UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM FARMÁCIA

JANAÍNA KOELZER

Avaliação do efeito anti-inflamatório e antibacteriano da *Lotus corniculatus* v. São Gabriel

Florianópolis
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Dissertação apresentada ao curso de Pós-graduação em Farmácia do Centro de Ciências da Saúde da Universidade Federal de Santa Catarina como requisito parcial para obtenção do título de Mestre em Farmácia, sob a orientação da Profa. Dra. Tânia Silvia Fröde.

Florianópolis
2009

"Ser feliz é reconhecer que vale a pena viver apesar de todos os desafios, incompreensões e períodos de crise

Ser feliz é deixar de ser vítima dos problemas e se tornar um autor da própria história

É atravessar desertos fora de si, mas ser capaz de encontrar um oásis no recôndito da sua alma

É agradecer a Deus a cada manhã pelo milagre da vida

Ser feliz é não ter medo dos próprios sentimentos

É saber falar de si mesmo

É ter coragem para ouvir um "Não"

É ter segurança para receber uma crítica, mesmo que injusta

Pedras no caminho?

Guardo todas, um dia vou construir um castelo..."

(Fernando Pessoa)

Aos meus pais, Vera e Gilberto, por todo amor, apoio e confiança em mim depositada.

Ao meu marido Jerônimo, por todo amor, apoio, companheirismo e sacrifício, de ambas as partes, para a realização desse trabalho.

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Obrigada a todos vocês por fazerem parte da minha vida.

SUMÁRIO

LISTA DE ABREVIAÇÕES

A_1, A_{2A}, A_{2B} e A_3	Receptores de Adenosina
AcOEt	Fração acetato de etila obtida do extrato bruto da *Lotus corniculatus* v. São Gabriel
ADA	Adenosina-deaminase
AF	Fração resíduo aquoso obtido do extrato bruto da *Lotus corniculatus* v. São Gabriel
ATCC	American Type Culture Collection
B1	Receptor B1 da bradicinina
B2	Receptor B2 da bradicinina
BuOH	Fração butanólica obtida do extrato bruto da *Lotus corniculatus* v. São Gabriel
CBM	Concentração Bactericida Mínima
CD11b/CD18	Molécula de adesão do tipo β2-integrina expressa em neutrófilos
CE	Extrato bruto da *Lotus corniculatus* v. São Gabriel
Cg	Carragenina
CGS21680	Agonista seletivo do receptor A_{2A}
CIM	Concentração Inibitória Mínima
ConA	Concavalina A
COX-2	Ciclooxigenase tipo 2
DMSO	Dimetilsulfóxido
DPPH	2,2-difenil-1-picril-hidrazil
ECCO	European Culture Collection Organization
ELISA	Enzimaimunoensaio
EPAGRI	Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A.
EROS	Espécies Reativas de Oxigênio
EtOAc	Acetato de Etila
EtOH	Etanol
GTPase	Guanosina trifosfato hidrolase
HEX	Fração hexano obtida do extrato bruto da *Lotus corniculatus* v. São Gabriel
i.p.	Via intraperitoneal
i.pl.	Via intrapleural
i.v.	Via intravenosa
IFN-γ	Interferon gama
IL-4	Interleucina-4
IL-5	Interleucina-5
IL-10	Interleucina-10
IL-12	Interleucina-12
IL-1β	Interleucina-1 beta
IL-1 R1	Receptor de interleucina-1
IoM	Ionomicina
KC	Quimiocina para neutrófilo
LPS	Lipopolissacarídeo
MIP-2	Proteína inflamatória de macrófago 2
MK 886	Inibidor de leucotrienos
MPO	Mieloperoxidase
NADPH oxidase	Nicotinamida adenina dinucleotídeo fosfato oxidase
NF-κB	Fator de transcrição nuclear kappa B
NK	Célula Natural Killer
NO	Óxido nítrico
NO^x	Concentrações de nitrato/nitrito
NOS	Óxido nítrico sintase
eNOS	Óxido nítrico sintase constitutiva endotelial
iNOS	Óxido nítrico sintase induzida
nNOS	Óxido nítrico sintase constitutiva neuronal

PCA 4248	Inibidor seletivo do fator ativador de plaquetas
PCR	Proteína C Reativa
PGE_2	Prostaglandina E_2
PMA	Forbol-12-miristato-13-acetato
RAW 264.7	Linhagem de macrófagos de camundongos
SC-51	Inibidor seletivo de Inos
SOD	Superóxido desmutase
TCC	Cloridrato de tetrazolium-trifenil
TNF-α	Fator de necrose tumoral alfa
TNF RI	Receptor do tipo I do fator de necrose tumoral
TNF RII	Receptor do tipo II do fator de necrose tumoral
TPA	12-O-tetradecanoilforbol acetato
ZM241385	Antagonista seletivo do receptor A_{2A}
WHO	World Health Organization

LISTA DE FIGURAS

RESUMO

Avaliação do efeito anti-inflamatório e antibacteriano da *Lotus corniculatus* v. São Gabriel

A *Lotus corniculatus* (Fabaceae) é distribuída em muitas regiões do mundo e possui grande valor agronômico por seus constituintes: proantocianidinas, flavonoides e ácido oleanólico. Esta planta é utilizada como alimento para o gado proporcionando o aumento no ganho de peso e na produção de leite, além do controle de infecções intestinais parasitárias nos animais. O objetivo deste trabalho foi avaliar o efeito anti-inflamatório e antibacteriano do extrato bruto (CE) da *Lotus corniculatus* v. São Gabriel, frações (hexano (HEX), acetato de etila (AcOEt), butanólica (BuOH) e resíduo aquoso (AF)), e compostos isolados (canferitrin, ácido oleanólico e β-sitosterol). As partes aéreas da *Lotus corniculatus* foram secas a temperatura ambiente, e a partir desse material (620 g), foi extraído o extrato bruto (CE) por maceração com etanol 96% por um mês. O solvente foi evaporado resultando em 78 g de extrato bruto (CE). O extrato bruto foi fracionado por extração líquido-líquido utilizando solventes em ordem crescente de polaridade resultando nas frações hexano (HEX), acetato de etila (AcOEt), butanólica (BuOH) e resíduo aquoso (AF). A partir da fração HEX foram isolados o ácido oleanólico e o β-sitosterol, e a partir da fração AcOEt o canferitrin. Para a avaliação da atividade anti-inflamatória foi utilizado o modelo de pleurisia induzida pela carragenina, em camundongos, e foram avaliados os seguintes parâmetros inflamatórios: concentrações de leucócitos, exsudação, atividade da mieloperoxidase (MPO) e da adenosina-deaminase (ADA), além das concentrações de nitrito/nitrato (NO^x) e interleucina-1 beta (IL-1β). Nesse protocolo experimental, foram utilizados camundongos albinos suíços, os quais foram distribuídos em diferentes grupos e tratados com CE (100 – 400 mg/kg), HEX (50 – 200 mg/kg), AcOEt (100 – 400 mg/kg), BuOH (50 – 200 mg/kg), AF (25 – 200 mg/kg), canferitrin (50 – 100 mg/kg), ácido oleanólico (10 – 100 mg/kg) ou β-sitosterol (10 – 100 mg/kg) 0,5 h antes da administração da carragenina (1%, i.pl.). Os parâmetros inflamatórios foram avaliados 4 h após. Todos os animais, exceto nos experimentos que foram analisadas as atividades enzimáticas, foi administrado previamente (10 min.) Azul de Evans (25 mg/kg, i.v.) a fim de avaliar a exsudação. Dexametasona e indometacina foram utilizados como fármacos anti-inflamatórios de referência. Para a avaliação da atividade antibacteriana, foi utilizada a técnica de microdiluição em caldo para a determinação da concentração inibitória mínima (CIM) e da concentração bactericida mínima (CBM). Neste protocolo, o material vegetal, o extrato bruto (CE), frações e compostos isolados, foram dissolvidos em dimetilsulfóxido (DMSO) e transferidos para uma placa em diluição seriada e, a seguir, o inóculo bacteriano foi adicionado. Gentamicina foi utilizada como fármaco antibacteriano de referência. Diferenças estatísticas entre os grupos, para os parâmetros inflamatórios, foram determinadas pela análise dos testes t de Student ou de variância (ANOVA). Valores de $P < 0,05$ foram considerados significativos. O extrato bruto da *Lotus corniculatus* (200 – 400 mg/kg) e frações (50 – 200 mg/kg), assim como os compostos isolados (25 – 100 mg/kg) inibiram: leucócitos, neutrófilos, exsudação, a atividade da MPO e da ADA, além das concentrações de NO e IL-1β ($P < 0,05$). A indometacina e a dexametasona inibiram todos os parâmetros inflamatórios estudados ($P < 0,05$). Em relação à atividade antibacteriana, somente as frações HEX e AcOEt, e os compostos isolados ácido oleanólico e canferitrin, revelaram atividade bactericida e/ou bacteriostática. A fração HEX demonstrou moderada atividade antibacteriana (CIM = 100 μg mL^{-1}) para a bactéria *Bacillus cereus*, e fraca atividade antibacteriana (CIM = 600 a 1000 μg mL^{-1}) para as bactérias *Enterococcus faecalis, Staphylococcus aureus, Listeria monocytogenes, Staphylococcus epidermidis, Providencia alcalifaciens* e *Acinetobacter calcoaceticus.* A

fração AcOEt demonstrou fraca atividade antibacteriana (CIM = 800 µg mL^{-1}) para as bactérias *Enterococcus faecalis, Bacillus cereus* e *Acinetobacter calcoaceticus.* O ácido oleanólico revelou boa atividade antibacteriana para *Staphylococcus aureus* meticilina resistente (CIM = 100 µg mL^{-1}), *Listeria monocytogenes* e *Bacillus cereus* (CIM = 25 µg mL^{-1}), e o canferitrin demonstrou boa atividade antibacteriana para as bactérias: *Staphylococcus epidermidis, Shigella flexinerii, Salmonella typhimurium* e *Acinetobacter calcoaceticus* (CIM = 100 µg mL^{-1}). Este composto demonstrou excelente atividade antibacteriana para as bactérias *Enterococcus faecalis* (CIM = 3.9 µg mL^{-1}) e *Bacillus cereus* (CIM = 8.5 µg mL^{-1}). O extrato bruto, frações e compostos isolados da *Lotus corniculatus* v. São Gabriel demonstraram importante atividade anti-inflamatória e antibacteriana. Os compostos isolados: canferitrin, ácido oleanólico e β-sitosterol podem ser responsáveis por estes efeitos anti-inflamatório e/ou antibacteriano.

Palavras-chaves: *Lotus corniculatus,* atividade anti-inflamatória, pleurisia, mediadores da inflamação, atividade antibacteriana, canferitrin, ácido oleanólico, β-sitosterol.

ABSTRACT

Evaluation of anti-inflammatory and anti-bacterial effects of *Lotus corniculatus* v. São Gabriel

The *Lotus corniculatus* (Fabaceae) is distributed in many regions of the world and has a high agronomic value for its constituted: proanthocyanidins, flavonoids and oleanolic acid. This plant is used as food for cattle providing the enhancement of the weight and of milk production beyond the control of intestinal parasitic infections in the animals. The aim of this study was to evaluate the anti-inflammatory and anti-bacterial effects of crude extract (CE), fractions (hexane (HEX), ethyl acetate (AcOEt), n-butanol (BuOH) and aquous fraction (AF)), and isolated compounds (kaempferitrin, oleanolic acid and β-sitosterol) from *Lotus corniculatus* v. São Gabriel. The aerial parts of *Lotus corniculatus* were dried at room temperature and this material (620 g) the crude extract (CE) was extracted by maceration with ethanol 96% for one month. The solvent was evaporated resulting in 78 g of crude extract (CE). The crude extract was fractionated by liquid-liquid extraction using solvents in growing order of polarity resulting in hexane (HEX), ethyl acetate (AcOEt), n-butanol (BuOH) and aqueous fraction (AF). From the HEX fraction it was isolated the oleanolic acid and the β-sitosterol and from AcOEt fraction the kaempferitrin. To evaluate the anti-inflamatory activity, it was used the mouse model of pleurisy induced by carrageenan and the following inflammatory parameters were evaluated: leukocytes, exudation, myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities, as well as, nitrite/nitrate (NO^x) and interleukin-1 beta (IL-1β) levels. In this experimental protocol, swiss mice were used in the in vivo experiments, which were distributed in different groups and they were treated with CE (100 – 400 mg/kg), HEX (50 – 200 mg/kg), AcOEt (100 – 400 mg/kg), BuOH (50 – 200 mg/kg), RA (25 – 200 mg/kg), kaempferitrin (50 – 100 mg/kg), oleanolic acid (10 – 100 mg/kg) or β-sitosterol (10 – 100 mg/kg) 0,5 h before carrageenan (1%,i.pl.). The inflammatory parameters were evaluated 4 h after. All animals, except in the experiments that were analyzed enzymatic activities, the animals were previously pretreated (10 min.) with Evans blue dye (25 mg/kg, i.v.) in order to evaluate the exudation. Dexamethasone and indomethacin were used as anti-inflammatory reference drugs. To evaluate the anti-bacterial activity, it was used the microdilution in broth technical to determine the minimal inhibitory concentraton (MIC) and the minimal bactericidal concentration (MBC) methodology. In this protocol the crude extract (CE), fractions and isolated compounds were dissolved in dimethylsulfoxide (DMSO) and transferred to plate in serial dilution and, after, the bacterial inoculum were added. The gentamicin was used as the anti-bacterial reference drug. To the anti-inflammatory parameters, statistical differences between groups were determined by Student's t test and analysis of variance (ANOVA). Values of $P < 0.05$ were considered significant. The crude extract of *Lotus corniculatus* (200 – 400 mg/kg) and fractions (50 – 200 mg/kg), as well as its isolated compound (25 – 100 mg/kg) inhibited: lekocytes, neutrophils and exudation, the MPO and ADA activities, as well as NO and IL-1β levels ($P < 0.05$). Indomethacin and dexamethasone inhibited all the studied inflammatory parameters ($P < 0.05$). In regard to the anti-bacterial activity, only the HEX and AcOEt fractions and the isolated compounds, oleanolic acid and kaempferitrin, revealed anti-bacterial and/or bacteriostatic activities. The hexane fraction showed moderate anti-bacterial effect (MIC = 100 μg mL^{-1}) on *Bacillus cereus* and weak anti-bacterial effect (MIC = 600 to 1000 μg mL^{-1}) on *Enterococcus faecalis, Staphylococcus aureus, Listeria monocytogenes, Staphylococcus epidermidis, Providencia alcalifaciens* and *Acinetobacter calcoaceticus.* The AcOEt fraction demonstrated a weak anti-bacterial activity (MIC = 800 μg mL^{-1}) on *Enterococcus faecalis, Bacillus cereus* and *Acinetobacter calcoaceticus.*The Oleanolic acid, isolated

from hexane fraction, exhibited a good anti-bacterial activity on methycillin-resistant *Staphylococcus aureus* (MIC = 100 µg mL^{-1}), *Listeria monocytogenes* and *Bacillus cereus* (MIC = 25 µg mL^{-1}) and the kaempferitrin, a compound isolated from this fraction, demonstrated a good anti-bacterial effect on: *Staphylococcus epidermidis, Shigella flexinerii, Salmonella typhimurium* and *Acinetobacter calcoaceticus* (MIC = 100 µg mL^{-1}). This compound showed an excellent anti-bacterial activity on *Enterococcus faecalis* (MIC = 3.9 µg mL^{-1}) and *Bacillus cereus* (MIC = 8.5 µg mL^{-1}). The crude extract, fraction and isolated compound from *Lotus corniculatus* v. São Gabriel, demonstrated important anti-inflammatory and anti-bacterial activities. The isolated compounds: kampferitrin, oleanolic acid and β-sitosterol can be responsible for these anti-inflammatory and/or anti-bacterial effects.

Keywords: *Lotus corniculatus,* anti-inflammatory activity, pleurisy, mediators of inflammation, anti-bacterial activity, kaempferitrin, oleanolic acid, β-sitosterol.

1. INTRODUÇÃO

1.1. PLANTAS MEDICINAIS

A utilização de plantas para o tratamento, cura e prevenção de doenças ocorreu paralelamente à história da humanidade. No início do século passado, 80% de todos os medicamentos eram obtidos a partir de raízes, cascas e folhas de plantas (McCHESNEY; VENKATARAMAN; HENRI, 2007). Atualmente, os produtos naturais continuam sendo fontes significantes de medicamentos, sendo a utilização destes evidente em aproximadamente 60% dos medicamentos antitumorais e 75% dos medicamentos para o tratamento de doenças infecciosas, que são originários de produtos naturais ou derivados de produtos naturais (NEWMAN; CRAGG; SNADER, 2003). Nos países em desenvolvimento, assim como nos países desenvolvidos, o consumo de produtos à base de fontes naturais tem aumentado de forma significativa (JUNIOR; PINTO; MACIEL, 2005). Segundo dados da Organização Mundial de Saúde (World Health Organization: WHO), 50% da população da Europa e da América do Norte já utilizaram produtos de origem natural pelo menos uma vez. No Canadá este percentual sobe para 70%, e na China, 30-50% dos medicamentos utilizados na terapia são de origem natural (WHO, 2008).

No Brasil, as plantas medicinais da flora nativa são consumidas com pouca ou nenhuma comprovação das propriedades farmacológicas e muitas vezes essas plantas são, inclusive, utilizadas para fins medicinais diferentes daqueles utilizados pelos silvícolas (JUNIOR; PINTO; MACIEL, 2005).

A elucidação dos componentes ativos presentes nas plantas, bem como o estudo do mecanismo de ação, vem sendo um dos maiores desafios para os pesquisadores, principalmente nas áreas de bioquímica e de farmacologia (CALIXTO, 2005). Por exemplo: estudos demonstraram importantes atividades anti-inflamatória e antioxidantes de plantas comumente utilizadas para o tratamento da asma, da doença de Alzheimer, da artrite reumatóide e da aterosclerose (KAPLAN et al., 2007)

Na realidade, a pesquisa relacionada às propriedades químicas e farmacológicas de plantas medicinais está voltada à procura de substâncias com atividade biológica, no sentido de promover uma base científica à medicina popular (GIORGETTI; NEGRI; RODRIGUES, 2007) ou para a obtenção de novas estruturas

químicas de interesse para a indústria farmacêutica, com atividades farmacológicas específicas, podendo resultar em novos fármacos.

1.2. GÊNERO *Lotus*

A *Lotus corniculatus* v. São Gabriel (Fabaceae) é conhecida popularmente como "Cornichão" no Brasil. Esta planta é uma leguminosa perene hiberno-primaveril, de origem européia e mediterrânea, porém distribuída, em nível mundial, em diferentes regiões do mundo, com exceção daquelas muito frias e de áreas tropicais. No Brasil, o único cultivar disponível está localizado na cidade de São Gabriel, desenvolvido pela Estação Experimental de São Gabriel, no Estado do Rio Grande do Sul, tendo seu cultivo expandido para outros países da América do Sul (SOSTER; SCHEFFER-BASSO; DALL'GNOL, 2004).

A *Lotus corniculatus* é utilizada como forrageira, no pasto e na silagem, por possuir grande quantidade de proantocianidinas, também denominada de taninos condensados (DIXON; XIE; SHARMA, 2005). As proantocianidinas são polímeros de flavonoides considerados importantes, tendo em vista suas atividades biológicas, como: antitumoral (KANDIL et al., 2002), antioxidante (BAGCHI et al., 2000), imunomodulatória (LIN; KUO; CHOU, 2002), analgésica e anti-inflamatória (SUBARNAS; WAGNER, 2000). Nos animais, as proantocianidinas são importantes substâncias, uma vez que promovem aumento de: 1) absorção de aminoácidos, como a metionina e a cisteína, 2) taxa de ovulação, 3) ganho de peso e 4) e produção de leite (WAGHORN; McNABB, 2003).

Outros constituintes já identificados nas folhas e flores da *Lotus corniculatus* foram os flavonoides (kaempferol e quercetina) (REYNAUD; LUSSIGNOL, 2005). Além disso, existem estudos que demonstraram que os flavonoides apresentam propriedades antiinflamatória, antitumoral, antimicrobiana, imunomodulatória e antitrombótica (KIM et al., 2004). Em relação ao efeito anti-inflamatório destes constituintes, García-Mendiavilla et al. (2007) demonstraram que estas substâncias inibiram: óxido nítrico sintase induzida (iNOS), a ciclooxigenase-2 (COX-2), a liberação da proteína C reativa (PCR), e o fator nuclear kappa B (NF-κB) (GARCÍA-MENDIAVILLA et al.,2007).

Outros compostos também já foram isolados da *Lotus corniculatus*, como o ácido oleanólico e as saponinas (WALTER, 1961). O ácido oleanólico é um

composto triterpenóide que se encontra distribuído nas plantas e possui atividades: hepatoprotetoras, antitumoral, cardioprotetora, antioxidante e imunomodulatória (LIU, 2005). As saponinas são conhecidas por exibirem também atividade antiinflamatória (KANG et al., 2005), antioxidante e antitumoral (RAO; SUNG, 1995).

Na França, o chá das folhas da *Lotus corniculatus* é utilizado como sedativo e espasmolítico. Baseados no fato de que a planta possui grande quantidade de polifenóis, Trouillas et al. (2003) demonstraram que o extrato hidroalcólico da *Lotus corniculatus* possui atividade antioxidante e antiproliferativa (TROUILLAS et al., 2003).

Uma vez que o trabalho teve como objetivo avaliar o efeito anti-inflamatório e antimicrobiano da *Lotus corniculatus* v. São Gabriel, é válido comentar sobre o processo inflamatório e a atividade antimicrobiana de produtos naturais.

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Figura 1. *Lotus corniculatus* L. var São Gabriel (Cornichão)

1.3. PROCESSO INFLAMATÓRIO

A reação inflamatória é um mecanismo que consiste em uma cascata de reações celulares e vasculares, que ocorre como resposta a um estímulo e que tem como objetivo o reparo tecidual ou a geração de novos tecidos, na tentativa de proteger o organismo (SCHMID-SCHÖBEIN, 2006). A lesão tecidual ou o trauma

promove a liberação de mediadores químicos endógenos que são responsáveis pelos sinais clínicos da inflamação: dor, calor, rubor e tumor (HOFSETH, 2008).

Os mediadores exógenos da inflamação, como por exemplo, peptídeos microbianos, agem como agentes quimiotáticos para os neutrófilos. Essas células fagocíticas formam os fagolisossomos por meio da fusão com grânulos lisossomais, os quais contêm enzimas e espécies reativas de oxigênio (EROS) (HOFSETH, 2008) na tentativa de destruir os microorganismos ou degradar células mortas (SERHAN; CHIANG; DYKE, 2008).

Os neutrófilos são células fagocíticas presentes na resposta imune inata, constituem a primeira linha de defesa do organismo ao patógeno. Estas células possuem a habilidade de fagocitar o agente estranho devido aos conteúdos citoplasmáticos, os quais são lesivos aos patógenos (SEGAL, 2005).

Dentre as principais enzimas relacionadas ao processo inflamatório destaca-se a mieloperoxidase (MPO), que é sintetizada e secretada pelos neutrófilos e corresponde a 5% do total de proteínas dessa célula (LAU; BALDUS, 2006). A MPO também pode ser encontrada em monócitos e macrófagos, mas os monócitos ao longo do tempo perdem a capacidade de sintetizar a MPO durante o processo de maturação, quando estas células se diferenciam em macrófagos (FAITH et al., 2008).

A mieloperoxidase (MPO) é armazenada nos grânulos azurófilos dos polimorfonucleares e monócitos e durante a ativação celular é liberada dentro do vacúolo fagocítico, assim como no espaço extracelular (WINTERBOURN; VISSERS; KETTLE, 2000). Essa enzima cataliza a formação do ácido hipocloroso e radicais tirosil (LORIA et al., 2008). Essa reação ocorre a partir da liberação de íons cloreto (Cl^-) e de peróxido de hidrogênio (H_2O_2) presentes nos fagossomos. Essa substância é sintetizada, principalmente, no processo do metabolismo oxidativo também denominado de "*burst respiratório*" que ocorre nas células fagocíticas, em especial nos neutrófilos, por meio da enzima nicotinamida adenina dinucleotídeo fosfato (NADPH) oxidase (KLEBANOFF, 2005; ARATANI, 2006). A NADPH oxidase converte o oxigênio molecular (O_2) no ânion superóxido (O_2^-), o qual é convertido em H_2O_2 pela enzima superóxido desmutase (SOD) (WINTERBOURN et al., 2006). Este radical, juntamente com o HOCl e outras EROS é responsável pela atividade microbicida da MPO no interior dos fagossomas dos neutrófilos (LAU; BALDUS, 2006). O aumento nas concentrações de MPO, tanto nos tecidos como no plasma, é

utilizado como marcador de leucocitose neutrofílica, em condições de inflamação e sepse (FAITH et al., 2008). Além disso, a MPO também está envolvida na modulação da sinalização vascular e nas funções vasodilatadoras, via liberação de óxido nítrico (NO), durante o processo inflamatório (EISERICH et al., 2002).

Outras células também presentes na resposta imune inata são os mononucleares, que compreendem os monócitos. Estas células que liberam vários mediadores, dentre eles as citocinas e EROS. Além disso, estas células, nos tecidos, diferenciam-se em macrófagos (DALE; BOXER; LILES, 2008). As funções biológicas dos monócitos/macrófagos ativados são inúmeras: captura, processamento e morte de antígenos não-específicos e ativação de linfócitos (HASKO et al., 2007). Os macrófagos pertencem a uma população heterogênea de fagócitos mononucleares e possuem papel importante no comando e na execução do processo inflamatório, liberando também citocinas e EROS (STOUT; SUTTLES, 2004).

A enzima adenosina-deaminase (ADA) também merece destaque devido a sua participação no processo inflamatório. A adenosina-deaminase catalisa a reação de desaminação da adenosina e 2-desoxiadenosina em inosina e desoxinosina, e inosina que posteriormente é degradada em ácido úrico (VANNONI et al., 2004). Várias células são importantes fontes de adenosina, como por exemplo: os neutrófilos, os mononucleares e as células endoteliais, que liberam grandes quantidades de adenosina em situações de estresse oxidativo, inflamação e/ou infecção (CONLON; LAW, 2004; HASKO et al., 2007).

A adenosina foi primeiramente reconhecida como um regulador fisiológico do tônus vascular coronariano, no entanto descobriu-se que a adenosina regula funções celulares por meio da ligação ao receptor específico na superfície da membrana celular (HASKO; CRONSTEIN, 2004; HASKO et al., 2008). Já foram identificados quatro diferentes subtipos de receptores para adenosina (A_1, A_{2A}, A_{2B}, A_3) (JACOBSON; GAO, 2006). Várias atividades anti-inflamatórias já foram descritas para a adenosina, como: inibição da maturação e proliferação das células mononucleares (LANDELLS et al., 2000) e inibição do *"burst respiratório*" em monócitos/macrófagos (THIELE et al., 2004).

O óxido nítrico (NO) é também um importante mediador liberado na resposta inflamatória. A enzima óxido nítrico sintase (NOS) converte a L-arginina em L-citrulina e NO (TRIPATHI et al., 2007). O NO é liberado por várias células:

monócitos, mastócitos, neutrófilos, macrófagos, células endoteliais, epiteliais, da musculatura lisa vascular e fibroblastos (PERANZONI et al., 2008). O NO é uma molécula importante e versátil na regulação do tônus vascular, na neurotransmissão e na inflamação (HOFSETH, 2008). Esta molécula está envolvida também na imunidade inata como um agente tóxico contra organismos infecciosos. No entanto, o NO também pode regular a morte e a função dos linfócitos (BOGDAN et al., 2000). O NO induz a apoptose de macrófagos, a adesão dos neutrófilos e a síntese de citocinas pelos leucócitos (TRIPATHI et al., 2007).

Estudos já identificaram várias isoformas da enzima óxido nítrico sintase: eNOS, iNOS e nNOS. A eNOS e a nNOS são expressas em: neurônios, macrófagos, células dendriticas, células natural killer (NK) e linfócitos B e T, e sua ativação é dependente da liberação de cálcio calmodulina (GARCIA; STEIN, 2006; PERANZONI et al., 2008). A iNOS, também expressa em macrófagos, células dendríticas, células natural killer e linfócitos B e T, é a forma induzida da enzima, e sua expressão é regulada principalmente através da liberação de citocinas por células ativadas (TRIPATHI et al., 2007). O NO liberado por essa via possui papel importante na defesa do organismo por sua atividade antimicrobiana (PACHER; BECKMAN; LIAUDET, 2007). Estudos revelaram que as espécies reativas de NO possuem atividade antimicrobiana por meio da formação de metabólitos, como por exemplo: peroxinitrito, 5-nitrosotiol e dióxido de nitrogênio, que são capazes de promover lesão ao DNA do microorganismo (GARCIA; STEIN, 2006; PACHER; BECKMAN; LIAUDET, 2007).

As citocinas são outros importantes mediadores da resposta inflamatória. Elas interagem com os receptores de membrana específicos nas células na tentativa de regular a resposta inflamatória (TAYAL; KALRA, 2008). Estes mediadores, dependendo do estímulo, possuem efeitos pró ou anti-inflamatórios. O balanço entre as citocinas têm um papel fundamental na defesa do hospedeiro. Entre as principais citocinas pró-inflamatórias secretadas pelas células fagocíticas ativadas, inclui-se a interleucina-1 beta (IL-1β) (KIM; MOUDGIL, 2008).

A IL-1β é secretada principalmente por monócitos, macrófagos e linfócitos, mas também por neutrófilos e células dendríticas, e promove a proliferação e a diferenciação das células do sistema imune inato e adaptativo (JOOSTEN; VAN DEN BERG, 2006; OLIVEIRA et al., 2008). Além disso, a liberação da IL-1β está relacionada a doenças de caráter inflamatório, como a artrite reumatóide

(JOOSTEN; VAN DEN BERG, 2006), a osteoartrite (PUJOL et al., 2008), a aterosclerose (JAWIEN, 2008) e a sepse (KURT et al., 2007).

1.4. MODELOS DE INFLAMAÇÃO

Para a avaliação do mecanismo de ação anti-inflamatória de diferentes fármacos e/ou plantas, vários modelos de inflamação já foram descritos, como a pleurisia, a bolsa de ar, o edema de pata, a artrite e o implante de esponjas embebidas em agentes irritantes (SEDGWICK; LEES, 1986).

A inflamação local induzida pela carragenina (pleurisia ou edema de pata) é um modelo muito utilizado para estudar os mediadores envolvidos nas reações vasculares associadas com a inflamação aguda e/ou crônica (CRISAFULLI et al., 2006). O modelo de inflamação da pleurisia é caracterizado pelo aumento de leucócitos do tipo neutrófilos e da exsudação que ocorre 4 h após a administração da carragenina na cavidade pleural de camundongos. No fluído da cavidade pleural de animais inflamados por carragenina ocorre a liberação de vários mediadores pró-inflamatórios como citocinas e espécies reativas de oxigênio (SALEH; CALIXTO; MEDEIROS, 1999; MARIOTTO et al., 2008).

Neste trabalho, optou-se pelo modelo experimental de pleurisia, uma vez que esta técnica foi padronizada em nosso laboratório, é de fácil execução e é considerado um bom modelo para avaliar mecanismo de ação de plantas ou fármacos que possuem propriedades anti-inflamatórias.

1.5. ATIVIDADE ANTIBACTERIANA

Desde a descoberta dos antimicrobianos, em 1930, até 1980, acreditava-se que os antibióticos eram capazes de curar todas as infecções bacterianas. Entretanto, desde 1980, o crescente aumento da resistência aos agentes antimicrobianos e o surgimento de microorganismos multiresistentes tem sido motivo de crescente preocupação. Entretanto, a verdadeira magnitude desse problema somente é observada quando se tornam evidentes as poucas perspectivas para o desenvolvimento de novos antibióticos a curto e médio prazo (DROND; JUSTRIBÓ, 2007).

Apesar de a indústria farmacêutica produzir um número significativo de novos antibióticos nas últimas décadas, a resistência microbiana a esses fármacos também aumentou. Em geral, as bactérias naturalmente induzem o processo de mutação genética tornando-se resistentes aos antimicrobianos usualmente utilizados. Além disso, algumas bactérias são capazes de transmitir esta resistência a outras bactérias (COHEN, 1992). O problema da resistência bacteriana é crescente e a perspectiva futura do uso de fármacos com propriedades antibacterianas é incerta. Dessa forma, torna-se, importante adotar medidas para a resolução desse problema, dentre elas: o controle no uso indiscriminado de antibióticos, o desenvolvimento de pesquisas para melhor compreensão dos mecanismos genéticos da resistência microbiana e o desenvolvimento de novos medicamentos, sintéticos ou de origem natural, com atividade antimicrobiana (DROND; JUSTRIBÓ, 2007; COS et al., 2006).

A pesquisa direcionada a busca de novas substâncias antimicrobianas deve ser contínua e várias fontes devem ser exploradas, pois, além das substâncias químicas, os produtos naturais ainda são as maiores fontes de agentes terapêuticos inovadores, inclusive para as doenças infecciosas (COS et al., 2006).

É importante ressaltar que quando se pesquisa produtos naturais com possível atividade antibacteriana, é necessário utilizar microrganismos padronizados que podem ser adquiridos da American Type Culture Collection (ATCC), European Culture Collections Organisation (ECCO), entre outras (VANDEN BERGHE; VLIETINCK, 1991). Os métodos de triagem atualmente disponíveis para detectar atividade antibacteriana de produtos naturais enquadram-se dentro de três grupos: 1) métodos de difusão; 2) métodos bioautográficos; e 3) métodos de diluição. Os ensaios de difusão e/ou bioautográficos são considerados qualitativos. Já os métodos de diluição podem ser considerados semiquantitativos ou quantitativos (COS et al., 2006). Nesse trabalho foi utilizada a técnica de Microdiluição em Caldo para a determinação da concentração inibitória mínima (CIM), uma vez que esta apresenta como vantagens a possibilidade de: 1) quantificar a potência do materal teste, 2) avaliar a eficácia de compostos polares e apolares, 3) avaliar a eficácia de compostos com alto peso molecular, e também, ser uma técnica de fácil execução, alta reprodutibilidade e sensível (COS et al., 2006; GAUTAM; SAKLANI; JACHAK, 2007).

OBJETIVOS

2.1. OBJETIVO GERAL

Estudar o mecanismo de ação anti-inflamatória e antibacteriano do extrato bruto, frações e compostos isolados das partes aéreas da *Lotus corniculatus* v. São Gabriel.

2.2. OBJETIVOS ESPECÍFICOS

I. Avaliar o efeito anti-inflamatório do extrato bruto, frações e compostos isolados sobre a migração dos leucócitos (polimorfonucleares e mononucleares) e a exsudação no modelo de pleurisia, induzido pela carragenina, em camundongos.

II. Investigar o efeito do material vegetal sobre a atividade da mieloperoxidase (MPO) e da adenosina-deaminase (ADA), bem como as concentrações de nitrito/nitrato (NO^x) e da interleucina-1 beta (IL-1β), no modelo de pleurisia, induzido pela carragenina , em camundongos..

III. Avaliar a atividade antibacteriana do material vegetal.

3. ARTIGO SUBMETIDO À PUBLICAÇÃO – FOOD CHEMISTRY

Evaluation of the Anti-inflammatory Efficacy of *Lotus corniculatus*

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Abstract

The anti-inflammatory effects of the crude extract (CE) of *Lotus corniculatus* v. São Gabriel and its derived hexane (HEX), ethyl acetate (AcOEt), n-butanol (BuOH) and aqueous (AF) fractions, and isolated compounds kaempferitrin, oleanolic acid and β-sitosterol, in a mouse model of pleurisy induced by carrageenan were investigated. Swiss mice were used in the *in vivo* experiments. The crude extract of *Lotus corniculatus* and its derived fractions, and also its isolated compounds, inhibited leukocytes, exudation, and myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities, as well as nitrite/nitrate concentration and interleukin-1 beta (IL-1β) level ($p < 0.05$). *Lotus corniculatus* showed important anti-inflammatory activity by inhibition not only of leukocytes and/or exudation, but also of pro-inflammatory enzymes and mediators such as MPO, ADA and IL-1β and its constituents kaempferitrin, oleanolic acid and β-sitosterol may well account for it.

Keywords: *Lotus corniculatus*, Anti-inflammatory Activity, Pleurisy, Mediators of Inflammation, Mice.

1. Introduction

Plants are a good source of useful anti-inflammatory agents. The continuing search for novel anti-inflammatory substances especially from plants with historically documented or pharmacological properties, holds considerable nutraceutical and/or pharmaceutical promise (Kaplan, Mutlu, Benson, Fields, Banan, & Kesshavarzian, 2007). *Lotus corniculatus* v. São Gabriel (Fabaceae), also known as “Cornichão” in Brazil, is used for cattle grazing pasture and has a potential benefit of silage supplementation for increased milksolids yield in summer when low pasture growth rates and quality may otherwise limit production (Aerts, Barry, & McNabb, 1999). There are many nutritional effects of *Lotus corniculatus*, such as increase of essential amino acid absorption (Waghorn, Ulyatt, John, & Fisher, 1987), ovulation rate, and production of milk protein lactose (Wang, Douglas, Waghorn, Barry, & Foote, 1996). This herb is also used as an important anti-helmintic substance in cattle (Aerts, Barry, & McNabb, 1999).

Studies had demonstrated that these effects are due to the presence of secondary metabolites named proanthocyanidins, or condensed tannins (Xie & Dixon, 2005). The proanthocynidins also have importantaha antioxidant (Kandil et al., 2002), immunomodulatory (Lin, Kuo, & Chou, 2002), analgesic and anti-inflammatory effects (Subarnas & Wagner, 2000).

Previous investigations into the chemical composition of this herb have identified the following compounds: flavonoids (kaempferol and quercetin) (Reynaud & Lussignol, 2005), oleanolic acid and saponins (Walter, 1961). Studies have also reported that kaempferol and quercetin modulate the inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), reactive C-protein (CRP), and nuclear factor kappa B (NF-κB) (García-Mediavilla et al., 2007). Other studies have shown that oleanolic acid and saponins possess anti-inflammatory, (Giner-Larza et al., 2001; Wang, Gao, Kou, Zhu, & Yu, 2008) and antitumoral properties (Chen et al., 2008; Wang et al., 2008).

Motivated by rare studies of the anti-inflammatory properties of *Lotus corniculatus,* the crude extract and derived fractions of this herb was studied by

analyzing its effects upon leukocyte migration, exudation concentration and myeloperoxidase/ adenosine-deaminase activities, as well as nitrite/nitrate concentration and interleukin-1 beta levels, in the inflammation induced by carrageenan in the mouse model of pleurisy. We also isolated and identified the components from *Lotus corniculatus* that were responsible for the anti-inflammatory activity.

2. Material and Methods

2.1 Plant material

Lotus corniculatus L. var. São Gabriel was collected in November 2006 in Lages, Santa Catarina State, Brazil. The material was identified by the botanist Prof. Dr. Daniel de Barcelos Falkenberg at the Department of Botany of the Federal University of Santa Catarina, Florianópolis, SC, Brazil. A voucher specimen was deposited in the Herbarium at the same university (FLOR 18.770).

2.2 Preparation of plant extracts

The aerial parts of *Lotus corniculatus* var. São Gabriel were air-dried protected from light at room temperature (25^{o}C) for one week. Subsequently the dried aerial parts (620g) was grounded into particles (1.5 mm of diameter) using a knife mill (Mill TE-651, Tecnal, Piracicaba, SP). The grounded material was placed into a plastic tube contained 5 L of ethanol 96% (plant 1:8, w/v) at room temperature for two days. In the next step, the ethanol was removed and evaporated under reduced pressure (600 mm Hg) (Vacuum Q-355A2, Quimis, Diadema, SP) using a rotavapor apparatus at 55^{o}C to obtain the crude extract. This procedure of extraction was done exhaustively more three times in one week until 12-fold in a one month to obtained the maximal yield of the crude extract. Finally this procedure yelded 78 g of the crude extract.

The CE was fractionated by liquid-liquid extraction using solvents in growing order of polarity, resulting in hexane (HEX: 7.82 g), ethyl acetate (AcOEt: 11.4 g), n-butanol (BuOH: 5.24 g) and aqueous (AF: 30.8 g) fractions.

2.3 Preliminary phytochemical analysis

A preliminary phytochemical screening of the crude extract of *Lotus corniculatus* was carried out to detect the presence of phenols, tannins, anthocyanins, anthocyanidins, flavonoids, xanthones, steroids, triterpenes and saponins using colourimetric reactions following the methodologies described by WHO, 1984.

2.4 Isolation and identification of the compounds

The hexane fraction was subjected to silica gel column chromatography and eluted with a gradient of HEX/EtOAc, resulting in the isolation of two terpenoids: β-sitosterol as colourless crystals, m.p 137-139ºC, 76 mg, from fraction eluted with HEX/EtOAc (90:10,v/v), and oleanolic acid, as a white powder, m.p. 279-282 ^{o} C, 25 mg, from HEX/EtOAc (70:30, v/v) . The chromatographyc fractionation on silica gel of the EtOAc fraction afforded a crude flavonoid from EtOAc/EtOH (50:50, v/v) eluate that was further purified by flash chromatography using ethyl acetate/water/formic acid/acetic acid- (70:20:3:2, v/v/v/v) as eluent, yielding 45 mg of kaempferitrin as a yellow needle crystals, m.p. 198.5-201.3 ^{o}C. The structures of the known compounds were identified by their spectroscopic data (^{1}H NMR, ^{13}C NMR (Varian AS-400 -Palo-Alto, CA, USA), and IR- Perkin Elmer FTIR 16 PC, Beaconsfield, England) measurement, comparison with spectral data obtained from the literature (Hung & Yen, 2001; Urgaonkar & Shaw, 2007), and co-TLC with authentic samples.

2.5 Structure elucidation of the compounds

The chemical structure of each isolated compound was determined on the basis of its physical characteristics, spectral data produced by infrared

analysis (Perkin Elmer FTIR 16 PC, Beaconsfield, England) and nuclear magnetic resonance (^{1}H and ^{13}C-NMR) recorded on a Varian AS-400 (Palo-Alto, CA, USA) spectrometer operating at 400 and 100 MHz, respectively. Thyn Layer Chromatography (TLC) was carried out on silica gel 60 F_{254} plates (Macherey-Nagel, Düren, Germany). Finally, the structures of the compounds isolated were confirmed by comparing with reference data previously reported from available reliable sources.

2.6 Animals

Swiss mice, weighing 18-25 g, were housed under standardized conditions (room at constant temperature (22 ± 2℃) with alternating 12 h periods of light and darkness), humidity 50-60%, and they were fed on a standard mouse diet with water *ad libitum* before use. This study was approved by the Committee for Ethics in Animal Research of the Federal University of Santa Catarina (Protocol number – PP00180), and experiments were performed in accordance with the norms of the Brazilian College of Animal Experimentation.

2.7 Experimental protocol

Initially, for analysis of the dose-response curve, different groups of animals were treated with different doses of crude extract (CE: 100 - 400 mg/kg) of *Lotus corniculatus* var. São Gabriel or its derived fractions or isolated compounds: hexane fraction (HEX: 50 - 200 mg/kg), ethyl acetate fraction (AcOEt: 100 - 400 mg/kg), n-butanol fraction (BuOH: 50 - 200 mg/kg), aqueous fraction (AF: 25 - 200 mg/kg), kaempferitrin (50 and 100 mg/kg), oleanoic acid (10 - 100 mg/kg) or β-sitosterol (10 - 100 mg/kg) administered by intraperitoneal route (i.p.) 0.5 h before pleurisy induction by carrageenan (Cg 1%) that was administered by intrapleural route (i.pl.). In parallel, some animals received an injection of either sterile saline (NaCl, 0.9%) (negative - control group) or carrageenan (positive - control group) administered by intrapleural (i.pl.) route. After 4 h the animals were killed with an overdose of ether, the thorax was opened, and the pleural cavity was washed with 1.0 mL of sterile phosphate buffered saline (PBS) (pH 7.6), composition: NaCl (130 mmol), Na_2HPO_4: (5

mmol), KH_2PO_4 (1 mmol) and distillated water (1000 ml) containing heparin (20 IU/mL). Leukocytes and exudation were then evaluated.

In another set of experiments employed to establish the time course profile, different groups of animals were pre-treated with a single dose of CE (200 mg/kg), HEX (100 mg/kg), AcOEt (200 mg/kg), BuOH (100 mg/kg), AF (100 mg/kg), kaempferitrim (100 mg/kg), oleanolic acid (50 mg/kg) or β-sitosterol (50 mg/kg) administered at different time points (0.5 - 4 h) and the same inflammatory parameters were evaluated 4 h after carrageenan administration.

After choosing the best dose and period of pre-treatment required for the crude extract of *Lotus corniculatus* and its derived fractions, as well as its isolated compounds, that inhibit leukocytes and/or exudation, different groups of animals were treated with CE (200 or 400 mg/kg), HEX (100 or 200 mg/kg), AcOEt (200 or 800 mg/kg), BuOH (100 mg/kg), AF (50 or 400 mg/kg), kaempferitrin (100 mg/kg), oleanolic acid (50 mg/kg) or β-sitosterol (50 mg/kg) administered 0.5 h prior to pleurisy induction to analyze their effects upon myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities, as well as nitrite/nitrate concentration (NO_x) and interleukin-1 beta (IL-1β) levels.

Dexamethasone (potent inhibitor of phospholipase A_2, of the expression of both induced NOS and of COX-2, among others, 0.5 mg/kg) and indomethacin (cyclooxygenase inhibitors, 5 mg/kg, i.p.) administered by intraperitoneal route (i.p.) 0.5 h before pleurisy induction, were used as anti-inflammatory drugs.

2.8 Quantification of leukocyte migration and exudation

After killing the animals, samples of the fluid leakage of the pleural cavity were collected to determine the total and differential leukocyte contents, and exudation. Total leukocyte counts were determined in a Newbauer chamber, and cytospin preparations of fluid leakage were stained with May-Grünwald-Giemsa for the differential count (Saleh, Calixto, & Medeiros, 1996). The degree of exudation was determined by measuring the amount of Evans blue dye

extravasation. Thus, in each experimental group, animals were challenged 0.5 h before the inflammation induction with a solution of Evans blue dye (25 mg/kg) administered by intravenous route (i.v.) in order to evaluate the exudation in the pleural cavity. On the day of the experiment, a batch of stored samples was thawed at room temperature and the amount of dye was estimated by colourimetric using an Elisa plate reader (Organon Teknika, Roseland, NJ, USA) at 620 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50 µg/mL.

2.9 Quantification of nitrite/nitrate concentration

Nitric oxide and its breakdown products nitrite (NO_2^-) and nitrate (NO_3^-) were measured using the Griess method (Green, Wagner, Glowski, Skipper, Wishnok, & Tannenbaum, 1982). Samples of exudates from the pleural cavity were collected, separated and stored at -20ºC. Nitrite/nitrate concentration was determined and the concentrations were estimated by means of colourimetric measurement at 450 nm on an ELISA plate reader (Organon, Teknika, Roseland, NY, USA) by interpolation from a standard curve (0-150 µM). The results were expressed as µM.

2.10 Quantification of myeloperoxidase activity

Standard samples with different concentrations of myeloperoxidase (from human neutrophils, Sigma: M6908, St. Louis, MO, USA) were prepared in order to obtain a standard curve in the range of 0.07-140 mU/mL. Pleural cavity fluid samples (40 µL) and standards were transferred to cuvettes and the reaction was initiated with the addition of 360 µL of assay buffer (0.167 mg/mL of o-dianisidine (3, 3'-dimethoxybenzidine; fast blue B) dihydrochloride and 0.0005% H_2O_2). The reaction was stopped with sodium azide 1%. Afterwards, the samples were centrifuged at 50 x g for 5 min, the supernatants were separated, and the rates of changes in absorbance at 520 nm were determined. The mieloperoxidase activity was estimated by interpolation from the standard curve by means of colourimetric measurements on an ELISA plate reader (Organon

Teknika, Roseland, NJ, USA) (Rao, Curie, Scaffer, & Isakson, 1993). The results were expressed as mU/mL.

2.11 Quantification of adenosine deaminase activity

Initially, standard samples (final volume of 500 µL) with different volume concentrations of $NaH_2PO_4.H_20$ (35 mM), $Na_2HPO_4.H_2O$ (15 mM) and NH_3SO_4 (15 mM) were prepared in order to obtain a standard curve in the range of 10-50 U/L. Pleural cavity fluid samples (20 µL) were transferred to cuvettes and the reaction was initiated by the addition of adenosine phosphate buffered solution (pH 6.5, 500 µL, composition: $NaH_2PO_4.H_2O$ (35 mM), $Na_2HPO_4.12H_2O$ (15 mM) and adenosine (0.5 mM)). After incubation for 1 hour at 37°C, the reaction was halted with the addition of a solution (1000 µL) of phenol (1 mM) and nitroprussiate (0.17 mM), plus alkaline buffer (1000 µL: NaCl: 11 mM). This solution (final volume 2000 µL) was also added to the cuvettes with the different standard samples. Afterwards, the rate of change in absorbance at 620 nm was determined. ADA activity was estimated by interpolation from the standard curve by means of colourimetric measurements on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) (Giusti & Galanti, 1984). The results were expressed as U/L.

2.12 Quantification of IL-1β levels

For analysis of IL-1β levels, samples of exudates were collected and immediately prepared for the analysis of cytokine levels. In this protocol, commercially available kits were used with monoclonal-specific antibodies for each cytokine. The cytokine level was measured by enzyme-linked immunosorbent assay (ELISA), using the kits according to the manufacturers' instructions. The range of values detected by this assay was: IL-1β (100 - 6400 pg/mL). The intra- and inter-assay coefficients of variation (CV) for IL-1β were: intra CV: IL-1β = 6.2 ± 0.4%; inter CV: IL-1β = 5.1 ± 0.6%, with a sensitivity value of IL-1β = 1.7 pg/mL. The cytokine concentration was estimated by means of colourimetric measurements at 450 nm on an ELISA plate reader (Organon Teknika, Roseland, NJ, USA) by interpolation from a standard curve.

2.13 Drugs

The following drugs and reagents were used: carrageenan (degree IV), human neutrophil myeloperoxidase, indomethacin, (Sigma Chemical Co., St. Louis, MO, USA), dexamethasone, (Ache pharmaceutical laboratories S.A., São Paulo, SP, Brazil), and Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of rat IL-1β. Organic solvents: Acetone, Chloroform, n-Hexane, Ethyl acetate, n-Butanol, Methanol, and Ethanol, all analytical grade, were purchased from Synth (Diadema, SP, Brazil). Other reagents used were also of analytical grade and were obtained from different commercial sources.

2.14 Statistical Analysis

The data is reported as the mean ± SEM. Significant differences between groups were determined by two-way analysis of variance (ANOVA) followed by Student's-Newman-Keuls post-hoc tests, and the significant difference was set at $p < 0.05$.

3. Results

3.1 Phytochemical Analysis

In this study, preliminary phytochemical analysis showed that the crude extract of *Lotus corniculatus* var. São Gabriel contained a significant amount of flavonoids, steroids and terpenoids. Representing the steroids and terpenoids classes, we isolated the β-sitosterol (Compound **1**) (Fig. 1A) and the oleanolic acid (Compound **2**:) (Fig. 1B) from hexane fraction. In the ethyl acetate fraction, the flavonoid *O*-heteroside kaempferitrin (Compound **3**) (Fig. 1C) as representative of flavonoids was isolated. These compounds isolated from the specie *L. corniculatus* not reported previously to this variety. β-sitosterol represented 2,08% and the oleanolic acid 0.67% of the hexane fraction. Further, the kaempferitrin represented 0.51% of the ethyl acetate fraction.

3.2 Effects of crude the extract of Lotus corniculatus v. São Gabriel, its derived fractions and isolated compounds upon leukocyte migration and exudation

The crude extract of *Lotus corniculatus* (CE: 200 and 400 mg/kg) significantly decreased leukocyte migration from 38.7 ± 5.8 to 48.0 ± 7.7% ($p < 0.01$), neutrophils from 37.7 ± 5.2 to 46.4 ± 8.4% ($p < 0.01$), mononuclears from 44.2 ± 9.4 to 57.3 ± 11.6% ($p < 0.05$), and exudation from 25.4 ± 5.3 to 50.5 ± 2.2% ($p < 0.05$). CE (100 mg/kg) did not modify the studied inflammatory parameters ($p > 0.05$) (Table 1).

The hexane fraction (HEX: 100 and 200 mg/kg) significantly suppressed leukocytes from 26.1 ± 5.0 to 53.2 ± 12.5% ($p < 0.01$) and neutrophils from 21.8 ± 6.7 to 68.3 ± 17.3% ($p < 0.05$). HEX (100 mg/kg) also inhibited mononuclears by 45.5 ± 12.2% ($p < 0.05$) and at the dose of 200 mg/kg, HEX also inhibited exudation by 51.7 ± 12.0% ($p < 0.05$). The HEX fraction (50 mg/kg) did not alter any of the studied inflammatory parameters ($p > 0.05$) (Table 1).

The ethyl acetate fraction (AcOEt: 200 and 400 mg/kg) produced a significant inhibition of leukocyte migration from 21.0 ± 6.5 to 41.9 ± 8.7% ($p < 0.05$), neutrophils from 20.9 ± 7.4 to 56.1 ± 5.8% ($p < 0.05$) and exudation from 33.6 ± 6.6 to 34.8 ± 10.9% ($p < 0.05$). Nevertheless, the AcOEt fraction (100 mg/kg) did not inhibit these inflammatory parameters ($p > 0.05$). Mononuclears also were not inhibited by the AcOEt fraction ($p > 0.05$) (Table 1).

The n-butanol fraction (BuOH: 100 and 200 mg/kg) produced a significant decrease of leukocytes from 34.9 ± 9.1 to 39.2 ± 4.9% ($p < 0.01$) and neutrophils from 36.0 ± 5.8 to 36.3 ± 8.8% ($p < 0.01$). The BuOH fraction (200 mg/kg) also decreased mononuclears by 54.8 ± 6.2% ($p < 0.05$), and the dose of 50 mg/kg did not vary the leukocyte content ($p > 0.05$), but caused a significant enhancement of exudation by 50.7 ± 16.0% ($p < 0.05$) (Table 1).

The aqueous fraction (AF: 50 - 200 mg/kg) caused a significant inhibition of leukocytes from 31.6 ± 8.1 to 43.0 ± 5.8% ($p < 0.05$), and neutrophils from 30.7 ± 8.5 to 44.2 ± 6.7% ($p < 0.01$), but failed to reduce mononuclears ($p > 0.05$). The AF fraction (200 mg/kg) was also effective in inhibiting exudation by

44.7 ± 3.8% ($p < 0.05$). The doses of 25 and 50 mg/kg of this fraction increased exudation from 31.4 ± 12.3% to 38.2 ± 5.9% ($p < 0.05$). The AF fraction (25 mg/kg) also did not inhibit total and differential leukocytes ($p > 0.05$) (Table 1).

The study of the effect of the isolated compounds showed that kaempferitrin, at the dose of 100 mg/kg, was also effective in significantly inhibiting leukocytes by 35.5 ± 8.0% and neutrophils by 33.7 ± 8.8% ($p < 0.05$) (Table 2).This compound (50 and 100 mg/kg) also inhibited mononuclears by 65.2 ± 20.9% and 43.5 ± 5.7%, and exudation by 33.3 ± 9.8% and 37.0 ± 3.3% ($p < 0.05$) (Table 2).

The oleanolic acid (25 - 100 mg/kg) significantly suppressed leukocytes from 21.2 ± 5.8 to 52.4 ± 11.2% ($p < 0.05$) and neutrophils from 27.9 ± 7.5 to 71.6 ± 2.8% ($p < 0.05$). This compound failed to change mononuclears ($p > 0.05$). The dose of 10 mg/kg of this compound did not inhibit the inflammation caused by carrageenan ($p > 0.05$). Under the same conditions, oleanolic acid (25 and 50 mg/kg) reduced exudation levels from 35.3 ± 1.9 to 42.6 ± 3.9% ($p < 0.05$) (Table 2).

The β-sitosterol (25 - 100 mg/kg) significantly decreased leukocytes from 15.1 ± 1.9 to 33.9 ± 9.4% ($p < 0.05$) and neutrophils from 19.1 ± 2.9 to 36.7 ± 12.6% ($p < 0.05$). This compound did not modify mononuclears ($p > 0.05$), but at doses of 25 and 50 mg/kg it significantly inhibited the exudation from 28.2 ± 10.0% to 33.1 ± 2.3% ($p < 0.05$). The dose of 10 mg/kg did not vary the inflammatory parameters ($p > 0.05$) (Table 2).

The time course profile for the crude extract of *Lotus corniculatus* and its derived fractions, as well as its isolated compounds, showed that they were effective in inhibiting the studied inflammatory parameters when they were administered 0.5 h before carrageenan. It is important to note that the BuOH fraction (100 mg/kg), as well as the AcOEt fraction (200 mg/kg), had a long-lasting anti-inflammatory effect, since they were able to decrease the inflammation caused by carrageenan for up to 2 h of pre-treatment (results not shown).

As expected, dexamethasone (0.5 mg/kg, i.p.) and indomethacin (5.0 mg/kg, i.p.) significantly inhibited leukocytes by 71.7 ± 5.0% and 63.5 ± 5.0% (p

< 0.01), neutrophils by 73.8 ± 4.2% and 64.9 ± 5.0% ($p < 0.01$), mononuclears by 60.7 ± 2.0% and 57.1 ±1.0% ($p < 0.05$), and exudation by 43.3 ± 7.8% and 31.0 ± 5.0% ($p < 0.05$), in the inflammation response induced by carrageenan, respectively (Tables 1 and 2).

3.3 Effects of the crude extract of Lotus corniculatus v. São Gabriel, its derived fractions and isolated compounds upon myeloperoxidase and adenosine-deaminase activities

The pre-treatment (0.5 h) of animals with crude extract of *Lotus corniculatus* and its derived fractions, and also its isolated compounds, caused a significant decrease in myeloperoxidase activity (% of inhibition: CE (200 mg/kg): 63.7 ± 6.0, HEX (100 mg/kg): 47.4 ± 11.6, AcOEt (200 mg/kg): 57.9 ± 4.4, AF (100 mg/kg): 34.3 ± 12.6, kaempferitrin (100 mg/kg): 90.6 ± 3.4, oleanolic acid (50 mg/kg): 45.5 ± 21.6 and β-sitosterol (50 mg/kg): 73.0 ± 13.1) ($p < 0.05$), and adenosine-deaminase activities (% of inhibition: CE (200 mg/kg): 67.5 ± 6.8, HEX (100 mg/kg): 54.4 ± 15.0, AcOEt (200 mg/kg): 94.0 ± 1.4, BuOH (100 mg/kg): 67.1 ± 9.0, AF (50 mg/kg): 64.5 ± 8.8, kaempferitrin (100 mg/kg): 77.1 ± 7.9, β-sitosterol (50 mg/kg): 68.7 ± 6.0) ($P < 0.01$), except for the BuOH fraction that did not inhibit MPO, and oleanolic acid that did not decrease ADA activities ($p > 0.05$) (Table 3).

Dexamethasone and indomethacin were effective in inhibiting myeloperoxidase by 59.4 ± 7.0% and 64.4 ± 7.0% ($p < 0.01$), and adenosine-deaminase activities by 71.4 ± 6.0% and 64.4 ± 6.0%, respectively ($p > 0.05$) (Table 3).

3.4 Effects of the crude extract of Lotus corniculatus v. São Gabriel, its derived fractions and isolated compounds upon IL-1β levels

The crude extract of *Lotus corniculatus* and its derived fractions, as well as its isolated compounds, caused a significant decrease of IL-1β (% of

inhibition: CE (200 mg/kg): 24.9 ± 6.9, HEX (100 mg/kg): 43.2 ± 8.6, AcOEt (200 mg/kg): 36.9 ± 24.0, BuOH (100 mg/kg): 38.1 ± 17.2, AF (100 mg/kg): 35.9 ± 2.6, and kaempferitrim (100 mg/kg): 61.1 ± 13.8) ($p < 0.05$). The oleanolic acid (50 mg/kg) caused a significant increase of the IL-1β levels by 61.4 ± 19.7% ($p < 0.05$), and β-sitosterol (50 mg/kg) did not alter the IL-1β level ($p > 0.05$) (Table 3).

Dexamethasone and indomethacin also significantly inhibited the IL-1β level by 55.5 ± 6.0% and 49.8 ± 6.0%, respectively ($p < 0.05$) (Table 3).

3.5 Effects of the crude extract of Lotus corniculatus v. São Gabriel, and its derived fractions and isolated compounds upon nitrite/nitrate concentration

Although the best dose of the crude extract of *Lotus corniculatus* and its derived fractions was determined, only higher doses caused a significant decrease of nitrite/nitrate concentration (% of inhibition: CE (400 mg/kg): 30.4 ± 5.6, HEX (200 mg/kg): 34.3 ± 9.9, AcOEt (800 mg/kg): 38.4 ± 5.4 and AF (400 mg/kg): 44.1 ± 4.2) ($p < 0.05$). The isolated compounds also inhibited this inflammatory parameter (% of inhibition: kaempferitrin (100 mg/kg): 32.9 ± 4.2, oleanolic acid (50 mg/kg): 35.9 ± 5.6 and β-sitosterol (50 mg/kg): 35.2 ± 3.2) ($p < 0.05$). Dexamethasone and indomethacin pre-treatment of animals presented an inhibitory effect on nitrite/nitrate concentration by 75.6 ± 2.6% and 50.0 ± 1.3%, respectively ($p < 0.01$) (Table 4).

4. Discussion

Data from this study indicates that the crude extract of *Lotus corniculatus* and its derived fractions had an important anti-inflammatory effect in a murine model of pleurisy. Although different doses of *Lotus corniculatus* and its derived fractions were necessary to inhibit this inflammatory reaction. The results show that this herb exhibits a distinct inhibitory profile when compared to conventional drugs, such as indomethacin and dexamethasone.

The anti-inflammatory effect of *Lotus corniculatus* was more pronounced in relation to the inhibition of leukocytes and exudation. To understand the modulation of leukocytes by this herb, we also studied its effect upon MPO and ADA activities, which are known to be markers of activated neutrophils and mononuclears, respectively (Fröde & Medeiros, 2001). Our results demonstrated that the crude extract of *Lotus corniculatus* and its derived fractions significantly attenuated both MPO and ADA activities. These results reveled that *Lotus corniculatus* not only inhibited the leukocyte influx to the site of the inflammatory response, but also the activated leukocytes (Fröde & Medeiros, 2001).

The role of IL-1β in the inflammatory response is well known. The effect of *Lotus corniculatus* upon IL-1-β levels was also analyzed. In this experiment we observed a significant inhibition of this important mediator by *Lotus corniculatus* and its derived fractions.

Nitric oxide (NO) is another important pro-inflammatory substance that is released in the acute and chronic inflammatory response and is related to the exudation and cellular chemotaxis (Tripathi, Tripathi, Kashyap & Singh, 2007). Once again, *Lotus corniculatus* showed an anti-inflammatory response, since the crude extract of this herb and its derived fractions, except for BuOH, caused a significant decrease of nitrite/nitrate concentration.

All the studied fractions isolated from *Lotus corniculatus* presented an important anti-inflammatory effect. The distinct biological effect may be linked to differences among chemical structures. This fact is observed mainly in the HEX and AcOEt fractions, which revealed a more pronounced anti-inflammatory response than the other fractions, since they were able to inhibit all the studied inflammatory parameters. On the contrary, BuOH did not inhibit either MPO activity or nitrite/nitrate concentration.

In an attempt to evaluate the anti-inflammatory potential of the isolated compounds from these fractions, first we isolated kaempferitrin from the AcOEt fraction, and β-sitosterol and oleanolic acid from the HEX fraction

Likewise, we tested the effects of these compounds upon the same studied pro-inflammatory parameters. Our results revealed that all compounds

inhibited leukocytes, neutrophils, exsudation, and MPO activity, as well as NO levels.

Although, β-sitosterol was a main compound isolated from *Lotus corniculatus*, kaempferitrin demonstrated a better anti-inflammatory effect since it was 3.8-fold, 1.5-fold and 1.3-fold more effective in inhibiting MPO, ADA and IL-1β levels, respectively, than indomethain, and 4.3-fold and 1.2-fold more effective in inhibiting MPO and ADA activities than dexamethasone.

Similar results have also been observed with kaempferitrin that inhibits nitric oxide levels, as well as pro-inflammatory cytokines such as TNF-α and IL-12, in murine macrophages stimulated by LPS/IFN-γ (Fang, Rao, & Tzeng, 2005).

In relation to oleanolic acid, studies from literature have demonstrated the anti-inflammatory effect of this substance by reducing the paw edema induced by dextran, and also inhibiting the nitric oxide release via down-regulation of NF-κB in murine macrophage cells induced by LPS (Singh, Singh, Bani, Gupta, & Banerjee, 1992; Suh et al., 2007).

For β-sitosterol, studies have demonstrated important anti-inflammatory activity not only by inhibiting IL-12 from human Jukart T cells stimulated by concanavalin (ConA) or by phorbol-12-myristate-13-acetate plus ionomycin (PMA + IoM) (Aherne & O'Brien, 2008), but also by decreasing the eosinophil influx, mucus secretion, and IL-4/ IL-5 expression in a murine model of asthma induced by ovalbumin (Yuk et al., 2007), and the ear oedema induced by acetone in mice (Mavar-Manga, Haddad, Pieters, Baccelli, Penge, & Quetin-Leclercp, 2008).

In conclusion, *Lotus corniculatus* showed an important anti-inflammatory property, and its constituents kaempferitrin, oleanolic acid and β-sitosterol may well account for it. These compounds have potential as novel lead compounds for the future development of therapeutic intervention in the treatment of patients with inflammatory disorders.

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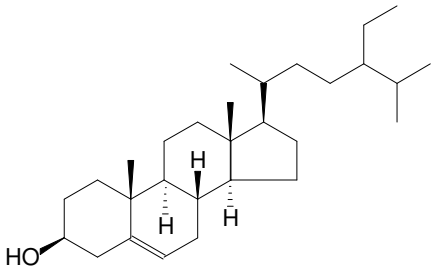
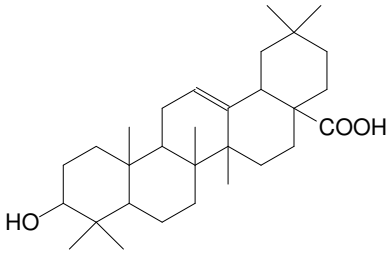
A



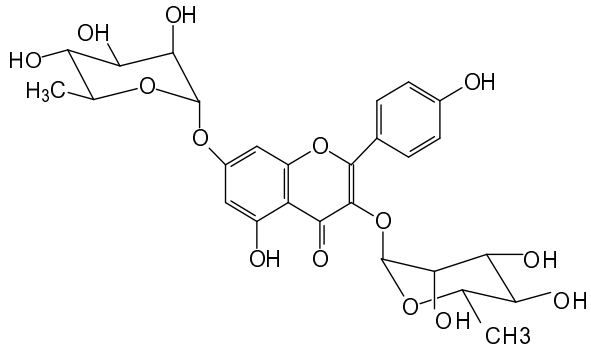
Compound **1** (β-sitosterol)

B



Compound **2** (Oleanolic acid)

C



Compound **3** (Kaempferitrin)

Fig. 1: The chemical structures of β-sitosterol (A), oleanolic acid (B) isolated from n-hexane fraction and Kaempferitrin (C) isolated from ethyl acetate fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae) aerial parts.

Table 1 – Effects of the crude extract of *Lotus corniculatus* and its derived fractions upon leukocyte migration and exudation in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/Doses (mg/kg)	**Leukocytes ($x10^6$)**	**Neutrophils ($x10^6$)**	**Mononuclears cells ($x10^6$)**	**Exudation (µg/mL)**
C[a]	6.20 ± 0.30	5.08 ± 0.30	1.12 ± 0.20	11.50 ± 1.00
CE 100[b]	5.77 ± 0.60	4.85 ± 0.50	0.91 ± 0.10	9.03 ± 0.50
CE 200[b]	3.80 ± 0.40**	3.17 ± 0.20**	0.63 ± 0.10*	8.58 ± 0.60*
CE 400[b]	3.21 ± 0.50**	2.73 ± 0.40**	0.48 ± 0.10**	5.70 ± 0.20**
HEX 50[b]	5.23 ± 1.20	4.60 ± 1.00	0.63 ± 0.20	8.91 ± 2.10
HEX 100[b]	4.58 ± 0.30**	3.97 ± 0.30*	0.61 ± 0.10*	8.85 ± 1.50
HEX 200[b]	2.90 ± 0.70**	1.62 ± 0.80**	1.28 ± 0.40	5.56 ± 1.40*
AcOEt 100[b]	6.97 ± 0.70	5.56 ± 0.90	1.49 ± 0.10	10.6 ± 1.80
AcOEt 200[b]	4.91 ± 0.40*	4.02 ± 0.30*	0.89 ± 0.10	7.64 ± 0.70*
AcOEt 400[b]	3.38 ± 0.70**	2.26 ± 0.40**	1.12 ± 0.40	7.11 ± 1.40*
BuOH 50[b]	7.04 ± 0.40	6.14 ± 0.40	0.90 ± 0.10	17.30 ±1.80*
BuOH 100[b]	4.07 ± 0.50**	3.36 ± 0.50**	0.71 ± 0.09	9.59 ± 1.10
BuOH 200[b]	3.77 ± 0.30**	3.26 ± 0.30**	0.51 ± 0.07*	9.48 ± 0.60
AF 25[b]	5.70 ± 0.30	5.01 ± 0.20	0.68 ± 0.10	15.90 ± 0.70*
AF 50[b]	4.24 ± 0.50*	3.53 ± 0.40**	0.71 ± 0.10	15.10 ± 1.40*
AF 100[b]	3.73 ± 0.80**	2.84 ± 0.60**	0.90 ± 0.20	10.40 ± 1.20
AF 200[b]	3.52 ± 0.30**	2.83 ± 0.30**	0.68 ± 0.20	6.37 ± 0.40**
Dex 0.5[b]	1.75 ± 0.30**	1.33 ± 0.20**	0.44 ± 0.10**	6.51 ± 0.50**
Indo 5[b]	2.26 ± 0.30**	1.78 ± 0.20**	0.48 ± 0.20*	7.93 ± 0.50*

The crude extract (CE: 100 to 400 mg/kg) of *Lotus corniculatus* and its derived fractions, hexane (HEX: 50 - 200 mg/kg), ethyl acetate fraction (AcOEt: 100 - 400 mg/kg), n-butanol fraction (BuOH: 50 - 200 mg/kg) or aqueous fraction (AF: 25 - 200 mg/kg) administered 0.5 h before the pleurisy induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = response in animals pre-treated with indomethacin (5.0 mg/kg). * $p < 0.05$ and ** $p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. N = 5 animals.

Table 2 – Effects of the isolated compounds of *Lotus corniculatus* upon leukocytes migration and exudation in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/Doses (mg/kg)	**Leukocytes ($x10^6$)**	**Neutrophils ($x10^6$)**	**Mononuclear cells ($x10^6$)**	**Exudation (µg/mL)**
C[a]	6.20 ± 0.30	5.08 ± 0.30	1.12 ± 0.20	11.50 ± 1.00
Kaempferitrin 50[b]	7.26 ± 0.80	6.87 ± 0.70	0.39 ± 0.20*	7.68 ± 1.10*
Kaempferitrin 100[b]	4.00 ± 0.50*	3.37 ± 0.40*	0.63 ± 0.10*	7.25 ± 0.40*
Oleanolic acid 10[b]	6.15 ± 0.60	4.83 ± 0.40	1.32 ± 0.20	9.20 ± 1.10
Oleanolic acid 25[b]	4.88 ± 0.30*	3.66 ± 0.40*	1.23 ± 0.30	7.44 ± 0.20*
Oleanolic acid 50[b]	3.08 ± 0.60**	2.42 ± 0.60**	0.66 ± 0.10	6.61 ± 0.40*
Oleanolic acid 100[b]	2.95 ± 0.70**	1.45 ± 0.10**	1.50 ± 0.60	10.80 ± 1.20
β-sitosterol 10[b]	5.63 ± 0.10	4.09 ± 0.60	1.55 ± 0.50	8.42 ± 0.90
β-sitosterol 25[b]	5.27 ± 0.10*	4.11 ± 0.10*	1.16 ± 0.10	7.69 ± 0.20*
β-sitosterol 50[b]	4.42 ± 0.70**	3.66 ± 0.70**	0.76 ± 0.10	8.26 ± 1.10*
β-sitosterol 100[b]	4.10 ± 0.60**	3.22 ± 0.60**	0.88 ± 0.30	9.38 ± 1.70
Dex 0.5[b]	1.75 ± 0.30**	1.33 ± 0.20**	0.44 ± 0.10**	6.51 ± 0.50**
Indo 5[b]	2.26 ± 0.30**	1.78 ± 0.20**	0.48 ± 0.20*	7.93 ± 0.50*

Kaempferitrin (50 and 100 mg/kg), Oleanolic acid (10 - 100 mg/kg) and β-sitosterol (10 - 100 mg/kg) isolated from *Lotus corniculatus* administered 0.5 h before the pleurisy induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = response in animals pre-treated with indomethacin (5.0 mg/kg). * $p < 0.05$ and ** $p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. N = 5 animals.

Table 3 – Effects of crude extract of *Lotus corniculatus,* its derived fractions and isolated compounds upon myeloperoxidase and adenosine-deaminase activities, and IL-1β levels in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/Doses (mg/kg)	**MPO (mU/mL)**	**ADA (U/L)**	**IL-1β (pg/mL)**
C[a]	334.00 ± 36.7	9.80 ± 0.30	1160.00 ± 119.00
CE 200[b]	121.00 ± 19.90**	3.20 ± 0.60**	871.00 ± 80.00*
HEX 100[b]	176.00 ± 38.80*	4.49 ± 1.40**	659.00 ± 100.00*
AcOEt 200[b]	141.00 ± 14.80**	0.63 ± 0.10**	732.00 ± 279.00*
BuOH 100[b]	303.00 ± 45.70	3.24 ± 0.80**	719.00 ± 200.00*
AF 50[b]	281.00 ± 35.70	3.50 ± 0.80**	-
AF 100[b]	220.00 ± 42.10*	3.21 ± 0.90**	744.00 ± 30.00*
Kampferitrin 100[b]	31.50 ± 11.30**	2.25 ± 0.70**	451.00 ± 160.00*
Oleanolic acid 50[b]	182.00 ± 72.20**	8.23 ± 3.70	1870.00 ± 229.00*
β-sitosterol 50[b]	85.80 ± 56.30**	3.06 ± 0.60**	1040.00 ± 213.00
Dex 0.5[b]	135.80 ± 14.60**	2.80 ± 0.70**	516.00 ± 47.30*
Indo 5[b]	120.10 ± 13.70**	3.48 ± 0.10**	586.00 ± 20.00*

The crude extract (CE: 200 mg/kg) of *Lotus corniculatus* and its derived fractions, hexane (HEX: 100 mg/kg), ethyl acetate fraction (AcOEt: 200 mg/kg), n-butanol fraction (BuOH: 100 mg/kg) or aqueous fraction (AF: 50 0r 100 mg/kg), and isolated compounds, kaempferitrin (100 mg/kg), oleanolic acid (50 mg/kg) and β-sitosterol (50 mg/kg) administered 0.5 h before the inflammation induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = response in animals pre-treated with indomethacin (5.0 mg/kg). * $p < 0.05$ and ** $p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. N = 5 animals.

Table 4- Effects of the crude extract of *Lotus corniculatus,* its derived fractions and isolated compounds upon nitrite/nitrate concnetration in the inflammation induced by carrageenan in the mouse model of pleurisy.

Groups/Doses (mg/kg)	**NO^x (µM)**
C[a]	16.40 ± 1.50
CE 400[b]	11.40 ± 0.90*
HEX 200[b]	10.80 ± 1.60*
AcOEt 800[b]	10.10 ± 0.90**
BuOH 100[b]	14.90 ± 0.60
AF 400[b]	9.16 ± 0.70**
Kaempferitrin 100[b]	11.0 ± 0.70*
Oleanolic acid 50[b]	10.5 ± 0.90*
β-sitosterol 50[b]	10.6 ± 0.50*
Dex 0.5[b]	4.00 ±0.76**
Indo 5[b]	8.21 ± 1.48**

The crude extract (CE: 400 mg/kg) of *Lotus corniculatus* and its derived fractions, hexane (HEX: 200 mg/kg), ethyl acetate fraction (AcOEt: 800 mg/kg), n-butanol fraction (BuOH: 100 mg/kg), aqueous fraction (AF: 400 mg/kg), and isolated compounds, kaempferitrin (100 mg/kg), oleanolic acid (50 mg/kg) and β-sitosterol (50 mg/kg) administered 0.5 h before the pleurisy induction by carrageenan (1%). C = response in animals treated only with carrageenan. Dex = response in animals pre-treated with dexamethasone (0.5 mg/kg). Indo = response in animals pre-treated with indomethacin (5.0 mg/k). * $p < 0.05$ and ** $p < 0.01$. The data is reported as the mean ± SEM. a = administered by intrapleural route, b = administered by intraperitoneal route. N = 5 animals.

4. ARTIGO SUBMETIDO À PUBLICAÇÃO – JOURNAL OF PHARMACY AND PHARMACOLOGY

Anti-bacterial activity of *Lotus corniculatus* var. São Gabriel

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Abstract

Introduction: *Lotus corniculatus* (Fabaceae) is distributed in many regions and has a high agronomic value constituted by characteristics such as enhancement of the weight of ruminants and control of intestinal parasitic infections. In our study we evaluated the anti-bacterial activity of crude extract, fractions and isolated compounds from *Lotus corniculatus* var. São Gabriel using the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). Results: The crude extract did not show any important anti-bacterial activity. On the other hand, the hexane fraction showed moderate anti-bacterial effect (MIC = 100 µg mL^{-1}) on *Bacillus cereus* and weak anti-bacterial effect (MIC = 600 to 1000 µg mL^{-1}) on *Enterococcus faecalis, Staphylococcus aureus, Listeria monocytogenes, Staphylococcus epidermidis, Providencia alcalifaciens* and *Acinetobacter calcoaceticus.* Oleanolic acid isolated from hexane fraction exhibited a good anti-bacterial activity on methycillin-resistant *Staphylococcus aureus* (MIC = 100 µg mL^{-1}), *Listeria monocytogenes* and *Bacillus cereus* (MIC = 25 µg mL^{-1}). The other fraction, ethyl acetate, also demonstrated a weak anti-bacterial activity (MIC = 800 µg mL^{-1}) on *Enterococcus faecalis, Bacillus cereus* and *Acinetobacter calcoaceticus,* but Kaempferitrin, a compound isolated from this fraction, demonstrated a good anti-bacterial effect on *Staphylococcus epidermidis, Shigella flexinerii, Salmonella typhimurium* and *Acinetobacter calcoaceticus* (MIC = 100 µg mL^{-1}). Furthermore, this compound showed an excellent anti-bacterial activity on *Enterococcus faecalis* (MIC = 3.9 µg mL^{-1}) and *Bacillus cereus* (MIC = 8.5 µg mL^{-1}). Conclusion: These results qualify Kaempferitrin and oleanolic acid as potential sources for the development a new anti-bacterial drug.

Introduction

In developing countries, infectious disease remains the cause of high mortality. This fact is associated with the increase in bacterial resistance to the available antibiotic agents and also the new opportunistic pathogens, especially those that infect the immune system-debilitated host population (Planta 2007).

Nowadays, much attention is being paid to determining the anti-bacterial activities of plant extracts prior to those already found in folk medicine. In this context, plants produce a variety of compounds named “secondary metabolites” that have many biological activities such as: analgesic and anti-inflammatory (Roldão et al 2008; Yam et al 2008), antiviral and anti-fungicidal (Maregesi et al 2008), anti-ulcer (Roldão et al 2008), anti-cancer (Tong et al 2008), anti-thrombotic and anti-platelet (Jin et al 2007) and anti-bacterial effects (da Silva et al 2008).

Lotus corniculatus (Fabaceae), also known as “bird’s trefoil”, is distributed in many regions of the world, and has a high agronomic value since it is used as a forage plant to increase the weight of ruminants (Sivakumaran et al 2006) and to control intestinal parasitic infection (Marley et al 2006). In Brazil, *Lotus corniculatus* var. São Gabriel is cultivated in the southern region of the country and it is also used as forage to reduce feed intake and to increase the digestibility in ruminants (Min et al 2002).

In relation to phytochemical studies of the *Lotus* species, there are some reports showing important constituents including flavonoids (Reynaud & Lussignol 2005), anthocyanins (Robbins et al 2003), sterols (Abdel-Ghani et al 2001), tannins (Hedqvist et al 2000), alkaloids (Rizk et al 1986) and cyanogenic compounds (Goverde et al 2008). Nevertheless, there are few reports concerning the investigation of the anti-bacterial activity of the genus *Lotus* (Mahasneh 2002), including *Lotus corniculatus* (Abdel-Ghani et al 2001). In this study we investigated the anti-bacterial activity of the crude extract, fractions and purified compounds from *Lotus corniculatus* var. São Gabriel. We also identified which compounds were responsible for this biological activity.

Materials and Methods

Plant materials

Lotus corniculatus var. São Gabriel was collected in November 2006, in Lages, Santa Catarina State, Brazil, at the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina S.A. (EPAGRI). The material was identified by the botanist Prof. Dr. Daniel de Barcelos Falkenberg of the Botany Department at the Federal University of Santa Catarina, Florianópolis, SC, Brazil. A voucher specimen is deposited in the Herbarium at the same university (FLOR 18.770).

Preparation of plant extracts

The aerial parts of *Lotus corniculatus* var. São Gabriel were dried at room temperature with air circulation. The dried and ground material (620 g) was extracted by maceration with ethanol 96% for one month. The solvent was evaporated under low pressure at 55^{o}C to dryness, yielding 78 g of crude extract (CE). The CE was fractionated by liquid-liquid extraction using solvents in growing order of polarity, resulting in hexanic (HEX: 7.82 g), ethyl acetate (AcOEt: 11.4 g), butanolic (BuOH: 5.24 g) and aqueous (AF: 30.8 g) fractions.

Preliminary phytochemical

In a preliminary phytochemical screening of the crude extract of *Lotus corniculatus,* we used a colourimetric reaction according to standard methods and the presence of phenols, tannins, antocyannin, antocyanidines, flavonoids, xantones, steroids, triterpenes and saponins was identified (Matos 1997).

Chomatografic separation and isolation of constituents

The hexane fraction was chromatographed using silica gel column chromatography with a HEX/EtOAc gradient resulting in the isolation of two terpenoids: a fraction eluted with HEX/EtOAc (90/10) affording 76 mg of a white crystal powder (Compound 1), and HEX/EtOAc (70/30) producing 25 mg of a white powder (Compound 2). From ethyl acetate fraction, after silica gel column chromatography eluted with EtOAc/EtOH (50/50), followed by purification of the

flavonoidic fraction with flash chromatography, 45 mg of a yellow powder (Compound 3) was isolated using a system of solvents (ethyl acetate/water/formic acid/acetic acid - 70:20:3:2, v/v/v/v).

Structure elucidation of the compounds

The chemical structure of each isolated compound was determined on the basis of its physical characteristics and spectral data produced by infrared analysis recorded on a Perkin Elmer FTIR 16PC infrared instrument. Analysis was carried out with KBr pellets and the results were registered in centimeters^{-1} (cm^{-1}). Nuclear magnetic resonance (^{1}H and ^{13}C-NMR) was recorded on a Varian AS-400 spectrometer operating at 300 and 100 MHz respectively. Thyl thin layer chromatography (TLC) was carried out on silica gel 60 F_{254} plates (Macherey-Nagel, Germany). Finally, the structures of the three isolated compounds were confirmed by comparison with reference data previously reported from available literature (Mahato & Kundu 1994; Kovganko et al 2000; Pizzolatti et al 2004).

Bacterial strains

The microorganisms used in the anti-bacterial tests were Gram-Positive bacteria: *Bacillus cereus* from the American Type Collection Culture (ATCC 11778), *Enterococcus faecalis* from ATCC 29912, *Listeria monocytogenes* from ATCC 35152, *Staphylococcus aureus* from ATCC 25923, *Staphylococcus epidermidis* from ATCC 12228 and Methicillin-Resistant *Staphylococcus aureus* (MRSA) from ATCC 43300 and Gram-Negative bacteria; *Acinetobacter baumanii* from ATCC 17978, *Acinetobacter calcoaceticus* from ATCC 19606, *Escherichia coli* from ATCC 25922, *Klebsiella pneumoniae* from ATCC 31488, *Pseudomonas aeruginosa* from ATCC 27853, *Proteus mirabilis* from ATCC 25933, *Providencia alcalifaciens* from ATCC 9886, *Salmonella typhimurium* from ATCC 14028 and *Shigella flexineri* from ATCC 12022. The identification of strains was confirmed by the use of biochemical profiles according to the recommendation of the Manual of Clinical Microbiology (Murray et al 2003).

Anti-bacterial assay

The direct anti-bacterial effect was evaluated by the broth microdilution method as recommended by the Clinical Laboratory Standards Institute (National Committee for Clinical Laboratory Standards - CLSI 2008) for determination of the MIC (Minimal Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration) of the crude extract of *Lotus corniculatus* var. São Gabriel and its derived fractions and isolated compounds. Crude extract, fractions and isolated compounds were dissolved in Dimethylsufoxide (DMSO) (starting from 10 mg mL^{-1} for the crude extract and fractions and 1 mg mL^{-1} for the isolated compounds). These solutions were transferred to 96-well plates (100 µL/well) and serially diluted in Mueller-Hinton broth (100 µL/well). The inoculum (5 µL) containing a 5 x 10^8 colony-forming unit per mL (CFU/mL) of each microorganism was added to each well. A number of wells were reserved on each plate for extract sterile control (no inoculum added), positive control (no extract added) and reference drug control (inoculum with gentamicin from 100 to 0.1 µg mL^{-1}) (Sarker et al 2007). Plates were aerobically incubated for 18–24 hr at 35^o C, and 10 µl of methanol solution (5 mg mL^{-1}) of 2,3,5 triphenyl-tetrazolium chloride (TTC, Vetec, São Paulo, Brazil) was added to each well to detect the active bacterial metabolism.

The MIC was defined as the lowest concentration of crude extract, fractions or compounds that visibly inhibited growth of bacterial spots detected with TTC (Rahman et al 2004; Abdillahi et al 2008).

To determine the Minimal Bactericidal Concentration (MBC), 10 µL of aliquots broth were taken from each well and plated in Muller-Hinton agar for 24 h at 37^{o}C. MBC represents the concentration necessary to kill 99.9 % or more of the initial inoculum (Bosio et al 2000).

To evaluate the anti-bacterial activity of the crude extract and fractions, an MIC below 100 µg mL^{-1} was considered as an excellent effect, from 100 to 500 µg mL^{-1} as moderate, from 500 to 1000 µg mL^{-1} as weak, and over 1000 µg mL^{-1} as inactive (Machado et al 2005). For isolated compounds, an MIC below 10 µg mL^{-1} was excellent, 10 to 100 µg mL^{-1} was good, and over 100 µg mL^{-1} was inactive (Ríos & Recio 2005). If the MBC was up to three-fold the dilution of the MIC, the anti-bacterial activity was considered to be bacteriostatic, and if the MBC was lower than three-fold the dilution of the MIC, the anti-bacterial activity was considered to be bactericidal (Okusa et al 2007).

Chemicals

Purchases were as follows. Muller Hinton broth and agar from Oxoid (Hampshire, UK); gentamicine from Laboratório Chile (Santiago, CHILE); 2,3,5-triphenyltetrazolium chloride TTC fromVetec (São Paulo, SP, Brazil); organic solvents: acetone, chloroform, n-Hexane, ethyl acetate, n-Butanol, methanol, and ethanol (all analytical grade) from Synth (Diadema, SP, Brazil); sheep's blood (Newprov, Curitiba, PR, Brazil); Dimethylsulfoxide – DMSO from Sigma–Aldrich (St. Louis, USA). Other reagents used were of analytical grade and were obtained from different commercial sources.

Results

Phytochemical analysis

Preliminary phytochemical analysis showed that the crude extract of *Lotus corniculatus* var. São Gabriel had a significant presence of flavonoids, steroids and terpenoids. From hexane fraction, we isolated the Compound 1 that was identified as β-sitosterol (Figure 1A). The second compound (Compound 2), also isolated from the same fraction, was identified as oleanolic acid (Figure 1B). Finally, from ethyl acetate fraction we isolated a flavonoid *O*-heteroside (Compound 3), that was identified as Kaempferitrin (Figure 1C).

Anti-bacterial analysis

The anti-bacterial activities of the crude extract, aqueous and butanolic fractions against all bacteria tested were considered inactive since none of them showed anti-bacterial activity up to 1000 µg mL^{-1}. On the other hand, the hexane fraction demonstrated a moderate anti-bacterial activity (MIC = 100 µg mL^{-1}) in the gram-positive bacteria *Bacillus cereus* (Table 1) and a weak anti-bacterial effect (MIC = 600, 800 or 1000 µg mL^{-1}) on *Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus* and *Staphylococcus epidermidis* and the gram-negative bacteria *Acinetobacter calcoaceticus* and *Providencia alcalifaciens*.

Ethyl acetate fraction (AcOEt) also had a weak anti-bacterial activity (MIC = 800 µg mL^{-1}) on the gram-positive bacteria *Bacillus cereus* and *Enterococcus faecalis* and the gram-negative bacterium *Acinetobacter calcoaceticus* (Table 1).

All these effects were considered to be of bacteriostatic action, since the MBCs were at a dilution of more than three-fold that of the MICs (Tables 1 and 2). Further, the hexane and ethyl acetate fractions demonstrated better anti-bacterial activity than the other studied fractions.

Subsequently, we wondered which active constituents could be responsible for the anti-bacterial activity of *Lotus corniculatus* var. São Gabriel. First of all, we isolated Compound 1 (β-sitosterol) (Figure 1A) and Compound 2 (oleanolic acid) (Figure 1B) from the hexane fraction and Compound 3 (Kaempferitrin) (Figure 1C) from the ethyl acetate fraction. The second step was to investigate the anti-bacterial effect of these three isolated compounds using the same methodology. Compound 1 (Figure 1A) did not show any significant anti-bacterial activity. On the other hand, Compound 2 (Figure 1B) showed a good anti-bacterial activity (MICs from 25 to 100 µg mL^{-1}) on the gram-positive bacteria *Bacillus cereus, Listeria monocytogenes,* and the methicillin-resistant *Staphylococcus aureus* (Table 1). Compound 3 (Figure 1C) also showed a good anti-bacterial activity (MICs from 25 to 100 µg mL^{-1}) on the gram-positive bacterium *Staphylococcus epidermidis* and the gram-negative bacteria *Acinetobacter calcoaceticus, Shiguella flexinerii* and *Salmonella typhimurium.* These effects were considered to be bacteriostatic (Tables 1 and 2).

Surprisingly, the best activity was observed with this Kaempferitrin against two gram-positive bacteria, *Bacillus cereus* and *Enterococcus faecalis,* with an excellent anti-bacterial activity (MIC = 8.5 µg mL^{-1} and 3.9 µg mL^{-1}, respectively). In this case, the anti-bacterial activity was considered to be bacteriostatic for *Bacillus cereus* and bactericidal for *Enterococcus faecalis* (Tables 1 and 2).

Discussion

There are few reports about the anti-bacterial activity of *Lotus corniculatus* concerning the *Lotus corniculatus* var. *ternuifolius* that showed anti-bacterial activity against gram-positive and gram–negative bacteria. Our results demonstrated that *Lotus corniculatus* var. São Gabriel exhibited an important anti-bacterial activity and this effect was more pronounced with hexane and ethyl acetate fractions. The

phytochemical analysis of the crude extract showed the presence of flavonoids, steroids and tepenoids. Similar results have been presented by other authors who have also demonstrated these compounds in *Lotus corniculatus* var. ternuifolis (Abdel-Ghani et al 2001). One hypothesis to explain the anti-bacterial effect observed in the hexane fraction could be the presence of terpenoids. This is in accordance with other studies that have also demonstrated anti-bacterial properties of terpenoids (Marthanda Murthy et al 2005). Studies have shown that the anti-bacterial effect of terpenoids is due to their ability to disrupt the membranes of the bacteria which leads to the death of the microorganisms (Cowan 1999).

After analysis of the hexane fraction we isolated a terpenoid, identified as oleanolic acid, that showed moderate anti-bacterial activity against three gram-positive bacteria, a fact that had also been demonstrated by other authors showing the important anti-bacterial activity of this compound against the same gram-positive bacteria as those tested in our experiments (Woldemichael et al 2003; Horiuchi et al 2007).

Another substance that revealed important anti-bacterial activity was the flavonoid isolated from ethyl acetate fraction. Other studies have also demonstrated the presence of flavonoids in aerial parts of japonicus and alpine varieties of *Lotus* (Reynaud & Lussignol, 2005). In addition to their ability to cause DNA damage in bacteria (Urgaonkar et al 2007), it is well known that flavonoids possess important anti-bacterial activity that acts via different mechanisms of action, such as complexing with the bacterial cell wall and decreasing microbial growth (Cushnie & Lamb 2005), inhibiting the activity of the DNA topoisomerase II (DNA gyrase) (Piddock et al 1990), promoting the inhibition of bacterial cell division (Vollmer 2006), and inhibiting the GTPase activity (Urgaonkar et al 2005). The flavonoid *O*-heteroside Kaempferitrin showed excellent anti-bacterial activity against two gram-positive bacteria comparable to the reference antibiotic gentamicine, and this result is also in accordance with Abdel-Ghani and co-workers (2001) who have also demonstrated important anti-bacterial activity of Kaempferitrin against both gram-positive and gram negative bacteria.

Another compound isolated from hexane fraction was β-sitosterol, a sterol that does not show a significant anti-bacterial activity. These results are also in accordance with other authors who have shown that this compound has slight anti-

bacterial activity against *Escherichia coli* and absence of activity against some gram-positive bacteria (Nazif 2002).

Conclusion

Our results demonstrated a potent anti-bacterial activity of *Lotus corniculatus* var. São Gabriel that can be attributed to isolated compounds such Kaempferitrin and oleanolic acid. Furthermore, Kaempferitrin demonstrated a good anti-bacterial effect in some gram-negative bacteria and excellent anti-bacterial effect against two gram-positive bacteria. These results qualify Kaempferitrin and oleanolic acid as being sources for the development of a new anti-bacterial drug from natural product.

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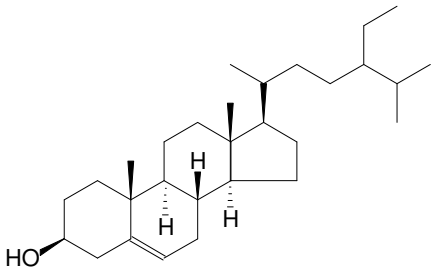
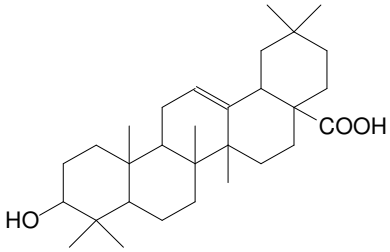
A



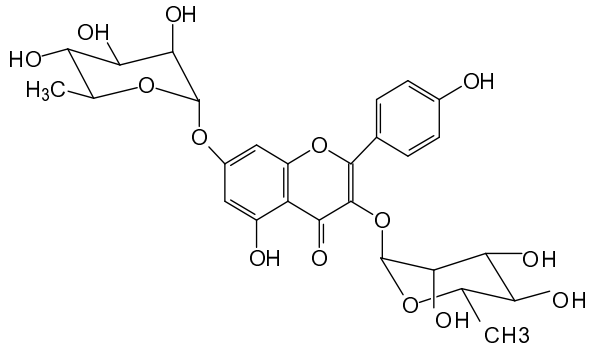
Compound 1 (β-sitosterol)

B



Compound 2 (Oleanolic acid)

C



Compound 3 (Kaempferitrin)

Figure 1: The chemical structures of β-sitosterol (A), oleanolic acid (B) isolated from n-hexane fraction and Kaempferitrin (C) isolated from ethyl acetate fraction of *Lotus corniculatus* var. São Gabriel (Fabaceae) aerial parts.

Table 1: Minimal inhibitory concentrations (MICs: µg mL^{-1}) of crude extract, fractions and isolated compounds of aerial parts from *Lotus corniculatus* var. São Gabriel

	CE	HEX	AF	BuOH	AcOEt	Comp. 1	Comp. 2	Comp. 3	GE
Gram-Positive bacteria									
Bacillus cereus ATCC 11778	>1000	100	>1000	>1000	800	>1000	25	8,5	0,2
Enterococcus faecalis ATCC 29912	1000	600	>1000	>1000	800	300	300	3,9	6,0
Listeria monocytogenes ATCC 35152	>1000	800	>1000	>1000	>1000	500	25	300	0,2
MRSA ATCC 43300*	>1000	>1000	>1000	>1000	>1000	500	100	200	100
Staphylococcus epidermidis ATCC 12228	>1000	800	>1000	>1000	>1000	500	600	100	0,1
Staphylococcus aureus ATCC 25923	>1000	1000	>1000	>1000	>1000	500	800	200	1,0
Gram-Negative bacteria									
Acinetobacter baumanii ATCC 17978	>1000	>1000	>1000	>1000	>1000	>1000	>1000	500	6,0
Acinetobacter calcoaceticus ATCC 19606	>1000	600	>1000	>1000	800	800	600	100	6,0
Escherichia coli ATCC 25922	>1000	>1000	>1000	>1000	>1000	>1000	>1000	500	6,0
Klebsiella pneumoniae ATCC 31488	>1000	>1000	>1000	>1000	>1000	>1000	>1000	500	1,0
Proteus mirabilis ATCC 25933	>1000	>1000	>1000	>1000	>1000	500	200	200	12,0
Providencia alcalifaciens ATCC 9886	>1000	800	>1000	>1000	>1000	800	500	500	2,0
Pseudomonas aeruginosa ATCC 27853	>1000	>1000	>1000	>1000	>1000	800	800	500	1,0
Salmonella typhimurium ATCC 14028	>1000	>1000	>1000	>1000	>1000	500	200	100	6,0
Shigella flexinerii ATCC 12022	>1000	>1000	>1000	>1000	>1000	400	200	100	3,1

CE = Hidroalchoolic crude extract; HEX = n-Hexane extract; AF = aqueous extract; BuOH = butanol extract; AcOEt = ethyl acetate extract; Comp. 1 = β sitosterol; Comp. 2 = Oleanolic acid and Comp. 3 = Kaempferitrin; GE = Gentamicine; * Methicillin-resistant *Staphylococcus aureus*; ATCC – *American type collection culture* (data from three experiments).

Table 2 Minimal bactericidal concentrations (MBCs: µg mL^{-1}) of crude extract, fractions and isolated compounds of aerial parts from *Lotus corniculatus* var. São Gabriel

	CE	HEX	AF	BuOH	AcOEt	Comp. 1	Comp. 2	Comp. 3	GE
Gram-Positive bacteria									
Bacillus cereus ATCC 11778	>1000	> 1000	>1000	>1000	>1000	>1000	200	34	0,4
Enterococcus faecalis ATCC 29912	>1000	>1000	>1000	>1000	>1000	900	>1000	7,4	12,0
Listeria monocytogenes ATCC 35152	>1000	>1000	>1000	>1000	>1000	>1000	200	900	0,2
MRSA ATCC 43300*	>1000	>1000	>1000	>1000	>1000	>1000	800	800	100
Staphylococcus aureus ATCC 25923	>1000	>1000	>1000	>1000	>1000	>1000	> 1000	800	4,0
Staphylococcus epidermidis ATCC 1222	>1000	>1000	>1000	>1000	>1000	1000	>1000	400	0,2
Gram-Negative bacteria									
Acinetobacter baumanii ATCC 17978	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	24,0
Acinetobacter calcoaceticus ATCC 1960	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	12,0
Escherichia coli ATCC 25922	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	12,0
Klebsiella pneumoniae ATCC 31488	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	4,0
Proteus mirabilis ATCC 25933	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	24,0
Providencia alcalifaciens ATCC 9886	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	8,0
Pseudomonas aeruginosa ATCC 27853	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	4,0
Salmonella typhimurium ATCC 14028	>1000	>1000	>1000	>1000	>1000	>1000	>1000	800	12,0
Shigella flexinerii ATCC 12022	>1000	>1000	>1000	>1000	>1000	>1000	>1000	800	3,1

CE = Hidroalchoolic crude extract; HEX = n-Hexane extract; AF = aqueous extract; BuOH = butanol extract; AcOEt = ethyl acetate extract; Comp. 1 = β sitosterol; Comp. 2 = Oleanolic acid and Comp. 3 = Kaempferitrin; GE = Gentamicine; *Methicillin-resistant *Staphylococcus aureus*; ATCC – *American type collection culture* (data from three experiments).

5. DISCUSSÃO

Os resultados deste trabalho demonstraram que o extrato bruto hidroalcólico (CE), obtido das partes aéreas da *Lotus corniculatus* v. São Gabriel foi efetivo em inibir a migração leucocitária, principalmente às custas da inibição de neutrófilos, células consideradas marcadores da resposta inflamatória aguda (KELLY et al., 2007).

Além do extrato bruto hidroalcólico, as frações: hexano (HEX), acetato de etila (AcOEt), butanólica (BuOH) e resíduo aquoso (AF) também demonstraram importante efeito anti-inflamatório, inibindo a migração dos leucócitos e dos neutrófilos. É importante salientar que a fração AF demonstrou melhor efeito anti-inflamatório comparada as frações HEX, AcOEt e BuOH, tendo em vista que dose inferior (50 mg/kg) foi efetiva em inibir a migração dos leucócitos quando comparada as outras frações, as quais também inibiram este parâmetro, mas em doses superiores (100 e 200 mg/kg).

O extrato bruto hidroalcólico e as frações isoladas também inibiram a atividade da enzima adenosina-deaminase (ADA), a qual está relacionada à ativação dos mononucleares (FRÖDE; MEDEIROS, 2001; HASKO et al., 2008). Estudos de biologia molecular realizado por Zhong et al. (2003) identificaram a expressão dos receptores de adenosina (A_{2A}, A_{2B} e A_3) em mastócitos no pulmão de camundongos (ZHONG et al., 2003). Fozard et al. (2002) demonstraram o tratamento prévio de ratos com agonista seletivo do receptor A_{2A} (CGS21680), houve diminuição da infiltração de leucócitos, da atividade da MPO e da peroxidase de eosinófilos, no lavado broncoalveolar, em modelo de asma alérgica induzido por ovoalbumina. Esses efeitos foram inibidos quando os animais receberam o tratamento prévio com o antagonista seletivo para o receptor de adenosina A_{2A} (ZM241385) (FOZARD et al., 2002). Haskó et al. (2006) demonstraram que ratos tratados previamente com o agonista seletivo do receptor A_{2A} (CGS21680) apresentaram diminuição da infiltração de neutrófilos, edema e atividade da MPO, no lavado broncoalveolar, em modelo de isquemia/reperfusão induzido por choque hemorrágico (HASKÓ et al., 2006). Esses resultados sugerem a participação da adenosina na resposta inflamatória via receptor A_{2A} (HASKÓ et al., 2000)

O extrato bruto hidroalcólico e as frações HEX, AcOEt e AF foram efetivos em inibir também a atividade da MPO, enzima considerada marcador da ativação neutrofílica (LAU; BALDUS, 2006). As frações HEX e AF demonstraram também melhor efeito anti-inflamatório com relação a esse parâmetro, uma vez que, a dose de 100 mg/kg inibiu esta enzima pró-inflamatória quando comparada a fração AcOEt, a qual foi efetiva em inibir a atividade da MPO somente em dose superior de 200 mg/kg.

Estudos utilizando modelos experimentais, já demonstraram a participação da MPO no processo inflamatório, inclusive no modelo de pleurisia induzida pela carragenina (FRÖDE; MEDEIROS, 2001; MENEGAZZI et al., 2008) e no modelo de inflamação do duodeno, induzida por terenbetina, ambos em camundongos (FAITH et al., 2008). Lau et al. (2005) também já evidenciaram o envolvimento da MPO no processo inflamatório. Nesse estudo observou-se que polimorfonucleares humanos quando estimulados *in vitro* com a MPO houve aumento na ativação da proteína quinase ativadora de miógeno (p38), do fator nuclear kappa B (NF-κB) e da expressão de moléculas de adesão do tipo integrinas (CD11b/CD18). Esses resultados revelaram que a MPO pode ativar os neutrófilos também via proteina tirosina quinase (LAU et al., 2005).

É importante ressaltar que a liberação da MPO está relacionada também com a liberação do NO (LAU; BALDUS, 2006). Aoi et al. (2008) demonstraram o aumento da atividade da MPO e da concentração de NO por meio da ativação das enzimas iNOS e eNOS, em modelo de colite induzido por dextran, em ratos (AOI et al., 2008). Estudos realizados em animais *knockout* para a MPO, observou-se que a administração de LPS promoveu o aumento da liberação de NO. Esses resultados sugerem que tanto a ativação da MPO, como a liberação de NO, podem ter uma via comum de sinalização (EISERICH et al., 2002).

Em nossos experimentos, os animais tratados previamente com o extrato bruto hidroalcólico, frações e compostos isolados da *Lotus corniculatus* v. São Gabriel apresentaram também a diminuição nas concentrações de nitrito/nitrato.

O óxido nítrico é um importante mediador da resposta inflamatória e é liberado em grande quantidade por meio da ativação da enzima iNOS (GARCIA; STEIN, 2006). Muitos estudos já evidenciaram o papel da iNOS em modelos experimentais de inflamação crônica das vias aéreas. Prado et al. (2006) demonstraram em modelo de inflamação pulmonar crônica em cobaias, estimulados

com ovoalbumina, que os animais tratados com inibidor seletivo de iNOS (1400W), ocorreu: 1) diminuição na concentração do óxido nítrico exalado, 2) inibição da infiltração de eosinófilos e de mononucleares e 3) diminuição da deposição de fibras de colágeno e elásticas em tecido não cartilaginoso nas paredes das vias aéreas dos animais (PRADO et al., 2006). Eynott et al. (2002) demonstraram ainda em modelo de asma alérgica induzido por ovoalbumina em ratos, que quando os animais foram tratados previamente com inibidor seletivo de iNOS (SC-51), ocorreu a diminuição de: 1) óxido nítrico exalado, 2) hiperresponsividade brônquica e 3) infiltração de neutrófilos e de eosinófilos, no lavado broncoalveolar (EYNOTT et al., 2002).

O efeito anti-inflamatório do material vegetal foi caracterizado também pela inibição da exsudação, exceto para a fração butanólica (BuOH). A exsudação possui papel fundamental na resposta inflamatória na mucosa das vias aéreas, pois consiste no primeiro mecanismo de defesa como um fator potencialmente pró-inflamatório, e pode ser implicado como um marcador específico da resposta inflamatória, já que a exsudação reflete o quanto a mucosa é lesada por essa resposta. Na asma brônquica, a exsudação tem um papel importante, já que este promove a amplificação da resposta inflamatória . Esta amplificação deve-se ao fato da liberação a nível local de vários mediadores pró-inflamatórios, como por exemplo: histamina, bradicinina, leucotrienos, fator ativador de plaquetas e fator de necrose tumoral (TNF-α) (PERSSON et al., 1998; GREIFF et al., 2003).

Os resultados demonstraram que o extrato bruto hidroalcólico e frações também inibiram a concentração de IL-1β na inflamação induzida pela carragenina. Estudos já demonstraram a participação da IL-1β na inflamação em modelos experimentais. Oliveira et al. (2007) demonstraram que o tratamento prévio de ratos com inibidor seletivo do fator ativador de plaquetas (PCA 4248), inibidor de leucotrienos (MK 886) ou dexametasona inibiram a migração de leucócitos e neutrófilos na cavidade peritoneal, em modelo de peritonite induzido por IL-1β (OLIVEIRA et al., 2007). Cardell et al. (2008) demonstraram ainda que a inalação de IL-1β promoveu o aumento na expressão dos receptores IL-1 R1, TNF RI e TNF RII nas células epiteliais do pulmão, e de TNF RI e TNF RII nas células da musculatura lisa da traquéia, em modelo de asma, bem como em cultura de células da musculatura lisa da traquéia de camundongos. Esses resultados evidenciaram a participação da IL-1β na hiperrresponsividade das vias aéreas, em camundongos

(CARDELL et al., 2008). Zhang, Adner e Cardell (2007) demonstraram em estudo *in vitro,* utilizando células da musculatura lisa da traquéia de camundongos estimuladas com IL-1β e/ou TNF-α, o aumento na expressão dos receptores de bradicinina B1 e B2 nessas células. A IL-1β aumentou ainda a expressão do RNAm do TNF-α nas células epiteliais e nas células da musculatura lisa da traquéia de camundongos. Esses resultados indicam que a IL-1β e o TNF-α participam da hiperresponsividade no processo inflamatório das vias aéreas, em células da musculatura lisa e epiteliais da traquéia de camundongos (ZHANG; ADNER; CARDELL, 2007).

Com relação aos compostos isolados da *Lotus corniculatus* v. São Gabriel, o ácido oleanólico e o β-sitosterol, isolados da fração HEX, apresentaram melhor atividade anti-inflamatória por meio da inibição de leucócitos e da exsudação comparados ao canferitrin, isolado da fração AcOEt.

Entretanto, o canferitrin demonstrou melhor efeito anti-inflamatório em inibir a atividade da MPO e da ADA, e as concentrações de IL-1β em relação aos outros compostos e fármacos de referência, uma vez que o canferitrin inibiu 3,8 vezes mais a MPO, 1,5 vezes mais a ADA e 1,3 vezes mais a concentração de IL-1β, comparado à indometacina ($P < 0,05$). Além disso, este composto inibiu 4,3 e 1,2 vezes mais a atividade da MPO e da ADA, quando comparado à dexametasona, respectivamente ($P < 0,05$).

Os resultados encontrados estão de acordo com o trabalho realizado por Trouillas et al. (2003), o qual demonstraram em estudos *in vitro* que o extrato hidroalcólico da *Lotus corniculatus* na presença dos radicais 2,2-difenil-1-picril-hidrazil (DPPH), ânion superóxido (O_2^-), e radical hidroxil (OH^-), inibiu a atividade oxidante desses radicais por meio do seqüestro dos mesmos, evidenciando a propriedade antioxidante do extrato (TROUILLAS et al., 2003).

Resultados semelhantes foram encontrados por Fang et al. (2005), os quais demonstraram que o canferitrin diminuiu as concentrações de NO, TNF-α e IL-12 em macrófagos peritoneais de camundongos estimulados por LPS/IFN-γ (FANG; RAO; TZENG, 2005). Estudo *in vitro* realizado por Regasini et al. (2008), demonstraram ainda que o canferitrin inibiu a peroxidação do guaiacol na presença de H_2O_2 e da MPO. Este resultado indica que o composto inibiu indiretamente a atividade da MPO (REGASINI et al., 2008).

O ácido oleanólico, um composto triterpenóide, também possui atividade anti-inflamatória. Singh et al. (1992) demonstraram que este composto inibiu a exsudação e a infiltração de leucócitos no modelo de pleurisia induzida por carragenina, em camundongos (SINGH et al., 1992). Esse composto inibiu ainda a enzima iNOS, via inibição da ativação do NF-κB, em macrófagos de camundongos (RAW 264.7) estimulados por LPS (SUH et al., 2007).

Em relação ao β-sitosterol, um fitoesterol presente em muitas plantas, sabe-se que esse composto também possui atividade anti-inflamatória. Gómez et al. (1999) demonstraram que esse composto inibiu o edema, a migração de neutrófilos e atividade da MPO, no modelo de inflamação de edema de orelha induzido por 12-O-tetradecanoilforbol acetato (TPA), em camundongos (GÓMEZ et al., 1999). Yuk et al. (2007) também demonstraram que o tratamento de camundongos com β-sitosterol diminuiu o influxo de eosinófilos, a secreção de muco e a expressão de IL-4 e IL-5, no lavado broncoalveolar, no modelo de asma alérgica induzida por ovoalbumina (YUK et al., 2007). O β-sitosterol também foi efetivo em inibir a exsudação, no modelo de edema de orelha induzido por acetona, em camundongos (MAVAR-MANGA et al., 2008). Essa substância também inibiu a liberação de IL-12 em células T Jukart humanas estimuladas por concavalina (ConA) ou por forbol-12-miristato-13-acetato e ionomicina (PMA + IoM) (AHERNE; O' BRIEN, 2008).

Desta forma, os resultados obtidos com a *Lotus corniculatus* v. São Gabriel demonstraram importante atividade anti-inflamatória, e seus constituintes, canferitrin, ácido oleanólico e β-sitosterol podem ser os responsáveis por essa atividade.

Além da atividade anti-inflamatória demonstrada pela *Lotus corniculatus* v. São Gabriel, esta planta também apresentou atividade antibacteriana. O extrato bruto hidroalcóolico, e as frações AF e BuOH foram considerados inativos para as bactérias testadas, por terem demonstrado CIM superiores a 1000 µg/mL. Segundo os critérios de Machado et al. (2005), a fração HEX apresentou fraca atividade antibacteriana para *Enterococcus faecalis, Staphylococcus epidermidis e Listeria monocytogenes,* e atividade moderada para *Bacillus cereus,* todas estas bactérias gram-positivas.

O β-sitosterol, segundo Rios e Recio (2005), não demonstrou atividade antibacteriana significativa para as bactérias testadas. Esses resultados são corroborados por Hess et al., (1995) que demonstraram que o β-sitosterol não foi efetivo em inibir o crescimento bacteriano da *E. coli* e *S. aureus* (HESS et al., 1995).

O ácido oleanólico demonstrou atividade antimicrobiana considerada moderada para as bactérias Gram-positivas: *Bacilus cereus* (CIM = 25 µg/mL) e *Listeria monocytogenes* (CIM = 25 µg/mL). Este resultado, em particular, pode ser explicado, em parte, devido à atividade antibacteriana observada pela fração HEX para as mesmas bactérias. Segundo Cowan (1999), um dos prováveis mecanismos para o efeito antibacteriano do ácido oleanólico seria por meio da ruptura da membrana das bactérias Gram-positivas (COWAN, 1999).

Outra fração que apresentou fraca atividade antibacteriana foi a AcOEt para as bactérias Gram-positivas: *Enterococcus faecalis* e *Bacillus cereus*, e para a bactéria Gram-negativa: *Acinetobacter calcoaceticus* com valores de CIM 800 µg/mL. Esta atividade pode ser atribuída à presença significativa de flavonoides nesta fração, dos quais já se conhece que um dos mecanismos de ação antimicrobiana é via inibição da parede celular, impedindo o crescimento e a multiplicação do microrganismo (COWAN, 1999; CUSHNIE; LAMB, 2005).

O composto majoritário da fração AcOEt, o flavonóide canferitrin, demonstrou excelente atividade antibacteriana (Rios & Récio, 2005) para aos microrganismos Gram-positivos: *Enterococcus faecalis* (3,9 µg/mL) e *Bacilus cereus* (8,5 µg/mL). Abdel-Ghani et al. (2001) já relataram a atividade antimicrobiana deste mesmo flavonóide, e segundo os autores, este efeito foi comparado ao cloranfenicol, a penicilina, a oxitetraciclina e a gentamicina, considerados os mais potentes antibióticos de uso comercial para o tratamento de infecções induzidas por bactérias Gram-positivas e Gram-negativas (ABDEL-GHANI et al., 2001). O provável mecanismo de ação antibacteriano do canferitrin foi proposto por diversos autores, que demonstraram que esse flavonóide inibiu a enzima topoisomerase II (PIDDOCK; WALTERS; DIVER, 1991), induziu a lesão do DNA (NORMAN; HANSEN; SORENSEN, 2006) e inibiu a divisão celular bacteriana (VOLLMER, 2006).

Os resultados encontrados são bastante promissores no que se diz respeito ao tratamento futuro de infecções bacterianas causadas pelo *Enterococcus faecalis* multiresistentes, já que atualmente esta bactéria está entre os principais e mais preocupantes causadores de infecções hospitalares nos Estados Unidos da América e outros países desenvolvidos, além de ser também um problema emergente nos hospitais brasileiros (JUNIOR et al., 2007). O tratamento das infecções causadas por esta bactéria é bastante limitado, devido à presença de mecanismos de resistência intrínsecos apresentados por esse microrganismo. Em

geral, os *Enterococcus sp.* demonstraram resistência intrínseca às cefalosporinas, lincosamidas, e muitos antibióticos β-lactâmicos sintéticos, como as penicilinas resistentes as penicilamases. Algumas espécies de *Enterococcus* também demonstram resistência às baixas concentrações de aminoglicosídeos, uma vez que os antibióticos dessa classe são pouco absorvidos no trato gastrointestinal (GIRAFFA, 2002). É importante salientar que os índices de resistência desta bactéria frente à vancomicina e a teicoplanina (glicopeptídeos utilizados como última opção de tratamento em casos de infecções graves causadas por *Enterococcus sp*.) estão aumentando significativamente (DESHPANDE et al., 2007; CHOU et al., 2008). Essas informações tornam os resultados encontrados promissores, já que não existem muitas opções de tratamento para infecções graves causadas pelo *Enterococcus*.

Além disso, o canferitrin apresentou excelente atividade contra à bactéria *Bacillus cereus*, o qual é responsável por grande número de intoxicações alimentares relacionadas ao acondicionamento inadequado de alimentos processados e cozidos, como por exemplo, carnes e cereais. Embora não seja uma bactéria que apresente resistência aos antibióticos, a infecção causada por este microrganismo é uma importante causa de óbitos de crianças em países subdesenvolvidos (OMBUI; KAGIKO; ARIMI, 2001; OMBUI; NDUHIU, 2005).

6. CONCLUSÕES

I. O mecanismo de ação anti-inflamatório da *Lotus corniculatus* v. São Gabriel parece estar relacionado à inibição da infiltração de leucócitos, bem como diminuição da MPO, ADA, NO e IL-1β.

II. Os compostos canferitrin, ácido oleanólico e o β-sitosterol parecem ser os responsáveis pelo efeito anti-inflamatório apresentado pela *Lotus corniculatus* v. São Gabriel;

III. Dentre os compostos isolados da *Lotus corniculatus* v. São Gabriel, o canferitrin demonstrou melhor atividade antibacteriana;

IV. Os compostos, canferitrin, ácido oleanólico, bem como o β-sitosterol, podem ser importantes candidatos ao desenvolvimento de fármacos com potencial atividade anti-inflamatória e/ou antimicrobiana.

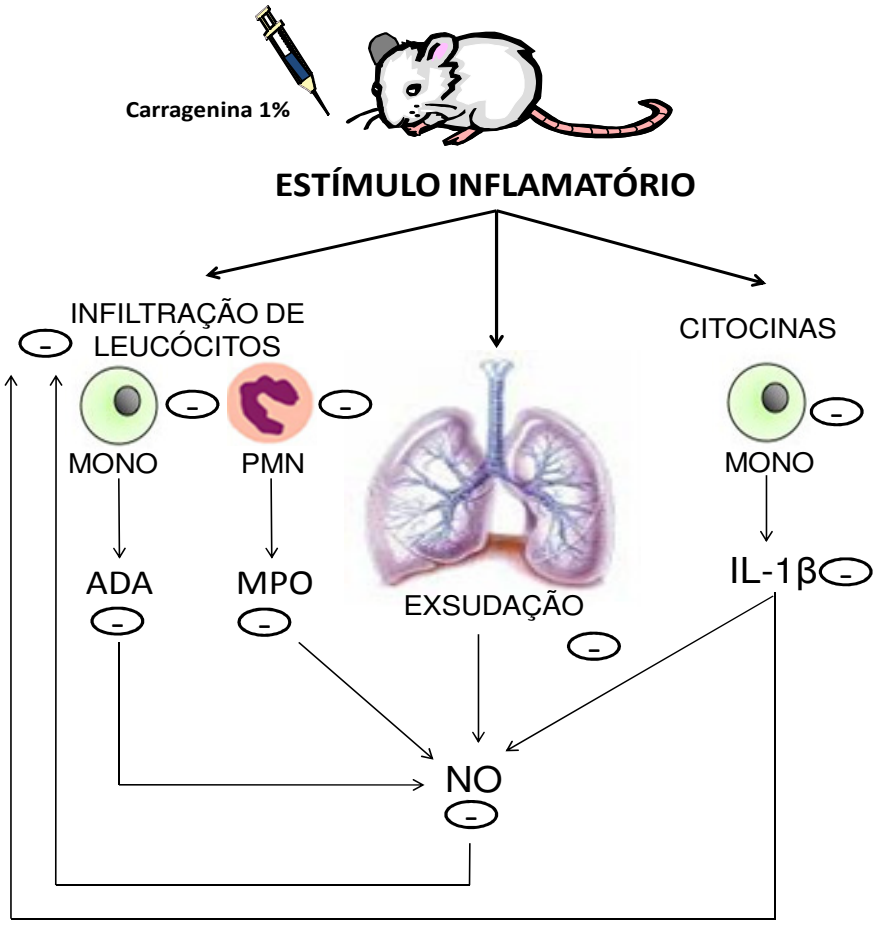


Figura 2: Mecanismo de ação anti-inflamatória proposto para a *Lotus corniculatus v.* São Gabriel. ADA: Adenosina-deaminase, MPO: Mieloperoxidase, IL-1β: Interleucina-1 beta, TNF-α: Fator de necrose tumoral alfa, NO: Óxido nítrico, (-): efeito de inibição.

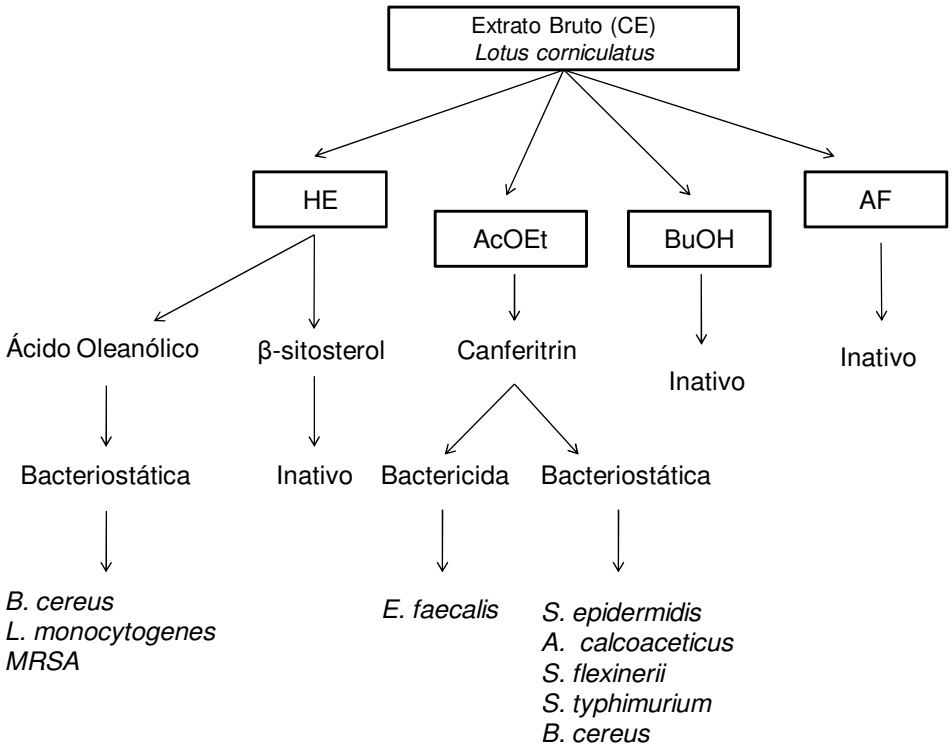


Figura 3: Efeito antibacteriano da *Lotus corniculatus* v. São Gabriel

7. PERSPECTIVAS

I. Avaliar o efeito do extrato bruto, frações e compostos isolados da *Lotus corniculatus* v. São Gabriel no modelo de pleurisia, em camundongos, utilizando diferentes agentes flogísticos, como a bradicinina, substância P e histamina;

II. Avaliar o mecanismo de ação do extrato bruto, frações e compostos isolados da *Lotus corniculatus* v. São Gabriel quando administrados oralmente, em modelo de inflamação;

III. Avaliar a toxicidade do extrato bruto da *Lotus corniculatus* v. São Gabriel *in vivo*;

IV. Avaliar a atividade antimicrobiana do ácido oleanólico e do canferitrin frente às bactérias em que estes apresentaram ótimos resultados, mas em cepas isoladas de pacientes que apresentem resistências aos antibióticos tradicionalmente utilizados para o tratamento das infecções;

V. Realizar modificações estruturais no ácido oleanólico e canferitrin e verificar o aumento da atividade anti-inflamatória e/ou antimicrobiana.

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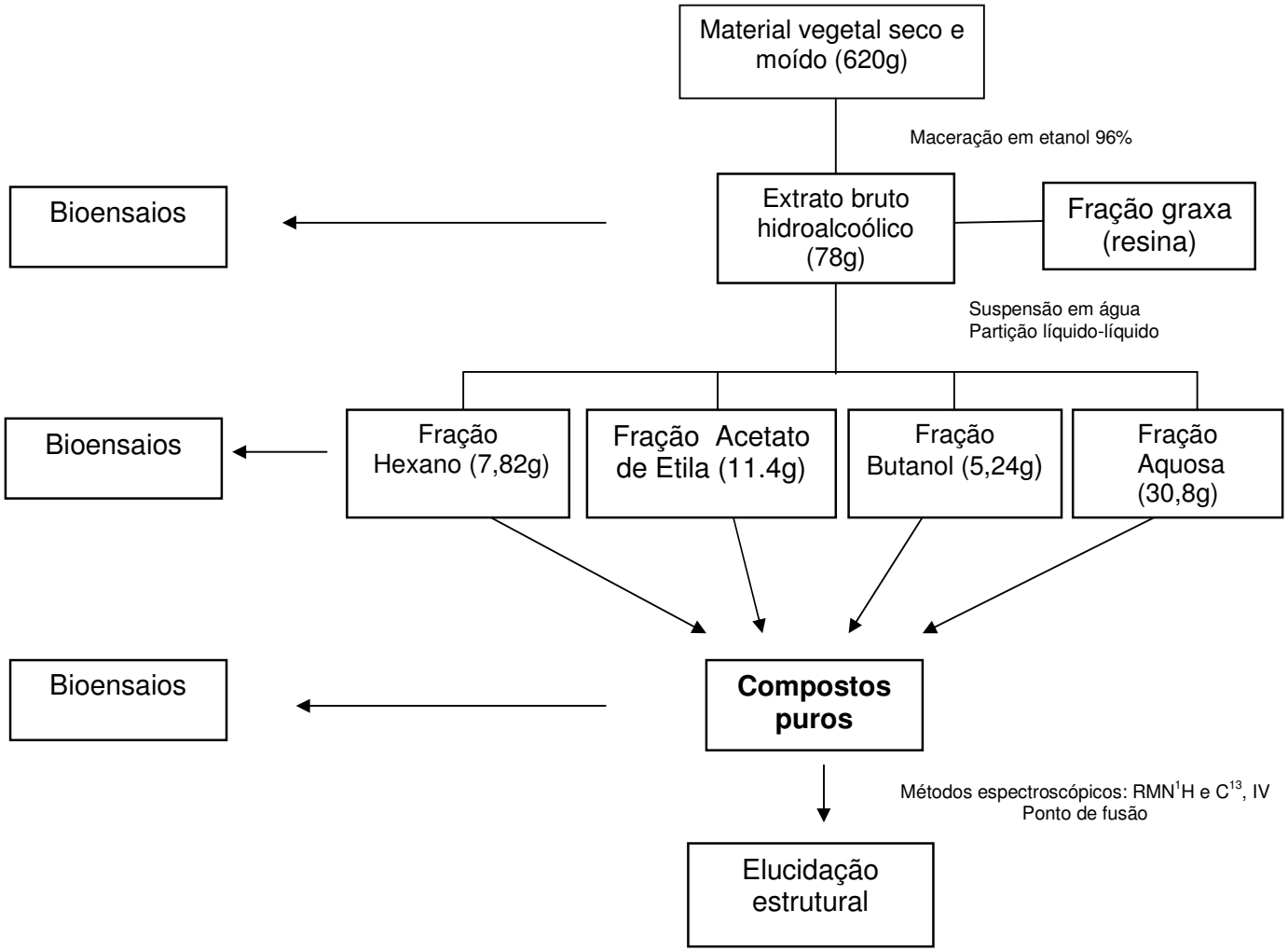
ANEXO 1

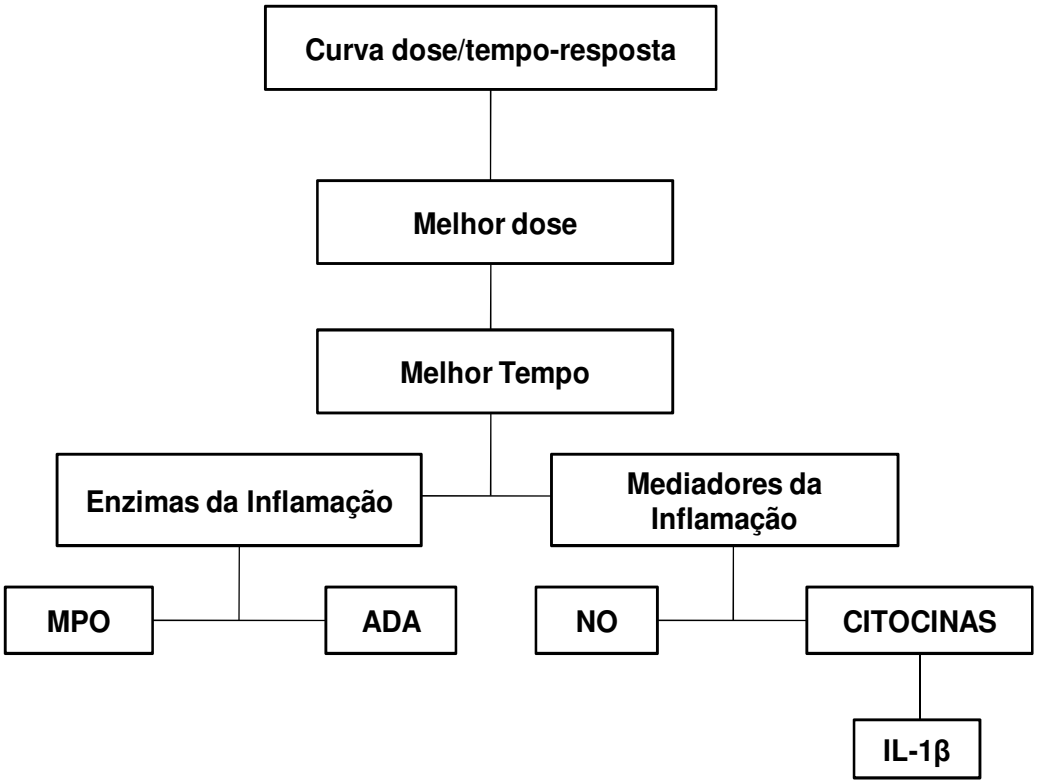
Protocolo de extração do extrato bruto, frações e isolamento dos compostos da *Lotus corniculatus* v. São Gabriel



Anexo 1: Esquema de fracionamento da espécie *Lotus corniculatus* v. São Gabriel

ANEXO 2

Protocolo do estudo do extrato bruto, frações e compostos isolados da *Lotus corniculatus* v. São Gabriel, no modelo da pleurisia induzida pela carragenina, em camundongos



Anexo 2 - Protocolo do estudo do extrato bruto, frações e compostos isolados da *Lotus corniculatus* v. São Gabriel, no modelo da pleurisia induzida pela carragenina, em camundongos.

ANEXO 3

Protocolo e cadastro da Comissão de Ética no Uso de Animais