Flávia Bonamin

Elucidação dos mecanismos de ação do monoterpeno β-mirceno sobre as úlceras pépticas

ORIENTADORA: Profa. Adj. Clélia Akiko Hiruma-Lima
CO-ORIENTADORA: Profa. Dra. Lúcia Regina Machado da Rocha

Tese apresentada ao Departamento de Farmacologia do Instituto de Biociências de Botucatu como requisito para a obtenção do Título de Doutor em Ciências Biológicas (AC: Farmacologia).

Botucatu/SP
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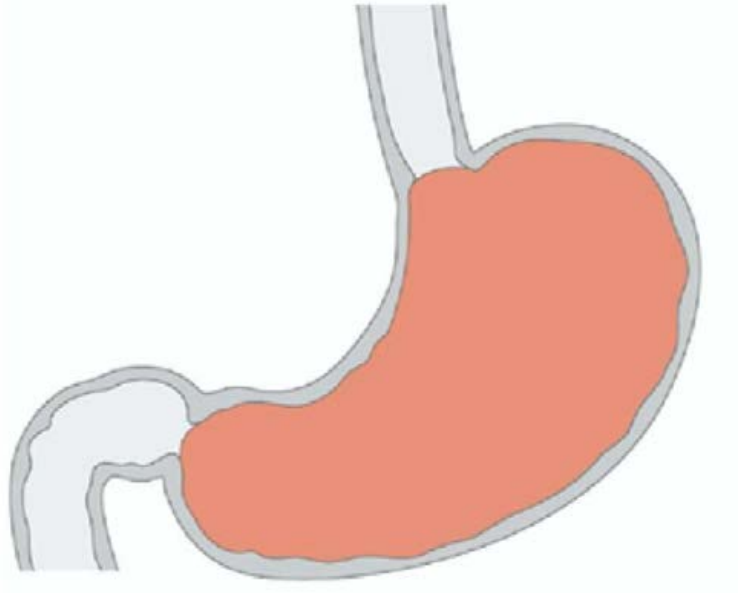

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Orientador: Clélia Akiko Hiruma-lima
Coorientador: Lúcia Regina Machado da Rocha
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Tese apresentada ao Departamento de Farmacologia do Instituto de Biociências de Botucatu como requisito para a obtenção do Título de Doutor em Ciências Biológicas (AC: Farmacologia).

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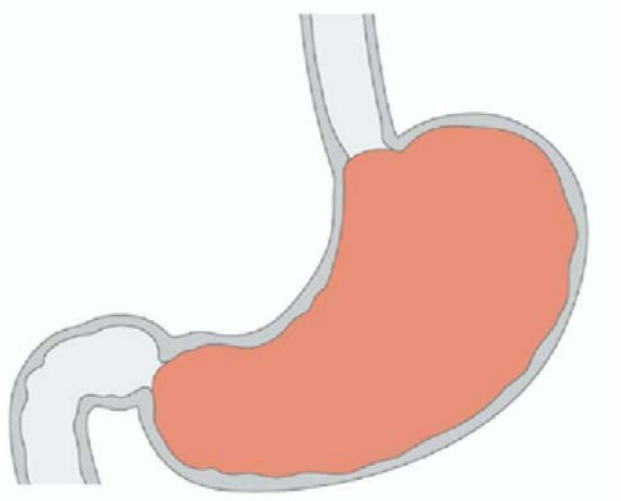
Botucatu/SP
2014

Auxílio Financeiro: **CAPES**
(Coordenação de Aperfeiçoamento de Pessoal de Nível Superior)

"A coisa mais bela que o homem pode experimentar é o mistério. É esta a emoção fundamental que está na raiz de toda ciência e arte. O homem que desconhece esse encanto está, por assim dizer, morto e tem os olhos extintos..."

Albert Einstein



Dedicatória

"A Família não nasce pronta; constrói-se aos poucos,
e é o melhor laboratório do amor. Em casa,
entre pais e filhos, pode-se aprender a amar,
pode-se experimentar com profundidade
a grande aventura de amar sem medo.
A família pode ser o ambiente mais
apropriado para uma maravilhosa
experiência de amor".

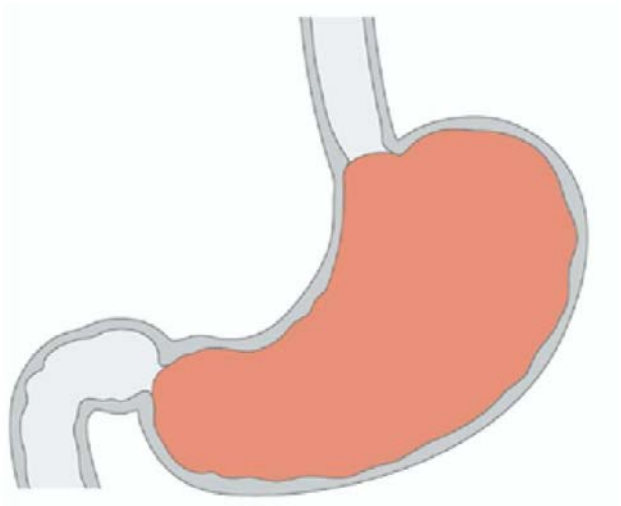
Mazenildo F. Pereira

Aos meus pais José Carlos e Juliana,
Queridos guerreiros, exemplo de vida, dedicação e perseverança!

“...Te amo con toda mi fe, sin medida...”

Maná

Ao Celso SaK’S,
Meu AMOR!



Agradecimentos

"E eu poderia suportar, embora não sem dor, que
tivessem morrido todos os meus amores, mas
enlouqueceria se morressem todos os meus amigos!
Até mesmo aqueles que não percebem o quanto
são meus amigos e o quanto minha vida
depende de suas existências...
A alguns deles não procuro, basta-me saber que eles existem.
Esta mera condição me encoraja a seguir
em frente pela vida."

Amigos – Vinicius de Moraes

Este trabalho não teria sido elaborado sem o auxílio de diversas pessoas às quais quero expressar meus sinceros agradecimentos. Começo então agradecendo,

Às minhas ***Orientadora e Co-orientadora*** *Profa. Adj. Clélia Akiko Hiruma-Lima e Profa. Dra. Lúcia R. M. da Rocha por todos os ensinamentos e por me ajudarem a realizar mais este sonho!!!Ahh...e também ao cãozinho* ***Magoo****...rsrs.*

Aos meus companheiros de laboratório *sempre presentes nos momentos importantes e ao técnico e amigo Hélio Kushima por toda a ajuda durante a realização do meu projeto de doutorado.*

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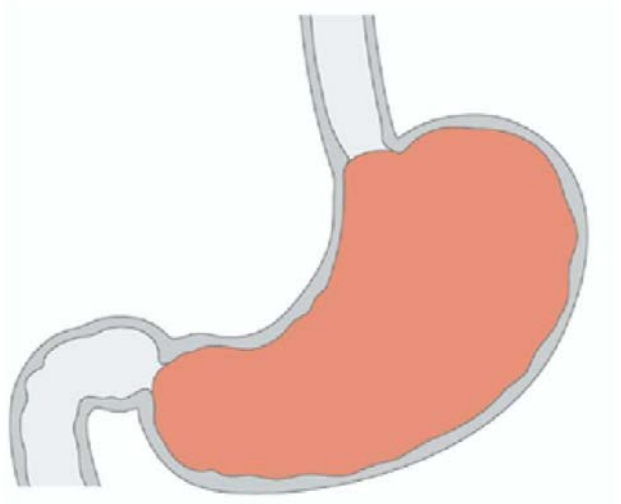
Muito Obrigada!!!

“Lute com determinação, abrace a vida com paixão, perca com classe e vença com ousadia, porque o mundo pertence a quem se atreve e a vida é muito para ser insignificante”.

Charles Chaplin



Prólogo

Prólogo

O projeto de doutorado possibilitou a formação específica em Farmacologia, com os estudos direcionados para a caracterização de atividade farmacológica de produtos naturais, sendo o objeto alvo dos estudos as úlceras pépticas. Durante a execução do projeto de doutorado, várias outras atividades foram realizadas, no intuito de enriquecer a formação profissional do aluno.

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1. BONAMIN, Flávia ; MORAES, T. M.; Kushima, H.; SILVA, M. A.; ROZZA, A. L.; Pellizzon, C.H.; Bauab, T.; ROCHA, L. R. M.; Wagner Vilegas; HIRUMA-LIMA, C. A. Can a Strychnos species be used as antiulcer agent? Ulcer healing action from alkaloid fraction of *Strychnos pseudoquina* St. Hil. (Loganiaceae). Journal of Ethnopharmacology, 2011.

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4. FALCÃO, Heloina De Sousa; MAIA, Gabriela Lemos De Azevedo; BONAMIN, Flávia; KUSHIMA, Hélio; MORAES, Thiago Mello; HIRUMA LIMA, Clélia Akiko; TAKAYAMA, Christiane; FERREIRA, Anderson Luiz; SOUZA BRITO, Alba Regina Monteiro; AGRA, Maria De Fátima; BARBOSA FILHO, José Maria; BATISTA, Leônia Maria. Gastroprotective mechanisms of the chloroform and ethyl acetate phases of *Praxelis clematidea* (Griseb.) R.M. King H. Robinson (Asteraceae). Natural Medicines, 2012.

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1. BONAMIN, Flávia; COSTA, C. A. R. A.; ROCHA, L. R. M.; HIRUMA-LIMA, C. A. Avaliação do monoterpeno β-mirceno nas úlceras gástrica e duodenal e seus mecanismos de ação. In: 11th ISE Congress and I Encuentro Hispano-Portugués de Etnobiología, 2010, Albacete/ESPANHA. Revista de Fitoterapia. Barcelona: Ediciones Rol, v. 10. p. 68-68, 2010.

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1. BONAMIN, Flávia; ROCHA, L. R. M.; HIRUMA-LIMA, C. A. Efeito do β-Mirceno sobre os níveis de glutationa e de mieloperoxidase na mucosa gástrica de ratos lesados por etanol absoluto e indometacina. In: XXI Simpósio de Plantas Medicinais do Brasil, 2010, João Pessoa/Paraíba. Anais do XXI Simpósio de Plantas Medicinais do Brasil, 2010.

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Cursos

1. Curso de Animales de laboratório: (Carga horária: 80h). Universidad de Buenos Aires, **2011**.
2. III Curso de Biologia Molecular Genotyping - Teórico e Prático - Marcadores Moleculares - Marcadores Moleculares, **2011**.

Cursos e palestras ministradas

1. BONAMIN, Flávia. **Curso de Atualização em Plantas Medicinais**. Encontro Nacional dos Estudantes de Biomedicina (ENBM), 2011.
2. BONAMIN, Flávia. Palestra **"Plantas Medicinais - Fitoterápicos"**. Universidade Federal do Mato Grosso (UFMT), 2011.

Participação em Bancas de Comissões Julgadoras

1. BONAMIN, Flávia. Comissão Julgadora de Trabalhos do XXIII Congresso de Iniciação Científica - CIC - UNESP. 2011. Universidade Estadual Paulista Júlio de Mesquita Filho.
2. BONAMIN, Flávia. Comissão Julgadora de Trabalhos do XXIV Congresso de Iniciação Científica da UNESP. 2012. Universidade Estadual Paulista Júlio de Mesquita Filho.

Organização de eventos científicos

1. BONAMIN, Flávia. III SIMFAR-Simpósio da Farmacologia. **2013**.
2. BONAMIN, Flávia. I Curso de Inverno em Farmacologia e Biotecnologia. **2013**.
3. BONAMIN, Flávia. II SIMFAR-Simpósio da Farmacologia. **2012**.
4. BONAMIN, Flávia. I SIMFAR-Simpósio de Farmacologia. **2011**.
5. BONAMIN, Flávia. Expositora das atividades do projeto de Extensão Universitária "Difundindo e popularizando a Ciência na UNESP: Interação entre Pós-Graduação e Ensino Básico", durante o evento "VENHA CONHECER O IB". **2011**.
6. BONAMIN, Flávia. IX Workshop da Pos-Graduação & X Workshop de Genética, **2010**.

Participação em eventos científicos

1. Simpósio Internacional de Plantas Medicinais e Nutracêuticos, **2012.**
2. Simpósio de Plantas Medicinais do Brasil, **2012.**
3. XIV Jornada de Toxicologia, **2012**.
4. Curso de Reprodução de A a Z, **2012**.
5. Ciência ao cair da tarde: Palestra com Miguel Nicolelis, **2012**.
6. I Simpósio de Farmacologia, **2011.**
7. 7th Biota Symposium, in the 7th Biota Program Assessment Meeting in the 4th Bioprospecta Program Assessment Meeting, **2011.**
8. III Curso de Biologia Molecular Genotyping - Teórico e Prático - Marcadores Moleculares - Marcadores Moleculares, **2011**.
9. Curso Reprodução de A a Z. Curso Reprodução de A a Z, **2011.**
10. Curso Pipetas GILSON: como aumentar a vida útil e obter melhores resultados, **2011**.
11. Curso de Animales de Laboratorio - Universidad de Buenos Aires, **2011**.
12. 11th ISE Congress and I Encuentro Hispano-Portugués de Etnobiología, **2010**.
13. XXI Simpósio de Plantas Medicinais do Brasil, **2010**.
14. IX Workshop da Pós-Graduação & X Workshop de Genética. **2010**.

Menção Honrosa

1. Menção Honrosa como Pôster no III SIMFAR - Simpósio da Farmacologia, Universidade Estadual Paulista – UNESP, **2013**.
2. Menção Honrosa como Pôster no XXI Simpósio de Plantas Medicinais do Brasil, XXI Simpósio de Plantas Medicinais do Brasil, **2010**.

Docência

Docente da disciplina de Farmacologia dos cursos de Enfermagem e Biologia da Faculdade Eduvale Avaré (Avaré/SP) (admitida em **2011**). Vínculo autorizado e devidamente aprovado pela CAPES e pelo Conselho do Programa de Pós-graduação em Farmacologia - IBB/UNESP.

Disciplinas Cursadas

1) Métodos Biofísicos de Avalição da Motilidade Gastrintestinal **(conceito A)**

2) Tópicos de Atualização em Ciências **(conceito A)**

3) Tópicos Especiais em Farmacologia: Farmacologia de plantas medicinais com atividade antinociceptiva (analgesica) e/ou antiinflamatória **(conceito A)**

4) Interação entre a Pós-Graduação e o Ensino Básico de Ciências e Biologia **(conceito A)**

5) Farmacologia Avançada **(conceito A)**

6) Classificação de Receptores Farmacológicos **(conceito A)**

7) Interação entre a Pós-Graduação e o Ensino Básico de Ciências e Biologia **(conceito A)**

8) Tópicos Especiais em Farmacologia: Métodos de Purificação e análise de Produtos Naturais **(conceito A)**

9) Tópicos Especiais em Farmacologia: Abordagem experimental e computacional para investigar a estrutura e função de macromoléculas biológicas e suas interações **(conceito A)**

LISTA DE ABREVIATURAS

HCl – Ácido Clorídrico

cAMP – Adenosina 3',5'- monofosfato cíclico)

CCK_2 – Receptores de colecistocinina

M_3 – Receptores de colecistocinina subtipo 3

Ca^{+2} – Cálcio

H^+/K^+-ATPase – Bomba de prótons ou bomba protônica

H_2 – Receptor de Histamina subtipo 2

Ach – Acetilcolina

H^+ - Hidrogênio

K^+ - Potássio

Cl^- - Cloreto

SST_2 – Receptor de somatostatina subtipo 2

Gi – Proteína G inibitória

PGs – Prostaglandinas

EP_3 – Receptor de prostaglandina

COX_1 – Enzima ciclooxigenase 1

COX_2 - Enzima ciclooxigenase 2

MEC – Matriz extracelular

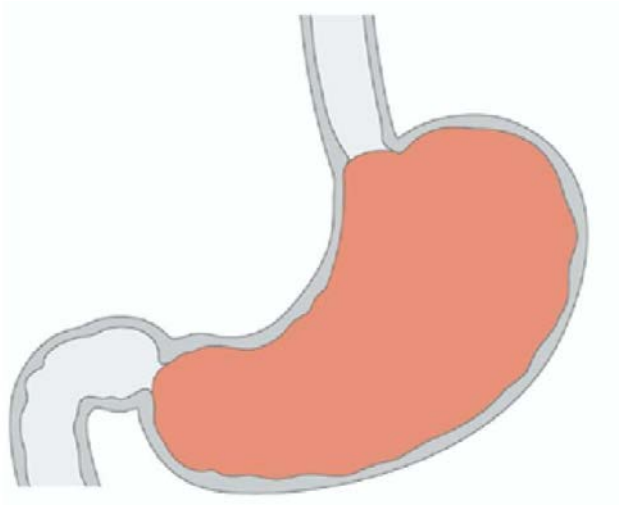
MMP_2 – Metaloproteinase de matriz 2

MMP_9 – Metaloproteinase de matriz 9

TIMPs – Inibidores teciduais de metaloproteinases

DAINEs – Drogas Antiinflamatórias Não-Esteroidais

IBPs – Inibidores de Bomba de Prótons



Resumo Geral da Tese

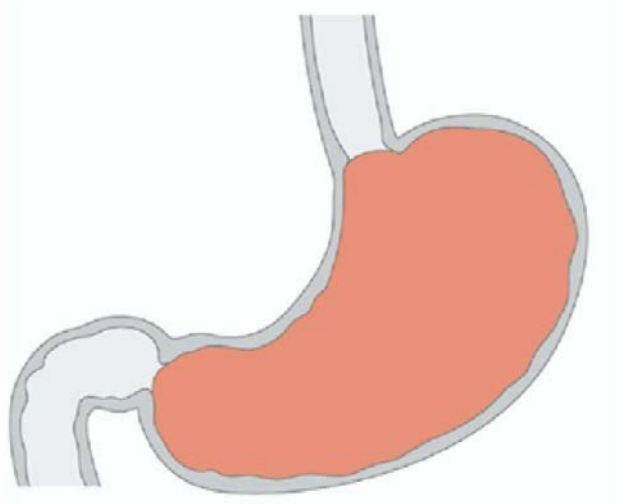
RESUMO

Resumo Geral da Tese

A doença ulcerosa péptica é um comprometimento da mucosa gástrica e duodenal de pacientes decorrente do ataque de fatores lesivos endógenos e/ou exógenos sobre este tecido. Dentre os fatores endógenos que favorecem o surgimento desta doença, destacam-se o enfraquecimento da barreira mucosa e o déficit de seus componentes protetores (como o muco e o bicarbonato) que, conseqüentemente contribuem para o avanço dos fatores lesivos sobre a mucosa péptica desprotegida. Entretanto, o freqüente e progressivo contato que os indivíduos têm com os fatores exógenos, como o uso constante de medicamentos (drogas antiinflamatórias não-esteroidais, antibióticos, dentre outros), o aumento na produção de ácido gástrico e o ataque da bactéria *Helicobacter pylori* são apontados como os mais freqüentes indutores de lesões ulcerosas. O estudo de novos compostos oriundos de plantas medicinais potencializa a descoberta de novos protótipos que podem ser utilizados na terapêutica. Estudos anteriores realizados no Laboratório de Ensaios Biológicos com Produtos Naturais (UNESP/Botucatu) caracterizaram fitoquimicamente o óleo essencial de *Citrus aurantium*, uma espécie medicinal que possui fortes indicativos de uso popular para gastrites e distúrbios do trato gastrintestinal, apontando a presença do monoterpeno β-mirceno (1.43%) como um dos constituintes minoritários de sua composição. A proteção promovido pelo β-mirceno, na dose de 7.5 mg/kg, variou de 60 a 86% de proteção da mucosa gástrica e duodenal o que representa uma ação equivalente a 13 vezes à promovida pela cimetidina (dose 100 mg/kg) ou 4 vezes o efeito do lansoprazol (30 mg/kg), duas drogas antiulcerogênicas padrão usadas na terapêutica. A etapa seguinte foi elucidar outros mecanismos de ação deste monoterpeno que envolveu a caracterização se sua propriedade antissecretória e cicatrizante gástrica. Para um melhor entendimento do trabalho, os resultados foram divididos em dois capítulos. No capítulo 1 encontra-se o trabalho intitulado "*β-myrcene, a monoterpene with antissecretory gastric activity and protective against gastroesophageal reflux: role of calcium channels and gastrin in the gastroprotection*". Este estudo mostra os resultados da atividade do β-mirceno em diversos modelos experimentais que caracterizam a secreção ácida gástrica e elucida os mecanismos de ação antissecretório do monoterpeno. O β-mirceno promove a diminuição da acidez gástrica por meio da diminuição dos níveis séricos de gastrina, atua através dos canais de cálcio e K^+atp para modular a secreção ácida. Além disso, este monoterpeno combate os efeitos do refluxo gastroesofágico e reduz a peroxidação lipídica na mucosa.No capítulo 2, encontra-se o trabalho intitulado *The effects of monoterpene β-myrcene on gastric healing activity: role of cytokines, metalloproteinases of matrix -2 and -9 and gastric contractions recovery*. Este estudo evidencia o efeito cicatrizante do β-mirceno e seus mecanismos de ação caracterizados atuação sobre as metaloproteinases de matriz 2 (MMP-2), diminuição dos níveis de MPO, envolvimento de interleucinas e de COX-1 e COX-2. O efeito do β-mirceno sobre a mucosa gástrica também envolve o reestabelecimento das contrações gástricas após a ulceração. Os resultados indicaram que β-mirceno (7,5 mg/kg) tem importante atividade cicatrizante gástrica por via oral com a diminuição significativa de lesões gástricas induzidas por ácido acético, não mostrando nenhum sinal de toxicidade durante o tempo de tratamento (7 ou 14 dias de tratamento). Investigou-se a regulação das MMPs (MMP-2 e -9) na úlcera gástrica induzida por ácido acético de animais submetidos a tratamentos durante 7 e 14 dias com β-mirceno. Nos grupos de animais tratados durante 7 dias, a atividade da enzima MMP-2 estava presente em todos os grupos, porém a mesma se mostrou maior no grupo tratado com veículo e menor nos grupos tratados com o lansoprazol, β-mirceno e sham (18,5%, 25% e 26%, respectivamente).
Foi observada a atividade da MMP-9 em todos os grupos tratados durante 14 dias, mas não de

forma significativa. Além disso, os níveis de interleucina IL-1β foi significativamente maior nos estômagos ulceradas enquanto o tratamento com β-mirceno (14 dias)reduziu os níveis de interleucina IL-1β no tecido gástrico. O mesmo ocorreu com os níveis de MPO, no qual se observou diminuição significativa nos estômagos tratados com o monoterpeno β-mirceno (30%) em relação ao veículo. A atividade cicatrizante do monoterpeno envolve igualmente a participação da COX-1 (com 7 dias de tratamento) e COX-2 (com 14 dias de tratamento), bem como à recuperação (após 7 dias) das contrações gástricas depois da ulceração com restabelecimento de 40% da frequência e 88% da amplitude das contrações. Com base em todos os resultados obtidos nesta tese, pode-se conclui que o β-mirceno é um composto de origem natural que possui uma efetiva ação cicatrizante da mucosa gástrica e apresenta uma considerável atividade antissecretória, ambas ações foram desprovidas de efeitos tóxicos.

Palavras-chave: úlcera gástrica; β-mirceno; monoterpeno; antissecretório; cicatrizante.



Introdução Geral da Tese

INTRODUÇÃO

CONSIDERAÇÕES INICIAIS

A humanidade tem convivido com as úlceras pépticas desde tempos remotos. Talvez a primeira descrição dessa doença tenha sido nos pilares do templo de Esculápio em Epidaurus por volta do século IV a.C.: "um homem com úlcera no estômago. Ele incubou e teve uma visão; o Deus pareceu ordenar a seus seguidores que o capturassem e o mantivesse prisioneiro, que ele poderia operar o seu estômago. Assim, o mesmo fugiu, mas foi capturado e amarrado a uma aldrava. Em seguida Esculápio abriu-lhe o estômago, retirou a úlcera, costurou-o novamente e soltou suas amarras". Muitas pessoas ilustres sofreram de indigestão e úlceras, inclusive o imperador romano Marco Aurélio, cuja morte foi atribuída por alguns a uma úlcera perfurada e cujo médico era o próprio Galeno. A neutralização ácida foi reconhecida como um tratamento eficaz há mais de doze séculos por Paulus Aeginata, o qual prescreveu uma mistura de solos de Samos e Lemnos e leite, não diferente dos esquemas terapêuticos com leite e antiácidos usados em meados do século XX".

O parágrafo acima retirado de HOOGERWERF & PASRICHA em "Goodman & Gilman's The Pharmacological basis of the therapeutic" (2005), citando SMITH & RIVERS (1953), mostra o longo histórico das úlceras pépticas e sua importância na história da humanidade.

Úlceras pépticas, uma das mais importantes causas de morbidade em todo o mundo, são áreas lesionadas da mucosa gástrica e duodenal resultante de diferentes fatores, incluindo redução do fluxo sanguíneo local, diminuição na produção de prostaglandinas, bem como decorrente de um aumento da liberação de ácido gástrico e espécies reativas de oxigênio na mucosa gástrica e duodenal (LAU et al., 2011;. TARNAWSKI et al., 2012.; THORSEN et al., 2013). Esta lesão se estende através da muscularis mucosae até camadas mais profundas (submucosa ou a muscularis propria). Assim, ao atingir estas camadas, pode haver o rompimento de vasos sanguíneos e dessa forma ocasionar hemorragia (YEOMANS & NAESDAL, 2008).

A úlcera péptica é considerada um problema grave e é altamente prevalente em todo o mundo com uma incidência crescente ano após ano. Ulcerações e sangramentos gástricos têm sido atribuídos muitas vezes a um aumento na secreção de ácido gástrico, que destrói a barreira mucosa e penetra na camada de muco, permitindo que agressores endógenos e exógenos promovam lesões na superfície do epitélio celular (GUSTAFSON & WELLING, 2010).

A incidência e prevalência das úlceras pépticas são difíceis de serem estimadas devido a sua característica extremamente dinâmica. Nesse contexto, a subjetividade sintomática e a semelhança do quadro clínico com vários tipos de dispepsia geram dados contraditórios na

literatura científica. No entanto, mesmo considerando estas dificuldades, D'ACAMPORA e colaboradores (2008) comentam que a incidência de ulcera péptica sobre a população no Brasil varia de 1 a 20%. Apesar de ser uma doença extremamente freqüente, não se conhece de modo preciso sua real incidência neste país.

A regulação da secreção ácida gástrica é um processo complexo, mediado por mecanismos neurais, hormonais, parácrinos e autócrinos em níveis central e periférico os quais convergem para a etapa final da secreção de ácido clorídrico (HCl): a atividade da enzima H^+/K^+- ATPase nas células parietais gástricas (SCHUBERT, 2004).

O trato gastrointestinal desenvolve atividades como digestão, proteção e motilidade por meio da secreção de ácido, enzimas digestivas, muco, bicarbonato, bem como controle na absorção de água, nutrientes e eletrólitos. Essas atividades são reguladas pelo sistema nervoso composto de fibras neuronais extrínsecas (neurônios sensoriais e eferentes do sistema nervoso autônomo parassimpático e simpático), intrínsecas (sistema nervoso entérico), mediadores parácrinos, autócrinos e endócrinos (KOEPPEN & STANTON, 2009).

O sistema nervoso autônomo parassimpático regula as funções gastrointestinais por meio da liberação de acetilcolina, o simpático atua via ação de noradrenalina, enquanto o sistema nervoso entérico libera neuropeptídeos que agem controlando a secreção gastrointestinal via plexo submucoso, localizado na camada submucosa e a motilidade pelo plexo mioentérico localizado entre as camadas muscular longitudinal e circular. Dentre os principais hormônios que controlam o funcionamento do trato gastrointestinal, podem ser citados a histamina, gastrina, somatostatina, colecistocinina, secretina, peptídeo YY e serotonina (KOEPPEN & STANTON, 2009). Outros peptídeos que regulam a ingestão de alimentos, incluindo a grelina, Orexina-A e especialmente o recém-descoberto nesfatin-1, de forma semelhante à leptina e ao peptídeo YY, não são apenas envolvidos na secreção endócrina e regulação da saciedade e da fome, mas também participar de outros mecanismos fisiológicos, incluindo a atividade secretória gástrica e pancreática e também motora do trato gastrointestinal bem como a proteção da mucosa gástrica contra agentes agressores à ela (SZLACHCIC et al., 2013).

Os receptores desses secretagogos (Histamina - H_2, Acetilcolina - M_3 e de Gastrina - CCK_2) foram identificados anatômica e farmacologicamente na membrana basolateral da célula parietal. Existem duas vias principais de sinalização na célula parietal: a via dependente do cAMP e a via dependente do Ca^{2+}(**Figura 1**). Enquanto a histamina utiliza a primeira via, a gastrina e a acetilcolina (Ach) exercem seus efeitos por meio da última. A via dependente de cAMP resulta na fosforilação das proteínas efetoras da célula parietal e a via dependente do Ca^{2+} leva a um

aumento do Ca^{2+} citosólico. Ambas as vias ativam a H^+/K^+-ATPase (a bomba protônica), que consiste em uma grande subunidade α e uma subunidade β menor. Essa bomba produz o maior gradiente iônico conhecido nos vertebrados, com um pH intracelular de cerca de 7,3 e um pH intracanalicular de mais ou menos 0,8 (HOOGERWERF & PASRICHA, 2005).

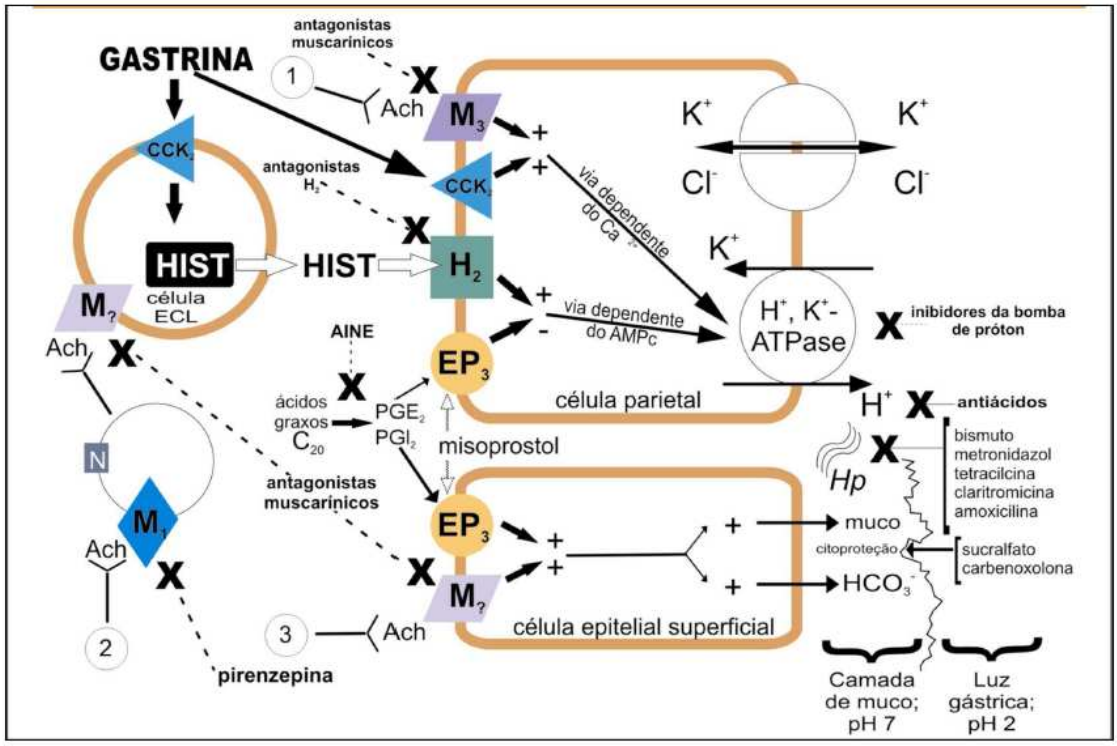


Figura 1. Regulação fisiológica e farmacológica das secreções gástricas: a base para o tratamento da doença ulcerosa péptica. Adaptado de HOOGERWERF & PASRICHA, (2005).

Quando não estimulada, a atividade da H^+/K^+-ATPase é mantida predominantemente dentro de túbulo-vesiculas citoplasmáticas. Sob estimulação, estas vesículas se fundem com a membrana plasmática apical, resultando em extensiva secreção. Ao cessar a secreção, H^+/K^+-ATPase é recolhida da membrana apical, e restabelecida no seu compartimento túbulo-vesicular. Não se sabe ao certo o mecanismo exato desta regulação, mas os dados sugerem que microfilamentos de actina, GTPases e também proteínas de ancoragem/fusão sejam os responsáveis por este mecanismo (SCHUBERT & PEURA, 2008).

A secreção de prótons ocorre nas células parietais pela troca de íons hidrogênio H^+ por íons potássio K^+ através da enzima H^+/K^+-ATPase. Este mecanismo ocorre juntamente com extrusão de Cl^- via canais de cloreto e potássio na membrana apical. Esses canais são alvos freqüentes de estudos de drogas anti-secretoras (SCHUBERT & PEURA, 2008).

Para controlar a secreção de ácido gástrico e de fluido intestinal, as células D localizadas no estômago e duodeno secretam somatostatina que por ação endócrina e parácrina atuam sobre os receptores SST_2 acoplados a proteína inibitória Gi localizadas nas membranas plasmáticas das células G, enterocromafins e parietal (YANG et al., 2010). A somatostatina também pode ser liberada pelas fibras nervosas intrínsecas e inibe o peristaltismo gastrointestinal, diminui a secreção biliar e a proliferação celular (SUN et al., 2002).

Outro mediador endógeno responsável pela regulação da acidez gástrica são as prostaglandinas (PGs) que estimulam os receptores EP_3 localizados nas células parietais (SCHUBERT & PEURA, 2008).

MECANISMOS DE DEFESA DA MUCOSA

Para se proteger da ação corrosiva do ácido e proteolítica da pepsina, a mucosa gastroduodenal faz uso de diversos mecanismos de defesa os quais abrangem processos dinâmicos que incluem mecanismos locais e neuro-hormonais (LAINE et al., 2008; WALLACE, 2008).

Didaticamente, a defesa da mucosa pode ser dividida em fatores pré-epiteliais, epiteliais, sub-epiteliais, e da defesa imune (**Figura 2**).

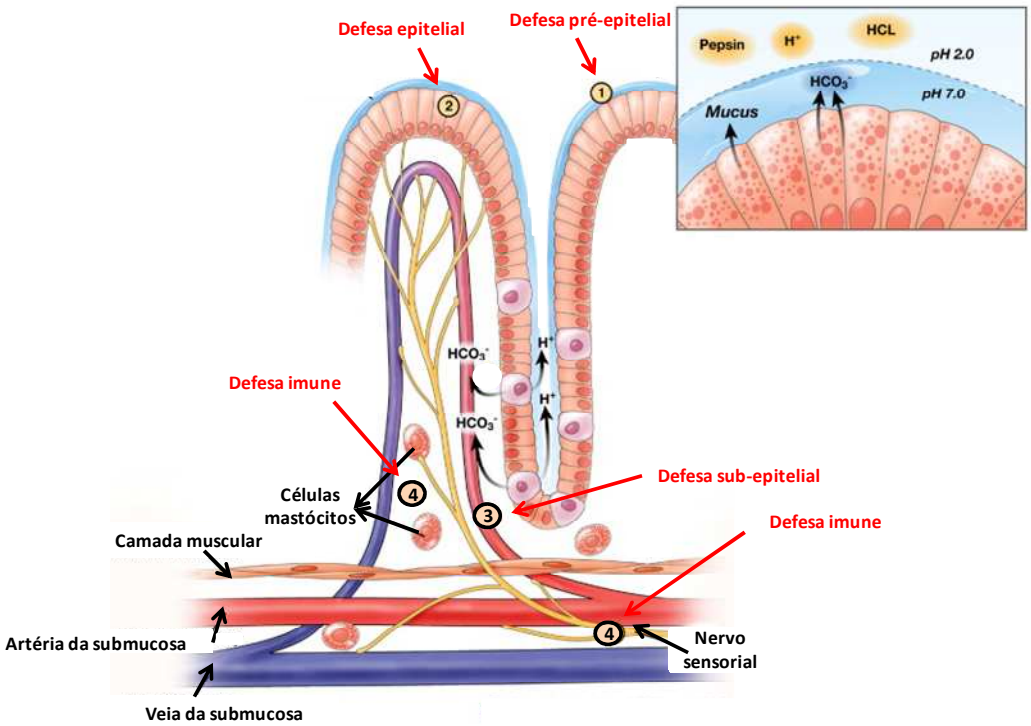


Figura 2. Ilustração dos fatores de defesa da mucosa (Adaptado de LAINE et al., 2008).

Os fatores de defesa pré-epiteliais (n.1 da figura 2) representam os diferentes mecanismos de proteção que ocorrem no lúmen. Estes fatores incluem a barreira física de muco-bicarbonato e os fosfolipídeos que revestem a camada celular, impedindo a ação do ácido e pepsina sobre a superfície epitelial. Os fatores de defesa epiteliais (n.2 da figura 2) são recrutados quando o epitélio superficial é lesado com rápida migração das células do colo glandular para a área afetada, cobrindo a lesão. O fator de crescimento epidérmico estimula a síntese de DNA e o desenvolvimento celular. Como principal fator de defesa subepitelial (n.3 da figura 2) tem-se o fluxo sangüíneo. Este é o principal carreador de substâncias lesivas como H^+ e toxinas, e fornece nutrientes impedindo a lesão celular (LAINE et al., 2008). Outro importante fator de defesa da mucosa é a resposta imune (n.4 da figura 2). Este fator exerce proteção e reparo celular através de células de alerta como mastócitos e macrófagos que, por sua vez, mobilizam todo sistema imunológico celular para uma resposta inflamatória de proteção (WALLACE, 2008).

O epitélio gástrico humano renova-se completamente a cada 2-4 dias. Conforme a **Figura 3**, e o processo de cicatrização da mucosa na úlcera gastrointestinal requer reconstituição da estrutura glandular epitelial (migração, proliferação e re-epitelização), restauração da lâmina própria incluindo uma rede microvascular na mucosa (angiogênese), restauração de nervos e células de tecidos conjuntivos (deposição de matriz fibrosa) levando a formação de uma cicatriz (TARNAWSKI, 2005).

Figura 3. Representação esquemática da úlcera gástrica e seus dois maiores componentes envolvidos na cicatrização: a margem da úlcera (re-epitelização) e o tecido de granulação (angiogênese e reconstrução glandular) (Adaptado de TARNAWSKI 2005).

Histologicamente, a úlcera gástrica é formada por uma margem chamada "zona de cicatrização", em que o tecido não necrosado e as glândulas dessa região começam a dilatar e as células de revestimento sofrem diferenciação. E formada uma base de tecido granuloso constituído de tecido conectivo (fibroblastos, macrófagos e microvasos) que surgem em um período de 48–72 horas após a lesão e são continuamente modulados (região de re-epitelização). A angiogênese facilita esse processo por liberar oxigênio e nutrientes para este local (TARNAWSKI, 2005).

A migração de fibroblastos e a sua proliferação são estimuladas por fatores de crescimento e citocinas, como TNF-α e IL-1 derivados de células inflamatórias, células endoteliais ativadas e macrófagos. Os fatores de crescimento epidermal (EGF), endotélio vascular (VEGF), de fibroblasto (FGF), do hepatócito (HGF), derivado de plaquetas (PDGF) e tumoral β (TGF-β), juntamente com a ação de prostaglandinas produzidas pela enzima ciclooxigenase 2 (COX-2) ativadas por IL-1, estimulam a diferenciação, proliferação e migração celular (TARNAWSKI, 2005).

As prostaglandinas geradas em uma úlcera gástrica, pela COX-2, parecem ter um papel fundamental na sua cicatrização, estimulando a proliferação celular, promovendo angiogênese e a restauração da integridade da mucosa. Por outro lado, as prostaglandinas produzidas pela COX-1 (constitutivas), são responsáveis pela regulação do fluxo sanguíneo da mucosa e secreção epitelial de muco e HCO_3^- (KONTUREK et al., 2005).

Para WALLACE (2005), a contribuição de COX-2 para a defesa da mucosa gástrica e o processo de cicatrização está cada vez mais clara, o que pode levar ao desenvolvimento de novas terapias. A expressão de COX-2 controla mecanismos de proteção da mucosa como re-epitelização, angiogênese e citoproteção adaptativa.

Além das ciclooxigenases, outro fator envolvido na cicatrização de úlceras gástricas é a matriz extracelular. A matriz extracelular (MEC) é um complexo de proteínas fibrilares, proteoglicanos e glicosaminoglicanos essencial para a arquitetura, fisiologia e biomecânica dos tecidos. A MEC é composta por uma rede de colágeno, especificamente os colágenos tipo I e III, elastina, fibronectina e laminina. Juntas, essas proteínas garantem a integridade estrutural, a flexibilidade e a adesão às células por apresentarem domínios de interação com receptores específicos da membrana celular. (SPINALE, 2002; ADAIR-KIRK & SENIOR, 2008).

A função biológica da MEC é o suporte estrutural capaz de manter a arquitetura do tecido e preencher os espaços entre as células. Além disso, confere adesão e forma, permitindo a migração, a proliferação e a diferenciação das células adjacentes (ADAIR-KIRK & SENIOR, 2008). Alterações na MEC, como mudanças no equilíbrio entre sua síntese e degradação e na

continuidade da rede de colágeno fibrilar são as características essenciais do remodelamento tecidual que ocorre em muitos processos patológicos (ROY et al., 2011). Dessa forma, a manutenção da integridade e da organização da MEC é de grande importância e tem recebido destaque em pesquisas científicas.

Componentes protéicos da MEC têm sido estudados devido à importante participação no remodelamento do tecido conjuntivo. Dentre esses componentes, destacam-se as metaloproteinases de matriz (MMPs). As MMPs compreendem um amplo grupo de endopeptidases zinco-dependentes, classificadas de acordo com sua afinidade por determinado substrato. Com base especificamente em sua estrutura primária, as MMPs são categorizadas em colagenases, gelatinases, estromelisinas, matrilisinas, MMPs de membrana, entre outras (GEURTS et al., 2011). Uma das funções dessas endopeptidases é a degradação de componentes da matriz extracelular, sendo reguladores importantes de uma variedade de processos fisiológicos. Além disso, atuam na resposta imune clivando citocinas e quimiocinas e, assim, participando ativamente da inflamação (VANHOUTTE et al., 2006; KUPAI et al., 2010).

Por serem capazes de degradar componentes protéicos da MEC, as MMPs participam de uma variedade de processos fisiológicos como angiogênese, reparação tecidual, morfogênese, mobilização de células tronco e cicatrização de feridas (VANHOUTTE et al., 2006; KUPAI et al., 2010).

As MMPs podem ser divididas em grupos de acordo com sua especificidade a determinado substrato, as colagenases que degradam colágeno intersticial, as gelatinases A e B (MMP-2 e MMP-9) que degradam gelatina, colágeno do tipo IV, V, VII e IX, elastina e fibronectina, stromelisinas 1-3 que possui uma ampla especificidade e, por fim, as MMPs do tipo membrana (VISSE & NAGASE, 2003). Essas enzimas são sintetizadas e secretadas por células epiteliais gástricas, macrófagos e neutrófilos, e sua ativação é dependente de vários fatores como proteases celulares e os inibidores teciduais de metaloproteinases (TIMPs) (KUPAI, 2010).

A MMP-2 está envolvida no processo de ulceração e remodelagem da matriz extracelular, enquanto a MMP-9 está relacionada principalmente ao processo inflamatório. SHAHIN e colaboradores (2001) mostraram que a primeira observação da expressão do RNA-MMP-2 ocorre 24 horas após a indução da lesão, o que coincide com a remoção do tecido necrosado, e que 48 horas após a lesão ocorre um aumento na expressão, que ocorre conjuntamente com a ampliação do diâmetro da lesão, após a retirada total do tecido necrosado. Esses dados mostram que a MMP-2 tem um potencial de destruição, promovendo o processo de ulceração através da degradação local da matriz e de proteólise tecidual, sendo que a presença desta metaloproteinase

é constatada na parede gástrica até 3 semanas após a indução da lesão. Parte disso se dá porque elas são necessárias para que ocorra a migração de fibroblastos, neutrófilos e macrófagos para a área lesada, para que esses efetuem o reparo. No 30º dia após a lesão, a atividade enzimática diminui e a síntese de colágeno é neutralizada. Nesta fase existe um equilíbrio entre a degradação tecidual e o processo de reconstrução que evita a deposição excessiva de colágeno, que levaria a formação de uma cicatriz (SHAHIN et al., 2001).

A qualidade de restauração da mucosa gastrointestinal na região lesionada é determinante na cicatrização a fim de evitar recorrências na região adjacente frente aos agentes ulcerosos. Estudos relataram que uma re-epitelização rápida e grosseira causa anormalidades no tecido, como altura reduzida do epitélio, dilatação das glândulas, aumento de tecido conectivo e uma rede microvascular desorganizada. Assim, a cicatrização da úlcera é um processo complexo e bem regulado de preenchimento da mucosa lesada com proliferação, migração de células dos tecidos epitelial e conjuntivo. Este processo inclui re-estabelecimento da superfície contínua de camada epitelial, estruturas glandulares, microvasos e tecido conjuntivo no interior da cicatriz (TARNAWSKI, 2005).

TERAPÊUTICA MEDICAMENTOSA E SUAS LIMITAÇÕES

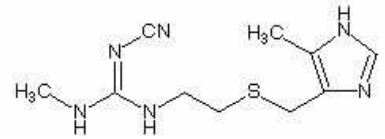
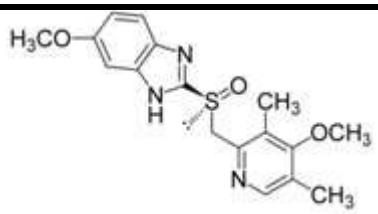
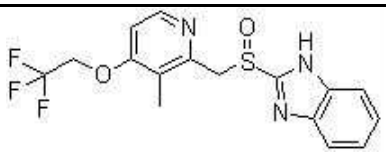
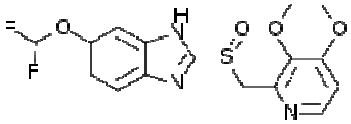
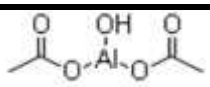
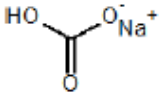
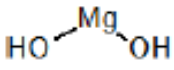
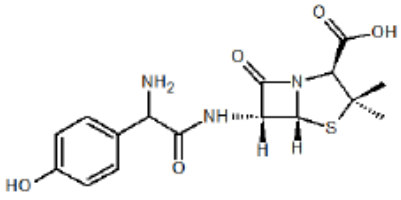
HOOGERWERF & PASRICHA em "Goodman & Gilman's The Pharmacological basis of the therapeutic" (2005), citando SMITH & RIVERS (1953) chamam a atenção para os enormes avanços no conhecimento da patogênese e no tratamento dos quadros clínicos de úlceras pépticas, entretanto a descoberta da *Helicobacter pylori* (TYRGAT & RAUWS, 1986) e dos inibidores protônicos (SIEPLER et al., 1986) são os últimos grandes acontecimentos e ambos ocorreram no século passado. Do ponto de vista clínico, o controle da acidez nestes casos, bem como o tratamento anti *H. pylori* parece estar bem estabelecido. No entanto alguns avanços ainda devem ser feitos, tendo em vista que os antinflamatórios não esteroidais – os DAINES – são os maiores responsáveis pelo surgimento de úlceras pépticas, depois do *H. pylori* (FIORUCCI et al., 2009).

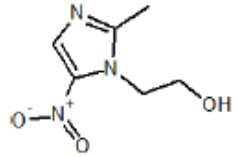
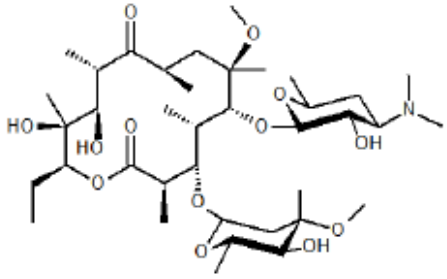
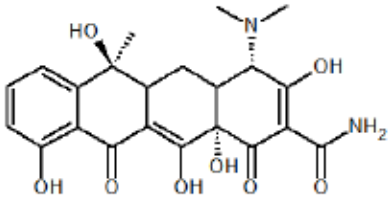
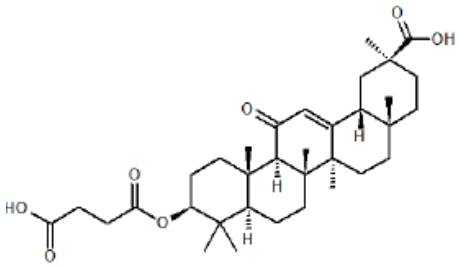
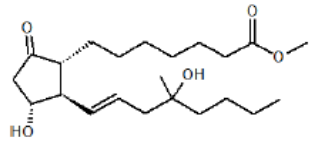
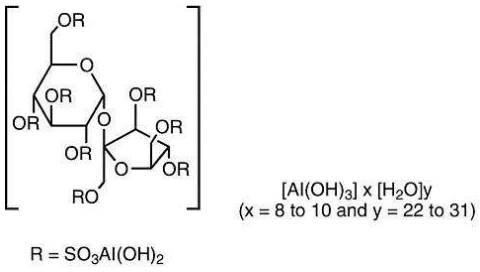
Na tentativa de proteger a mucosa do ácido gástrico, aumentar a velocidade e qualidade da cicatrização e prevenir a recorrência da úlcera, a farmacoterapia utiliza diversas alternativas terapêuticas que são direcionadas à redução dos fatores de agressão, neutralização ácida, supressão ácida, ou indução de mecanismos citoprotetores (AIHARA et al., 2003).

Nesse contexto, vários fármacos, como os antiácidos, antagonistas dos receptores histaminérgicos e colinérgicos, inibidores da bomba protônica (H^+/K^+ATPase), citoprotetores, além

dos fármacos com a função de erradicar a *H. pylori* têm sido desenvolvidos, e muito deles ativamente utilizados no tratamento de doenças ulcerosas pépticas (JAIN et al., 2007). Na **Tabela 1** estão dispostos alguns exemplos de fármacos utilizados na terapia da úlcera gástrica, bem como sua estrutura química, e classificação farmacológica.

Tabela 1. Exemplos de fármacos utilizados na terapêutica da úlcera péptica, classificação farmacológica e estrutura química.

Classificação farmacológica	Fármaco	Estrutura química
Antagonistas dos receptores H_2	Cimetidina	
	Ranitidina	
Inibidores da bomba de prótons	Omeprazol	
	Lansoprazol	
	Pantoprazol	
Antiácidos	Hidróxido de alumínio	
	Bicarbonato de sódio	
	Hidróxido de magnésio	
Antibióticos	Amoxicilina	

	Metronidazol	
Antibióticos	Claritromicina	
	Tetraciclina	
Citoprotetores	Carbenoxolona	
	Misoprostol	
	Sucralfato	

Entretanto, o uso disseminado e por tempo prolongado, como normalmente requer a terapêutica com estes fármacos, têm permitido um acúmulo de informações que revelam a presença de efeitos colaterais (KATZUNG, 2010).

Os fármacos ativos na neutralização ácida, como o bicarbonato de sódio, hidróxido de magnésio e alumínio, agem apenas no alívio dos sintomas, não sendo efetivos no processo de cura da mucosa. Seu princípio terapêutico encontra-se embasado em sua capacidade de reagir com HCl^- formando H_2O e CO_2^-. Embora, eficazes no alívio dos sintomas da doença, possuem efeitos indesejáveis como flatulência, náuseas, diarréia ou constipação (AIHARA et al., 2003; JAIN et al., 2007).

Por outro lado, os supressores ácidos são capazes de promover a cura das lesões, e por isso são muito utilizados no tratamento da doença. Dentre eles, destacam-se os anticolinérgicos como a pirenzepina e talenzepina, capazes de reduzir a produção basal de ácido em cerca de 50%. No entanto, seu uso está cada vez mais restrito devido aos efeitos colaterais tais como taquicardia, boca seca, visão turva e constipação (AIHARA et al., 2003; HOOGERWERF & PARSRICHA, 2005; JAIN et al., 2007).

Outra classe de supressores ácidos são os antagonistas de receptores histaminérgicos do tipo H_2 como a cimetidina, ranitidina, famotidina, nizatidina e roxotidina. A ação destes medicamentos envolve a inibição da interação da histamina com receptores H_2, reduzindo o volume do suco gástrico, bem como a concentração de íons H^+. No entanto, o uso prolongado dessas substâncias também pode provocar efeitos adversos, sendo a diarréia, cefaléia, tontura, fadiga, dor muscular e prisão de ventre os efeitos colaterais mais freqüentes (AIHARA et al., 2003; HOOGERWERF & PARSRICHA, 2005; JAIN et al., 2007). Em adição, casos de ginecomastia em homens e galactorréia em mulheres devido à ligação da cimetidina aos receptores androgênicos e à inibição da hidroxilação do estradiol catalisada pelo citocromo P450, também foram descritos. Em pacientes do sexo masculino há também relatos de redução na contagem de espermatozóides e impotência reversível em usuários deste medicamento (HOOGEWERF & PARSRICHA, 2005).

Os inibidores da bomba de prótons (omeprazol, lansoprazol, rabeprazol, pantoprazol e esomeprazol) são os mais potentes supressores ácidos do estômago e têm sido largamente utilizados por atuarem como inibidor do sangramento gástrico, como coadjuvantes na erradicação do *H. pylori*, bem como na profilaxia de recidivas do episódio ulceroso. Eles atuam ligando-se a H^+/K^+ATPase localizada na membrana da célula parietal e inibindo a liberação de íons H^+ para lúmen gástrico (JAIN et al., 2007).

Atualmente sabe-se que os inibidores da bomba de prótons podem participar de interações com outros fármacos interferindo negativamente na ação terapêutica dos mesmos (MARCOLIN et al., 2004). Somado a isso ZLABEK & ANDERSON (2002) descrevem reações adversas graves como trombocitopenia; RA & TOBE (2004) citam a nefrite intersticial aguda; FISHER & LE

COUTEUR (2001) descrevem a nefrotoxicidade e hepatotoxicidade e GONZALEZ et al. (2002) relatam a presença de reações anafiláticas em usuários dos inibidores da bomba de prótons.

Além dos antiácidos e supressores ácidos, outra classe de fármacos são os citoprotetores. Têm esse nome devido à capacidade que esses fármacos possuem em acentuar os fatores protetores da mucosa. São exemplos: o sucralfato (estimula a síntese de prostaglandinas, a produção local de fator de crescimento epidérmico, e a absorção de pepsina), o misoprostol (análogo das prostaglandinas que atua inibindo a secreção de acido, aumentando o fluxo sanguíneo, e a secreção de muco e bicarbonato, mas ocasiona diarreia, cólicas abdominais e abortos), e da carbenoxolona (age modificando a composição da quantidade de muco gástrico). Embora também possuam efetividade no tratamento da doença, há relatos de efeitos adversos como, por exemplo, o uso prolongado de carbenoxolona causa hipertensão e retenção de líquidos, fazendo com que ela seja utilizada apenas como ferramenta farmacológica (JAIN et al., 2007).

As terapias que envolvem a associação de antimicrobianas com drogas antissecretórias são usadas com sucesso no tratamento de úlceras pépticas devido infecção por *H. pylori*. Nesse contexto, o uso de metronidazol, compostos bismúticos e tetraciclina ou amoxicilina é recomendado. No entanto, os esquemas terapêuticos propostos, até o momento, demonstram alguns inconvenientes, principalmente no que concernem os efeitos adversos e o custo do tratamento, fatores que vem contribuindo contrariamente a adesão ao tratamento (EISIG et al., 2003). JAIN et al. (2007) e MAJUMDAR et al. (2007) relatam o aparecimento de náuseas, diarreias e tonturas em pacientes que fazem uso desta associação de medicamentos.

Em adição aos efeitos adversos proporcionados pelos fármacos atuais, OCHI et al. (2005) apontam outras falhas da terapia medicamentosa, como é o caso da crescente resistência das bactérias *H. pylori* aos antibióticos utilizados, constituindo relevantes fatores no aparecimento de recidivas. Corroborando com estes dados, EGAN et al. (2007) discutem que apesar de múltiplos esquemas terapêuticos terem sido clinicamente testados nos últimos anos, ainda não surgiu um tratamento ideal para erradicação do *H. pylori*. Os regimes em que se utilizam dois fármacos (sendo um inibidor de bomba de prótons e um antimicrobiano) não atingem 100% de erradicação, por isso utiliza-se a associação de três fármacos, o qual em múltiplos ensaios clínicos europeus mostrou-se eficaz em mais de 90% dos casos. Dos três fármacos, um é obrigatoriamente inibidor de bomba de prótons (IBP) ou um antagonista dos receptores H_2 da histamina, sendo os IBP's utilizados com maior frequência.

Outro fator relevante é a não efetividade completa dos medicamentos antiulcerogênicos atuais. SZABO & VINCZE (2000) relatam que úlceras cicatrizadas espontaneamente ou depois do

uso de cimetidina são precariamente vascularizadas e têm de 2-3 vezes menos densidade de vasos sanguíneos novos do que no tecido normal em seu entorno.

Embora a supressão da secreção ácida tenha sido um pilar para a promoção da cicatrização de úlceras gástricas por três décadas, há um grande interesse por novos fármacos que promovam maior velocidade e qualidade na cicatrização (WALLACE, 2005). Assim sendo, parece que o desbalanço entre os fatores agressores e defensores da mucosa estão sendo tratados de outra maneira em pesquisas recentes. Os fatores defensores participariam do controle da acidez e o fortalecimento desses fatores propiciariam um novo horizonte de perspectivas para o tratamento da doença ulcerosa péptica (DAJANI & KLAMUT, 2000; WALLACE, 2005).

Somado a estes fatores, estudos também revelam que uma ínfima parcela da população possui úlcera idiopática, ou seja, cuja origem é desconhecida (COELHO, 2003). Para estes, traçar um tratamento eficaz é praticamente impossível.

Portanto, conclui-se que o estudo das úlceras pépticas constitui ainda um grande desafio e torna-se necessário o desenvolvimento de novos agentes terapêuticos mais eficazes e menos tóxicos para o seu tratamento.

BUSCA POR NOVAS MOLÉCULAS FARMACOLOGICAMENTE ATIVAS

Segundo SCHMEDA-HIRSCHMANN & YESILADA (2005), dentre as diversas fontes de novas moléculas farmacologicamente ativas no tratamento e prevenção de úlceras pépticas, as originarias de produtos naturais são consideradas fontes importantes de novas moléculas. Dessa forma, muitos produtos derivados de plantas apresentam-se como uma fonte atraente seja como fonte de desenvolvimentos de novos fármacos, ou como produto adjuvante do tratamento (ARRIETA et al., 2003); e de acordo ZAYACHKIVSKA et al. (2005) muitos têm mostrado resultados promissores na clínica.

Nesse contexto, estudos têm mostrado que substâncias de origem vegetal, dentre elas os terpenóides, têm apresentado atividade antiúlcera bastante expressiva (BORRELLI & IZZO, 2000; RODRIGUEZ et al., 2004; MARQUES et al., 2006).

β-mirceno (7-metil-3-metileno-1,6-octadieno) é um monoterpeno com cheiro agradável encontradas em muitas plantas, incluindo capim-limão (*Cymbopogom citratus*), lúpulo e Verbena (GÜNTHER & ALTHAUSEN, 1949), bem como na composição de óleo essencial obtida a partir de cascas de *Citrus aurantium* (MORAES et al., 2009). Ele é utilizado na produção de perfumes e é também um componente de óleos essenciais que têm sido utilizados como aditivos aromatizantes na fabricação de bebidas alcoólicas e na indústria alimentar (LEUNG, 1980).

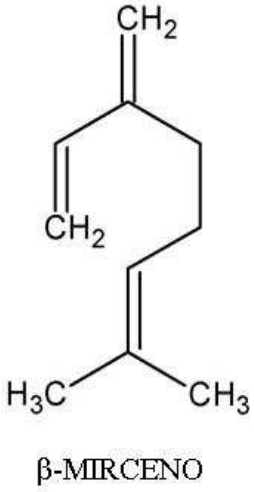


Figura 4) Estrutura química do monoterpeno β-mirceno

JUSTIFICATIVA

Estudos têm mostrado que substâncias de origem vegetal, dentre elas os terpenóides, têm apresentado atividade antiúlcera bastante significativa (BORRELLI & IZZO, 2000; RODRIGUEZ et al., 2004; MARQUES et al., 2006).

β-mirceno (7-metil-3-metileno-1,6-octadieno) é um monoterpeno com cheiro agradável encontradas em muitas plantas, incluindo capim-limão (*Cymbopogom citratus*), lúpulo e Verbena (GÜNTHER & ALTHAUSEN, 1949), bem como na composição de óleo essencial obtida a partir de cascas de *Citrus aurantium* (MORAES et al., 2009). Ele é utilizado na produção de perfumes e é também um componente de óleos essenciais que têm sido utilizados como aditivos aromatizantes na fabricação de bebidas alcoólicas e na indústria alimentar (LEUNG, 1980).

Além das grandes utilizações do monoterpeno β-mirceno na indústria alimentar, resultados previos tem demonstrado o grande potencial gastroprotetor do β-mirceno. Bonamin et al. (2014) demonstraram que β-mirceno protege a mucosa gástrica e duodenal dos efeitos lesivos induzidos por etanol, DAINEs, estresse, cisteamina. β-mirceno atua através de ativação de mecanismos antioxidantes que favorece o fortalecimento da barreira mucosa (**Figura 4)**.

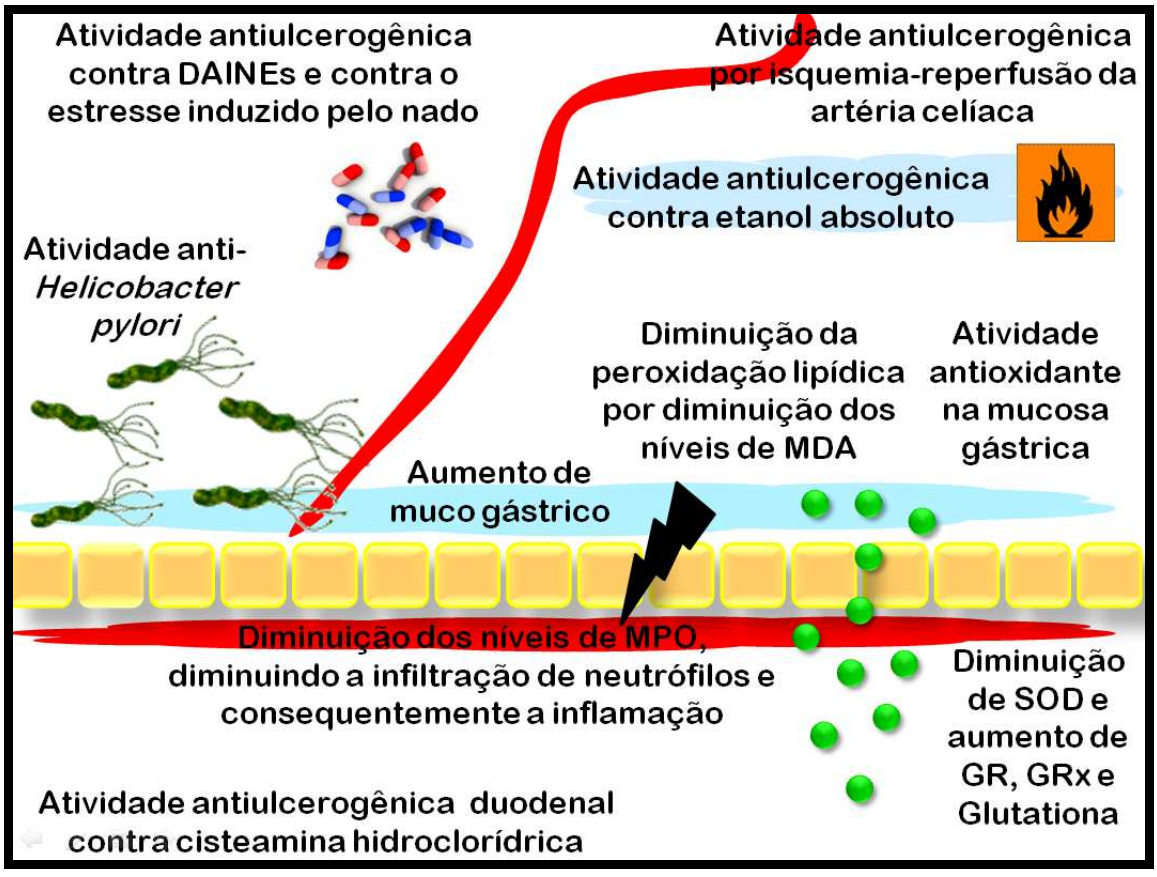
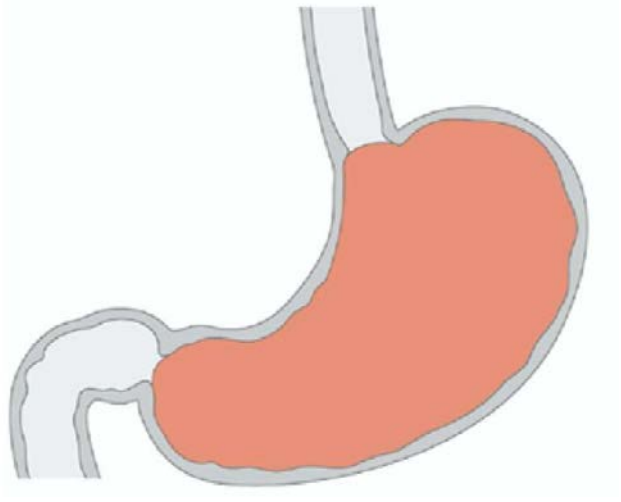


Figura 5: Resumo das atividades já encontradas com o monoterpeno β-mirceno

Esses resultados encontrados durante o desenvolvimento do mestrado nos instigaram a continuar a investigá-lo ainda mais. O passo seguinte foi avaliar se este monoterpeno seria responsável também por uma atividade sobre o ácido gástrico. Considerando a relevância da reconstrução da mucosa após uma lesão, é fundamental a continuidade dos estudos para avaliar a ação cicatrizante do β-mirceno...

E os estudos continuaram...



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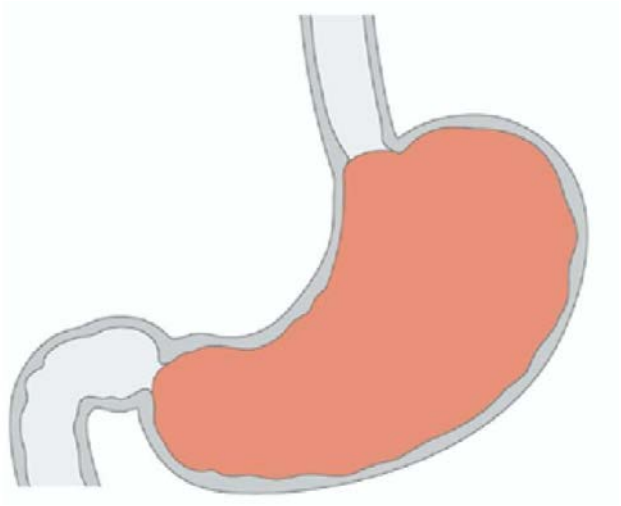


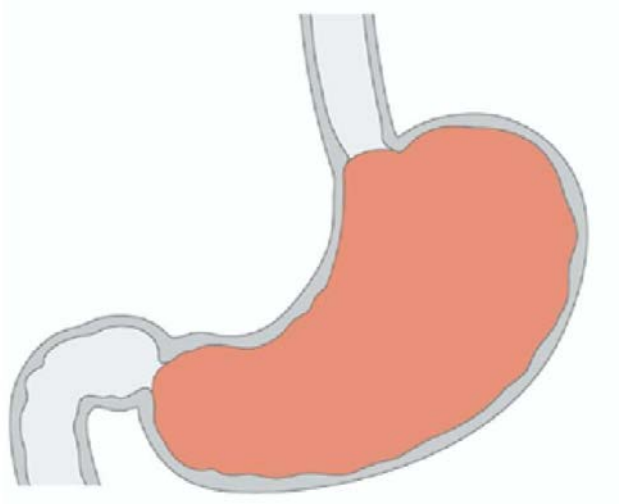
Apresentação dos Capítulos

APRESENTAÇÃO

Optamos por apresentar os resultados em 2 capítulos distintos com o objetivo de otimizar os resultados na forma de artigos para publicação em revistas científicas.

No **capítulo 1** encontra-se o trabalho intitulado "β-myrcene, a monoterpene with antissecretory gastric activity and protective against Gastroesophageal reflux: Role of calcium channels and gastrin in the gastroprotection". Este estudo mostra os resultados de atividade antissecretória do monoterpeno β-mirceno em diversos modelos de atividade sobre a secreção ácida gástrica elucidando seus mecanismos de ação antissecretórios caracterizado pela diminuição da acidez gástrica por meio da diminuição dos níveis séricos de gastrina, envolvimento dos canais de cálcio e pela abertura dos canais de Katp. Além disso, este monoterpeno apresenta redução significativa na atividade no combate ao refluxo gastroesofágico induzido por esofagite, diminuindo os níveis de peroxidação lipídica na mucosa.

No **capítulo 2** encontra-se o trabalho intitulado "The effects of monoterpene β-myrcene on gastric healing activity: role of cytokines, metalloproteinases of matrix -2 and -9 and gastric contractions recovery". Este estudo evidencia o efeito cicatrizante do monoterpeno β-mirceno e seus mecanismos de ação cicatrizantes caracterizados pelas metaloproteinases de matriz 2 (MMP-2), diminuição dos níveis de MPO, envolvimento de interleucinas e de COX-1 e COX-2 e reestabelecimento das contrações gástricas após a ulceração.



Capítulo I

β-myrcene, a monoterpene with antisecretory gastric activity and protects against gastroesophageal reflux: the role of calcium channels and gastrin in gastroprotection

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Abstract: The monoterpene β-myrcene is widely used in cosmetics, food and beverages, and it is normally found in the essential oils of *Citrus* fruits, such as *Citrus aurantium*. The aim of this study was to determine the antisecretory activity of β-myrcene, determine its mechanism of action and assess the effect of this monoterpene in experimental gastroesophageal reflux assay. Male wistar rats (180-220 g) or male swiss mice (25-30g) were used to generate the following models: pyloric ligation-induced ulcers and the evaluation of gastric acid secretion stimulated by secretagogues; K^+atp channels and the involvement of Ca^{+2} channels; in vitro determination of H^+/K^+-ATPase enzymatic activity inhibition; gastroesophageal reflux induced by esophagitis, lipid peroxidation (MDA) and gastrin measurement. Animals were treated with a positive control (lansoprazole (30mg/kg) or omeprazole (345µg/ml), vehicle (8% Tween 80®) or β-myrcene (7.5mg/kg). The results are expressed as the mean ± S.E.M. Statistical significance was determined by one-way analysis of variance followed by Dunnett's or Tukey's test. P-values<0.05 were considered to be statistically significant. The oral and intraduodenal administration of β-myrcene (9.11 ± 0.96 and 6.40 ± 0.24, respectively) reduced the H^+ concentration compared to the vehicle (13.33 ± 1.23 and 19.20 ± 1.24 mEq/mL/4h, respectively). To verify the mechanisms responsible for this action, gastric acid secretion was stimulated by secretagogues: histamine, bethanechol or pentagastrin. β-myrcene was only active against pentagastrin, so gastrin was measured in the plasma. Thus, treatment with β-myrcene reduced gastrin secretion compared with the control group (10.89 ± 3.99) pmol/L. Furthermore, treatment with the K^+atp channel blocker, glibenclamide (5mg/kg), significantly reduced the gastroprotective effects of β-myrcene (39.80±5.30) mm^2 and diazoxide (3mg/kg) (62.20±8.15) mm^2 when compared to the groups pretreated with saline, indicating a role for K^+atp channels in gastroprotection. Moreover, β-myrcene displayed activity through the intraduodenal route at a dose of 7.5mg/kg, as it significantly decreased esophageal lesions (13,21±3,551) mm^2 and decreased lipid peroxidation levels (5,332 ± 0,2352) nmol TBARS/mg protein compared to the vehicle group (141,0 ± 55,33 and 10,75 ± 1,558, respectively). Thus, this study indicates that β-myrcene displays important antisecretory activity modulated by the action of gastrin.

Keywords: β-myrcene; essential oils; gastric acid secretion; gastric ulcer, esophagitis

1.Introduction

Gastric ulcers are the result of numerous aggressive factors (acid secretion, *Helicobacter pylori*, stress, use of nonsteroidal antiinflammatory agents and ethanol) and excessing protective mechanisms (mucus-bicarbonate, prostaglandin release, and mucosal microcirculation) (Ham & Kaunitz, 2007).

Gastrointestinal lesions, peptic ulcers, gastroesophageal reflux induced by esophagitis, Zollinger-Ellison syndrome and gastritis, have become a major focus of experimental and clinical research, with a commercial emphasis to development of new therapeutic agents (Whittle, 2004).

Acid-related disorders are highly prevalent in the developed world, and they significantly impact patient quality of life and place a heavy burden on health care systems (Cylwik et al., 2005). Gastric acid has been identified as an important pathogenic factor for most prevalent gastrointestinal disorders, including duodenal ulcer and gastroesophageal reflux disease (Schubert et al., 2008; Shin et al., 2008). Gastric H^+/K^+-ATPase, which is localized in gastric membrane vesicles and catalyzes the electro-neutral exchange of intracellular H^+ and extracellular K^+ coupled with the hydrolysis of cytoplasmic ATP, is the proton pump responsible for the final step of acid secretion in the stomach (Shin et al., 2009; Weidemüller & Hauser et al., 2009). Drugs that are used to treat acid-related diseases primarily act through inhibiting H^+/K^+-ATPase activity (Shin et al., 2008, Suzuki et al., 2008; Palmer et al., 2010).

The principal stimulants of acid secretion are histamine, gastrin, and acetylcholine. These agents interact with receptors that are coupled to two major signal transduction pathways, i.e., adenylate cyclase and intracellular calcium. Both the intracellular cAMP and calcium-dependent signaling systems activate downstream kinases, ultimately leading to fusion and activation of the H^+/K^+-ATPase proton pump (Shubert et al., 2008). Because the gastric proton pump is located at the end of the signaling cascade, its inhibition especially efficient for controlling gastric acid secretion, and the development of H^+/K^+-ATPase inhibitors for the treatment of acid-related diseases has attracted considerable interest (Bamford et al., 2009).

The introduction of H_2 receptor antagonists in 1977, followed by proton pump inhibitors targeted to the gastric H^+/K^+-ATPase in 1989, reflected a major medical therapeutic breakthrough for the treatment of peptic ulcers and gastroesophageal reflux disease and resulted in more rapid lesion healing and symptom relief (Fellenius et al., 1971; Black et al., 1972).

It was postulated that the K^+atp channels are involved in a variety of pathophysiologic functions in the stomach, including the regulation of blood flow, gastric acid secretion and stomach contractility (Toroudi, 1999). Peskar et al. (2002) demonstrated that the K^+atp channel blocker, glibenclamide, inhibited the

gastroprotective effect of several agents. Thus, regulating the opening and closing of K^+atp channels in the stomach represents a defense mechanism that the gastric mucosa uses against external aggressions.

Besides ulcers, another disease related to gastric acid secretion is gastroesophageal reflux disease. Gastroesophageal reflux disease occurs when gastric acid flows back into the esophagus, resulting in erosion of the esophageal mucosal epithelium. Gastric secretion inhibitors, such as proton pump inhibitors, can alleviate gastroesophageal reflux disease symptoms (Fass et al., 2012). The prevalence of this disease is increasing worldwide, and it is currently the most prevalent gastrointestinal disorder. This disease is responsible for a substantial proportion of health care expenditure in the developed world (Hanauer et al., 2009).

It was reported that an increase in lipid peroxides was observed in the esophageal mucosa of patients with reflux esophagitis or Barrett's esophagus, depending on the severity of their symptoms, indicating that free radicals/active oxygen species are involved in the pathogenesis of reflux esophagitis (Olyaee et al., 1995). Lipid peroxidation is the introduction of a functional group containing two catenated oxygen atoms, O-O, into unsaturated fatty acids through a free radical reaction (Pasha, 2010).

Cell membranes and organelles contain large amounts of polyunsaturated fatty acids. Membrane fluidity is related to the presence of unsaturated chains of phospholipids and cholesterol. Damage to this lipid layer tends to decrease membrane fluidity. The attack of some reactive oxygen species (ROS), which subtract a hydrogen atom from the methylene group of the chain polyunsaturated fatty acids, initiates the process of lipid peroxidation (Halliwell & Gutteridge, 1991).

Lipid peroxidation is a binding process connected with the formation of aldehydes. Malondialdehyde (MDA) (Niedworok & Bielaszka, 2007) is the end product of lipid peroxidation, and it is a good marker of free radical-mediated damage and oxidative stress (Likidlilid et al., 2010). MDA has been utilized as a suitable biomarker for lipid peroxidation (Sofo et al., 2004) because it reacts with the free amino groups of proteins, phospholipid, and nucleic acids, leading to structural modifications that induce immune system dysfunction (Lee et al., 2004). Vitamin E supplements significantly decrease MDA concentrations (Agarwal & Prabakaran, 2005).

According to Schmeda-Yesilada & Hirschmann (2005), among the plethora of sources of new, pharmacologically active molecules for the prevention and treatment of peptic ulcers, those originating from natural products are very important. Thus, many plant products are attractive sources for the development of new drugs or treatment adjuvants (Arrieta et al., 2003). Zayachkivska et al. (2005) reported that many such products have shown promising results in the clinic.

In this context, studies have demonstrated that substances of vegetable origin, including terpenoids, display significant antiulcer activity (Borrelli & Izzo, 2000; Rodriguez et al., 2004; Marques et al., 2006; Bonamin et al., 2014).

Terpenoids, designated as such because their biosynthetic origin is derived from isoprene units, constitute a wide variety of plant substances. The most abundant terpene compounds are the monoterpenes (90% oil) and sesquiterpenes (Cardoso et al., 2000).

β-myrcene (7-methyl-3-methylene-1,6-octadiene) is a pleasant-smelling monoterpene that is found in many plants, including lemongrass, hops and verbena (Günther & Althausen, 1949), and it is also present in essential oils obtained from the fresh fruit peels of *Citrus aurantium* (Moraes et al., 2009). β-myrcene is used in perfume production, and it is also a constituent of essential oils that have long been used as flavoring additives for the manufacture of alcoholic beverages and in the food industry (Leung, 1980).

In addition to the major uses of β-myrcene in the food industry, their effects against peptic ulcers are very important. Bonamin et al. (2014) demonstrated the gastroprotective activity of β-myrcene in experimental models of peptic ulcers induced by ethanol, NSAIDs, stress, and cysteamine. β-myrcene presented considerable antioxidant activity, maintaining GSH levels in gastric mucosa as protection. To establish a general profile for the gastroprotective activity of β-myrcene, we tested three doses (3.75, 7.5 and 11.25mg/kg body weight) to select one that produced the best gastroprotective effect. These doses of β-myrcene were selected based on the effective antiulcer dose of essential oil from *Citrus aurantium* (250mg/kg) and the percentage of β-myrcene present in this composition (1.43%) (Moraes et al., 2009). Therefore, the aim of this study was to determine the antisecretory activity of β-myrcene, its mechanisms of action, and its ability to protect against gastroesophageal reflux induced by esophagitis and lipid peroxidation in rats and mice.

2. Material & Methods

2.1. Animals and evaluation of the lesions

Male Wistar rats (180–250 g) were supplied by the Central Animal House of the Unesp. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of dark/light (12h/12h), humidity (60 ± 1.0%) and temperature (22 ± 1°C). The animals were fasted before all experimental procedures because the standard drugs and β-myrcene were administered orally (gavage). The animals were housed in cages with raised floors of wide mesh to prevent coprophagy. All experiments were performed in the morning and followed the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993). Our protocol was approved by the Institutional Animal Care and Use Committee from Unesp (CEUA n°363). After each experiment, the animals were killed with CO_2 gas. Their stomachs were cut along the greater curvature, excised, gently rinsed under tap water, pressed onto a glass plate,

and scanned so that the lesions could be counted using the AVSoft program. The results were expressed as the total ulcerated area (mm^2). In addition to the animal groups treated with vehicle (negative control), antiulcer drugs (positive control), or β-myrcene, we included a sham group, in which the animals were subjected to the same procedure as the other groups, but without the use of agents that induce injury.

2.2.Drugs and treatment

Ketamine hydrochloride, Xylazine hydrochloride, ethanol, Lansoprazole, Cimetidine, Atropine, Histamine, Bethanechol, Pentagastrin, Glibenclamide, Diazoxide, Nifedipine and Calcium Hydrochloride were purchase from Sigma Co (USA). β-myrcene was purchased from Acros Organics (USA), and it was dissolved in 8% Tween 80® aqueous solution and administered by orally (p.o.). The vehicle control group was given 8% Tween 80® aqueous solution (10mL/Kg).

2.3.Determination of gastric acid secretion

The gastric acid secretion assay was performed according to the method described by Shay et al (1945). Male Wistar rats (n=10) were randomly divided into six groups and fasted for 12h with free access to water. The determination of gastric secretion was performed according to the following two routes: oral route (three groups): thirty minutes after the oral administration of β-myrcene (7.5mg/kg, which is the dose that provided the best gastroprotective results), rats were given Lansoprazole (30mg/kg) as a positive control or vehicle (8% tween 80®, 10mL/Kg); and Intraduodenal route (three groups): male rats (n=10) were fasted for 12h with free access to water. Immediately after pylorus ligature, β-myrcene (7.5mg/kg), Lansoprazole (30mg/kg) or 8% Tween 80® (10mL/Kg) was administered via the intraduodenal route. All animals were killed 4 h later, their stomachs were removed and the gastric secretion was collected. The volume of the gastric juice supernatant was determined in a graduated cylinder, and the total acidity was determined by titration with 0.1N NaOH, using 2% phenolphthalein as acid–base indicator (mEq/mL/4 h).

2.4.Determination of gastric acid secretion in rats stimulated with Histamine, Pentagastrin and Bethanechol

Animals were separated into the following eight groups: 1, negative control (vehicle - 8% Tween 80®); 2, monoterpene β-myrcene; 3, Histamine; 4, Histamine plus Cimetidine; 5, Histamine plus β-myrcene; 6, Bethanechol; 7, Bethanechol plus atropine; 8, Bethanechol plus β-myrcene; 9, Pentagastrin; and 10, Pentagastrin plus β-myrcene. A pylorus ligature was carefully performed in fasted male rats under anesthesia, and β-myrcene (7.5mg/kg, i.d.), vehicle (Tween 80® at 8%, 10mL/Kg i.d.), Cimetidine (100mg/kg, i.d.) or atropine (3mg/kg, s.c.) was administered at the moment of the ligature proceeding. One hour after pylorus ligation (Shay et al., 1945), the animals received histamine (20mg/kg, s.c.) bethanechol (2.5mg/kg, s.c.) or Pentagastrin (0.4mg/kg s.c.). Four hours after pylorus ligation, the animals were killed,

their stomachs were opened and the gastric secretion was collected. The volume of the gastric juice and total acidity were determined as previously described.

2.5.Gastrin measurement

To determine the gastrin levels in the plasma of animals subjected to pylorus ligature, blood was collected by decapitation and centrifuged, and the plasma was analyzed for gastrin levels using a dosage immunoenzymatic kit (Enzo Life Sciences). The samples were read in an ELISA biosystem reader at 405nm. The results were expressed as pmol/l.

2.6.Effects of Nifedipine and Calcium Hydrochloride pretreatments on β-myrcene gastroprotection

To verify the role of intracellular calcium in the gastroprotective effect of β-myrcene against ethanol-induced mucosal damage, the influences of Nifedipine and/or calcium hydrochloride were investigated (Lira et al., 2009). The following groups of mice were treated: negative control (8% Tween 80®), β-myrcene (7.5mg/kg, p.o.), Nifedipine (40mg/kg, i.p.), calcium hydrochloride (50mg/kg, i.p.), Nifedipine + β-myrcene, or Nifedipine + calcium hydrochloride. One hour after β-myrcene treatment, 40min after Nifedipine treatment, or 30 min after calcium hydrochloride administration, all animals received 0.2ml absolute ethanol by oral gavage. Thirty minutes later, the animals were killed, and their stomachs were removed to evaluate the gastric lesions, as previously described.

2.7.In vitro determination of H^+/K^+- ATPase enzymatic activity

Gastric H^+/K^+-ATPase was prepared by ultra-centrifugation and gradient separation. Briefly, mucosal tissues from the corpus region of the rabbit stomach were scraped, suspended in 10 volumes of homogenizing buffer (HB) (250 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA and 2 mM HEPES, pH 7.4) containing a protease inhibitor (composition in mM: AEBSF 104, aprotinin 0.08, leupeptin 2, bestatin 4, pepstatin A 1.5, and E-64 1.4), homogenized (20 up/down strokes) with a motor-driven Teflon pestle (1500 rpm) and centrifuged (20,000×g for 20min). The supernatant was removed and centrifuged at 100,000×g for 60min. The resulting pellet was homogenized in HB, placed on a 30% sucrose cushion and centrifuged at 100,000×g for 2h. The gastric vesicular fraction containing the H^+, K^+-ATPase remaining at the isotonic 30% sucrose interface was collected and stored at −70°C. All procedures were performed at 4°C. Protein content was determined in a 96-well plate using the BCATM protein assay kit (PIERCE, Rockford, IL, USA) using bovine serum albumin as a standard, according to the supplier's instructions. Enzyme activity was determined by incubating 4 g of protein with monoterpene β-myrcene (10, 100, 1000 g/ml), Oubaine (728 µg/ml) or omeprazole (175 g/ml) in a reaction mixture containing 1 mM ATP, 20 mM KCl, 2 mM MgCl2 and 50 mM Tris–HCl (pH 7.4) at 37°C for 20 min. The reactions were terminated by the addition of 60 µl ice cold 50% trichloroacetic acid. The amount of inorganic phosphate formed was quantified according to the method of Fiske and Subbarow (1925). Activity was calculated using the extinction coefficient of Pi (ε = 11,000/(M

cm)). All experiments were performed in triplicate, and the results were expressed as the % inhibition of enzymatic activity (Kubo et al., 1995).

2.8.Protein determination

The protein concentration of gastric ATPase was determined in a 96-well plate using a commercial BCA protein assay kit (Pierce, Rockford, IL, USA), with bovine serum albumin as the standard.

2.9.Role of K^+atp channels in the gastroprotective effect of β-myrcene

Groups of mice (n=8) were pretreated with vehicle (8% Tween 80®, 10ml/kg), β-myrcene (7.5mg/kg, p.o.), diazoxide (3mg/kg, i.p.), or the combinations with glibenclamide (5mg/kg, i.p.), prior to the oral administration of 1 ml of absolute ethanol. Monoterpene and diazoxide were administered 1 h or 30 min, respectively, prior to ethanol or glibenclamide. Glibenclamide was administered 30 min before β-myrcene (Olinda et al., 2008).

2.10.Gastroesophageal reflux induced by esofagitis

All groups of male rats (n=10) were fasted for 12h with free access to water. Then, the animals were anaesthetized with ketamine hydrochloride and xylazine hydrochloride (10 and 5mg/kg, i.p., respectively), and the rats remained sedated throughout the procedure. An incision was made approximately 3 cm to the left of the abdomen, the stomach was located and ligatures were performed to induce esophagitis. Two ligatures were introduced using sutures, one in the pyloric region and the other in the transition region between the fundus and the body of the stomach, to prevent gastric contents from secreting out of the duodenum, while allowing gastric contents into the esophagus, respectively (Nakamura et al., 1982). Immediately after ligature, β-myrcene (7.5mg/kg), Lansoprazole (30mg/kg) and vehicle (8% Tween 80®) (10 mL/Kg) were administered via an intraduodenal route. Then, the abdomen was sutured, and the animals were placed in boxes to recover from surgery. The animals were killed, and their stomachs were removed to evaluate gastric lesions, as described previously.

2.11.Effects of monoterpene β-myrcene on Lipid peroxidation (Malondialdehyde - MDA)

Malondialdehyde (MDA) is an end product of lipid peroxidation. Samples from the glandular portion of the stomach (approximately 200mg) were ground, homogenized in 1ml of phosphate buffer containing 0.1 M sodium (pH 7.4) and centrifuged at 12,000 rpm at 4°C for 15 minutes. The supernatants were collected and stored in Eppendorf tubes at -80°C. Each homogenate was thawed at 1:2 and diluted in KCl (0.15 M.) Then, 0.1 ml of sodium dodecyl sulfate 8.1%, 0.75 ml acetic acid 20% (pH=3.5, adjusted with NaOH), 0.75 ml of thiobarbituric acid (TBARS) 0.8% to 0.25ml of the diluted sample were KCl mixed, and distilled water was added to obtain a volume of 2ml. All samples were shaken for 1 min and placed in a 95°C water bath with stirring for 1 hour. After this period, the samples were cooled to room temperature with 0.5ml of distilled

water (total volume of 2.5ml). Then, the samples were subjected to continuous agitation for 1 minute and centrifuged at 12,000 rpm for 10 minutes. In this model, malondialdehyde reacts with thiobarbituric acid to produce a pink color. The samples were pipetted in duplicate on microplates and read at 532nm using a spectrophotometer. The results were expressed as nmol TBARS/mg protein (Ohkawa et al., 1979).

3.Results

3.1.Gastric acid secretion

β-myrcene displayed a clear gastroprotective effect compared to the vehicle-treated group. β-myrcene (7.5mg/kg), administered via the intraduodenal (id.) and oral (v.o.) routes, inhibited the hydrogen concentration by 67%, thereby reducing the acidity of the contents and increasing the pH significantly (0.69 units) (Table 1).

The i.d. administration of Lansoprazol, a proton pump inhibitor, prevented the hydrogen concentration in gastric contents by 83% and prevented the decrease of pH (3.16 units) (Table 1).

3.2.Gastric acid secretion stimulated by secretagogues

Histamine (20mg/kg), administered subcutaneously (sc.), stimulated the release of gastric acid secretion; the gastric contents increased by 31% 4h after pylorus ligation. Histamine also decreased the pH of gastric contents by 0.69 units. In the control group, the gastric content volume was 7.01 ± 0.7 g, pH 1.29 ± 0.04, and the hydrogen concentration in gastric contents was 7.04 ± 0.35 μEq $[H^+]$/ml/4h (Table 2).

β-myrcene (7.5mg/kg), administered intraduodenally (id.) one hour before stimulation of gastric acid secretion by histamine, failed to prevent the increase of gastric content exerted by histamine. In addition, β-myrcene failed to reduce the acidity of the gastric content, and it not changed the pH (Table 2).

The administration of Cimetidine, an H_2-receptor antagonist, prevented the increase of gastric contents and the hydrogen concentration in the gastric content by 53.2 and 29%, respectively. It also prevented the decrease of pH by 0.65 units (Table 2).

Bethanechol administration (2.5mg/kg, s.c.) stimulated gastric acid secretion 4 h after pylorus ligation and increased the gastric contents by 16.4%. Bethanechol also reduced the pH of the gastric content by 0.49 units (Figures Table 2). In the control group, the gastric content was 8.33 ± 0.24 g, the pH was 1.02 ± 0.02, and the hydrogen concentration in the gastric content was 9.55 ± 0.39 μEq $[H^+]$/ml/4h (Table 2).

β-myrcene (7.5mg/kg), administered intraduodenally (id.) 1 hour before the stimulation of gastric secretion by bethanechol, failed to prevent the increase in gastric content volume. In addition, β-myrcene failed to reduce the acidity of this content and did not alter the pH of the solution (Table 2).

The administration of atropine, a muscarinic antagonist, prevented increases in both the gastric content volume and the hydrogen concentration in the gastric content by 54.4% and 43%, respectively. Bethanechol also reduced the pH of the gastric contents by 0.41 units (Table 2).

Pentagastrin (0.4mg/kg), administered subcutaneously (sc.), stimulated the release of gastric acid secretion 4h after ligation of the pylorus, thereby increasing the gastric content volume and the hydrogen concentration in the gastric content by 20% and 28%, respectively. Pentagastrin also reduced the pH of the gastric content by 0.36 units. In the control group, the gastric content was 8.31 ± 0.29 (g), pH 0.98 ± 0.06, and the hydrogen concentration in the gastric content was 8.76 ± 0.86 μEq $[H^+]$/ml/4h (Table 2).

β-myrcene (7.5mg/kg) administered intraduodenally (id.) one hour before the stimulation of gastric secretion by pentagastrin prevented an increase in the gastric content volume, reduced the hydrogen concentration in the gastric content and reduced the pH of gastric content by 46%, 27% and 0.91 units, respectively (Table 2), confirming its antisecretory activity.

3.3.Gastrin

Our previous results demonstrate that β-myrcene displays antisecretory activity when pretreated with pentagastrin, which acts on gastrin receptors (CCK). Thus, we evaluated whether β-myrcene acts through this pathway. To this end, we measured plasma gastrin levels in rats subjected to different treatments (ligature pylorus experimental model).

The administration of β-myrcene (7.5mg/kg, id.) 4h after pylorus ligation, which stimulates gastric secretion and gastrin levels, significantly reduced th eplasma levels of gastrin ($P<0.05$) (126.7 ± 8.380) compared to the vehicle (158.8 ± 9.498) (Figure 2). These data corroborate the antisecretory activity previously found using pentagastrin (Table 2).

3.4.Effects of Nifedipine and calcium hydrochloride pretreatments on β-myrcene gastroprotection

In mice pretreated with calcium hydrochloride (50mg/kg, i.p.) and monoterpene β-myrcene (7.5mg/kg), ethanol-induced gastric mucosal lesions were significantly reduced (Figure 2). Nifedipine pretreatment worsened ethanol-induced mucosal damage, whereas β-myrcene ($P<0.01$) and calcium hydrochloride ($P<0.01$) antagonized the damaging effect of Nifedipine on ethanol.

3.5.H+/K+ ATPase activity

ATP hydrolysis by the H^+/K^+- ATPase isolated from the gastric mucosa of rabbits treated with vehicle was 0,3070 ± 0,002 mM Pi mg/min. The monoterpene β-myrcene (10, 100 and 100 mg/ml) failed to reduce the H^+/K^+-ATPase activity (Figure 4).

The administration of Omeprazole (345µg/ml), a proton pump inhibitor, significantly reduced the H^+/K^+- ATPase activity ($P<0.05$) (Figure 4).

3.6.Effects of glibenclamide and diazoxide on β-myrcene gastroprotection

To assess the involvement of the K^+atp channels in the protective effect of β-myrcene, mice were pretreated with Glibenclamide (5.0mg/kg), a K^+atp channel blocker. Pretreatment with glibenclamide significantly reduced the gastroprotective effect of β-myrcene ($P<0.01$) and diazoxide (one opener of K^+atp channels) against ethanol compared to the groups pretreated with saline (Figure 2), indicating a role for these channels in the gastroprotective effect of β-myrcene.

3.7.Gastroesophageal reflux induced by esofagitis

To assess the protective effect of β-myrcene against gastroesophageal reflux induced by esophagitis, rats were pretreated with vehicle, lansoprazole (30mg/kg) and the monoterpene β-myrcene (7.5mg/kg) administered by intraduodenal (id., the same route that was successful in the pylorus ligature model) after surgery. The administration of lansoprazole ($P<0.01$) and β-myrcene group ($P<0.01$) demonstrated significant protection compared to the vehicle (Table 3).

3.8.Effects of monoterpene β-myrcene on Lipid peroxidation (MDA)

Next, we quantified the lipid peroxidation (MDA) level in the esophageal tissue of rats with Gastroesophageal Reflux induced by esophagitis. Lipid peroxidation (MDA) levels decreased in the presence of monoterpene β-myrcene ($P<0.01$) compared to either of the negative control groups (vehicle) (Table 3).

The lipid peroxidation levels were significantly reduced in the group treated with Lansoprazole (30mg/kg, a proton pump inhibitor) ($P<0.05$) and in the sham group ($p<0.001$) compared to the vehicle (Table 3).

4.Discussion

Animal models of peptic ulcer disease have provided valuable insights into early pre-ulcerogenic events and subsequent cellular and biochemical changes in the pathogenesis of gastric and duodenal ulceration. In 2009, Moraes et al. reported that the antiulcerogenic effect of *Citrus aurantium* essential oil was exclusively due to monoterpene limonene, its major compound. However, later studies indicated that this was a minority compound and that the monoterpene β-myrcene also displays antiulcer and antioxidant activities (Bonamin et al., 2014). This study provides novel and important insights into the effect of β-myrcene on peptic ulcers, and we clearly identified a protective role for β-myrcene against gastric ulceration in rodents. Thus, we measured the antisecretory activity of β-myrcene, evaluated its

antisecretory mechanisms of action and evaluated its protective potential against gastroesophageal reflux induced by esophagitis and lipid peroxidation.

Although many products are available for the treatment of gastric ulcers, including antacids, proton pump inhibitors, anticholinergics and histamine H_2-antagonists, most of these drugs produce numerous adverse reactions, such as gynecomastia, hematopoietic changes, acute interstitial nephritis (Ra & Tobe, 2004), thrombocytopenia (Zlabek & Anderson, 2002), anaphylaxis reactions (Gonzalez et al., 2002), nephrotoxicity and hepatotoxicity (Fisher & Le Couteur, 2001).

Gastric acid is an important factor for the development of ulceration in pylorus-ligated rats (Shay et al., 1945). Activation of the vagus-vagal reflux by stimulating pressure receptors in the antral gastric mucosa in the hypersecretion model of pylorus ligature is believed to increase gastric acid secretion (Brodie et al., 1966).

Gastric secretion is regulated by processes involving neural, endocrine and paracrine mechanisms acting on the cells of the stomach, resulting in the release of H^+ and Cl^- ions and water through the apical membrane of the parietal cells. In this process, three endogenous chemical mediators are involved that act directly on receptors on the parietal cells: histamine (H_2), pentagastrin (CCK2) and acetylcholine (M_3), or the activation of the antral G cells (Schubert, 2011).

Histamine, which is present in enterochromaffin cells, is released through a paracrine mechanism, stimulating acid secretion by interacting with H_2 and causing the release of cyclic AMP (Garcia et al., 1978). Acetylcholine (the second secretagogue evaluated) is secreted by vagal efferent neurons, initially as a result of stimulation by olfaction, vision, taste, or by chewing. As the presence of food stretches the stomach wall, local neurons present within the mucosa stimulate such secretions (Nakano et al., 2011).

Pentagastrin, the third secretagogue evaluated, is released by G cells in response to stimulation by proteins in food, including Ca^{2+}, Mg^{2+}, and Al^{3+} ions by vagal stimulation, and alkalinization of the antrum (Prinz et al., 1992; Dickinson, 2004). Activation of the gastric H^+/K^+-ATPase (proton pump) is the final step of acid secretion in both regulatory pathways (Yao & Forte, 2003; Schubert, 2009). In rats with ulcers, the gastrin level in the plasma, gastric juice and the antral mucosa tissue increase, while the somatostatin levels decrease (Sun et al., 2002). Considering the results obtained in this work, our studies indicate an antacid effect of monoterpene β-myrcene via gastrin (pentagastrin), but not by muscarinic receptors or histamine.

Edkins initially identified a bioactive mucosal agent from the gastric antrum that stimulated gastric acid secretion in 1905. Identification of this peptide, subsequently termed gastrin, represented the confirmation of Bayliss and Starling's hypothesis of chemical messengers in the gut mucosa capable of activating other cells (Modlin et al., 2005).

Excessive secretion of gastrin by a neuroendocrine cell neoplasm is responsible for the extreme acid hypersecretion of the Zollinger–Ellison syndrome. Milder elevations of plasma gastrin were also found to be associated with acid dysregulation in peptic ulcer disease, and subsequently demonstrated to be secondary to the *Helicobacter pylori* infection that underlies most peptic ulcer disease (Calam et al., 1997; Shin et al., 2008).

With the elucidation of the structure of gastrin, several peptide analogs of gastrin were synthesized, which could theoretically competitively inhibit gastrin stimulated acid secretion (Lebam et al., 1993; Shin et al., 2008).

In the review made by Makovek & D'Amato in 1997 the chemical structures of various gastrin antagonists were presented. Among them, the antagonists proglumide, Lorglumide and Spiroglumide were reported and what stood out most were their chemical structures. It is possible to compare these structures with the chemical structure of monoterpene β-myrcene, that is, the chemical structure of monoterpene resembles with the structure of these antagonists (Figure 1), which may explain its activity antisecretory via gastrin and the monoterpene may be also considered a possible antagonist of gastrin. That explains this important activity found here.

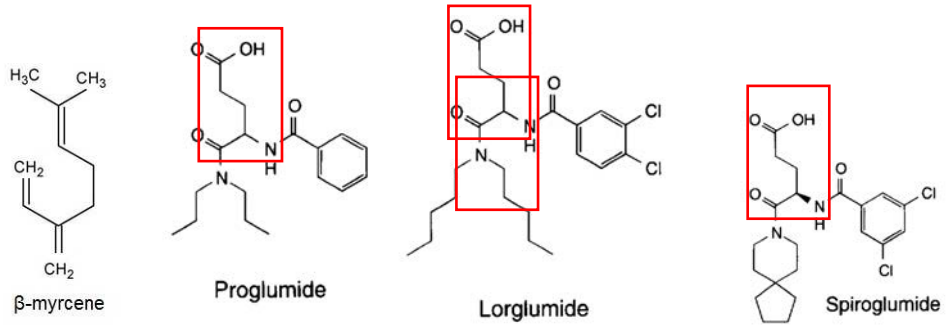

Figure 1: β-myrcene chemical structure and chemical structure of 3 antagonists of gastrin (Proglumide, Lorglumide and Spiroglumide (Makovec & D'Amato, 1997)).

It is known that the second messenger responsible for the action of gastrin is calcium (Schubert, 2004). Next, we verified the role of calcium in β-myrcene gastroprotection, as several studies suggest that intracellular calcium depletion in the gastric glandular mucosa may account for the ethanol ulceration and the ulcer-aggravating action of Nifedipine (Koo et al., 1986; Wong et al., 1991). Our results demonstrate that while calcium hydrochloride mimics effects of β-myrcene, Nifedipine, a calcium channel blocker, prevents the gastroprotection afforded both by β-myrcene and calcium hydrochloride, suggesting that

altered gastric cell calcium levels could be closely related to the mucosal lesions produced by ethanol. It is likely that β-myrcene affords gastroprotection by restoring gastric tissue calcium.

The H^+/K^+-ATPase is an electroneutral H^+ - K^+ exchange system is located in the membrane fraction of gastric parietal cells (Hervatin et al., 1987). This enzyme is responsible for HCl secretion in the gastric lumen. The results obtained with gastric microsomes isolated from rat stomach indicate that monoterpene β-myrcene could not inhibit H^+/K^+- ATPase activity, which is comparable to the reference drug, omeprazole ($P<0.05$), even an increase in enzyme inhibition in a concentration-dependent manner. These results suggest that monoterpene might be imparting anti-ulcer activity by decreasing acid and gastrin secretion, not via proton pump inhibition.

It has been postulated that K^+atp channels are involved in a variety of pathophysiologic functions of the stomach (Toroudi et al, 1999), including regulation of blood flow, gastric acid secretion and contractility. However, their direct effects on ATP-sensitive channels and the modulation of potassium gastroprotective events have not been fully investigated. Peskar (2002) demonstrated that the K^+atp channel blocker glibenclamide inhibits the gastroprotective effect of several agents. K^+atp channels openers were known to interfere with Ca^{++} ion influx and to antagonize mobilization of Ca^{++} bound to intracellular stores leading to reduction in free cytosolic Ca^{++} ion important for gastric acid and pepsin secretion (Bose et al., 2003). Thus, regulating the opening and closing of K^+atp channels and associate with calcium levels in the stomach, has finality as a defense mechanism for the gastric mucosa against external aggressions.

Diazoxide is a drug that activates and opens potassium channels in various tissues, causing hyperpolarization of the plasma membrane and reduced electrical activity (Jahangir et al., 2001). Other studies have reported that diazoxide in the stomach inhibited gastric mucosal lesions induced by ethanol, while glibenclamide (K^+atp channel inhibitor) increased ethanol-induced (Toroudi et al., 1999) gastric lesions. Another recent study (Nicolau et al., 2013) indicated that Glibenclamide alone reversed the gastric protective effect of Lawesson's reagent. However, glibenclamide plus diazoxide did not alter the effects of this reagent. These data suggest that Lawesson's reagent plays a protective role against ALD-induced gastric damage through mechanisms that depend, at least in part, on the activation of ATP-sensitive potassium (K^+atp) channels. In the present study, glibenclamide, a K^+atp channel blocker, significantly antagonized the protective effect of β-myrcene and diazoxide. Thus, these studies suggest that the regulation of K^+atp channel opening in the stomach could modulate the gastric mucosal defense against external aggressions.

This is the first study to report the effects of β-myrcene in an animal model of reflux-induced esophagitis. Gastroesophageal reflux disease is caused when gastric acid flows back into the esophagus, resulting in erosion of the esophageal mucosal epithelium (Fass et al., 2012). Our findings indicate that β-

myrcene (7.5mg/kg, id.) significantly decreased esophagus lesions ($P<0.05$) and decreased the lipid peroxidation levels ($P<0.01$) compared to the vehicle.

Thus, we conclude that the antisecretory action promoted by β-myrcene is due to decreased levels serum gastrin (can act as an antagonist of gastrin in the gastric mucosa), calcium channels involvement and the opening of K^+atp channels. Ultimately, ultimately β-myrcene presents significant activity against gastroesophageal reflux induced by esophagitis with decreased lipid peroxidation levels in the mucosa.

5.Abbreviations

MDA - Malondialdehyde

ROS - Oxygen species

H_2 - histamine receptor

CCK_2 - Cholecystokinin receptor

M_3 - muscarinic receptor

6.Conflict of Interests

The authors declare no conflicts of interest.

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8.Conclusion

This is the first study demonstrating the antisecretory activity and protective effect of β-myrcene against gastroesophageal reflux induced by esophagitis. Furthermore, β-myrcene may be used in combination with other nutraceuticals to manage acid secretion disease conditions.

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Legends

Table 1: Effect of monoterpene β-myrcene in rats subjected to pylorus ligature and evaluation of gastric juice parameters. Data are expressed as the mean ± S.E.M. Statistical significance was determined using ANOVA, followed by Dunnett's test; *P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

Table 2: Evaluation of the antisecretory mechanism of action of monoterpene β-myrcene in rats subjected to pylorus ligature stimulated by pentagastrin, bethanechol and histamine and evaluation of gastric juice parameters. Data are expressed as the mean ± S.E.M. Statistical significance was determined using ANOVA, followed by Dunnett's test; * P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

Table 3: Protective effect of the monoterpene β-myrcene against Gastroesophageal Reflux induced by esophagitis in rats and lipid peroxidation (MDA). Data are expressed as the mean ± S.E.M. Statistical significance was determined using ANOVA, followed by Dunnett's test; * P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

Figure 2: Determination of plasma gastrin levels in animals submitted to the pylorus ligation model. Data are expressed as the mean ± S.E.M. Statistical significance was determined using ANOVA, followed by Dunnett's test; * P<0.05, ***P<0.001 compared to vehicle.

Figure 3: Effects of nifedipine and calcium hydrochloride pretreatments on β-myrcene gastroprotection. Data are expressed as the mean ± S.E.M. Statistical significance was determined using ANOVA, followed by Dunnett's test; * P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

Figure 4: Effects of monoterpene β-myrcene on the gastric H^+/K^+- ATPase. Data are expressed as the mean ± S.E.M. Statistical significance was determined using ANOVA, followed by Dunnett's test; * P<0.05 compared to vehicle.

Figure 5: Role of K^+atp channels in the gastroprotective effect of β-myrcene. Data are expressed as the mean ± S.E.M. Statistical significance was determined using ANOVA, followed by Tukey's test; *P<0.05 and **P<0.01 compared to vehicle. Crossbars represent the mean and SEM, and significant differences (*P<0.05 and ***P<0.0001) indicate comparisons between the different pretreatments (saline + treatment + glibenclamide treatment).

Table 1

Route of administration	Treatments	pH (units)	Volume of gastric juice (mL/4h)	Hydrogen concentration $[H]^+$ (mEq/mL/4h)
Oral	Vehicle	1,00±0,06	6,43±0,52	13,33 ± 1,23
	Lansoprazole	3,57±0,17***	5,0±0,49	0,63 ± 0,07**
	β-myrcene	1,62±0,20*	6,5±0,43	9,11 ± 0,96**
Intraduodenal	Vehicle	1,02±0,06	7,6 ± 0,60	19,20 ± 1,24
	Lansoprazole	4,18±0,25***	7,0 ± 0,45	3,20 ± 0,20**
	β-myrcene	1,71±0,21*	6,4 ± 0,51	6,40 ± 0,24**

Table 2

Route of administration	Treatments	pH (units)	Volume of gastric juice (mL/4h)	Hydrogen concentration $[H]^+$ (mEq/mL/4h)
Intraduodenal	Vehicle	1,29±0,04	7,01±0,7	7,04±0,35
	β-myrcene	1,97±0,20*	4,01±0,43***	3,00 ± 0,31***
	Histamine	0,60±0,08*	9,18±0,35*	9,33 ± 0,35**
	Histamine+ Cimetidine	1,94±0,18*	3,28±0,38***	4,99 ± 0,67**
	Histamine+ β-myrcene	1,38±0,23	8,41±0,56	8,35 ± 0,15
Intraduodenal	Vehicle	1,02±0,06	8,33±0,35	9,55 ± 0,388
	β-myrcene	1,7±0,21*	5,41±0,34***	6,26 ± 0,34***
	Betanecol	0,53±0,07*	9,7±0,42*	11,07 ± 0,366*
	Betanecol+Atropine	1,43±0,20	3,8±0,26***	5,45 ± 0,39***
	Betanecol + β-myrcene	1,17±0,17	7,8±0,24	9,315 ± 0,318
Intraduodenal	Vehicle	0.98±0,06	8,33±0,35	8,762 ± 0,856
	β-myrcene	1,92±0,19***	5,41±0,34***	5,95 ± 0,434*
	Pentagastrin	0.62±0,07	9,96±0,38*	11,23 ± 0,488*
	Pentagastrin+ β-myrcene	1,89±0,13***	4,47±0,41***	6,402 ± 0,640*

Figure 2

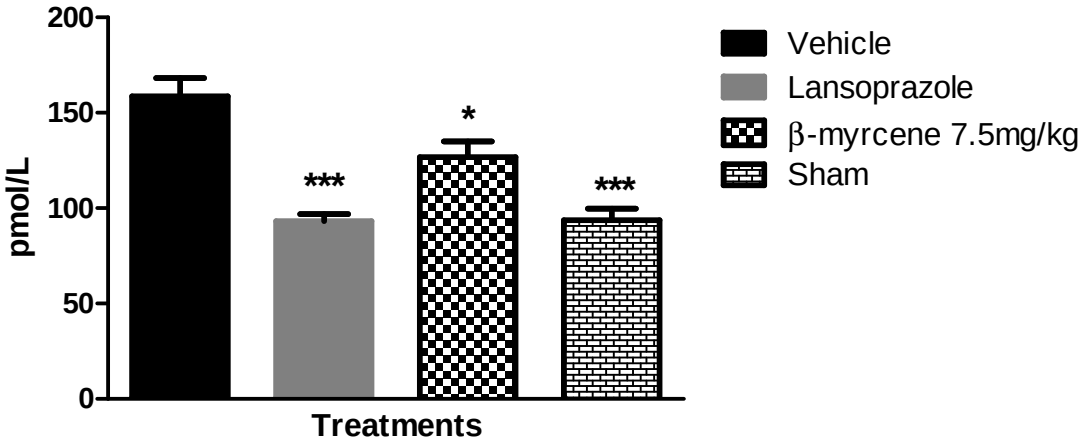

200
150
100
50
0
pmol/L
***
*
***
Treatments
Vehicle
Lansoprazole
β-myrcene 7.5mg/kg
Sham

Figure 3

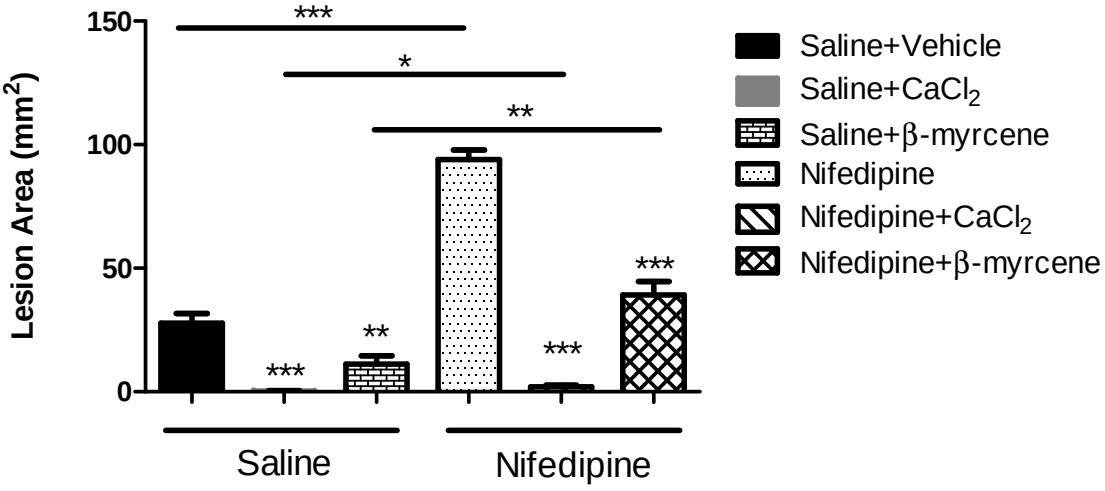

Lesion Area (mm²)
150
100
50
0
***
*
**
***
**
***
***
Saline
Nifedipine
Saline+Vehicle
Saline+CaCl2
Saline+β-myrcene
Nifedipine
Nifedipine+CaCl2
Nifedipine+β-myrcene

Figure 4

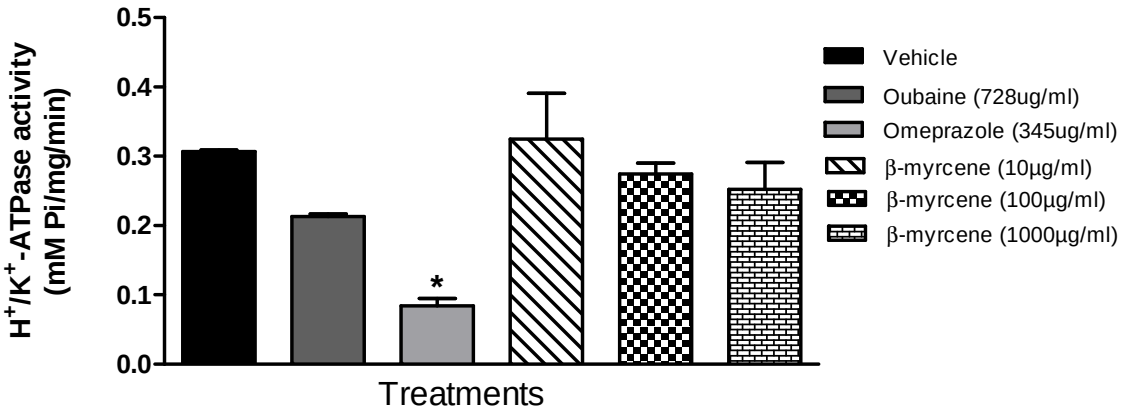

0.5
0.4
0.3
0.2
0.1
0.0
H+/K+-ATPase activity
(mM Pi/mg/min)
*
Treatments
Vehicle
Oubaine (728ug/ml)
Omeprazole (345ug/ml)
β-myrcene (10µg/ml)
β-myrcene (100µg/ml)
β-myrcene (1000µg/ml)

Figure 5

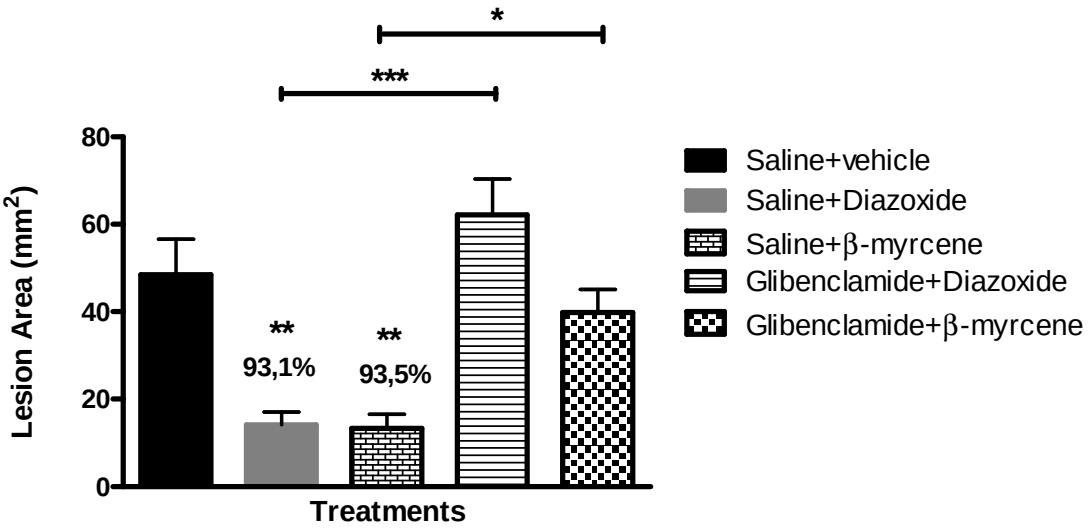
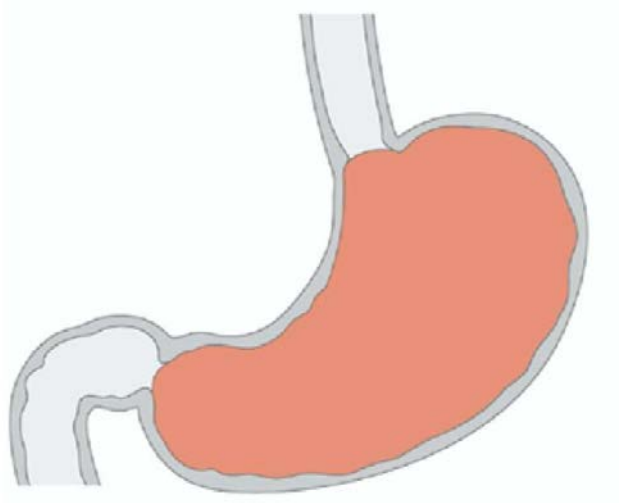

*
***
80
60
40
20
0
Lesion Area (mm2)
**
93,1%
**
93,5%
Treatments
Saline+vehicle
Saline+Diazoxide
Saline+β-myrcene
Glibenclamide+Diazoxide
Glibenclamide+β-myrcene

Table 3

Treatments	Lesion Area (mm^2)	Lipid peroxidation (MDA) nmol TBARS/mg protein
Vehicle	141,0± 55,33	10,75± 1,558
Lansoprazole (30mg/kg)	1,556± 0,5022**	6,725± 1,112*
β-myrcene (7.5mg/kg)	13,21± 3,551**	5,332± 0,2352**
Sham	0.0±0.0**	3,625± 0,4894***



Capítulo II

The effects of monoterpene β-myrcene on gastric healing activity: role of cytokines, matrix metalloproteinases-2 and -9 and gastric contraction recovery

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Abstract: The monoterpene β-myrcene has been widely used in cosmetics, foods and beverages and is normally found in essential oils from *citrus* fruit, such as *Citrus aurantium*. This medicinal plant has traditionally been used against gastritis and gastric disorders. This study aimed to determine the effects of the monoterpene β-myrcene on gastric healing after 7 and 14 days of treatment and evaluate the healing action mechanisms. The results indicated that β-myrcene has important gastric healing activity when administered orally at a dose of 7.5mg/kg, with a significant decrease of gastric lesions (47.2% and 47.1% respectively), and β-myrcene treatment exhibited no toxicity during the treatment period. We investigated the regulation of MMPs in chronic gastric ulceration induced by acetic acid and examined the activity and expression of MMP-2 and -9 in the stomach. In the groups treated for 7 days, MMP-2 and -9 activity was detected; MMP-2 activity was observed in all groups and was

higher in the vehicle group and lower in the groups treated for 7 days with lansoprazole or β-myrcene and the sham group (18.5%, 25% and 26%, respectively). In the groups treated for 14 days, non-significant MMP-9 activity was observed in all groups. Additionally, interleukin IL-1β levels were significantly higher in ulcerated stomachs, and β-myrcene treatment re-established the levels of interleukin IL-1β levels during chronic gastric ulceration after 14 days. MPO levels were also significantly elevated in ulcerated stomachs, and a significant decrease in MPO levels in the stomachs of animals treated with β-myrcene (30%) compared with vehicle. The healing activity of β-myrcene also involves COX-1 (with 7 days of treatment) and COX-2 (with 14 days of treatment); additionally, gastric contractions after ulceration were restored after 7 days of treatment by approximately 40% and 88% in frequency and contraction amplitude, respectively. We conclude that β-myrcene is an effective agent of natural origin that exhibits healing action on the gastric mucosa without the adverse effects presented by the current synthetic drugs.

Keywords: β-myrcene; essential oils*;* healing; gastric ulcer.

1. Introduction

An ulcer is a deep necrotic lesion that penetrates the gastrointestinal mucosa and muscularis mucosae. The gastric mucosa is continuously exposed to damaging agents that are involved in the pathogenesis of gastric ulcers. These damaging agents can be endogenous (e.g., hydrochloric acid, pepsin, refluxed bile, and reactive oxygen species) or exogenous (e.g., alcohol consumption, excessive coffee ingestion, and administration of non-steroidal anti-inflammatory drugs). The basic physiopathology of gastric ulcers results from an imbalance between damaging factors and cytoprotective factors, which include an intact mucus barrier, prostaglandins, adequate mucosal blood flow, activity of antioxidant compounds, and other mediators, although the exact ulcer etiology is complex and multifactorial (Laine et al., 2008).

Many experimental studies have been conducted to understand the etiology of ulcers; ulcer healing is an active and complicated process of reconstructing the mucosal architecture and involves inflammation, cell proliferation, re-epithelialization, formation of granulation tissue, interaction between various cells, matrix and tissue remodeling (Tarnawski et al., 2005).

Ulcer healing is a complex and tightly regulated process of filling the mucosal defect with proliferating and migrating epithelial and connective tissue cells. This process includes the re-establishment of the continuous surface epithelial layer, glandular epithelial structures, microvessels and connective tissue within the scar. Epithelial cells in the mucosa of the ulcer margin proliferate on and migrate to the granulation tissue to re-epithelialize the ulcerated area (Tarnawski et al., 2001).

The probable mechanism of action of the selected potential anti-ulcer compounds has yet to be elucidated. Reconstruction or rejuvenation of the mucosal architecture, which is needed for ulcer healing,

is dependent on elicitation of signaling molecules that can modulate the complete healing process, including the modulation of specific markers (Brzozowski et al., 2005).

Matrix metalloproteinases (MMPs), a group of zinc-dependent enzymes, remodel healthy connective tissue by specifically digesting collagen and other matrix molecules (Parks et al., 1998; Visse et al., 2003). In chronic inflammatory conditions, the extracellular secretion of MMPs substantially contributes to stromal tissue destruction (Parks et al., 1998; Kahari et al., 1999), and suppression of myeloperoxidase (MPO) activity, a marker of neutrophil infiltration during inflammation, was found to enhance gastric ulcer healing (Tsukimi et al., 1996). Gastric ulceration results from the imbalance between gastrotoxic agents and protective mechanisms, causing acute inflammation. Interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) are major proinflammatory cytokines that play an important role in the production of acute inflammation (Konturek et al., 2000; Li et al., 2000; Vanhoutte et al., 2006; Kupai et al., 2010; Polyakova et al., 2010).

According Schmeda-Yesilada and Hirschmann (2005), among many sources of new molecules pharmacologically active in the prevention and treatment of peptic ulcers, those originating from natural products are considered important sources of new molecules. Thus, many plant products are an attractive source for the development of new drugs or for use as a treatment adjuvant (Arrieta et al., 2003), and according to Zayachkivska et al. (2005), many have shown promising results in clinical settings.

In this context, previous studies have shown that substances of vegetable origin, such as terpenoids, have very significant antiulcer activity (Borrelli & Izzo, 2000; Rodriguez et al., 2004; Marques et al., 2006; Bonamin et al., 2014).

Terpenoids, so named because of biosynthetic origin is derived from isoprene units, comprise wide variety of plant substances. The terpene compounds most frequently encountered are monoterpenes (90% oil) and sesquiterpenes (Cardoso et al., 2000).

β-myrcene (7-methyl-3-methylene-1,6-octadiene) is a pleasant-smelling monoterpene found in many plants, including lemongrass, hops and verbena (Günther & Althausen, 1949), as well as in the composition of essential oil obtained from fresh fruit peels of *Citrus aurantium* (Moraes et al., 2009). It is used in the production of perfumes and is also a component of essential oils that have long been used as flavoring additives in the manufacturing of alcoholic beverages and the food industry (Leung, 1980).

In addition to the major uses of β-myrcene in the food industry, its effects against peptic ulcers are very important. Bonamin et al. (2014) showed that β-myrcene has gastroprotective activity using experimental models of peptic ulcers induced by ethanol, NSAIDs, stress, cysteamine (duodenal ulcer) and has considerable antioxidant activity, maintaining GSH levels in the gastric mucosa as protection. This study

aimed to determine the healing activity of β-myrcene after 7 and 14 days of treatment and evaluate its healing action mechanisms in rats.

2. Material & Methods

2.1. Animals and evaluation of the lesions

Male Wistar rats (180–250g) were supplied by the Central Animal House of the UNESP. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of dark/light (12h/12h), humidity (60 ± 1.0%) and temperature (22 ± 1°C). The animals were fasted before all experimental procedures while standard drugs and β-myrcene were administered orally (gavage). The animals were housed in cages with raised floors of wide mesh to prevent coprophagy. All experiments were performed in the morning and followed the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993). The experimental protocol was approved by the Institutional Animal Care and Use Committee from UNESP (CEUA n°363). After each experiment, the animals were killed with CO_2 gas, and the stomachs were excised, cut along the greater curvature, and gently rinsed under tap water. The stomachs were then pressed onto a glass plate and scanned so that the lesions could be counted using the AVSoft program. The results are expressed as the total ulcerated area (mm^2). In addition to the animal groups treated with vehicle (negative control), antiulcer drugs (positive control), or monoterpene β-myrcene, we also included a sham group, in which the animals were subjected to the same procedure as the other groups but without the use of injury-inducing agents.

2.2. Drugs and treatment

Pentobarbital, ketamine hydrochloride, xylazine hydrochloride, lansoprazole, acetic acid, myeloperoxidase, protease inhibitor cocktail, calcium chloride, Triton X-100, gelatin and Coomassie Brilliant Blue R-250 were purchase from Sigma Co (USA). β-myrcene was purchased from Acros Organics (USA) and dissolved in 8% Tween-80 aqueous solution administered by orally (p.o.). The vehicle control group was administered 8% Tween-80 aqueous solution (10 mL/kg).

2.3. Experimental models

2.3.1. Healing in acetic acid-induced gastric lesions

The experiment was performed according to the method described by Okabe et al. (1971, modified by Konturek et al., 1988). To evaluate the healing action of monoterpene β-myrcene, male Wistar rats (n = 8 for each experimental group) were anesthetized and had the abdomen opened to expose the stomach. A plastic 4.2 mm internal diameter tube was firmly applied to the serosal surface of the stomach wall, and 70 µl of a solution of acetic acid at 80% was applied for 20 seconds on the serosal surface of the stomach and completely removed; then, the abdomen was sutured. This process resulted in a chronic ulceration of the

mucosa and submucosa, with an approximate ulcer area of 13.8mm^2. The monoterpene β-myrcene (7.5mg/kg solubilized in the vehicle) was administered orally from day 1 after surgery and treated for 7 and 14 days. The positive control was the standard drug, lansoprazole (30mg/kg also dissolved in the vehicle), and negative control (vehicle), 8% Tween 80®, both administered orally. After 7 and 14 days, the animals were killed, and the stomach was removed for macroscopic examination. The total lesion area was scanned for analysis using the program AVSoft BioView Spectra, and strips of tissue were weighed and stored for biochemical determinations (myeloperoxidase, cytokines and matrix metalloproteinase).

2.3.2. Subacute toxicity

The toxicological analysis assessed the changes in the body weight of the animals of different groups, examined the organs morphometry to analyze behavioral changes and mortality, and compared the values of arcsine of organ weight/total weight of all animals. In addition, we evaluated serum biochemical parameters, such as ALT (Alanine transaminase), AST (Aspartate transaminase), gama-GT (gama-glutamyltransferase), creatinine and urea.

2.3.3. Biochemical determination of myeloperoxidase (MPO)

Samples of gastric tissue submitted to acetic acid agent were removed from the rats that underwent different treatments and then weighed and stored for subsequent biochemical analysis. Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al. (1984). Samples were suspended in 1mL of 50mM phosphate buffer with 0.5% hexadecyltrimethyl-ammonium bromide (pH 6.0) and minced with scissors for 15s on an ice-cold plate. The resultant suspension was diluted to a final ratio of 1:20 (w/v), homogenized for 1 min with an automatic Heidolph homogenizer, and then sonicated for 10s and subjected to three freeze-thaw cycles. The homogenates were then centrifuged at 7000× g at 4°C for 10 min, and the supernatants were assayed for MPO activity. The results are expressed as MPO units per gram (U/g) of wet tissue.

2.3.4. TNF-α, IL-1β and IL-10 measurements

To determine the TNF-α, IL-1β and IL-10 levels in tissue from animals submitted to acetic agents. The stomachs strips were collected, homogenized, and centrifuged, and the supernatant was analyzed using a kit dosage immunoenzymatic (R&D system) read in an ELISA biosystem reader at 450nm. The results are expressed as pg/mg of protein.

2.3.5. Metalloproteinases-2 and -9 analysis: extraction of total protein and zymography

Gastric ulcer samples from each experimental group were used to extract total protein. The extraction was carried out following the ratio of 30mg of tissue: 0.1ml of 50 mM Tris-HCl solution, pH7.5, containing 0.25% Triton X-100, 10 mM $CaCl_2$ and protease inhibitor cocktail (P-8849, Sigma-Aldrich, St

Louis, MO, USA) by crushing on the Polytron type homogenizer. The homogenate was centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant was collected and protein content was quantified by the Bradford method (1976). Samples of extracted proteins (28µg) of the gastric ulcer specimens from different experimental groups were subjected to electrophoresis under non-reducing conditions on 8% polyacrylamide gel copolymerized with 0.1% purified gelatin (Sigma). After electrophoresis, the gels were subjected to two washes of 15 minutes in a solution of 2.5% Triton X-100 to remove SDS and two washes of 5 minutes in 50mM Tris-HCl buffer, pH 8.0. Subsequently, gels were incubated in 50mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$ for 22 hours at 37°C. Finally, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO, USA). The relative molecular weight of the bands was determined according to the standard molecular weight (Precision Plus Protein™, BIO-RAD) used in electrophoresis. The bands obtained in zymography were scanned and analyzed by densitometry. The bands of gelatinase activity of MMP-2 and -9 was analyzed by obtaining the integrated optical density (IOD) of the bands using Image J software. Values were plotted in a histogram showing the ratio of treated groups IOD on the control group IOD value.

2.3.6. Expression of COX-1 and COX-2

The stomach was shaved and then centrifuged at 12,000 rpm at 4°C for 15 minutes. Protein concentration was quantified by Bradford (1976) method. Specific proteins (50 mg) were applied to polyacrylamide (SDS-PAGE) gels and subjected to electrophoresis with buffer solution (25mM Trisma base, 1.92mM glycine, 1% SDS). SDS-PAGE was initially submitted to 40V until the passage of the line marked by the stacking phase proteins (Stacking) and then 120V until the gel resolved. Then, the separated SDS-PAGE proteins were transferred to a nitrocellulose membrane in electrotransfer equipment, with the membranes soaked in transfer buffer (25mM, 192mM glycine, 20% methanol Trisma base) with a stored voltage constant of 90V for 2 hours. Nitrocellulose membranes containing the transferred proteins were incubated in blocking solution (150mM NaCl, 10mM Trisma base, 0.05% Tween 20®, skimmed milk powder 5%) for 1 hour to decrease the binding nonspecific protein. The membrane was then subjected to washings with basic buffer (150mM NaCl, 10mM Trisma base, 0.05% Tween 20®) at intervals of 10 minutes. The membrane was incubated at 4°C overnight with antibody specific to COX-1 and COX-2 (Cayman). The following morning, the nitrocellulose membrane was washed in basic buffer for 40 minutes and then incubated at room temperature for 1 hour with the secondary antibody (Thermo Scientific goat anti-rabbit IgG (H + L). To detect the immuno-reactive bands, the blot was exposed to chemiluminescent solution (ECL plus LumiGlo chemiluminescence substrate, Gaithersubrg Kirkegard and Perry, MD, USA) for 1 minute, followed by exposure to film to detect chemiluminescence (HyperfilmTMECLTM, Amersham Pharmacia Biotech, Buckinghamshire, England). The densities of the sample bands were captured on film for optical densitometric quantification using AVSoft BioView 4 software.

2.3.7. Alternated current biosusceptometry (ACB)

The ACB sensor consists of two pairs of induction coils composed of an excitation coil (outer) and detection coil (inner) in a first-order gradiometric configuration. Details on the instrumentation have been described previously (Américo et al., 2010). Briefly, ACB signal detection depends on the distance between the sensor and magnetic material; thus, changes in their relative position during contraction and relaxation can modulate the signal recorded by the sensor. In this study, signals resulting from magnetic marker fixed on gastric serosa, and its movements induced by contractions were detected by ACB. The instrumentation was developed to improve spatial resolution and sensitivity for laboratory animal studies (Américo et al., 2010; Quini et al., 2012).

2.3.8. Experimental procedure

After 12 hours of fasting, the animals were anesthetized with a composite solution of ketamine (30mg/kg) and xylazine (15mg/kg), and a laparotomy was performed. A magnetic marker (diameter 3.5mm; height 3.0mm) was implanted in the seromuscular layer by suturing it in the gastric corpus using 6/0 thread 3 cm away from the pylorus (Américo et al., 2010). The rats were allowed to recover for 10 days after surgery. Gastric contractility recordings were performed in three sessions (R1, R2 and R3). In the control conditions, gastric contractility was recorded after the induction of gastric ulcer by ethanol absolute and after 7 days of treatment with monoterpene β-myrcene. Rats were evaluated to acquire fed contractile patterns by ACB after ingestion of approximately 1g of standard chow. The animals were anesthetized (pentobarbital, 30mg/kg, Abbott Laboratories, Chicago, USA) and kept supine during the 40-minute measurement period. The magnetic sensor (Br4Science®, São Paulo, Brazil) was placed on the anterior surface of the abdomen, and a continuous ACB signal recording was started. Signals were acquired at a 20Hz/channel rate that was digitized using a multi-channel recorder (MP100 System; BIOPAC Inc., Santa Barbara, USA) and stored for further analyzes.

2.3.9. Histological analyses

After scanning, stomach samples were collected for histological slide preparation and stained with hematoxylin and eosin (HE).

3. Statistical analysis

The results are expressed as the mean ± S.E.M. Statistical significance was determined by a one-way analysis of variance, followed by Dunnett's test. $P<0.05$ was considered statistically significant.

4. Results

4.1. Lesion Area

Figures 1 and 2 show the percentage inhibition of gastric lesions induced by acetic acid in animals treated with vehicle, lansoprazole or the monoterpene β-myrcene for 7 and 14 days. These figures demonstrate the gastroprotective effect of β-myrcene at a dose of 7.5mg/kg, which promoted a protection of 47.2% ($P<0.05$) and 47.1% ($P<0.05$) at 7 and 14 days, respectively, compared with the vehicle-treated group. The gastroprotective effect of β-myrcene was also compared with the standard antiulcer drug lansoprazole at a dose of 30mg/kg (4-fold higher than that of β-myrcene). Lansoprazole induced a 74% ($P<0.01$) and 55% ($P<0.01$) inhibition at days 7 and 14, respectively, in gastric lesions compared with vehicle.

4.2. Subacute toxicity

Animals undergoing different treatments (vehicle, lansoprazole and β-myrcene) in the acetic acid-gastric ulcer model exhibited important toxicity factors, including body weight changes over 14 days (data not show), mortality, and vital organ weight (Table 1). The 14-day change in body weight with β-myrcene treatment did not significantly differ from the vehicle group, and the average weights of vital organs and visceral conditions were normal and comparable to those of the control group ($P>0.05$). No premature animal death was observed. Table 2 also presents results obtained by biochemical analyses of serum organized by treatments, and no alterations in these parameters were observed.

4.3. Biochemical determination of myeloperoxidase (MPO)

Table 3 shows the gastroprotective effect of β-myrcene in the acetic acid-induced rat model of gastric ulcers and the biochemical parameters of gastric mucosal tissue in terms of myeloperoxidase (MPO), IL-1β, IL-10 and TNF-α levels. Treatment with β-myrcene was decreased MPO activity ($P<0.01$) in the gastric mucosa of rats compared with the vehicle-treated control group after 7 and 14 days of treatment.

4.4. TNF-α, IL-1β and IL-10 measurement

Treatment with β-myrcene increased IL-1β ($P<0.05$) after 7 days of treatment and decreased IL-1β activity ($P<0.05$) in the gastric mucosa of rats after 14 days of treatment compared with the vehicle-treated group. However, β-myrcene treatment increased IL-10 (an anti-inflammatory interleukin) in tissue after 7 days of treatment.

4.5. Determining MMP-2 and MMP-9 activity using zymography

Figure 3 shows the MMP-2 and -9 activity in stomach samples from animals submitted to the acetic acid model and treated for 7 or 14 consecutive days with vehicle, lansoprazole or β-myrcene. In the groups of animals treated for 7 days, MMP-9 activity was detected only in the vehicle, lansoprazole and β-myrcene group and was not observed in the sham group. MMP-2 activity was observed in all groups and was higher in the vehicle group and lower in the groups treated with lansoprazole and β-myrcene for 7 days and the sham group (18.5%, 25% and 26%, respectively). In the groups treated for 14 days, MMP-2 and -9 activity was observed in all groups; however, significant differences were not observed because local tissue remodeling had already started, and inflammation was already diminished.

4.6. Expression of COX-1 and COX-2

Figure 5 (A, B, C and D) shows graphs corresponding with the densitometric intensities obtained from the membranes used to analyze COX-1 and COX-2 using β-actin protein as the internal control. Densitometry was calculated for each membrane, and the densitometric values for proteins of interest were divided by the densitometry of β-actin in the same membrane.

Figures A and B show the expression of COX-1 after 7 and 14 days of treatment, respectively. Significant differences were observed between the groups treated with β-myrcene ($P<0.01$) and lansoprazole ($P<0.001$) and the sham ($P<0.001$) and the vehicle-treated group (A).

No significant differences were observed between groups at 14 days of treatment (B). Figures C and D show COX-2 expression after 7 and 14 days treatment, respectively; however, no significant differences was found after 14 days of treatment compared with the respective 7-day treatment group (C). However, as shown in Figure D, the expression of COX-2 was assessed 14 days of treatment, and a significant increase was found in the group treated with monoterpene β-myrcene ($P<0.05$).

4.7. Alternated current biosusceptometry (ACB)

Body weight, frequency and amplitude of gastric contractions are shown in Figure 4 (B). A significant reduction in these parameters was observed in the ulcerated rats. However, β-myrcene treatment increased body weight and the frequency of contractions similar to the controls but significantly greater than the ulcerated rats. β-myrcene treatment induced a significant increase in the amplitude of contractions compared with ulcerated rats and even when compared with the controls.

Alterations in motility from baseline were determined as percentages (Figure 4A). After ulceration, frequency was reduced by approximately 30%, and contraction amplitude was reduced by approximately 40% ($p<0.001$). From baseline, the increase in frequency after treatment was approximately 8%, and the increase in contraction amplitude was 48% ($p<0.001$). Considering the ulcerated animals, 7 days of

treatment with β-myrcene induced high recovery rates of approximately 40% and 88% for frequency and contraction amplitude, respectively.

4.8. Histological analyses

Microscopically, extensive deep damage induced by acetic acid was observed in the gastric mucosa of vehicle-treated control animals, in accordance with the macroscopic appearance of chronic gastric erosion (Figure 6, Panel B). Histological examination indicated that oral treatment of lansoprazole (30mg/kg) or β-myrcene (7.5mg/kg) promoted gastric lesion healing, with the base of the ulcer covered with regenerating mucosa, occasionally arranged into columnar structures above the granulation tissue, with high degree of vascularization (Figure 6, Panels C and D, respectively).

5. Discussion

An acetic acid-induced ulcer model was employed to investigate the mechanisms of gastric ulcer healing because the validity of this model for ulcer healing studies has been previously established (Tsukumi et al., 1994), and this model resembles human ulcers in terms of both pathological features and healing mechanisms (Okabe et al., 2005). Acetic acid consistently induces penetrating ulcers, beginning with vascular injury, ischemic necrosis, and then leading to ulceration (Bui et al., 1993). In addition, multi-mechanistic destructive routes, such as damaging of the gastric wall barrier, generation of free radicals, cell apoptosis, etc., also contributed to acetic acid-induced ulcer development.

The rat model of acetic acid-induced ulcers is the most similar model to gastric ulcers in humans (Okabe & Agamase, 2005). Ulcer healing can be divided into three stages: 0-3 days, 3-10 days and 10-20 days. Days 0-3 consist of ulcer development, including the development of necrotic tissue, ulcer implantation, inflammatory infiltration and ulcer margin formation. The rapid healing phase occurs during days 3-10 and involves epithelial cell migration and ulcer contraction. Slow healing occurs between days 10-20 and includes angiogenesis, granulation tissue remodeling and complete ulcer crater re-epithelization (Schmassmann, 1998).

Because we conducted 14 days of treatment, this chronic model was also used to evaluate subchronic toxicity. The results indicate that there was no change in total weight, organ weight, or blood biochemical parameters; therefore, no toxicity with β-myrcene treatment at dose of 7.5mg/Kg for 14 days was observed.

Histologically, gastric ulcers consist of the ulcer margin, which is formed by the adjacent non-edge necrosis that defines the injury, and the ulcer base, which is composed of necrotic tissue, as seen in Figure 6. The ulcer healing process is complex and involves migration, proliferation, re-epithelization, angiogenesis

and granulation tissue formation. These processes are controlled by growth factors, transcription factors and cytokines (Tarnawski et al., 1991).

Gastric ulcer induced by administration of 80% acetic acid exhibited elevated IL1-β levels, demonstrating that elevated pro-inflammatory cytokines levels induce interaction between leukocytes and endothelial cells. Administration of 80% acetic acid was previously suggested to induce macrophages to release pro-inflammatory cytokines (Konturek et al., 2000).

After gastric ulcer was induced by administration of 80% acetic acid, IL-1β levels on the gastric mucosa of animals treated with β-myrcene were elevated spontaneously on day 7 compared with the control groups (Table 3). This result may be due to the fact that when the gastric mucosa was damaged by acetic acid, the T and B lymphocytes in the submucosa beneath the damaged area that typically produce basal level of IL-1β were also damaged. The surviving macrophages were able to release of IL-1β in response to acetic acid injury. When inflammation occurs, IL-10 is synthesized. The increase in IL-10 levels reduced gastric inflammation through its feedback inhibition of IL-1β production. Therefore, IL-10 is spontaneously elevated in chronic gastric inflammation. Elevated IL-10 levels then reduce gastric tissue inflammation simultaneously (Eamlamnam et al., 2006).

Adhesion molecules play an important role in the recruitment of leukocytes to inflammation sites, leading to gastric mucosal injury (Wallace et al., 1991; Wallace et al., 1993). Leukocyte adhesion and/or aggregation can occlude microcirculation, resulting in ischemic mucosal injury (Wallace et al., 1993, Andrews et al., 1994). Leukocyte infiltration in gastric mucosa can cause tissue damage, leading the ulcerative lesions (Wada et al., 1996).

In the acetic acid model, gastric ulcer formation was associated with increases in MPO activity (Amagase et al., 2003). Considering that MPO is mainly produced by neutrophils, MPO activity is considered an index of neutrophil infiltration, which produce many enzymes and free radicals that damage the gastric mucosa, contributing to ulcer development (Bradley et al., 1982; Amagase et al., 2003; Potrich et al., 2010). We observed a significant increase in MPO activity in chronic gastric ulcers induced by acetic acid, suggesting neutrophil infiltration at the lesion site. We report here that β-myrcene decreases MPO activity in the gastric mucosa, contributing to the resolution of the inflammatory process caused by acetic acid and attenuating the gastric damage produced by leukocyte reactions.

As described by Wallace (2005), the contribution of COX-2 to the defense of gastric mucosa and healing is increasingly clear and may lead to the development of new therapies. COX-2 expression controls mucosal protection mechanisms, such as re-epithelialization, angiogenesis and adaptive cytoprotection. Drugs such as lansoprazole induce up-regulation of EGF and COX-2 in chronic treatments; drugs with this property are more successful at healing gastric ulcers (Poonam et al., 2005; Schmassman et al., 2005).

There are two known distinct isoforms of COX, termed COX-1 and COX-2. PGs generated by COX-2 in a gastric ulcer appear to play a key role in ulcer healing by stimulating cell proliferation, promoting angiogenesis and restoring mucosal integrity. However, PGs produced by COX-1 are responsible for the regulation of mucosal blood flow, mucus secretion and epithelial HCO3- (Konturek et al., 2005).

COX-1 and COX-2 expression was evaluated (Figures 5A, B, C and D). We found that COX-1 expression was altered in the groups treated with lansoprazole or β-myrcene and the sham group at 7 days of treatment, while COX-2 expression was at normal levels at 7 days treatment; however, COX-2 expressed was increased after 14 days of treatment in the β-myrcene-treated group.

Schmassman and colleagues (2005) showed that ulcer healing is interrupted when COX-2 is administered, demonstrating that the healing process is not only changed when COX-1 is suppressed. However, another important factor reported by these authors is that the ulcer does not develop spontaneously in animals when COX-1 or COX-2 suppressed; both isoforms must suppressed for ulcer formation to occur. The data obtained after 14 days of treatment with β-myrcene are consistent with the literature; faster healing of the β-myrcene-treated group is consistent with the increased COX-2 expression observed.

Additionally, the gastric mucosa of rats subjected to the chronic ulcer model showed better recovery from injury in the lansoprazole and β-myrcene groups after 14 days of treatment (Figure 6).

Ulcer healing therefore is a dynamic process of extracellular matrix (ECM) remodeling that is primarily influenced by MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs). A study on the role of omeprazole and ranitidine confirmed that inhibition of acid secretion could also enhance the healing process (Dharmani et al., 2003).

In our model, more significant activation of MMP-2 than MMP-9 was observed. Activated MMP-2 in acetic acid-induced ulcerous rats was down-regulated upon treatment with β-myrcene compared with the ulcer control groups. These results support the observation made previously by Baragi et al. (1997), who suggested that the increases in MMP activity during ulceration are down-regulated the ulcer healing.

In addition to this mechanism, gastric contraction analysis also revealed another probable pathway for β-myrcene in gastric protection. Thus, to confirm the healing activity of the β-myrcene, we evaluated gastric contractions of animals using ACB technique.

Gastric emptying and contractility are valuable tools for studying the integrated physiology of the stomach (Linke, 2008). Thus, motility appears to be an important parameter to evaluate during ulcer treatment, especially for demonstrating the efficacy of medicinal plants. ACB is a magnetic technique validated to register several parameters of GI motor function in rats (Américo et al., 2010; Quini et al.,

2012). ACB provides a mechanical record of contractility because it directly measures GI wall movements generated by smooth muscle (Américo et al., 2010).

The strong effects of gastric ulcers on gastric contractility are interesting because there are few studies focusing on this pathophysiological aspect. Our results show that β-myrcene treatment increases body weight and gastric motility similar to controls but significantly greater than ulcerated animals. These results corroborate the healing activity observed after 7 days treatment with β-myrcene. This monoterpene, in addition to presenting an important healing activity, can restore gastric contractions during treatment, aiding in the recovery of the mucosa and improving motility. To our knowledge, this is the first study to demonstrate the healing effects of β-myrcene on gastric ulcers, showing that β-myrcene may be important for the prevention and healing of peptic ulcers.

6. Conclusions

This gastric healing activity of the monoterpene β-myrcene was previously unknown. Furthermore, β-myrcene may be used in combination with other nutraceuticals for effective management of gastric disease conditions. Finally, considering that the modern approach to control gastric ulceration is to promote gastroprotection and stimulate epithelial cell proliferation, this monoterpene may be a useful source of new anti-ulcer compounds for improving the quality of ulcer healing.

7.Abbreviations

MPO – Myeloperoxidase

IL – Interleukin

IL-10 – Interleukin 10

TNF-α - tumor necrosis factor alpha

BAC - Biosusceptometry Alternating Current

COX-1 – Cyclooxygenase 1

COX-2 – Cyclooxygenase 2

GI – Gastrointestinal

MMP-2 – Metalloproteinase 2

MMP-9 - Metalloproteinase 9

ALT – Alanine transaminase

AST – Aspartate transaminase

Gama-gt - gama-glutamyltransferase

ACB - Alternating Current Biosusceptometry

8. Conflicts of Interest

All authors declare that they have no conflict of interests.

9. Acknowledgements

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Legends

Table 1: Effects of 7.5mg/kg, p.o., per day of β-myrcene administered for 14 days on body and organ weight. Data are expressed as the mean ± S.E.M. An ANOVA was performed, followed by Dunnett's tests. compared with vehicle.

Table 2: Effects of 7.5mg/kg, p.o., per day of β-myrcene administered for 14 days on serum biochemical parameters. Data are expressed as the mean ± S.E.M. An ANOVA was performed, followed by Dunnett's test. *P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

Table 3: Levels of MPO, IL-1β, TNF-α and IL-10 in samples of gastric tissue from animals treated for 14 days with β-myrcene at a dose of 7.5mg/kg per day after acetic acid-induced gastric ulcers. Data are expressed as the mean ± S.E.M. An ANOVA was performed, followed by Dunnett's test. *P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

Figure 1: Healing action on gastric ulcers after treatment with β-myrcene for 7 days. Mean ± S.E.M. of the ulcerative area (mm^2). The percentages indicate the reduction in the lesion area proportionate to the negative control. Data are expressed as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test, *P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

Figure 2: Healing action on gastric ulcers after treatment with β-myrcene for 14 days. Mean ± S.E.M. of the ulcerative area (mm^2). The percentages indicate the reduction in the lesion area proportionate to the negative control (vehicle). Data are expressed as the mean ± S.E.M. An ANOVA was conducted, followed by Dunnett's test. *P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

Figure 3: Gel gelatinase zymography showing the activity of matrix metalloproteinases-2 and -9. Vehicle group (line 1), lansoprazole group (line 2), β-myrcene group (line 3) and sham group (line 4). Data are expressed as the mean ± S.E.M. An ANOVA was performed, followed by Dunnett's test. *P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

Figure 4: A) Percentage changes from baseline for frequency (white bars) and contraction amplitude (crosshatched bars) during all procedures. B) Body weight, frequency and gastric contraction amplitude were recorded in the rats during all procedures. Data are expressed as the mean ± S.E.M. An ANOVA was performed, followed by Dunnett's test. * P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

Figure 5: Effect of oral administration of β-myrcene (7.5mg/kg) for 7 and 14 days on the expression of cyclooxygenase-1 (COX-1) (A and B) and cyclooxygenase-2 (COX-2) (C and D) during the healing of

lesion induced by acetic acid in rats. Data are expressed as the mean ± S.E.M. An ANOVA was performed, followed by Dunnett's test. *P<0.05, **P<0.01, ***P<0.001 compared with vehicle.

Figure 6: Photomicrographs of the stomachs of rats with ulcers induced by acetic acid after treatment with vehicle (8% Tween 80®, 10ml/Kg – panel B), lansoprazole (30mg/Kg – panel C), or β-myrcene (7.5mg/kg – panel D) for 14 days. The sham group is shown in panel A. Bars = 2 mm (A–D). Histological sections are magnified by 25x, where M indicates the ulcer margins and B indicates the ulcer base.

Figure 1

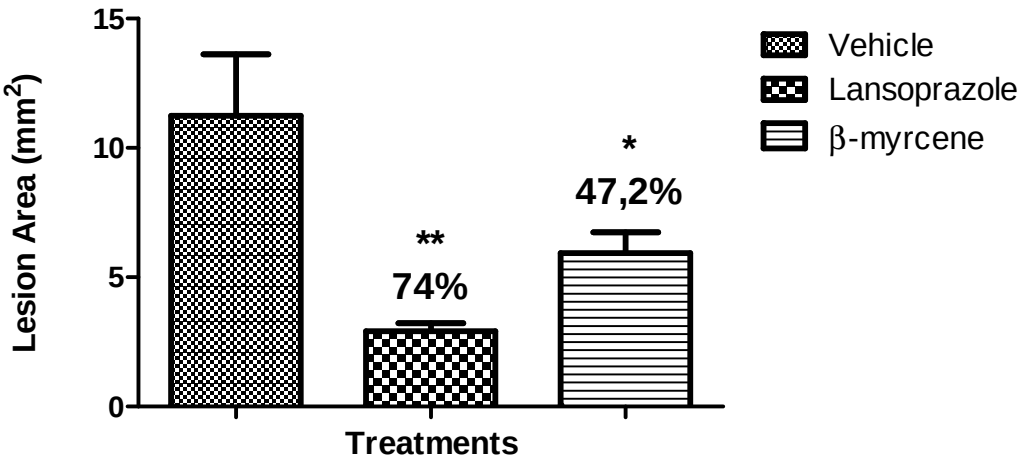

15
10
5
0
Lesion Area (mm2)
**
74%
*
47,2%
Treatments
Vehicle
Lansoprazole
β-myrcene

Figure 2

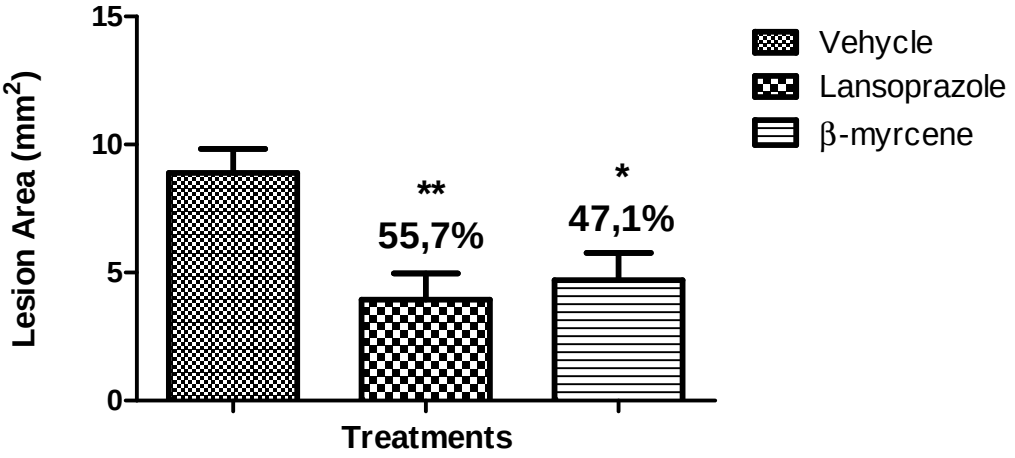
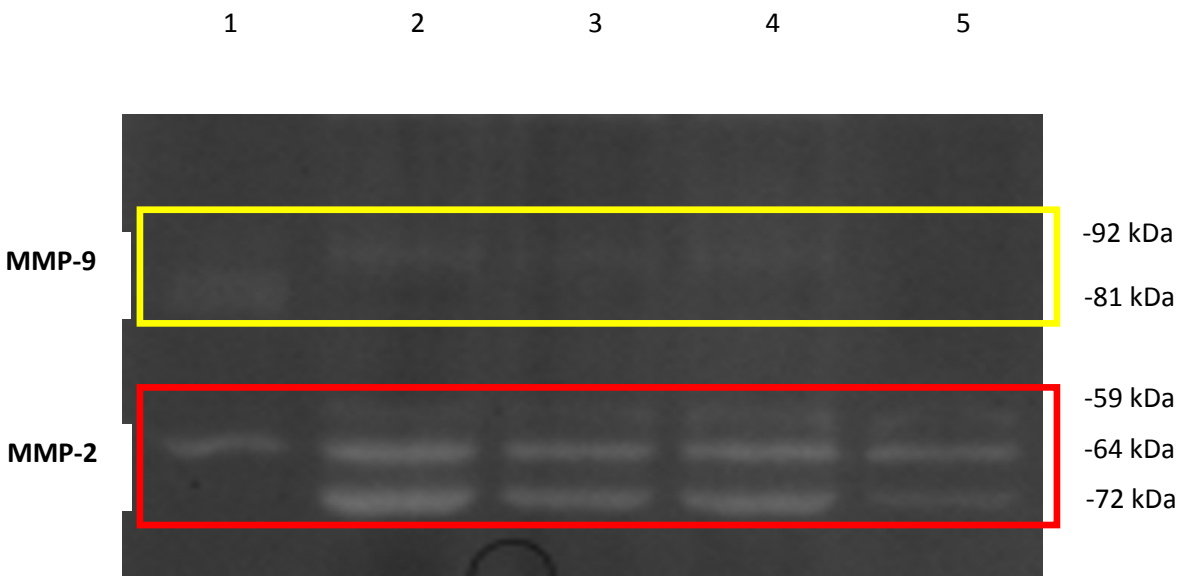
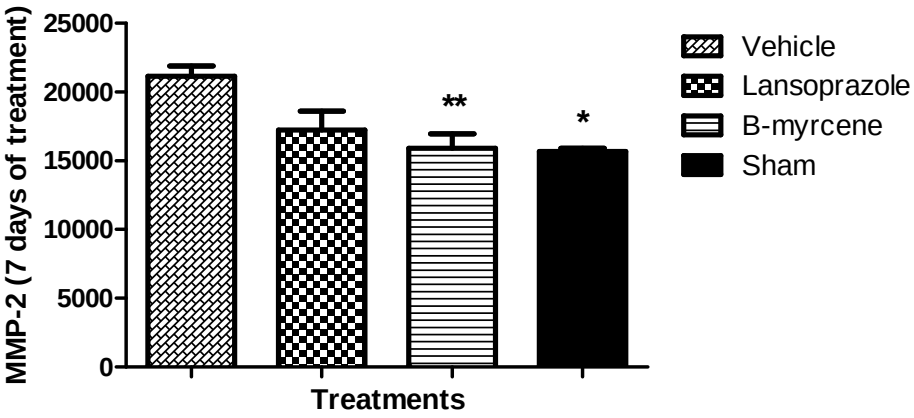

15
10
5
0
Lesion Area (mm2)
**
55,7%
*
47,1%
Treatments
Vehycle
Lansoprazole
β-myrcene

Table 2

Dosage	Treatments	Healing gastric – 7 days treatment	Healing gastric – 14 days treatment
MPO	Vehicle	3817±278.3	3451±338.5
	Lansoprazole	2567±237.3**	1966±336.7**
	β-myrcene	2940±227.0*	2385±231.4*
	Sham	1976±90.29***	1922±97.11**
TNF-α	Vehicle	0.8371± 0.07631	0.3565± 0.02701
	Lansoprazole	0.6248± 0.03696	0.2979± 0.02255
	β-myrcene	0.7623± 0.06938	0.2970± 0.02614
	Sham	0.5464± 008680*	0.1995±0.03193**
IL1-β	Vehicle	2.254± 0.06053	1.848± 0.1968
	Lansoprazole	2.106± 0.1520	1.277± 0.1030*
	β-myrcene	1.640± 0.1074*	1.300± 0.1425*
	Sham	1.576± 0.2179*	1.011± 0.04834**
IL-10	Vehicle	1.347± 0.2220	1.310± 0.2150
	Lansoprazole	1.520± 0.1647	1.019± 0.09236
	β-myrcene	2.404± 0.1937**	1.099± 0.1402
	Sham	2.405± 0.3044**	0.8386± 0.1301

Figure 3


1
2
3
4
5
MMP-9
MMP-2
-92 kDa
-81 kDa
-59 kDa
-64 kDa
-72 kDa


MMP-2 (7 days of treatment)
25000
20000
15000
10000
5000
0
**
*
Treatments
Vehicle
Lansoprazole
B-myrcene
Sham

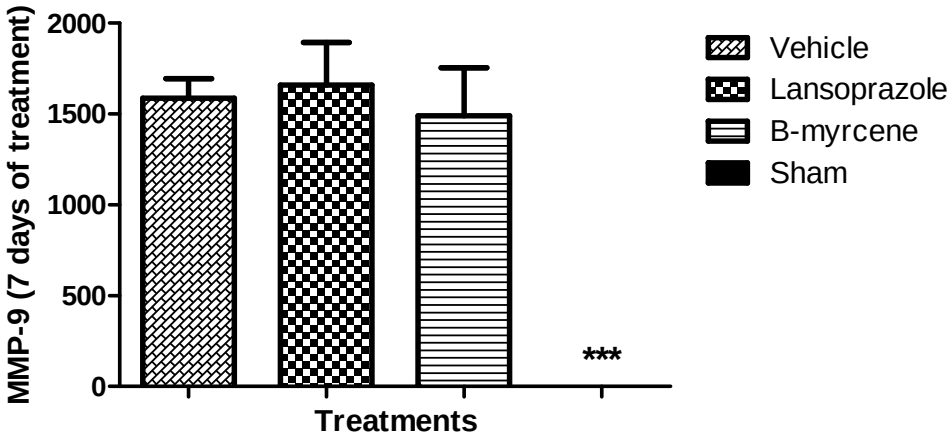
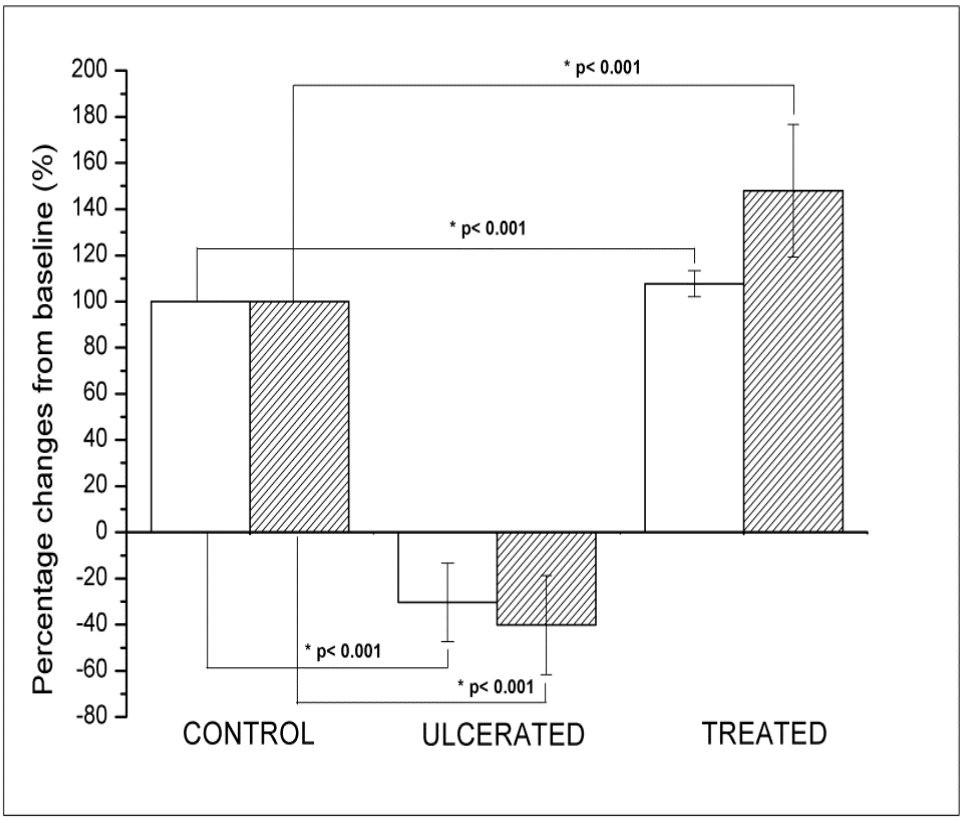

MMP-9 (7 days of treatment)
2000
1500
1000
500
0
***
Treatments
Vehicle
Lansoprazole
B-myrcene
Sham

Figure 4

A)

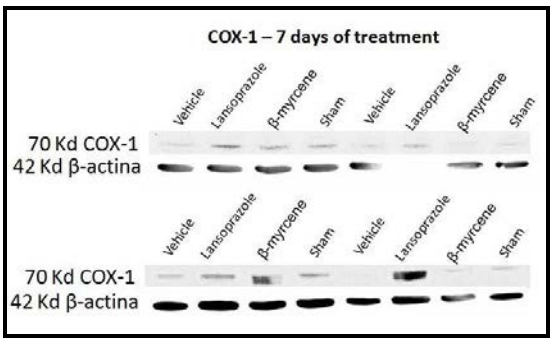


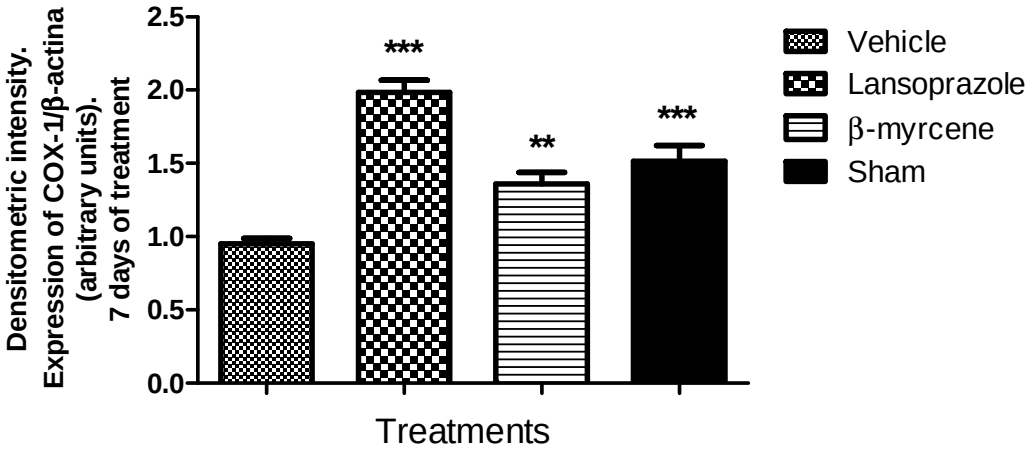
B)

	Control	Ulcerated	Treated with β-myrcene for 7 days
Body weight (g)	222.86±18.16	203.14±16.79**	225.29±12.50
ACB Frequency (cpm)	4.50±0.36	3.24±0.78*	4.74±0.48*
ACB Amplitude (V.s)	1.37±1.19	0.56±0.30****	1.46±0.98***

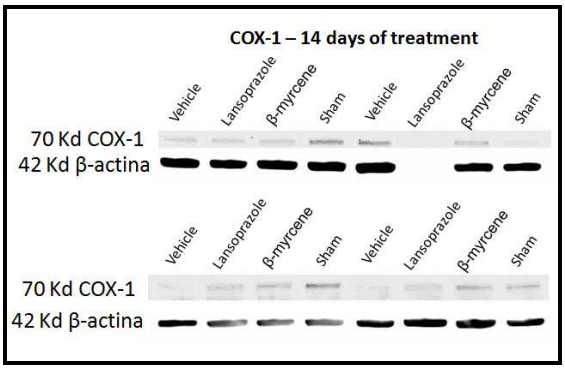
Figure 5

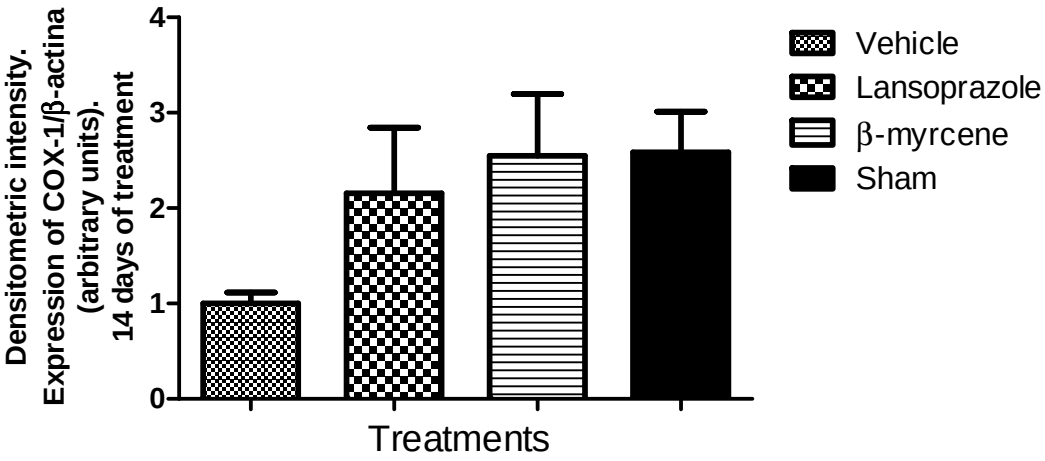
A)


COX-1 – 7 days of treatment
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
70 Kd COX-1
42 Kd β-actina
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
70 Kd COX-1
42 Kd β-actina

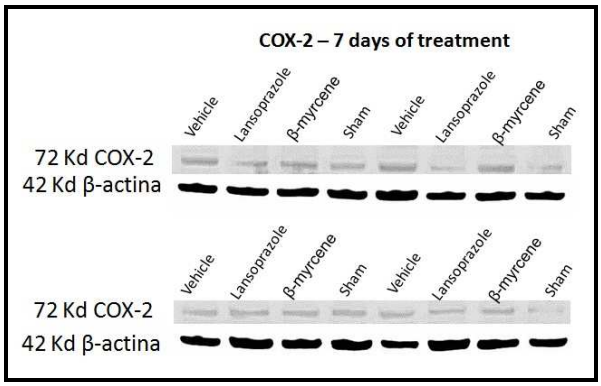

Densitometric intensity.
Expression of COX-1/β-actina
(arbitrary units).
7 days of treatment
2.5
2.0
1.5
1.0
0.5
0.0
***
**
***
Treatments
Vehicle
Lansoprazole
β-myrcene
Sham

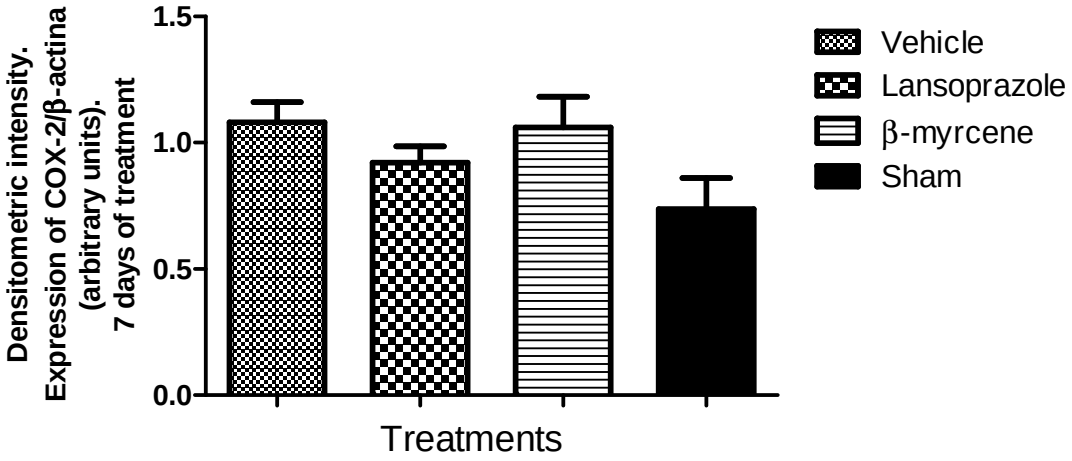
B)


COX-1 – 14 days of treatment
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
70 Kd COX-1
42 Kd β-actina
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
70 Kd COX-1
42 Kd β-actina

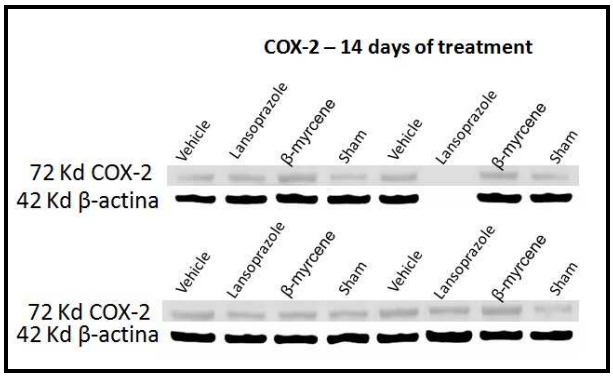

Densitometric intensity.
Expression of COX-1/β-actina
(arbitrary units).
14 days of treatment
4
3
2
1
0
Treatments
Vehicle
Lansoprazole
β-myrcene
Sham

C)


COX-2 – 7 days of treatment
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
72 Kd COX-2
42 Kd β-actina
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
72 Kd COX-2
42 Kd β-actina


Densitometric intensity.
Expression of COX-2/β-actina
(arbitrary units).
7 days of treatment
1.5
1.0
0.5
0.0
Treatments
Vehicle
Lansoprazole
β-myrcene
Sham

D)


COX-2 – 14 days of treatment
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
72 Kd COX-2
42 Kd β-actina
Vehicle
Lansoprazole
β-myrcene
Sham
Vehicle
Lansoprazole
β-myrcene
Sham
72 Kd COX-2
42 Kd β-actina

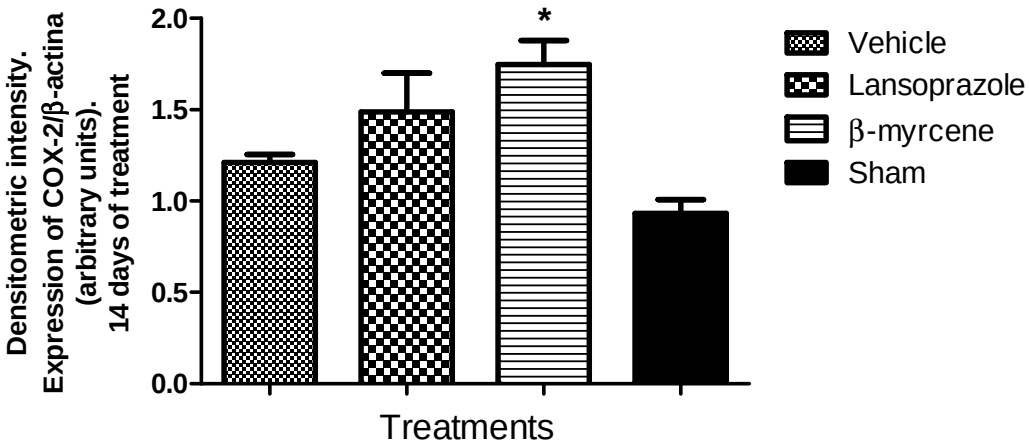
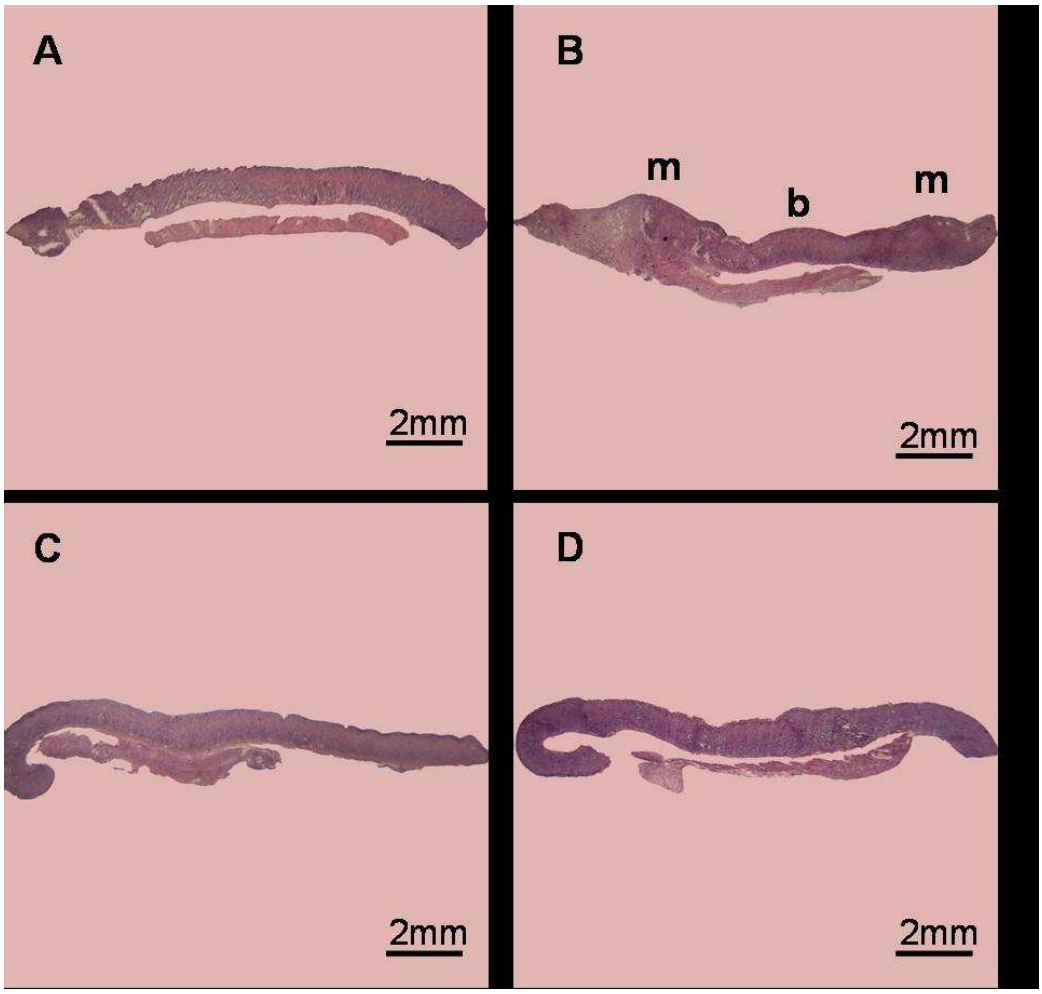
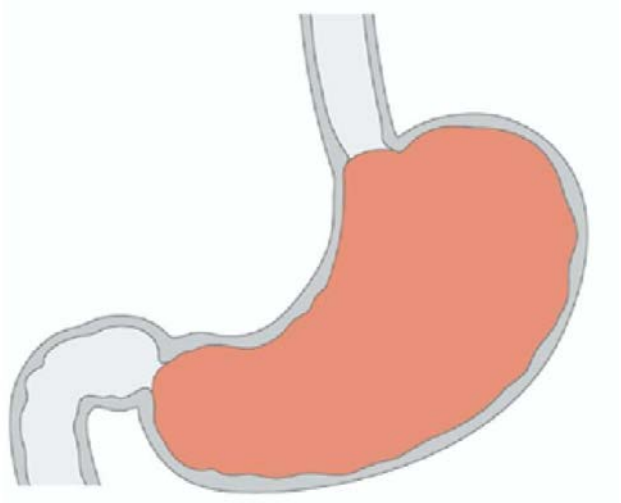

Densitometric intensity.
Expression of COX-2/β-actina
(arbitrary units).
14 days of treatment
2.0
1.5
1.0
0.5
0.0
*
Vehicle
Lansoprazole
β-myrcene
Sham
Treatments

Figure 6





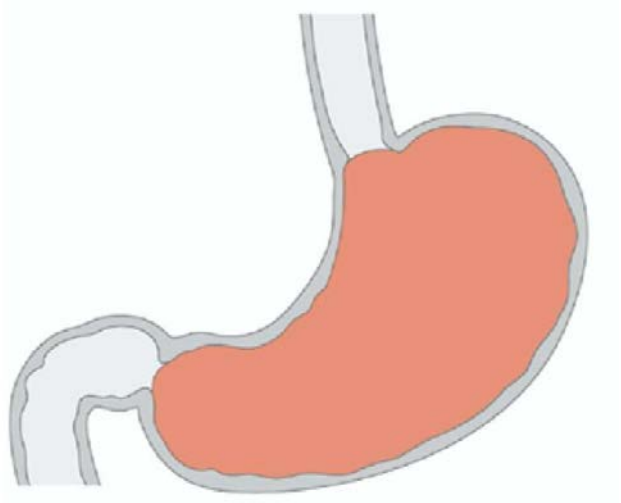
Conclusão Geral

CONCLUSÃO GERAL

À partir de todos estes dados levantados, podemos concluir que o β-mirceno é um interessante adjuvante gastroprotetor, pois ele é capaz de exercer ação cicatrizante frente ao ácido acético, um poderoso agente indutor de úlceras gástricas por meio da re-estabilização das contrações do estômago, reconstrução da mucosa gástrica conferida às metaloproteinases de matriz, envolvimento das enzimas COX-1 e COX-2, diminuição dos níveis de interleucinas pró-inflamatórias e de mieloperoxidase e por aumento dos níveis de interleucinas antiinflamatórias durante a cicatrização da úlcera gástrica induzida por ácido acético.

Aliado à isso o β-mirceno é capaz de diminuir a secreção de H^+ na mucosa gástrica, conferindo-lhe a importantíssima atividade antissecretória agindo principalmente pelo envolvimento dos canais de cálcio, abertura dos canais de K^+atp e promovendo a diminuição dos níveis de gastrina sérica, o qual é um importante hormônio secretagogo do estômago. Somado à isso, este monoterpeno apresenta atividade significativa contra o Refluxo Gastroesofágico induzida por Esofagite com importante queda dos níveis de peroxidação lipídica na mucosa esofágica.

Todos esses mecanismos fazem do β-mirceno um grande aliado na prevenção e cura de úlceras pépticas e também da doença do refluxo gastroesofágico.



Anexo I

Research Article

The effect of a minor constituent of essential oil from *Citrus aurantium*: the role of β-myrcene in preventing peptic ulcer disease

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ABSTRACT

The monoterpene β-myrcene has been widely used in cosmetics, food and beverages, and it is normally found in essential oil from citrus fruit. The aim of this study was to investigate the anti-ulcer effects of β-myrcene on experimental models of ulcers that are induced by ethanol, NSAIDs (non-steroidal anti-inflammatory drugs), stress, *Helicobacter pylori*, ischaemia-reperfusion injury (I/R) and cysteamine in order to compare with the essential oil of *Citrus aurantium* and its major compound limonene. The results indicate that the oral administration of β-myrcene at a dose of 7.50 mg/kg has important anti-ulcer activity with significantly decreased gastric and duodenal lesions as well as increased gastric mucus production. The results showed treatment with β-myrcene caused a significant increase in mucosal malondialdehyde level (MDA), an important index of oxidative tissue damage. The β-myrcene was also endowed with marked enhancement of antioxidant enzyme activity from GR system as evidenced by the decreased activity of superoxide dismutase (SOD) and increased levels of glutathione peroxidase (GPx), glutathione reductase (GR), and total glutathione in gastric tissue. Our results also shown that treatment with β-myrcene is not involved with thioredoxin reductase (TrxR) activity. Our results reveal, for the first time, the importance of β-myrcene as an inhibitor of gastric and duodenal ulcers and demonstrate that an increase in the levels of gastric mucosa defence factors is involved in the anti-ulcer activity of β-myrcene.

Keywords: β-myrcene; peptic ulcer; *Helicobacter pylori*; gastroprotection; antioxidant effects.

1. Introduction

Peptic ulcer disease (PUD) is an illness that affects many people worldwide. Despite advances in the understanding of PUD, its aetiology has not been completely elucidated. Factors that could increase the incidence of PUD in the global population include stress, high consumption of alcohol, smoking, *Helicobacter pylori* and ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) [1]. The use of NSAIDs, low-dose aspirin or dual anti-platelet therapy has become an important risk factor for recurrent ulcers and their associated complications [2]. The pathophysiology of PUD suggests that this disease is

the result of a disruption in the normal balance between aggressive factors (gastric acid, pepsin, and bile salts) and defensive aspects [prostaglandin (PG), mucosal blood flow, mucus, and bicarbonate] [3]. However, oxidative stress is an important factor underlying PUD, and reactive oxygen species (ROS) can cause intracellular damage. Studies have shown that ulcers are commonly associated with lipid peroxidation and oxidative damage in the mucosa because ROS may cause the oxidative damage of biological macromolecules such as DNA, protein and lipids. There is evidence that an adequate balance among antioxidant enzymes is important to minimise the damaging effect of ROS [4]. Endogenous antioxidant systems can inhibit oxidation by scavenging free radicals, decreasing the action of endogenous free radicals, or preventing lipid peroxidation. The field of antioxidant enzymes and free radicals is perceived to focus on the use of antioxidant supplements to prevent human diseases such as PUD [5].

β-myrcene (7-methyl-3-methylene-1,6-octadiene) is a pleasant-smelling monoterpene that is found in many plants, including lemongrass, hops and verbena [6], as well as in the composition of the essential oil that is obtained from fresh fruit peels of *Citrus aurantium* [7]. It is used in the production of perfumes, and it is also a constituent of essential oils that have long been used as flavouring additives in the manufacture of alcoholic beverages and in the food industry [8].

Moraes et al. [7] have previously described the anti-ulcer activity of limonene, which is a major compound (97.83 %) that is present in the essential oil of *C. aurantium*. They have also described the activity of the essential oil itself against gastric ulcers that were induced in young rats. The essential oil from *C. aurantium* at a dose of 250 mg/kg (p.o.) and limonene at 245 mg/kg had anti-ulcer activity against lesions that were induced by ethanol or NSAIDs in rats, and it did not interfere with gastric H^+ secretion, serum gastrin or the total glutathione level in the gastric mucosa. The gastric ulcer healing effects of the essential oil from *C. aurantium* was established in young and also middle-aged rats [9,10]. In addition to evidence of the anti-ulcer activity of the essential oil from *C. aurantium* and its major constituent limonene, the chromatographic analysis of samples from the essential oil indicated that β-myrcene was also present in the essential oil at a concentration of 1.43 % [7].

According to Wagner [11], only compounds that were present in high concentrations in a drug could be identified, and research focused on these main constituents with regard to chemical standardisation and pharmacological testing, even if it was not clear whether the compounds included the main therapeutically relevant active ingredients of the extract. Chen et al. [12] described the important contribution of minor constituents from *Rhizoma coptis* extract to cytotoxicity and anti-hyperglycaemic effects.

Aside from the widespread use of β-myrcene in the food industry, there are no reports about its effects on peptic ulcers. Therefore, the aim of the present study was to evaluate the anti-ulcer effects of β-myrcene in experimental models of peptic ulcers that were induced by different agents in rats and to evaluate the mechanisms of action underlying the effects of β-myrcene.

2. Material and Methods

2.1. Animals and evaluation of the lesions. Adult male Wistar rats (150 – 250 g) were supplied by the Central Animal House of the UNESP. The animals were fed a certified Nuvilab® (Nuvital) diet with free access to tap water under standard conditions of dark/light (12 h/12 h) and temperature (22 ± 1 °C). The animals were fasted before all experimental procedures because standard drugs and β-myrcene were administered orally (gavage). The animals were housed in cages with raised wide mesh floors to prevent coprophagy. All experiments were performed in the morning and followed the recommendations of the Canadian Council on Animal Care [13]. The employed protocol was approved by the Institutional Animal Care and Use Committee from UNESP (CEUA n° 18/05). After each experiment, the animals were sacrificed with CO_2 gas, their stomachs or duodenums were excised and cut along the greater curvature, gently rinsed under tap water, pressed onto a glass plate, and scanned so that the lesions could be counted with the aid of the AVSoft® program. The results were expressed as the total ulcerated area (mm^2). In addition to the animal groups that were treated with vehicle (negative control), anti-ulcer drugs (positive control), or β-myrcene. We also included a group called Sham, in which the animals were subjected to the same procedure as the other groups but without the use of agents that induced injury.

2.2. Drugs and treatment. Ketamine hydrochloride, xylazine hydrochloride, ethanol, lansoprazole, N-nitro-L-arginine methyl ester (L-NAME), indomethacin, carbenoxolone, N-ethylmaleimide (NEM), Alcian Blue, cysteamine hydrochloride, and cimetidine were purchased from Sigma Co (USA). β-myrcene was purchased from Acros Organics (USA) and dissolved in an 8 % Tween-80 aqueous solution that was administered orally (p.o.). The vehicle control group was administered an 8 % Tween-80 aqueous solution (10 mL/kg).

2.3. Anti-ulcer activity. *Ethanol-induced gastric ulcer in rats:* The method described by Robert et al. [14] was followed with slight modifications. Fasted rats (12 h) were randomly divided into five groups (n = 7) and given β-myrcene at doses of 3.75, 7.50 and 11.25 mg per kg body weight, 100 mg/kg carbenoxolone (positive control) or 8 % Tween-80 (vehicle). One hour later, all groups were treated orally with 1 mL absolute ethanol to induce gastric ulcers. After 1 h of induction, the animals were sacrificed and their stomachs were removed to evaluate the extent of the lesions, as previously described. Strips of the stomach (effective dose) were removed, weighed and stored for subsequent biochemical procedures. *Indomethacin-induced gastric ulcer in rats:* The procedure was performed according to the method described by Rainsford [15]. Rats were randomly divided into three groups (n = 7), and each group was given vehicle (8 % Tween-80), cimetidine (100 mg/kg) or β-myrcene (7.50 mg/kg). Thirty minutes later, all groups were orally given indomethacin (50 mg/kg) solubilised in sodium carbonate 0.5 % [16]. Six hours following the administration of the harmful agent (indomethacin), all animals were sacrificed, their stomachs were removed, and the gastric lesions were evaluated. *Stress-induced gastric ulcer in rats:* The method of Alder [17] was followed with slight modifications. The rats were randomly divided into 3 groups, and each group (n = 7) was treated orally with vehicle (8 % Tween-80), lansoprazole (30 mg/kg) or β-myrcene (7.50 mg/kg) for 14 consecutive days. On day 15, all groups were subjected to stress by forced swimming for 3 h in a glass cylinder (height 45 cm, diameter 25 cm) containing water at a height of 35 cm that was maintained at 23 ± 1 °C. Subsequently, the animals were sacrificed, and their stomachs were removed for evaluation of the gastric lesions, as previously described. *Gastric ulcer induced by ischaemia-reperfusion in rats:* The rats were divided into three groups (n = 7) and treated with vehicle, lansoprazole (30 mg/kg) or β-myrcene (7.50 mg/kg) 30 minutes before surgery (artery clamp). Then, the

animals were anaesthetised with ketamine hydrochloride and xylazine hydrochloride, and they remained sedated throughout the procedure [18]. An incision was made approximately 3 cm on the left side of the abdomen where the aorta and vena cava were first identified to facilitate the location of the coeliac artery. After identification, the coeliac artery was isolated and subjected to a process of cleaning and the removal of adhesions and any adipose tissue. A microvascular clamp was placed in the artery, and the artery remained clamped for 30 min. After this period of ischaemia, the clamp was removed (to allow reperfusion), and reperfusion was allowed to proceed for 60 min. At the end of this period, the animals were killed and their stomachs were removed for evaluation of the gastric lesions, as previously described. *Cysteamine-induced duodenal ulcer in rats:* The duodenal ulcers in rats were induced by oral administration of hydrochloric cysteamine. After fasting for 2 h, the rats were pre-treated with lansoprazole (30 mg/kg; positive control), vehicle (Tween 80® 8%; negative control), or β-myrcene (7.50 mg/kg) thirty minutes before the administration of cysteamine. Two doses of hydrochloric cysteamine (300 mg/kg in aqueous solution) were then given at an interval of 4 h (final dose of cysteamine: 600 mg/kg) [19]. Twelve hours after the onset of fasting, the animals were sacrificed, and their duodenums were removed and opened for the classification and analysis of the duodenal lesions.

2.4. Evaluation of mucosal protective factors. Determination of gastric mucus: Following the methodology described by Rafatullah et al. [20], after 12 h of fasting, rats were randomly divided into groups (n = 8) and treated with β-myrcene (7.50 mg/kg), carbenoxolone (200 mg/kg), or vehicle (8 % Tween 80) by oral administration. The treatment was performed 1 h before the ligature of the pylorus. Rats were anaesthetised and a longitudinal incision was made just below the xiphoid apophysis for the pylorus ligature. The animals were sacrificed after 4 h, and the glandular stomach was separated, weighed and immersed in Alcian Blue solution for the mucus quantification procedure. The absorbance was read with a spectrophotometer at 598 nm, and the results are expressed as mg Alcian blue/mL/g tissue. *Determination of prostaglandin E_2 levels:* To determine the effect of β-myrcene-treatment on the production of PGE_2, the method described by Curtis et al. [21] was used. Rats were randomly divided into groups (n = 6) and treated with vehicle, vehicle plus indomethacin

(30 mg/kg), β-myrcene (7.50 mg/kg) or β-myrcene plus indomethacin. After the procedures, the animals were sacrificed and their stomachs were removed. Scraped mucous membranes were punched and suspended in 1 mL phosphate buffer (10 mM, pH 7.4). The resulting solution was incubated at 37 °C for 20 min. The concentration of PGE_2 in the buffer was measured using an immunoenzymatic dosage kit (Amersham-RPN222), and the absorbance at 480 nm was read in an ELISA Biosystem reader. *Determination of the role of nitric oxide (NO) and sulfhydryl groups (SH) in gastric protection:* The procedure was performed according to the method described by Robert et al. [14] and Matsuda et al. [22]. Gastric mucosal lesions were induced in rats that were fasted for 12 h. The rats were randomly divided into nine groups (n = 6): three were pre-treated with saline (i.p.), three with 70 mg/kg NO synthase inhibitor (L-NAME), and three with 10 mg/kg SH depletor (NEM). The oral treatments were administered thirty minutes after pre-treatment. One group from each of the pre-treatment groups received saline, carbenoxolone 100 mg/kg or β-myrcene 7.50 mg/kg. Sixty minutes later, 1 mL absolute ethanol was given to each rat; all animals were sacrificed 1 h later. Their stomachs were removed to determine the gastric lesion area. *Biochemical determination of total glutathione content (GSH), glutathione peroxidase (GPx), glutathione reductase (Gr), superoxide dismutase (SOD), malondialdehyde (MDA), myeloperoxidase (MPO) and thioredoxin reductase (TrxR)*: Samples of duodenum or gastric tissue were removed from the rats that underwent different treatments and were subjected to different ulcerogenic agents; the samples were weighed and stored for subsequent biochemical analysis. MDA was measured according to the method described by Ohkawa et al. [23] and the results were expressed as nanomoles per gram (nmol/g) of wet tissue.The total glutathione content (GSH) was quantified using the recycling assay described by Anderson [24]. Samples were thawed, minced, diluted 1:20 (w/v) in ice-cold 5 % (w/v) trichloroacetic acid and homogenised. The homogenates were centrifuged at 7,000 × *g* for 15 min at 4 °C, and the supernatants were used to quantify GSH levels. The results are expressed as nanomoles per gram (nmol/g) of wet tissue. For the determination of glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD), the following methods were used: Yoshikawa et al. [25], Carlberg and Mannervik [26] and Winterbourn et al. [27], respectively. Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al. [28].

Samples were suspended in 1 mL 50 mM phosphate buffer containing 0.5 % hexadecyltrimethyl-ammonium bromide (pH 6.0) and minced with scissors for 15 s on an ice-cold plate. The resultant suspension was diluted to a final ratio of 1:20 (w/v), homogenised for 1 min with an automatic Heidolph homogeniser, then sonicated for 10 s and subjected to three freeze–thaw cycles. The homogenates were then centrifuged at 7,000 × *g* at 4 °C for 10 min, and the supernatants were assayed for MPO activity. The results are expressed as MPO units per gram (U/g) of wet tissue. TrxR was determine by method colorimetric assay kit for quantifying mammalian TrxR activity from gastric tissue homogenate as described by manufacturer (Cayman Chemical, U.S.A).

2.5. Determination of antibacterial effect. β-myrcene was tested for anti-*Helicobacter pylori* activity [29]. The strain of *H. pylori* (ATCC 43504) that was used was isolated from patients with duodenal ulcer disease. Frozen *Helicobacter pylori* isolate was thawed and grown on 5 % sheep's blood agar plates for 3 – 4 days at 37 °C in 10 % CO_2 and 98 % humidity. Each plate was swabbed with a sterile cotton-tipped applicator, and the cells were suspended in sterile saline to obtain a turbidity equivalent to a 2.0 McFarland standard. Muller-Hinton broth containing 10 % horse serum was added to all wells of a 96-well microtiter plate (Corning-USA). Each well was incubated with *H. pylori* at a final concentration of ~1 x 10^5 CFU/mL. The plates were incubated for 5 days in a microaerobic atmosphere at 37 °C. Following incubation, the plates were examined visually and spectrophotometrically, and the lowest concentration showing complete inhibition of growth was recorded as the MIC (Minimum Inhibitory Concentration).

2.6. Statistical analysis. The results are expressed as the mean ± S.E.M. Statistical significance was determined by one-way analysis of variance followed by Dunnett's or Tukey's test. *P*-values < 0.05 were considered to be statistically significant.

3. Results

To establish a general profile for the gastroprotective activity of β-myrcene, we tested three doses (3.75, 7.50 and 11.25 mg/kg body weight) to select the one that produced the best gastroprotective effect. These

doses of β-myrcene were selected based on the effective anti-ulcer dose of essential oil from *C. aurantium* (250 mg/kg) and the percentage of β-myrcene in this composition (1.43 %) [7]. Using the ethanol-induced ulcer model, the pre-treatment of rats with β-myrcene led to significant inhibition ($P < 0.001$) of gastric lesions at doses of 7.50 mg/kg (49.57 ± 5.54 mm^2) by 60 % when compared to the vehicle-treated control group (125.0 ± 2.30 mm^2); thus, the subsequent experiments were developed using only this dose of β-myrcene (Figure 1).

Figure 2 shows the gastroprotective effect of the monoterpene β-myrcene in the model of gastric ulcer that was induced by absolute ethanol in rats. It also shows the biochemical parameters of gastric mucosal tissue, total glutathione (GSH) and myeloperoxidase (MPO). The quantification of GSH level in the gastric tissue of rats that were treated with β-myrcene shows that the levels were increased in the presence of β-myrcene ($P < 0.01$) compared to GSH levels that were found in either the negative control group (vehicle) or in the positive control group ($P > 0.05$). The quantification of MPO levels in gastric mucosa shows that β-myrcene decreased the levels of MPO ($P < 0.01$). In animals with gastric ulcer treated with vehicle, MDA levels in gastric mucosa accounted for 4.11 ± 0.15 nmol/g tissue. Pre-treatment with β-myrcene significantly decreased ($P < 0.01$) the tissue accumulation of MDA promoted by ethanol (Table 1). We observed that administration of ethanol in animals that were pre-treated with β-myrcene increased the activity of GR ($P < 0.001$) and GPx ($P < 0.01$) and decreased the activity of SOD ($P < 0.01$) when compared with vehicle (Table 1). We also observed that administration of β-myrcene was not able to change the TrxR activity by administration of ethanol in rats. Only sham group (without lesion) was able to increase the TrxR activity.

β-myrcene also inhibited the formation of indomethacin-induced gastric lesions by 74 % compared to the vehicle-treated control group (Figure 3). The standard anti-ulcer drug, cimetidine (100 mg/kg), protected the stomach against indomethacin-induced damage by 99 % compared to the vehicle-treated group. The quantification of GSH and MPO levels indicated that only treatment with β-myrcene was able to induce a gastroprotective effect together with a decrease in the levels of MPO in the gastric mucosa ($P < 0.01$).

Figure 4 shows that β-myrcene was able to maintain basal levels of PGE_2 that were similar to those in the sham and vehicle-treated groups in pylorus ligature rats (gastric mucus model). However, when administered jointly with indomethacin, β-myrcene was not able to maintain the PGE_2 levels. Figure 5 shows the % inhibition of gastric lesions in animals that were treated with the monoterpene β-myrcene against gastric lesions that were induced by forced swim stress. This result clearly shows the gastroprotective effect of β-myrcene at a dose of 7.50 mg/kg that promoted a protection of 52.3 % ($P < 0.05$) compared to the vehicle-treated group. This gastroprotective effect of β-myrcene was also compared to the standard anti-ulcer drug lansoprazole at a dose of 30 mg/kg (4-fold higher than that of β-myrcene). Lansoprazole induced a 78 % inhibition in gastric lesions compared to vehicle. Treatment with β-myrcene increased GSH levels ($P < 0.01$) and decreased MPO activity ($P < 0.01$) in the gastric mucosa of rats when compared with the vehicle-treated control group.

In the model of gastric ulcer induced by ischaemia-reperfusion (I/R), β-myrcene caused a significant decrease in the ulcerative lesions, with gastric protection of 86 % compared to the vehicle-treated control group (Figure 6, $P < 0.001$). In the gastric tissue from animals that were subjected to the ischaemia-reperfusion model, the levels of GSH were significantly increased and those of MPO were significantly decreased ($P < 0.01$) following treatment with β-myrcene (7.50 mg/kg) compared to the vehicle-treated control group.

We observed that pre-treatment with L-NAME significantly reversed the gastroprotection that was exhibited by β-myrcene (Table 2). This result demonstrated that the nitric oxide (NO) pathway is important in the mechanism of action of gastroprotection by this monoterpene. Our results also show that pre-treatment with β-myrcene inhibited the cysteamine-induced duodenal lesion by 80 % compared to the control group (Figure 7). This protective effect of β-myrcene was accompanied by significant increases in GSH ($P < 0.01$), demonstrating the powerful antioxidant activity of this monoterpene in duodenal ulcers. We also observed a decrease in the MPO level ($P < 0.01$) upon treatment with β-myrcene compared to the vehicle-treated control group.

We quantified the adhered gastric mucus in pylorus ligature rats that were treated with β-myrcene. Table 1 shows gastric mucus production following different treatments compared to a negative control group. Treatment with β-myrcene caused a 50 % increase in the amount of adhered gastric mucus compared to the vehicle-treated group ($P < 0.05$); animals that were treated with carbenoxolone (positive control group) showed a 97 % increase in adhered gastric mucus. Administration of the inhibitor of endogenous SH compound formation (NEM) caused a significant increase in ethanol-induced gastric lesions (Table 2). Serial dilutions of β-myrcene showed that the minimum inhibitory concentration (MIC) of β-myrcene required to block the growth of *H. pylori in vitro* was 500 μg/mL.

4. Discussion

Alcohol has an important role in gastrointestinal diseases because it induces gastric mucosal injury by disrupting the barrier function of the mucus, the main protective barrier against gastric acid and pepsin [30]. High levels of ethanol in the gastric mucosa lead to increased epithelial permeability, changes in the potential difference of cells caused by the re-diffusion of H^+ ions through the injured mucosa, and damage to the mucosa due to vascular disorders and decreased blood flow [31]. Ethanol can also induce direct oxidative damage in gastric mucosal tissues by increasing superoxide anion and hydroxyl radical production and lipid peroxidation in the gastric mucosa [32]. Our results showed that β-myrcene at a dose of 7.50 mg/kg inhibited ethanol-induced gastric lesions in animals and maintained the biochemical parameters of gastric mucosal tissue, such as GSH and MPO levels, at normal levels that were similar to those of the sham group (without lesion gastric). GSH is an endogenous antioxidant, and its properties are related to the presence of the thiol group in its structure. Glutathione reacts with peroxides and toxic oxygen radicals to protect cells from damage. The quantification of GSH levels in the gastric tissue of rats that were treated with β-myrcene shows that the levels of total glutathione were increased in the presence of β-myrcene. This result clearly demonstrates that β-myrcene protected the gastric mucosa against damage caused by ethanol at a dose that was 13 times lower than the anti-ulcer drug carbenoxolone. The same results were also observed in the gastric mucosa of rats that were treated with limonene or the

essential oil from *C. aurantium* [7], but the difference between both results was the highest dose of the compounds (245 and 250 mg/kg, respectively) in relation to achieve by β-myrcene at dose of 7.50 mg/kg to achieve the same gastroprotective effects. Another important indicator of the quality of protection of the gastric mucosa by new drugs is the presence of the enzyme MPO in gastric tissue; MPO is a marker of infiltration/aggregation by neutrophils and its presence is increased in ulcerogenic lesions [33]. The quantification of MPO levels in gastric mucosa shows that treatment with β-myrcene decreased the levels of MPO (an inflammatory marker) in gastric tissue. These effects of β-myrcene strongly contribute to maintaining the integrity of the gastric mucosa.

Gastrointestinal damage induced by NSAIDs is one of the most frequently observed adverse side effects because these drugs exert therapeutic and toxic effects mainly through inhibiting COX and decreasing the levels of PGE_2 in the gastric mucosa. Consequently, this decreases mucus and bicarbonate secretion, reduces mucosal blood flow, causes vascular injury, induces leukocyte accumulation and reduces cell turnover [34]. In the present study, β-myrcene inhibited the formation of indomethacin-induced gastric lesions. MPO activity, which is a marker of granulocyte infiltration at the site of ulceration, indicated that there was an immediate accumulation of inflammatory cells in the ulcerated untreated rats. This result indicated that β-myrcene, better than cimetidine (histamine H2-receptor antagonist) protected the gastric mucosa against damage caused by indomethacin with a significant decrease in the levels of MPO in the gastric mucosa.

PGE_2 plays an important role in protecting the mucosa by stimulating the secretion of mucus and bicarbonate, maintaining mucosa blood flow and increasing the resistance of epithelial cells to damage by cytotoxins [35].In contrast to results obtained using limonene or the essential oil from *Citrus aurantium* [7] our results does not support the involvement of PGE_2 in the gastroprotective action of β-myrcene.

In addition to ethanol and NSAIDs, stress plays an important role in ulcerogenesis. The major factors that are involved in the development of stress-induced ulcers include increases in gastric acid secretion, pepsin activity and ROS as well as decreases in mucosal protection, mucus secretion, mucosal blood flow and NO [36]. Experimental models of stress-induced gastric ulcers, such as the forced swimming test and the water

immersion model, induce similar conditions of stress in human [37]. Our results clearly demonstrate the gastroprotective effect of β-myrcene when compared to the vehicle-treated group and β-myrcene also increased GSH levels and decreased MPO activity in the gastric mucosa.

Gastric stress ulcers are commonly associated with hypoxia, which reduces mucosal blood flow, and ischaemia has deleterious effects on the gastric mucosa [38]. The restoration of blood flow (reperfusion) after a period of ischaemia initiates a cascade of changes, including the release of local ROS and an increase in the adhesion of neutrophils to endothelial cells, which causes damage to the integrity of the mucosal lining; this phenomenon is known as ischaemia-reperfusion (I/R) injury [39]. β-myrcene caused a significant decrease in the I/R-induced ulcerative lesions. The levels of GSH were increased and those of MPO were decreased when compared to the vehicle-treated control group. The results that were obtained with β-myrcene in ischaemia-reperfusion (I/R) injury and the stress-induced ulcer model are evidence of the strong antioxidant effect of β-myrcene.

In addition to GSH, NO plays an important role in gastric mucosal defence through many actions, including the maintenance of mucosal blood flow. According to Wallace and Miller [40], NO is also an important regulator of mucus secretion in the stomach, and its effects are produced by the stimulation of guanylate cyclase in epithelial cells. Based on the positive results obtained in I/R model, we further evaluated the involvement of NO as an important factor in mediating the protection by β-myrcene using the NO inhibitor (L-NAME). We observed that pre-treatment with L-NAME significantly reversed the gastroprotection that was exhibited by β-myrcene. This result demonstrated that the NO pathway is important in the mechanism of action of β-myrcene highlight another different mechanism of action between essential oil and β-myrcene from *C. aurantium*.

Based on functional and morphological criteria, the animal models of duodenal ulceration that are induced by cysteamine are similar to human duodenal ulcer disease [41]. Cysteamine is a potent inducer of duodenal ulcers, and its cytotoxic effect partly depends on the generation of H_20_2 and ROS. This process increases the levels of the vasoconstrictor endothelin-1, which decreases the duodenal mucosal flow and causes tissue ischaemia and hypoxia [42]. Our results show that pre-treatment with β-myrcene inhibited the

cysteamine-induced duodenal lesion. This protective action of β-myrcene was accompanied by significant increases in GSH and a decrease in the MPO level. Because the origin of ulcers is directly related to the reduction of the mucus barrier, we propose that the gastroprotective action promoted by β-myrcene could be related to its ability to increase the amount of gastric mucus. Treatment with β-myrcene caused an increase in the amount of adhered gastric mucus compared to the vehicle-treated group. These results corroborate the hypothesis that one of the factors involved in gastric protection by β-myrcene is an increase in the production of continuous adherent mucus layer acts that acts as a barrier against luminal pepsin, thereby protecting the adjacent mucosa from proteolytic digestion [43].

In addition to the contribution of mucus to strengthen the gastric barrier, the maintenance of the integrity of the gastric mucosa depends on the formation of SH compounds that strengthen disulfide bonds and reduce oxygen free radical derivatives, thus protecting the cell [44]. Similar to what has been observed with limonene and essential oil from *C. aurantium*, the significant increase in lesions in animals that are pre-treated with the blocker of SH groups and treated with β-myrcene highlights the importance of SH compound formation in gastroprotection by this monoterpene.

Based on our results, β-myrcene protected the gastric and duodenal mucosa against several ulcerogenic agents, such as ethanol, stress, ischaemia-reperfusion and cysteamine. All of these damaging factors promote oxidative stress by increasing the formation of ROS and inducing the depletion of oxidative defences in the cell. MDA represents an end product of the peroxidation of polyunsaturated fatty acids and related esters within cell membranes and it is currently regarded as a reliable index of oxidative tissue damage [18].

The administration of ethanol was able to promote reduction of peroxidation lipidic (MDA) in ulcerated gastric tissue and increased the activities of GR and GPx and decreased the activity of SOD in animals that were treated with β-myrcene. On the other hand, Mei et al. [45] demonstrated that increased levels of GSH and preventative antioxidants, such as SOD and GPx, were an important first line of defence against the destructive action of oxidative damage. The administration of ethanol was able to promote reduction of peroxidation lipidic (MDA) in ulcerated gastric tissue and increased the activities of GR and GPx and

decreased the activity of SOD in animals that were treated with β-myrcene. Several key systems are involved with regulation of redox equilibrium proteins such as GR system (GSH, GPX and GR) and Trx/TrxR system (thioredoxin and thioredoxin reductase). The importance of Trx/TrxR system involves many aspects of cell function, particularly with respect to cell growth and protection against oxidant damage and apoptosis [46]. Our results show that treatment with β-myrcene maintains TrxR activity at low levels indicating the absence of the involvement of this system.

In the present study, the results indicated that β-myrcene maintained the baseline enzyme activity levels in the ethanol-induced gastric ulcer model. Treatment with β-myrcene attenuated the increased lipid peroxidative damage by decreasing MDA levels and increasing GSH levels, and also prevented the depletion of GSH, GR and GPx. These results are in agreement with obtained by Ciftci et al. [47] that observed antioxidant effects of β-myrcene but at highest dose (200 mg/kg) against in rats liver. Or results add new data to consolidate the action of β-myrcene as prevent the oxidative damage.

Therapies for eradicating the bacterium *H. pylori* have revolutionised the natural course of the development of gastric ulcers. Treatments against such infections have been relatively successful in eradicating infection in approximately 80 % of patients. Numerous studies suggest that the annual relapse rate – 80 % for duodenal ulcers and 60 % for gastric ulcers – is reduced to less than 5 % after treatment of the bacterium [48]. Highly significant results were obtained with this monoterpene. Our results using serial dilutions of β-myrcene showed that β-myrcene blocks the growth of *H. pylori in vitro* by minimum inhibitory concentration of 500 μg/mL. Therefore, *in vitro* evaluation of the antibiotic activity of the monoterpene β-myrcene against the bacterium *H. pylori* revealed another therapeutically important mechanism of action of β-myrcene.

5. Conclusion

This study highlights important biological features of terpenes (limonene and β-myrcene) that are present in the essential oil of *C. aurantium*. While the gastroprotective activity of limonene is related to the action of PGE_2 and gastric mucus as protective factors, this study presents protective activities of β-myrcene in

gastric and duodenal mucosa that involve the activation of important antioxidants, such as GSH, NO, SH, GPX and GR, that together, exert a substantial anti-ulcer activity. Thus, the monoterpene β-myrcene presents considerable gastroprotective activity and important antioxidant effects, which makes it relevant in the activity of the essential oil of *Citrus aurantium*, although it is a minor component of their composition. The hypothesis is that these monoterpenes (β-myrcene and limonene) have similar structures, and small differences in the structures of these monoterpenes may cause differences in their biological activity, as we have seen in this work and the study from Moraes et al. [7]. This anti-ulcer activity and the effective antioxidant potential of the monoterpene β-myrcene have been previously unpublished. Furthermore, β-myrcene could be used in combination with other nutraceuticals for the effective management of oxidative stress-induced disease conditions.

Abbreviations

NSAIDs - Non-Steroidal Anti-inflammatory Drugs

I/R - Ischaemia-Reperfusion Injury

GPx - Glutathione Peroxidase

SOD - Superoxide Dismutase

GR - Glutathione Reductase

PGE_2 - Prostaglandin E2

ROS - Reactive Oxygen Species

NEM - N-ethylmaleimide

L-NAME - N-nitro-L-arginine methyl ester

NO –Nitric Oxide

SH - Sulfhydryl Groups

MPO – Myeloperoxidase

GSH - Total Glutathione Content

TrxR - Thioredoxin reductase

Conflict of Interests

All authors declare that they have no conflict of interests.

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Figures and tables captions

Figure 1: Evaluation of different doses of monoterpene β-myrcene front the model of gastric ulcer induced by absolute ethanol in rats. Data are expressed as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test, *** $P < 0.001$ compared to vehicle (Tween 80® 8%).

Figure 2: Gastroprotective effect of the monoterpene β-myrcene in the model of gastric ulcer induced by absolute ethanol in rats. Columns represent the gastric lesion area from different groups after oral administration of ethanol (exception for Sham group), continuous traces represent GSH (glutathione) and discontinuous traces MPO (myeloperoxidase) levels. Data are expressed as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test * $P < 0.05$ and ** $P < 0.01$ (gastric lesion) $^{++}$ $P < 0.01$ (GSH) and $^{\#\#}$ $P < 0.01$ (MPO) when compared to vehicle.

Figure 3: Gastroprotective effect of the monoterpene β-myrcene in the model of NSAID-induced gastric ulcer in rats. Columns represent the gastric lesion area from different groups after oral administration of NSAID (exception for Sham group), continuous traces represent GSH (glutathione) and discontinuous traces MPO (myeloperoxidase) levels. Data are expressed as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test * $P < 0.05$ and ** $P < 0.01$ (gastric lesion) $^{++}$ $P < 0.01$ (MPO) when compared to vehicle.

Figure 4: Determination of levels of Prostaglandin E2 (PGE_2) in gastric mucosa. Data are expressed as the mean ± S.E.M. ANOVA: PGE_2 level is expressed in ng/µg protein. The different letters represent the significant differences. Tukey's test $P < 0.05$.

Figure 5: Gastroprotective effect of the monoterpene β-myrcene in the model of stress-induced gastric ulcer (forced swimming) in rats. Columns represent the gastric lesion area from different groups after stress (exception for Sham group), continuous traces represent GSH (glutathione) and discontinuous traces MPO

(myeloperoxidase) levels. Data are expressed as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test * $P < 0.05$ and ** $P < 0.01$ (gastric lesion), $^{++}$ $P < 0.01$ (GSH), $^{\#\#}$ $P < 0.01$ (MPO) when compared to vehicle.

Figure 6: Gastroprotective effect of the monoterpene β-myrcene in the model of gastric ulcer induced by ischaemia-reperfusion (I/R) in rats. Columns represent the gastric lesion area from different groups after I/R (exception for Sham group), continuous traces represent GSH (glutathione) and discontinuous traces MPO (myeloperoxidase) levels. Data are expressed as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test * $P < 0.05$ and ** $P < 0.01$ (gastric lesion), $^{++}$ $P < 0.01$ (GSH), $^{\#\#}$ $P < 0.01$ (MPO) when compared to vehicle.

Figure 7: Protective effect of the monoterpene β-myrcene in the model of duodenal ulcer induced by cysteamine in rats. Data are expressed as the mean ± S.E.M. Columns represent the duodenal lesion area from different groups administration of cysteamine (exception for Sham group), continuous traces represent GSH (glutathione) and discontinuous traces MPO (myeloperoxidase) levels. Data are expressed as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test ** $P < 0.01$ (gastric lesion), $^{+}$ $P < 0.05$ and $^{++}$ $P < 0.01$ (GSH), $^{\#\#}$ $P < 0.01$ (MPO) when compared to vehicle.

Table 1: Adhered gastric mucus, antioxidant enzymes and lipid peroxidation in rat stomach tissue administered with carbenoxolone and β-myrcene.

Table 2: Determining the role of the sulfhydryl group (SH) and nitric oxide (NO) in the gastric mucosa of the ethanol-induced model of gastric ulceration.

FIGURES

Figure 1

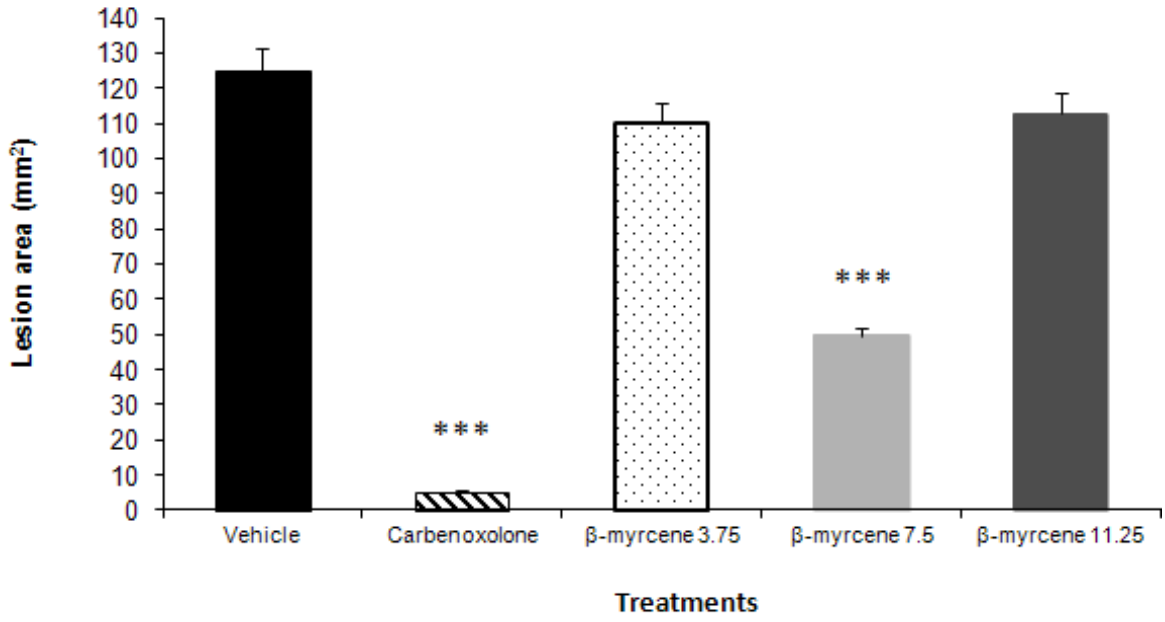

Figure 2

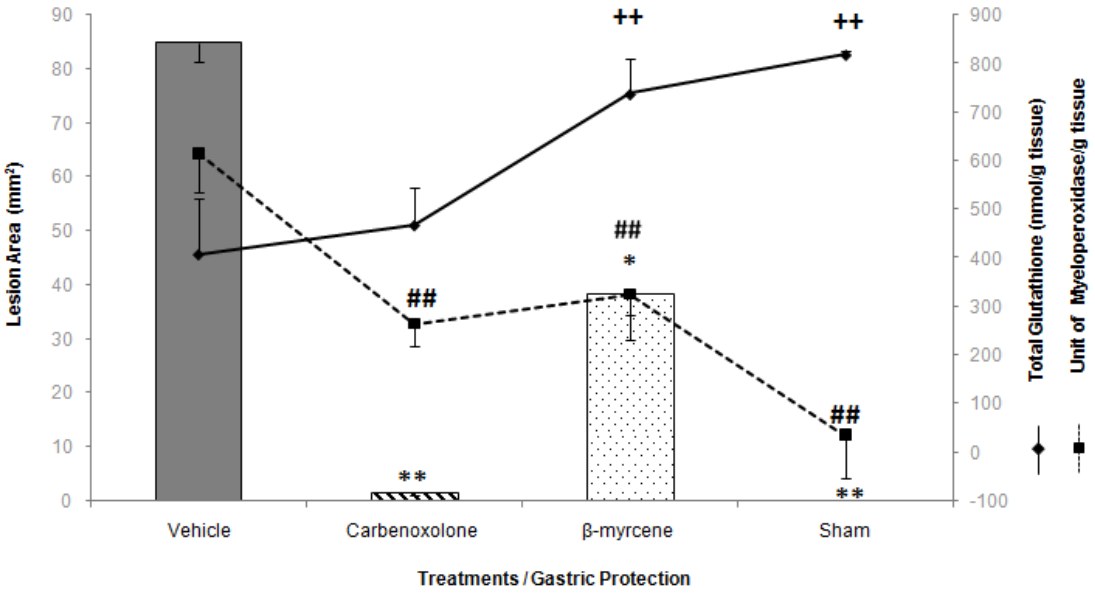
Lesion Area (mm²)
Total Glutathione (nmol/g tissue)
Unit of Myeloperoxidase/g tissue
Vehicle
Carbenoxolone
β-myrcene
Sham
Treatments / Gastric Protection
++
++
##
*
##
##
**
**

Figure 3

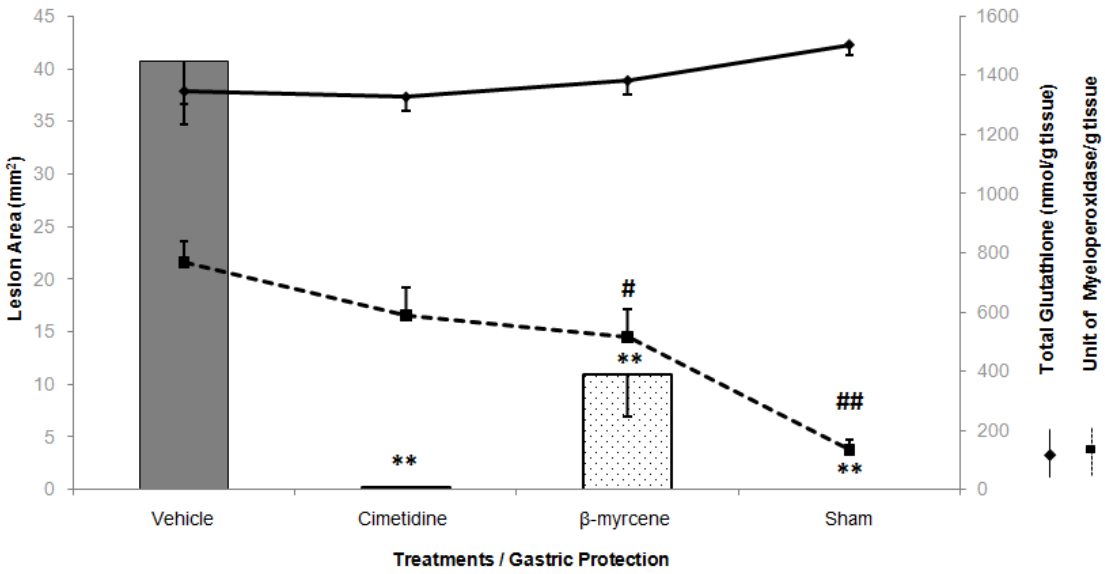
45
40
35
30
25
20
15
10
5
0
Lesion Area (mm²)
1600
1400
1200
1000
800
600
400
200
0
Total Glutathione (nmol/g tissue)
Unit of Myeloperoxidase/g tissue
#
**
**
**
##
Vehicle
Cimetidine
β-myrcene
Sham
Treatments / Gastric Protection

Figure 4

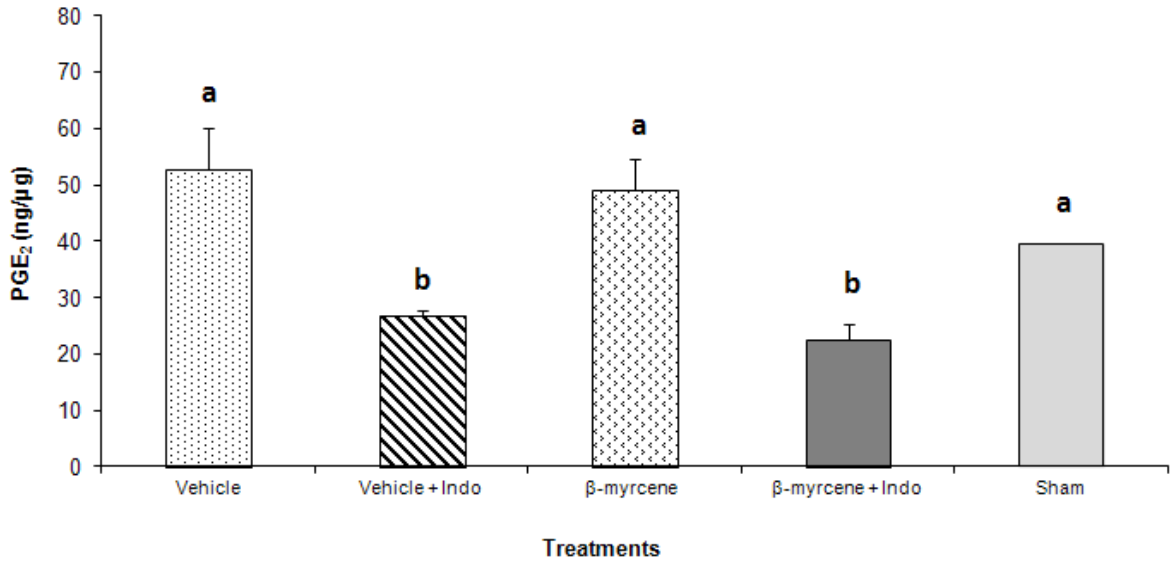
80
70
60
50
40
30
20
10
0
PGE2 (ng/µg)
a
b
a
b
a
Vehicle
Vehicle + Indo
β-myrcene
β-myrcene + Indo
Sham
Treatments

Figure 5

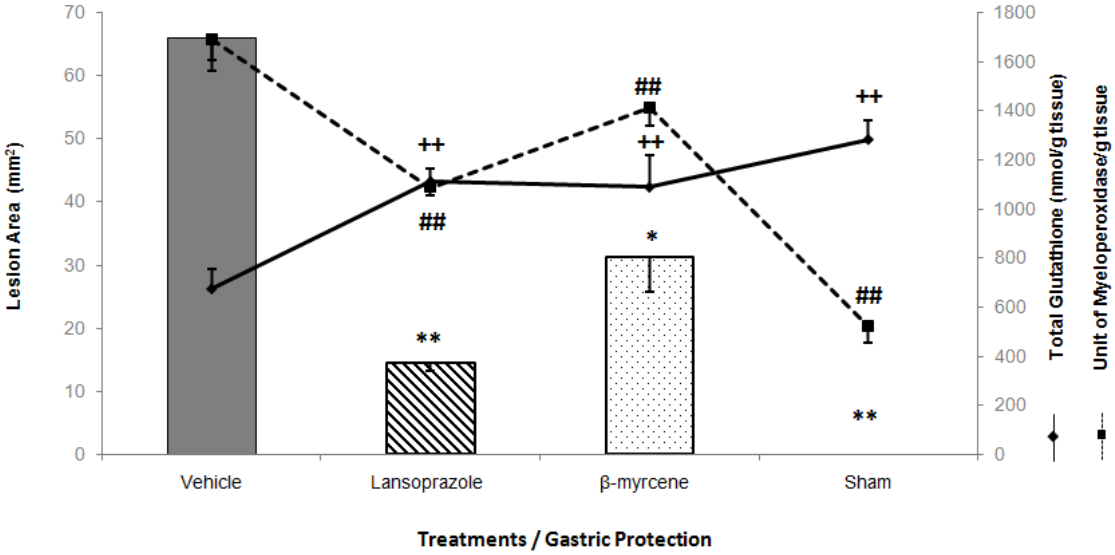
Lesion Area (mm²)
Total Glutathione (nmol/g tissue)
Unit of Myeloperoxidase/g tissue
Vehicle
Lansoprazole
β-myrcene
Sham
Treatments / Gastric Protection
++
##
*
**

Figure 6

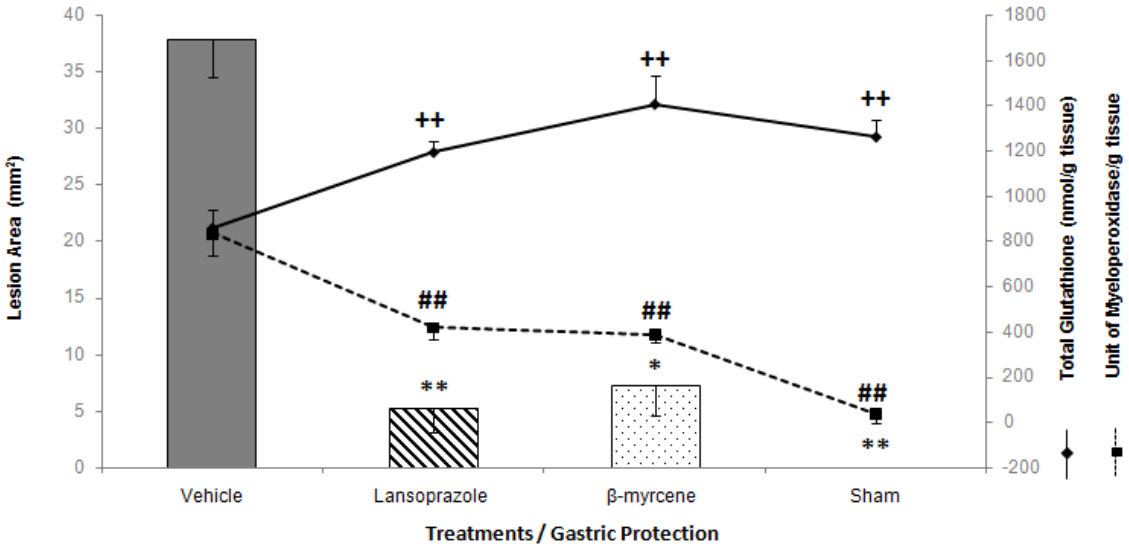
Lesion Area (mm²)
Total Glutathione (nmol/g tissue)
Unit of Myeloperoxidase/g tissue
Vehicle
Lansoprazole
β-myrcene
Sham
Treatments / Gastric Protection
++
##
**
*

Figure 7

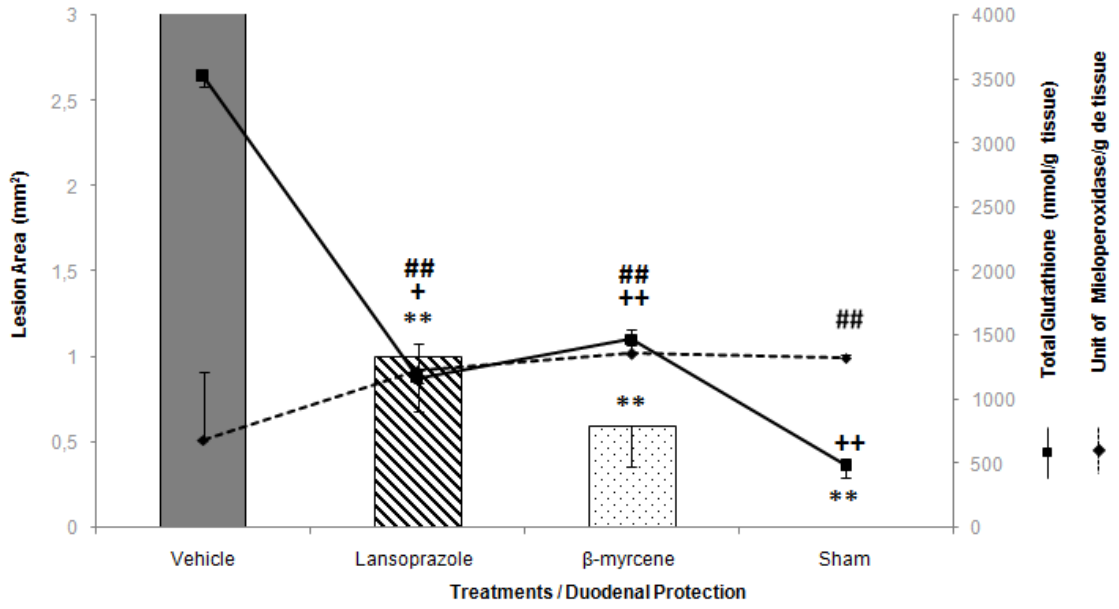
Lesion Area (mm²)
3
2,5
2
1,5
1
0,5
0
4000
3500
3000
2500
2000
1500
1000
500
0
Total Glutathione (nmol/g tissue)
Unit of Mieloperoxidase/g de tissue
##
+
**
##
++
**
##
++
**
Vehicle
Lansoprazole
β-myrcene
Sham
Treatments / Duodenal Protection

Table 2: Determining the role of the sulfhydryl group (SH) and nitric oxide (NO) in the gastric mucosa of the ethanol-induced model of gastric ulceration.

Method (animal)	Pretreatment (i.p.)	Treatment (p.o.)	Dose (mg/kg)	Ulcerative lesion (mm^2)	
	Saline	Vehicle	-	94.40 ± 15.94	
	Saline	Carbenoxolone	100	10.00 ± 3.64**	
	Saline	β-myrcene	7.50	51.00 ± 11.29*	
Nitric Oxide (NO)	L-NAME	Vehicle	-	235.33 ± 66.06	#
	L-NAME	Carbenoxolone	100	72.0 ± 15.07**	
	L-NAME	β-myrcene	7.50	499.67 ± 48.95**	###
Sulfhydryl	NEM	Vehicle	-	431.67 ± 29.29	###
Compounds (SH)	NEM	Carbenoxolone	100	96.67 ± 22.65**	
	NEM	β-myrcene	7.50	565.67 ± 19.93**	###

Data are presented as the mean ± S.E.M. ANOVA was carried out and followed by Dunnett's test. Ulcerative lesion: *P < 0.05, **P < 0.01, ***P < 0.001 compared to the vehicle-treated control group; # P < 0.05, ## P < 0.01, ### P < 0.001 compared to pre-treatments/saline + treatment groups.