-glicosídeos, uma vez que nenhum dos átomos do carbono da aglicona é absorvido nesta região (AGRAWAL, 1992). Resíduos de glicosil em C-3 da aglicona do sitosterol foi atribuído ao átomo de carbono com um desvio químico a 79,01 ppm. O resíduo glucosil em C-3 da aglicona aumenta o deslocamento do carbono em 6-7 ppm para campo baixo em comparação com o deslocamento deste carbono sem resíduo glucosil (FROHLICH et al., 2013). O resíduo de glicosil foi identificado como β-D-glicose através da comparação com o espectro de ^{13}C-RMN da literatura (AGRAWAL, 1992).

O último composto isolado de *S. buxifolia* foi o ácido ursólico (ácido 3β-hidroxi-urs-12-en-28-oico). Os dados espectrais obtidos através do ^{13}C RMN foram compatíveis com os dados já descritos na literatura para o ácido ursólico (SILVA et

al., 2008; HUANG et al., 2012). O ácido ursólico é um composto de ocorrência natural, triterpeno pentacíclico, amplamente distribuído em pantas alimentares e medicinais (HUANG et al., 2012). Este composto possui um amplo espectro de atividades biológicas, incluindo propriedades antibacteriana, antiviral, antitumoral, antimutagênica, cardiotônica, hepatoprotetora, antioxidante e imunomoduladora (FONRANAY et al., 2008; JANG et al., 2009; MA et al., 2005).

Extratos e frações de *S. buxifolia* possuem diversas propriedades, tais como, antioxidante, antiviral, antimicrobiana e cardiotônica (BOLIGON et al., 2009; DA SILVA et al., 2012), que podem estar relacionadas com a presença de ácido ursólico isolado e identificado na espécie.

6 CONCLUSÕES

- O extrato bruto e as frações das cascas do caule e folhas de *S. buxifolia* apresentaram em sua composição ácidos fenólicos (ácido gálico, ácido clorogênico e ácido cafeico) e flavonoides (rutina, quercetina, isoquercitrina, quercitrina e canferol).
- A partir da fração diclorometano das cascas do caule foi possível isolar triterpenos, sitosterol-3-*O*-β-D-glucosídeo e ácido ursólico, descritos pela primeira vez para esta espécie. Além disso, o β-sitosterol, já conhecido para esta espécie foi novamente isolado pelo nosso grupo de pesquisas.
- O óleo essencial de *S. buxifolia* foi obtido e caracterizado. Espatulenol, β-cubebene, germacreno D e carvacrol foram os principais componentes identificados no óleo essencial.
- *S. buxifolia* apresentou propriedades antimicrobianas, sendo efetiva frente a *K. pneumoniae, Micrococcus sp.*, *E. faecalis, C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. neoformans*, *M. pachydermatis*, *A. flavus*, *A. fumigatus, F. solani, M. smegmatis, M. avium e M. tuberculosis.*
- A fração acetato de etila das folhas e a fração n-butanol das cascas do caule de *S. buxifolia* apresentaram a mais promissora atividade antiviral (HSV-1).
- O extrato bruto de *S. buxifolia* (100, 200 e 400 mg/Kg) reverteu os danos gástricos causados pelo etanol em estômago de ratos, de maneira dose-dependente.

- A fração acetato de etila e diclorometano apresentaram as melhores atividades frente a *Paenibacillus* sp. Além disso, não mostraram toxicidade frente à *A. mellifera* após 15 dias de observação.

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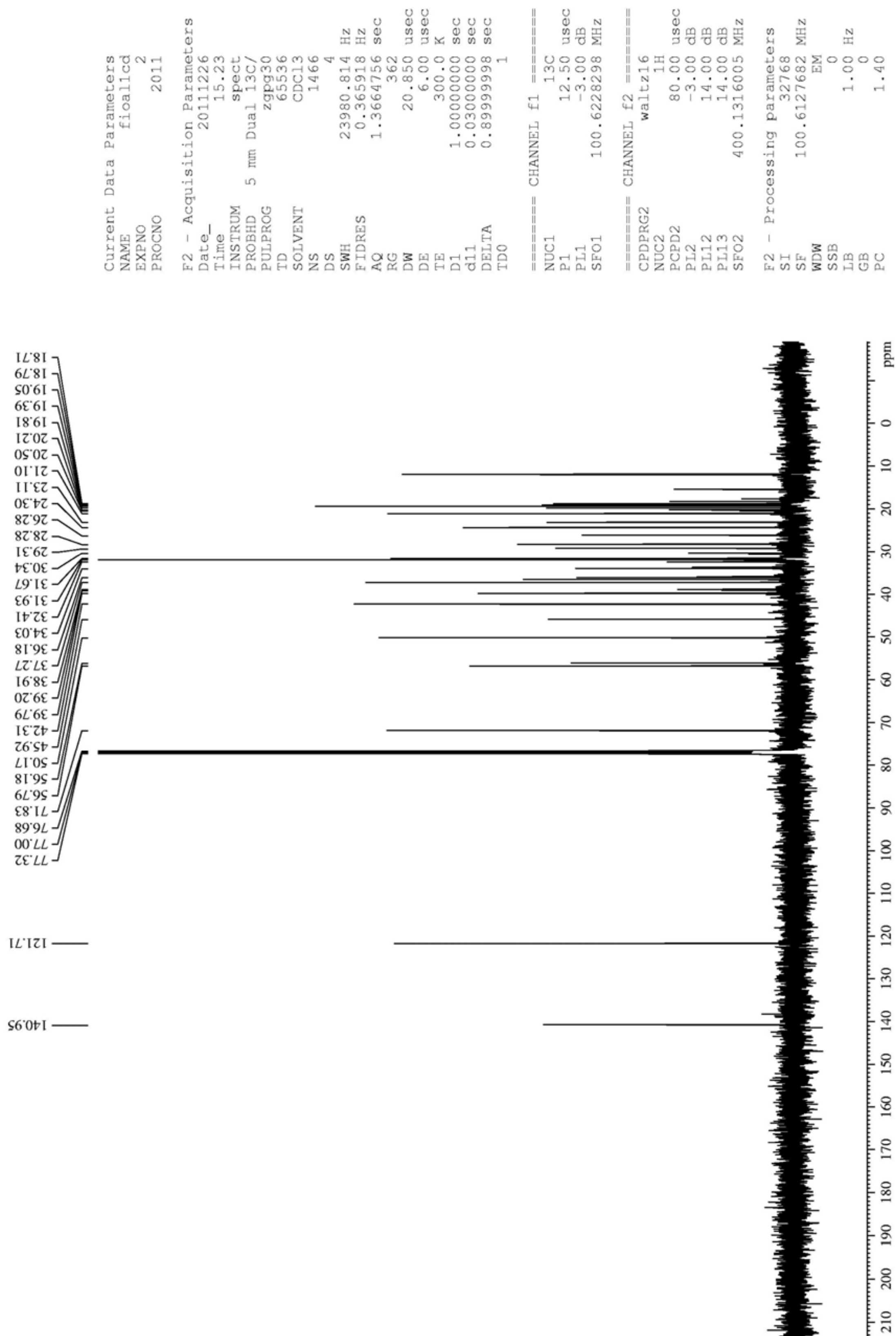
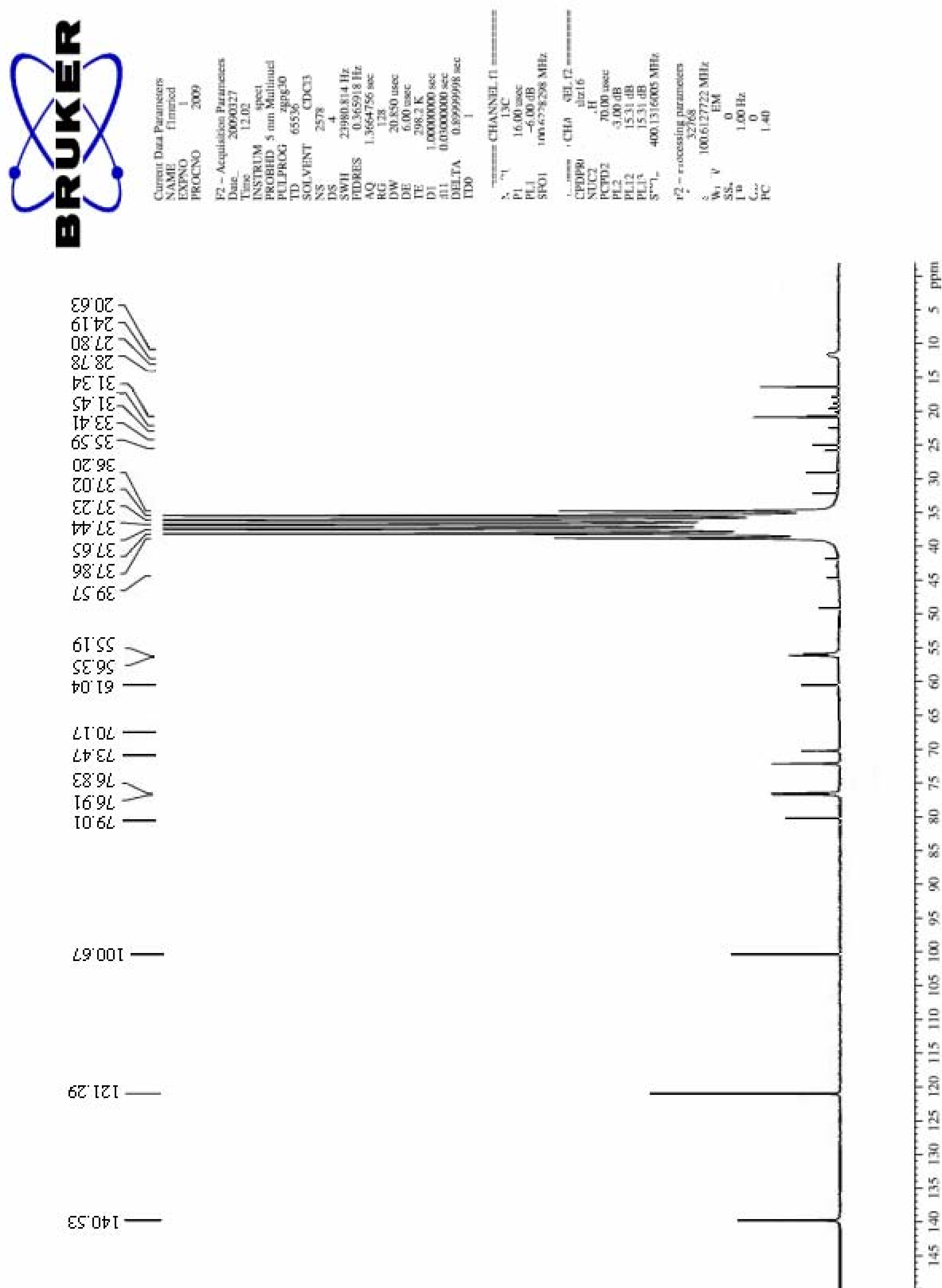
ANEXOS

ANEXO 01: Espectro de ^{13}C-RMN (100MHz, $CDCl_3$): β-Sitosterol



ANEXO 02: Espectro de ^{13}C-RMN (100MHz, DMSO): sitosterol-3-*O*-β-D-glucosídeo



ANEXO 03: Espectro de ^{13}C-RMN (100MHz, DMSO): Ácido ursólico