Universidade Estadual Paulista Júlio de Mesquita Filho
Instituto de Biociências
Programa de Pós-graduação em Ciências Biológicas
Área de Concentração Farmacologia
Curso de Doutorado

EFEITO DO (-)-MENTOL SOBRE A ÚLCERA GÁSTRICA: ESTUDO DO MECANISMO GASTROPROTETOR

ARIANE LEITE ROZZA

Orientadora: Profa. Dra. Cláudia Helena Pellizzon

Botucatu/SP
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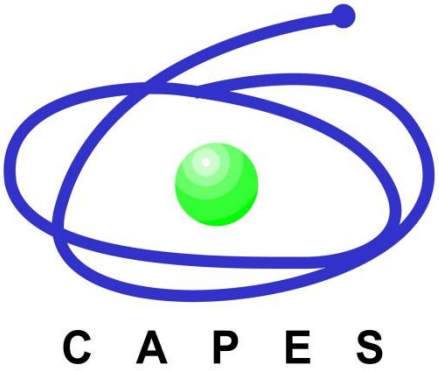

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Epígrafe

"Tenho a impressão de ser uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante dos meus olhos..."

Isaac Newton

Dedicatória

Aos meus pais, Remigio e Hilda, e minha irmã Mirela,

A minha orientadora Profa. Dra. Cláudia Helena Pellizzon.

"Só o que sonhamos é o que verdadeiramente somos,
porque o mais, por estar realizado,
pertence ao mundo e a toda a gente."

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"Confiança - o senhor sabe - não se tira das coisas feitas ou perfeitas:
ela rodeia é o quente da pessoa." Guimarães Rosa

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Aos amigos do centro cristão espírita Caminho da verdade,

A Deus, mestre, professor doutor.

"Em um mundo de peregrinos, quando os caminhos se cruzam, o mundo parece um lar durante algum tempo." Herman Hesse

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Prólogo

Atividades realizadas durante o curso de Doutorado (2009-2013) em Ciências Biológicas, área de concentração Farmacologia

Disciplinas cursadas

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2. Matriz extracelular animal
3. Tópicos de atualização em Ciências
4. Farmacologia do sistema nervoso autônomo simpático
5. Empreendedorismo
6. Estresse térmico e reprodução animal
7. Farmacologia avançada
8. Classificação de receptores farmacológicos
9. Mecanismos de controle e adaptação do trato gastrointestinal
10. Métodos de validação de novos fármacos e tópicos de atualização em plantas medicinais

Atividades de Ensino

02/2013 – 07/2013 Professora Bolsista da disciplina Farmacologia

02/2011 - 07/2011 Professora Bolsista da disciplina Histologia nos cursos de Biologia e Medicina

08/2010 - 12/2010 Estágio didático na disciplina Histologia no curso de Biomedicina

02/2010 - 07/2010 Estágio docência na disciplina Biologia Celular e Tecidual no curso de Física Médica

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2011 Honra ao Mérito, XXVI Reunião Anual da FeSBE

2010 Honra ao Mérito, XXV Reunião Anual da FeSBE

Participação em Eventos

1. 6th European Congress of Pharmacology EPHAR2012.

2. II Simpósio de Farmacologia da UNESP, 2012
3. 8th International Congress of Pharmaceutical Sciences CIFARP, 2011
4. XX Congresso Italo-Latinoamericano de Etnomedicina 'Prof Dr Francisco de Abreu Matos' - SILAE 2011
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Introdução

1. ÚLCERA GÁSTRICA

O estômago é um reservatório ativo que armazena, tritura e dispensa o alimento parcialmente digerido para o intestino para posterior digestão e absorção. Uma de suas principais funções é a secreção do ácido clorídrico (HCl), que tem a função de facilitar a digestão de proteínas através da conversão de pepsinogênio na enzima proteolítica pepsina. O ácido também facilita a absorção de ferro, cálcio, vitamina B12 e de alguns medicamentos, além de prevenir crescimento bacteriano e infecções entéricas (Choi et al., 2011; Pali-Scholl & Jensen-Jarolim, 2011).

O estômago é dividido em quatro regiões anatômicas (cárdia, fundo, corpo e piloro). Sua unidade básica funcional é a glândula gástrica, que se divide em três regiões: istmo, colo e base. Células-tronco e células mucosas em diferenciação estão localizadas no istmo, a partir de onde tais células migram e se diferenciam em célula parietal, mucosa do colo, zimogênica ou enteroendócrina. As células dominantes são as células parietais (secretoras de ácido) e células enteroendócrinas (secretoras de histamina). Entre os outros tipos celulares, encontram-se as células enterocromafins (produtoras de peptídeo natriurético atrial, serotonina e adrenomedulina) e células D (produtoras de somatostatina). A característica distintiva da mucosa pilórica é a célula G (produtora de gastrina) (Chu & Schuber, 2012).

A mucosa da região do corpo gástrico e, em menor extensão, do fundo e antro gástricos, contem as células parietais. A principal função das células parietais é produzir o acido clorídrico (HCl), que ativa a pepsina a partir do pepsinogênio, atua na digestão de proteínas e reduz a colonização bacteriana no estômago e intestino. As células parietais contém a bomba de prótons, ou H+/K+ATPase, que transporta H+ para o lúmen gástrico e K+ do lúmen para o interior da célula. Por causa da grande quantidade de energia necessária para a manutenção da bomba de prótons, a célula parietal contém a maior quantidade de mitocôndrias entre todas as células do corpo humano (Mercer & Robinson, 2008). Na célula parietal em descanso, as bombas estão contidas em vesículas intracelulares (Urushidani & Forte, 1997). Há um nível basal constante da produção de ácido clorídrico, mesmo quando a célula está em repouso, que corresponde a 10% da produção ácida da célula estimulada. No estado estimulado, as vesículas se fundem com a membrana apical da célula parietal, relocando as bombas protônicas para a superfície que está em contato com o lúmen gástrico (Urushidani & Forte, 1997; Mercer & Robinson, 2008). A representação esquemática da secreção ácida pela célula parietal está contida na figura 1.

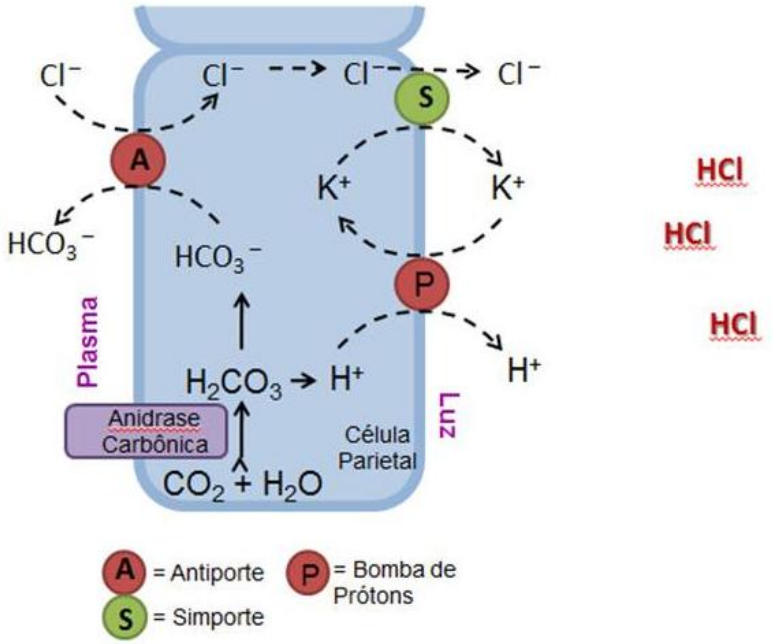

Figura 1: Ilustração esquemática da secreção do ácido clorídrico (HCl) pela célula parietal gástrica. A secreção envolve uma bomba de prótons (P), um carregador de simporte para K+ e Cl- e um antiporte para íons Cl- e HCO3- (modificado de Rang et al., 2011).

Há três tipos de receptores que, quando ativados, estimulam a secreção acida nas células parietais: o receptor muscarínico (M3), o receptor de colecistoquinina do tipo B (CCKB) e o receptor de histamina (H2) (Urushidani & Forte, 1997). Os três receptores estão localizados na membrana basolateral da célula parietal. O receptor M3 é estimulado pela acetilcolina, que é liberada através do estímulo da inervação parassimpática (Mercer & Robinson, 2008). O receptor CCKB é estimulado pela gastrina, produzida pelas células G, localizadas na região do antro gástrico (Mulholland, 2006; Mercer & Robinson, 2008). O receptor H2 é estimulado pela histamina, que é produzida pelas células enteroendócrinas, e sua liberação é estimulada pela gastrina e acetilcolina. O resultado final da ativação desses três receptores é a ativação da bomba protônica. (Mercer & Robinson, 2008).

O efeito inibitório na secreção de ácido gástrico é ativado pela somatostatina, que é produzida pelas células D presentes na mucosa fúndica e antral. A acetilcolina tem efeito inibitório sobre a liberação de somatostatina. A somatostatina inibe a secreção ácida atuando diretamente sobre a célula parietal, mas também indiretamente através de inibir a liberação de gastrina e histamina (Mercer & Robinson, 2008). A célula parietal contém outro receptor

inibitório da secreção para a PGE2, que inibe a produção ácida através da inibição da secreção de gastrina e estimula a secreção de somatostatina (Mulholland, 2006).

O trato gastrointestinal é um ambiente dinâmico que permite a entrada de nutrientes enquanto restringe a entrada de antígenos, toxinas e microrganismos, e responde a agentes luminais potencialmente prejudiciais, num processo de defesa da mucosa. A mucosa gástrica está sob constante ataque por fatores endógenos ou exógenos que induzem dano ao tecido gástrico, mas tais fatores são contrabalanceados por fatores gastroprotetores endógenos que mantém a integridade da mucosa (Wallace & Devchand, 2005).

Úlcera gástrica é uma erosão profunda na parede gástrica que penetra em toda a espessura da mucosa (Tarnawski, 2005). É uma doença comum de ocorrência global, com crescente incidência e prevalência, apesar de recentes avanços no campo médico. Anualmente, 14 milhões de pessoas apresentam úlceras, no mundo todo (Maity & Chattopadhyay, 2008).

O desenvolvimento das úlceras gástricas decorre do desequilíbrio entre fatores agressores à mucosa e a habilidade da mucosa em defender-se e curar-se (Repetto & Llesuy, 2002; Lu & Graham, 2006). Entre os fatores potencialmente agressores, incluem-se fatores endógenos (como secreção elevada de histamina e ácido clorídrico, bile, isquemia, leucotrienos, citocinas pró-inflamatórias, ativação de neutrófilos, espécies reativas de oxigênio e proteínas pró-apoptóticas) e fatores exógenos, como estresse (Cho et al., 1992), uso de drogas anti-inflamatórias não esteroidais (DAINEs) (Hawkey, 2000), álcool e colonização pela bactéria gram-negativa Helicobacter pylori (Peckenpaugh, 1997).

2. GASTROPROTEÇÃO

O estômago pode se defender de injúrias causadas por uma variedade de agentes irritantes e nocivos devido à ativação de várias linhas de defesa, no processo de defesa da mucosa (Brzozowski et al., 2005). Vários fatores permitem a mucosa permanecer intacta apesar de sua freqüente exposição à substâncias com larga variação de temperatura, pH e osmolaridade, assim como substâncias com ação citotóxica e produtos bacterianos capazes de causar reações inflamatórias (Wallace & Granger, 1996). Esse processo de defesa inclui mecanismos locais e neurohormonais. Entre os mecanismos locais, os mais importantes incluem a barreira de muco e bicarbonato, as células epiteliais de superfície e sua alta taxa de regeneração, adequada microcirculação no estômago, o fluxo alcalino, a geração contínua de prostaglandinas E2 e I2 (PGE2 e PGI2), a liberação de óxido nítrico (NO), a manutenção dos compostos sulfidrílicos não-proteicos (NP-SH), a atuação de enzimas antioxidantes e a

supressão do processo inflamatório (Tarnawski et al., 2001; Brzozowski et al., 2005; Laine et al., 2008).

A camada formada por muco, bicarbonato e fosfolipídeos surfactantes constitui a primeira linha de defesa da mucosa (Allen & Flemström, 2005). O muco é secretado pelas células epiteliais secretoras de muco e células mucosas do colo e seus principais constituintes são glicoproteínas, mucinas e água (Phillipson et al., 2008). Sua secreção é estimulada pelos hormônios gastrointestinais, incluindo gastrina e secretina, acetilcolina e também pela PGE2, que aumenta a viscosidade e o conteúdo de glicoproteínas no muco (Allen & Flemström, 2005).

Substâncias ulcerogênicas dissipam o muco e a camada de fosfolipídeos, levando a redifusão de ácido clorídrico, causando injúria na mucosa (Darling et al., 2004). A camada de muco retém os íons bicarbonato (HCO3-) secretados pelas células epiteliais, mantendo um microambiente de pH neutro na superfície das células e prevenindo a penetração da enzima pepsina e consequente proteólise do epitélio (Tarnawski et al., 2001; Valcheva-Kuzmanova et al., 2005). O muco também atua como lubrificante para reduzir o dano físico ao epitélio causado pelos materiais ingeridos e diminui a possibilidade de que bactérias tenham contato com o epitélio. A camada de muco é a única barreira pré-epitelial entre o lúmen e o epitélio. Quando ela está prejudicada, a próxima série de mecanismos protetores entra em ação (Wallace, 2008).

A próxima linha de defesa é formada por uma camada contínua de células epiteliais que, além da secreção de muco e bicarbonato, produzem prostaglandinas e proteínas heat-shock (HSPs). Devido à presença dos fosfolipídios na superfície, essas células são hidrofóbicas, repelindo agentes danosos solúveis em ácido ou água. Interconectadas por junções celulares, formam uma barreira que previne a redifusão de ácido e pepsina (Tarnawski, 1998; Allen & Flemström, 2005). As células parietais, que secretam ácido clorídrico no lúmen da glândula gástrica, simultaneamente secretam íons bicarbonato no interstício e lúmen dos capilares sanguíneos adjacentes através de sua membrana basolateral. Esse processo, chamado fluxo alcalino, neutraliza qualquer íon H+ que atinja a membrana basolateral e possa causar injúria na mucosa (Silen, 1993).

As HSPs são produzidas por essas células em resposta ao estresse celular, como aumento de temperatura, estresse oxidativo e agentes citotóxicos, prevenindo a denaturação de proteínas e consequentemente protegendo a célula de injúrias. As principais HSPs que atuam no estômago são a HSP-70 e a HSP-90 (Ishihara et al., 2011).

A contínua geração de PGE2 e PGI2 também é crucial para manutenção da integridade da mucosa e proteção contra agentes ulcerogênicos. Quase todos os mecanismos de defesa são estimulados ou facilitados pelas prostaglandinas. Elas inibem a secreção ácida, estimulam produção de muco e bicarbonato, estimulam o fluxo sanguíneo, aumentam a concentração de compostos sulfidrílicos não-proteicos (NP-SH) na mucosa e aceleram a regeneração epitelial e cicatrização da mucosa através do estímulo à proliferação celular (Brzozowski et al., 2005). Robert et al. (1979) concluíram que a prostaglandina aplicada exogenamente previne danos a mucosa gástrica induzidos por diferentes agentes, como etanol, ácido (HCl), base (NaOH), solução hiperosmótica (NaCl hipertônico), bile concentrada e até água fervente. A partir dessa descoberta, Robert et al. criaram a expressão citoproteção, definida como a habilidade do agente farmacológico de prevenir injúria a mucosa gástrica ou duodenal causada por uma variedade de agentes ulcerogênicos (Robert et al., 1979; Robert et al., 1983).

A adequada microcirculação na mucosa também é essencial para entrega de oxigênio e nutrientes e remoção de substâncias tóxicas. Quando a mucosa gástrica é exposta a um agente lesivo, há rápido aumento no fluxo sanguíneo, que permite remover ou diluir a concentração do agente lesivo. Essa resposta parece ser essencial para a defesa da mucosa, já que a restrição mecânica do fluxo sanguíneo leva a necrose hemorrágica (Laine et al., 2008). O aumento no fluxo sanguíneo na mucosa é mediado, pelo menos em parte, pelo NO, que é gerado pela enzima NO-sintase. A liberação de acetilcolina e de histamina, além de estimular a secreção ácida, tem efeitos vasodilatadores sobre a mucosa gástrica (Peskar, 2001; Holzer, 2006).

Os compostos NP-SH estimulam a formação de muco, ligam-se as espécies reativas de oxigênio (que estão envolvidas no dano tecidual) e ainda participam da síntese de proteínas (Banerjee et al., 2008). Agentes ulcerogênicos, como por exemplo o etanol, levam à queda na concentração de compostos NP-SH, que são neutralizados quando se ligam aos radicais livres produzidos pelo agente lesivo (Reyes-Trejo et al., 2008).

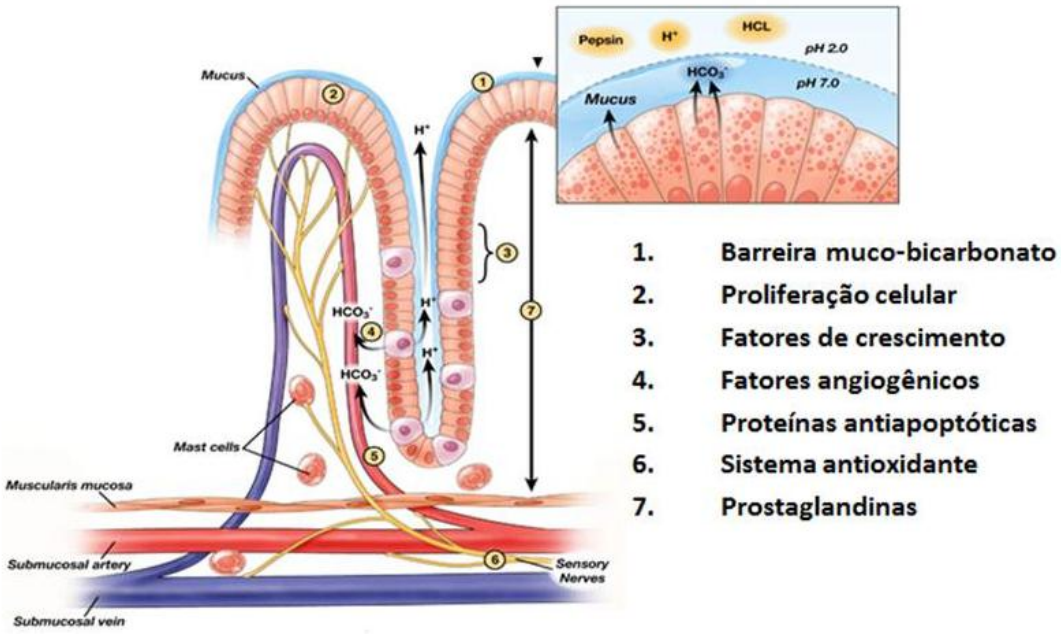

Figura 2: Representação esquemática de alguns dos processos de defesa da mucosa gástrica (modificado de Laine et al., 2008).

O contato do agente ulcerogênico com a mucosa do estômago induz um processo inflamatório local. A ativação dos neutrófilos que decorre da inflamação é uma das principais fontes de espécies reativas de oxigênio (ROS) (Kwiecien et al., 2002). Além disso, os neutrófilos produzem mieloperoxidase (MPO), enzima que libera radicais livres que danificam a mucosa, contribuindo para o desenvolvimento e manutenção da úlcera gástrica (Potrich et al., 2010). Para proteger os tecidos de injúrias causadas pelos radicais livres, as células contem enzimas antioxidantes, incluindo a glutationa peroxidase (GSH-Px), catalase, superóxido dismutase (SOD), glutationa redutase (GR) e o composto sulfidrílico glutationa (GSH).

A primeira enzima antioxidante a ser ativada na mucosa gástrica é a SOD, que catalisa a dismutação do ânion superóxido O2- em H2O2, que é menos nociva. O passo seguinte no metabolismo da H2O2 envolve a atividade da GSH-Px. A redução de H2O2 em H2O pela GSH-Px é acompanhada pela conversão da glutationa em sua forma reduzida (GSH) para sua forma oxidada (GSSH), que é convertida novamente a GSH pela atividade da enzima GR, usando NADPH como agente redutor (Kwicien et al., 2002). Compostos com ação

antioxidante podem bloquear a apoptose que levaria à necrose do tecido na região da úlcera gástrica (Amira et al., 2012).

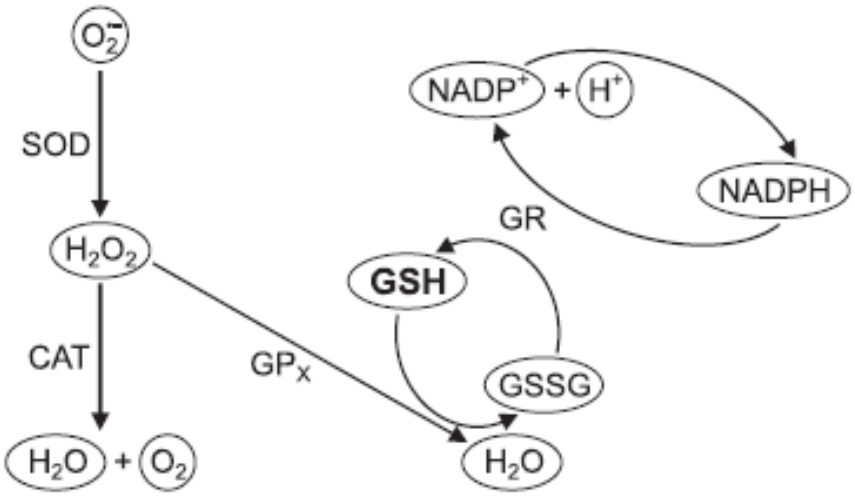

Figura 3: Representação da formação de espécies reativas de oxigênio no organismo. SOD: superóxido dismutase, CAT: catalase, GPx: glutationa peroxidase, GSH: glutationa, GSSG: glutationa na forma oxidada (retirado de Kwicien et al., 2002).

A defesa da mucosa gástrica é regulada também por fatores hormonais. Vários peptídeos, como gastrina, colecistoquinina e leptina tem função gastroprotetora através de variados mecanismo, como o estímulo da proliferação das células epiteliais (Peterson et al., 2010; Tanigawa et al., 2010).

Várias pesquisas tem mostrado o envolvimento da motilidade gastrointestinal no processo de defesa da mucosa. A motilidade normal ajuda a manter o fluxo sanguíneo regular, contribuindo no processo de gastroproteção (Tanaka et al., 2001).

3. PRINCIPAIS FATORES CAUSADORES DE ÚLCERA GÁSTRICA

O uso de DAINEs e a colonização por H. pylori no estômago são atualmente os principais fatores responsáveis pelo desenvolvimento de úlceras gástricas. DAINEs são os medicamentos mais utilizados nos Estados Unidos. Onze por cento da população faz uso regular de DAINE e um número muito maior faz uso intermitente dessa classe de medicamento (Paulose-Ram et al., 2005). Estudos comprovam que, após 10 minutos da ingestão de um comprimido contendo 650 mg de aspirina, 25% das células epiteliais apresentam danos quando observadas ao microscópio óptico e eletrônico (Baskin et al., 1976). Se analisado sob endoscopia, o estômago apresenta hemorragias subepiteliais dentro de 15

minutos após a ingestão do comprimido em 100% dos pacientes (Laine, 1996). Cinco por cento desses pacientes apresentam complicações severas no trato gastrointestinal, como perfurações e hemorragias (Yuan et al., 2006).

DAINEs são ácidos fracos, que se ligam a mucosa gástrica causando injúria local. O principal mecanismo de ação é a inibição sistêmica da ciclooxigenase (COX), uma enzima da via de produção das prostaglandinas a partir do ácido aracdônico. Existem duas isoformas, COX-1 e COX-2; DAINEs não seletivos bloqueiam as duas isoformas. A atuação dessas enzimas resulta na produção de PGE2, que protege a mucosa através da inibição da secreção ácida, estímulo na produção de muco e bicarbonato e aumento do fluxo sanguíneo (Ramsay & Carr, 2011), além de ter efeito trófico na mucosa, estimulando a proliferação celular (Pai et al., 2002). Estudos que utilizaram inibidores seletivos para COX-1 ou COX-2 evidenciaram que o efeito ulcerogênico das DAINEs decorre da inibição de ambas as isoformas (Takeuchi, 2012). A sequência de eventos que induz a formação de úlcera após administração de DAINE é a inibição da síntese de prostaglandina, indução da hipermotilidade gástrica, seguida por distúrbios microvasculares e ativação de neutrófilos (Laine et al., 2008).

Helicobacter pylori é uma bactéria gram-negativa flagelada que coloniza o estômago. A colônia de bactérias produz a enzima urease, que converte uréia em amônia (NH3) e dióxido de carbono (CO2), criando um microambiente que permite a sobrevivência das bactérias no baixo pH do estômago. A colonização pela bactéria H. pylori no estomago induz a formação de úlceras na mucosa através de diversos mecanismos. A colônia bacteriana produz enzimas que dissolvem a barreira de muco e também induz uma queda na população de células D, que baixa a produção de somatostatina e consequentemente aumenta a produção de gastrina e de ácido clorídrico (Ramsay & Carr, 2011). Mais de 50% da população mundial está infectada pela H. pylori, e essa infecção pode ser assintomática ou evoluir para gastrite, úlcera gástrica, úlcera duodenal, linfoma e adenocarcinoma. A erradicação da colônia de bactérias reestabelece a população de células D, resultando em queda na produção de ácido (Ramsay & Carr, 2011). O tratamento para erradicação consiste na chamada terapia tripla, que combina um inibidor de bomba protônica com os antibióticos claritromicina e amoxicilina. O sucesso dessa terapia está em torno de 80% (Calvet et al., 2000), mas ainda há a necessidade de uma terapia mais simples, que melhore o desconforto para o paciente, apresente custo reduzido e aumente a taxa de erradicação da bactéria (Yuan et al., 2006). À época de seu lançamento, tal terapia apresentava taxas de erradicação próximas a 90%, mas atualmente tem vivenciado um declínio, estando próxima a 70% (Paoluzi et al., 2010). Há várias razões que

justificam tal perda de eficácia, mas a principal delas é a crescente resistência da H. pylori aos antibióticos (Selgrad & Malfertheiner, 2011).

Antes da introdução do tratamento para erradicação da bactéria H. pylori no estômago, a úlcera gástrica afetava 10% da população mundial, com episódios cíclicos de cura e reincidência. O tratamento com erradicação da bactéria alterou esse ciclo da doença, entretanto, a crença „sem H. pylori, sem úlcera" está desatualizada (Arakawa et al., 2000). A prevalência de úlceras gástricas que não são induzidas por H. pylori nem por DAINEs, que podem ser chamadas idiopáticas, está próximo de 40% dos casos (Jyotheeswaran et al., 1998; Jang et al., 2008) e vem crescendo gradualmente nos últimos anos (Musumba et al., 2012). A taxa de reincidência da úlcera gástrica idiopática é maior que a reincidência das úlceras induzidas por H. pylori ou DAINE, e seu tratamento, por não combater uma causa específica, é mais difícil, mais longo e mais caro (Kang et al., 2012).

4. TERAPÊUTICA ATUAL E PERSPECTIVAS

O conhecimento sobre a doença mudou drasticamente com a descoberta da relação entre colonização estomacal por H. pylori e o desenvolvimento de úlcera gástrica, por Warren & Marshall (1983). Essa descoberta alterou a ideia de uma doença não apenas causada pelo ácido gástrico, mas também seria uma doença infecciosa, abrindo campo para intensivas pesquisas sobre a patogênese da doença. Além disso, essa pesquisa recebeu pleno reconhecimento em 2005, quando os autores foram laureados com o Prêmio Nobel. Entretanto, esse reconhecimento não fechou o capítulo sobre as úlceras gástricas. O tratamento da úlcera e principalmente de suas complicações ainda são desafios no campo da gastroenterologia (Garcia Rodriguez & Barreales, 2007).

As drogas atualmente usadas no tratamento de úlceras gástricas incluem antibióticos (nos casos de infecção por H. pylori), bloqueadores de receptores H2 (cimetidina, ranitidina), inibidores da bomba protônica (omeprazol, lansoprazol) e agentes citoprotetores (bismuto, sucralfato). A classe de drogas que obteve maior êxito é a que visa inibir a secreção ácida. Os antagonistas de receptores H2 revolucionaram a tratamento das úlceras, cicatrizando-as e mantendo-as em remissão durante o período em que eram administrados (Bianchi-Porro & Lazzaroni, 1992). Essa classe de drogas foi gradualmente substituída por uma classe de drogas mais potente na inibição da secreção ácida, os inibidores de bomba de prótons, que entraram no mercado em 1989. Tais medicamentos atuam bloqueando a bomba H+/K+ATPase nas células parietais (Fellenius et al., 1981). Já que a rapidez da cicatrização da úlcera estaria associada com o grau de supressão da produção ácida, os inibidores da

bomba protônica tornaram-se uma marca no tratamento das úlceras. Entretanto, após a fase de cicatrização, as úlceras geralmente reincidiam e, por muitos anos, a pratica padrão era manter o tratamento de supressão ácida para o paciente (Malfertheiner et al., 2009). Essas drogas geraram declínio nas taxa de morbidade, mas produzem muitos efeitos adversos, não impedem a reincidência da úlcera (DeVault & Talley, 2009), induzem tolerância (Komazawa et al., 2003) e geralmente tem um custo que não é acessível a grande parte da população (Maity & Chattopadhyay, 2008).

Assim, tem se tornado consenso que um fármaco antiulcerogênico deve atuar não apenas na supressão da secreção ácida, mas sim apresentar mecanismo de ação multifatorial. Um fármaco antiulcerogênico considerado ideal deve apresentar atividade antisecretória, antioxidante, antiapoptótica, anti-inflamatória e ainda estimular a proliferação celular e a angiogênese (Maity et al., 2009). A busca por esse composto ainda tem sido um desafio e tem impulsionado as pesquisas recentes da área (Maity & Chattopadhyay, 2008). Nesse sentido, extensivos trabalhos têm sido desenvolvidos com plantas e produtos naturais derivados de plantas. As plantas são fontes atrativas de novas biomoléculas, e elas têm mantido sua popularidade como tratamento para várias doenças por razões históricas e culturais. O interesse no uso de plantas para o tratamento de várias doenças é disseminado em várias partes do mundo.

Entre as principais classes de compostos químicos derivados de plantas que vem mostrando resultados promissores para a atividade antiulcerogênica, estão os carotenóides, terpenóides (Favier et al., 2005; Pertino et al., 2006) e os flavonóides (Alanko et al., 1999; Maity & Chattopadhyay, 2008).

O uso das espécies do gênero Mentha para fins medicinais é bastante disseminado. Suas folhas são utilizadas internamente para alívio dos sintomas de doenças dos tratos gastrointestinal e urinário, ou por via inalatória para alívio de sintomas de problemas respiratórios e tosse. De acordo com o conhecimento popular, seus benefícios incluem distúrbios biliares, dispepsia, enterite, flatulência, gastrite, úlcera péptica, cólica intestinal e síndrome do intestino irritável (Blumenthal et al.., 2000).

Mentol é o composto majoritário dos óleos essenciais do gênero Mentha. Apresenta fórmula molecular C10H20O e peso molecular de 156 kDa. É um terpeno cíclico que dá as plantas desse gênero seu aroma e sabor característicos. Atualmente, o consumo de mentol excede 700 toneladas por ano, e o produto puro movimenta cerca de 300 milhões de dólares (Croteau et al.., 2005). Sua aplicação é versátil e inclui produtos de higiene oral, confeitos, medicamentos, cosméticos, aromatizantes, além de estar presente na composição de fórmulas

antipruríticas, antissépticas, analgésicas e refrescantes (Eccles, 1994; Croteau et al., 2005). Ainda, o mentol apresenta comprovado efeito anti-nociceptivo, anestésico local (Haeseler et al., 2002), bactericida e fungicida (Işcan et al., 2002).

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Objetivos

OBJETIVOS

- Investigar e caracterizar o mecanismo de ação gastroprotetor do mentol contra diferentes agentes ulcerogênicos (etanol e indometacina),

- Investigar a atividade do mentol sobre a diarreia e motilidade intestinal,

- Investigar possível indução de efeito tóxico agudo do mentol em ratos.

Capítulo 1

Artigo a ser submetido para

Evidence-Based Complementary and Alternative Medicine (eCAM)

EFFECT OF MENTHOL IN EXPERIMENTALLY INDUCED ULCERS: PATHWAYS OF GASTROPROTECTION

ABSTRACT

Based on ethnopharmacological indications that Mentha species may be used in the treatment of gastrointestinal diseases, this study aimed to characterize the gastroprotective mechanisms of menthol (ME), the major compound of the essential oil from species of the genus Mentha. The gastroprotective action of ME was analyzed in gastric ulcers that were induced by ethanol or indomethacin in Wistar male rats. The mechanisms responsible for the gastroprotective effect were assessed by analyzing the amount of mucus secreted, involvement of non-protein sulfhydryl (NP-SH) compounds, involvement of calcium ion channels and NO/cGMP/K^+_{ATP} pathway, gastric antisecretory activity and the prostaglandin E_2 (PGE_2) production. The anti-diarrheal activity and acute toxicity of ME were also evaluated. Oral treatment with ME (50 mg/kg) offered 88.62% and 72.62% of gastroprotection against ethanol and indomethacin, respectively. There was an increased amount of mucus and PGE_2 production. The gastroprotective activity of ME involved NP-SH compounds and the stimulation of K^+_{ATP} channels, but not the activation of calcium ion channels or the production of NO. The oral administration of ME induced an antisecretory effect as it decreased the H^+ concentration in gastric juices. ME displayed anti-diarrheal and antiperistaltic activity. There were no signs of toxicity in the biochemical analyses performed in the rats‟ serum. These results demonstrated that ME provides gastroprotective and anti-diarrheal activities with no toxicity in rats.

Keywords: menthol, gastroprotection, gastric ulcer, diarrhea, acute toxicity

1. INTRODUCTION

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 (Laine et al., 2008).

There is a continuous search for a new bioactive compound derived from medicinal plants and natural products with gastroprotective and ulcer-healing properties. The new compound should be accessible, safe, and gastroprotective, and it should effectively heal the ulcer, thus avoiding its reoccurrence (Lallo et al., 2013). Plants have presented promising results in the treatment of gastric ulcers in several research projects worldwide (Schmeda-Hirschmann & Yesilada, 2005). Among the natural compounds that have been studied in recent studies, several terpenes have presented gastroprotective effects, such as limonene (Rozza et al., 2011), suaveolol (Vera-Arzave et al., 2012) and carvacrol (Silva et al., 2012). Menthol (ME) is a cyclic terpene with molecular weight 156 kDa and the formula $C_{10}H_{20}O$. ME is the main compound of the essential oil from the species of the genus mentha and is responsible for giving mentha species their distinctive smell and flavor. Among the optical isomers, (-)-menthol occurs most widely in nature and is endowed with the peculiar property of being a fragrance and flavor compound (Eccles, 1994). This study aimed to characterize the mechanism of action of ME against ethanol- and indomethacin-induced gastric ulcers in

rats. We also sought to evaluate the anti-diarrheal activity and the acute toxicity induced by ME.

2. MATERIAL AND METHODS

2.1. Menthol

(-)-Menthol (catalog #63660) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The chemical structure of ME is presented in figure 1.

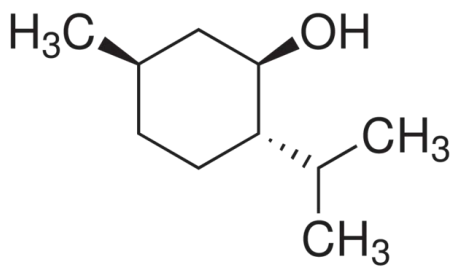

Figure 1: Chemical structure of (-)-menthol.

2.2. Animals

Male Wistar rats (200–250 g) from the Central Animal House of UNESP were fed a certified diet with free access to tap water under standard light-dark cycles (12 h dark–12 h light), humidity (60 ± 1%) and temperature (21 ± 2°C). All rats were fasted for at least 16 hours prior to each experiment because the treatments were orally administered. Rats were housed in cages with raised, wide-mesh floors to prevent coprophagy. All experimental protocols followed the recommendations of the Canadian Council on Animal Care and were approved by the UNESP Institutional Animal Care and Use Committee.

2.3. Experimental assays

2.3.1. Ethanol-induced gastric ulcers: determination of dose

Male Wistar rats were distributed into five groups (n=7) and then orally dosed with vehicle (10 mL/kg), carbenoxolone (100 mg/kg) or ME (25, 50 or 100 mg/kg). After 1 hour, the animals received an oral dose of 1 mL of absolute ethanol. One hour after ethanol treatment, the rats were euthanized, and their stomachs were removed (Robert et al., 1979). The stomachs were then opened along the greater curvature and washed. The flattened stomach samples were scanned, and the ulcer area (mm^2) was measured using the AVSoft BioView software. The lower effective dose from the 3 doses tested was adopted for all other assays.

After scanning, the mucosa of each stomach was gently scraped using two glass slices, homogenized in phosphate-buffered saline (PBS, 0.1 M, pH 7.4), and frozen at −80°C until biochemical analyses. Stomach samples were collected for histological slide preparation and were either stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) or used for immunohistochemical analyses. A microscopic score (Jahovic et al., 2007) was determined for the following parameters: epithelial desquamation, hemorrhage, glandular damage and eosinophilic infiltration. A scale ranging from 0 to 3 (0: none, 1: mild, 2: moderate and 3: severe) was used for each criterion. The highest possible score was 12.

2.3.2. Involvement of the NO/cGMP/K^+ATP pathway in gastroprotection

Rats were distributed into eight groups (n=7). Two groups of rats were subjected to intraperitoneal treatment with the following drugs: vehicle (8% tween 80, 10 mL/kg), L-NAME (N-nitro-L-arginine methyl ester 70 mg/kg, a NO synthase inhibitor), ODQ (1H[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one 10 mg/kg, a guanylate cyclase inhibitor) or glibenclamide (K^+_{ATP} channel blocker 3 mg/kg). One hour later, the vehicle (10 mL/kg) and

ME (50 mg/kg) were orally administered to four groups each (Chávez-Piña et al., 2011). After 60 minutes, all groups were orally treated with 1 mL of absolute ethanol for gastric ulcer induction. Animals were euthanized one hour after ethanol administration, and the stomachs were removed, opened along the greater curvature, scanned and the ulcer area (mm^2) was determined using the AVSoft BioView software.

2.3.3. Involvement of non-protein sulfhydryl compounds (NP-SH) or calcium ion channels in gastroprotection

Rats were distributed into six groups (n=7). Two groups of rats were subjected to intraperitoneal treatment with the following drugs: vehicle (8% tween 80, 10 mL/kg), NEM (N-ethylmaleimide 5 mg/kg, a NP-SH compounds blocker) or verapamil (a calcium channel blocker, 5 mg/kg). One hour later, the vehicle (10 mL/kg) and ME (50 mg/kg) were orally administered to three groups each. After 60 minutes, all groups were orally treated with 1 mL of absolute ethanol for gastric ulcer induction. Animals were killed one hour after ethanol administration, and the stomachs were removed, opened along the greater curvature, scanned and the ulcer area (mm^2) was determined using AVSoft BioView.

2.3.4. Evaluation of gastric juice parameters

Rats were randomly divided into six groups (n=7). Thirty minutes after oral treatment or immediately after the intra-duodenal administration of a single dose of vehicle (10 mL/kg), cimetidine (100 mg/kg) or ME (50 mg/kg), the rats were subjected to pyloric ligation (Shay et al., 1945). Four hours later, the animals were euthanized, the abdomen was opened and another ligature was placed around the esophagus, close to the diaphragm. The stomach was removed, and its contents were drained into a graduated centrifuge tube, which was then centrifuged at 2,000 g for 15 minutes. The total acid content of the gastric secretions was

determined by titration to pH 7.0 with 0.01 N NaOH using a digital burette. The total concentration of acid was expressed as mEq/mL/4 h.

2.3.5. Determination of mucus adherence to the gastric wall

After 24 hours of fasting, anesthetized rats (n=7) were subjected to longitudinal incisions slightly below the xiphoid apophysis for the placement of a pyloric ligature. The oral administration of vehicle, carbenoxolone (200 mg/kg) or ME (50 mg/kg) was performed 1 hour before the ligature. After 4 hours, the rats were euthanized, and the glandular portion of the stomach was weighed and immersed in Alcian Blue solution for the mucus quantification procedure. The absorbance was measured in a spectrophotometer at a wavelength of 598 nm, and the results were expressed as µg Alcian Blue/g tissue (Rafatullah et al., 1990).

2.3.6. Non-steroidal anti-inflammatory drug (NSAID)-induced gastric ulcers

Rats were distributed into three groups (n=7). Vehicle (10 mL/kg), cimetidine (100 mg/kg) or ME (50 mg/kg) were orally administered 30 minutes prior to the induction of gastric lesions by the oral administration of the ulcerogenic agent indomethacin (100 mg/kg). The animals were euthanized 5 hours after treatment with indomethacin (Puscas et al., 1997). The stomachs were removed, opened along the greater curvature and then scanned. The ulcer area (mm^2) was determined using AVSoft BioView.

2.3.7. Prostaglandin E_2 (PGE_2) assay

Rats were randomly divided into five groups (n=7): sham, vehicle, vehicle + indomethacin, ME (50 mg/kg) and ME (50 mg/kg) + indomethacin. The +indomethacin groups subcutaneosly received indomethacin (PGE_2 inhibitor) at 30 mg/kg, and the other

groups received vehicle. After 30 minutes, rats orally received vehicle or ME. The sham group received neither drug nor treatment. After 30 minutes, the rats were euthanized, and the stomachs were removed. Following stomach harvesting, the corpus was excised, weighed and suspended in 10 mM PBS at pH 7.4 (1 mL). The tissue was minced finely with scissors and incubated at 37°C for 20 minutes. After centrifugation at 9,000 g, the PGE_2 levels in the supernatant were measured by ELISA and read in spectrophotometer (420 nm). The results were expressed as ng/mL (Curtis et al., 1995).

2.3.9. Effect of menthol on castor oil-induced diarrhea

Three groups of rats were orally treated with vehicle (10 mL/kg), loperamide hydrochloride (3 mg/kg) or ME (50 mg/kg). After 30 minutes, each rat received 1 mL of castor oil orally. Immediately after ingesting the castor oil, each rat was kept in an individual cage, the floor of which was lined with blotting paper, and the rats were observed for 5 hours. The following parameters were then observed: onset of diarrhea, number of solid, semi-solid and watery feces and total frequency of fecal outputs. Each rat received an evacuation index (EI) expressed according to the formula: EI = 1×(no. solid stool) + 2×(no. semi-solid stool) + 3×(no. watery stool) (Crocci et al., 1994).

2.3.10. Effect of menthol on gastrointestinal motility

Rats were orally treated with vehicle (10 ml/kg), loperamide hydrochloride (4 mg/kg) or ME (50 mg/kg). After 20 minutes, each rat orally received 1 mL of charcoal meal (10% charcoal suspension in 5% aqueous gum Arabic). After 30 minutes, rats were euthanized, and the stomach and small intestine were removed. The distance between the charcoal meal and the pylorus was measured and correlated to the distance from the pylorus to the caecum (Calzada et al., 2010).

2.3.11. Acute toxicity

Male rats (n=10) orally received vehicle or a single acute dose of ME (500 mg/kg, corresponding to ten times the therapeutic dose) after 12 hours of fasting. Possible signs and symptoms associated with toxicity were observed at 0, 30, 60, 120, 180 and 240 minutes after the administration and then twice daily for the next 14 days. Body weights were noted daily. At the end of the period, the rats were euthanized, and the kidneys and liver were withdrawn, weighed and evaluated. Biochemical analyses were performed on the rats‟ serum to quantify AST (aspartate aminotransferase), ALT (alanine aminotransferase), γ-GT (gamma glutamyltransferase) and alkaline phosphatase to evaluate liver damage and creatinine and urea to evaluate kidney damage using the automated biochemical analyzer SBA-200, CELM, Brazil.

2.4. Statistical analysis

Parametric data were analyzed using an unpaired t-test or a one-way analysis of variance (ANOVA) followed by Dunnett‟s test and compared to the vehicle group or Tukey‟s test. The results were presented as the mean ± standard error of the mean (SEM). Nonparametric data (histology scoring) were analyzed using the Kruskal-Wallis (nonparametric ANOVA) test, followed by a Dunn multiple comparison test. The results were presented as the median (range). All analyses were performed using GraphPad InStat software. A value of $p<0.05$ was considered significant.

3. RESULTS

3.1. Ethanol-induced gastric ulcers

3.1.1. Gastric ulcer area

The vehicle group presented several hemorrhagic bands that were usually parallel to the long axis of the stomach, with an average ulcer area of 374.82 ± 12.75 mm^2. They were located mostly in the gastric corpus, and no visible lesions developed in the non-secretory part of the stomach. The two highest ME doses tested (i.e., 50 and 100 mg/kg) exhibited gastroprotective effects ($p<0.01$) when compared to the vehicle group. ME presented a gastroprotective effect of 20.06% for the lower dose (25 mg/kg), 88.62% for 50 mg/kg (ulcer area 42.64 ± 15.64 mm^2) and 98.42% for the highest dose, 100 mg/kg (ulcer area 5.91 ± 5.33 mm^2). However, according to Tukey"s test, there was no difference between the ulcer areas of the groups treated with 50 mg/kg or 100 mg/kg; therefore, the dose of 50 mg/kg was used for all subsequent experiments. The ulcer areas (mm^2) are represented in figure 2.

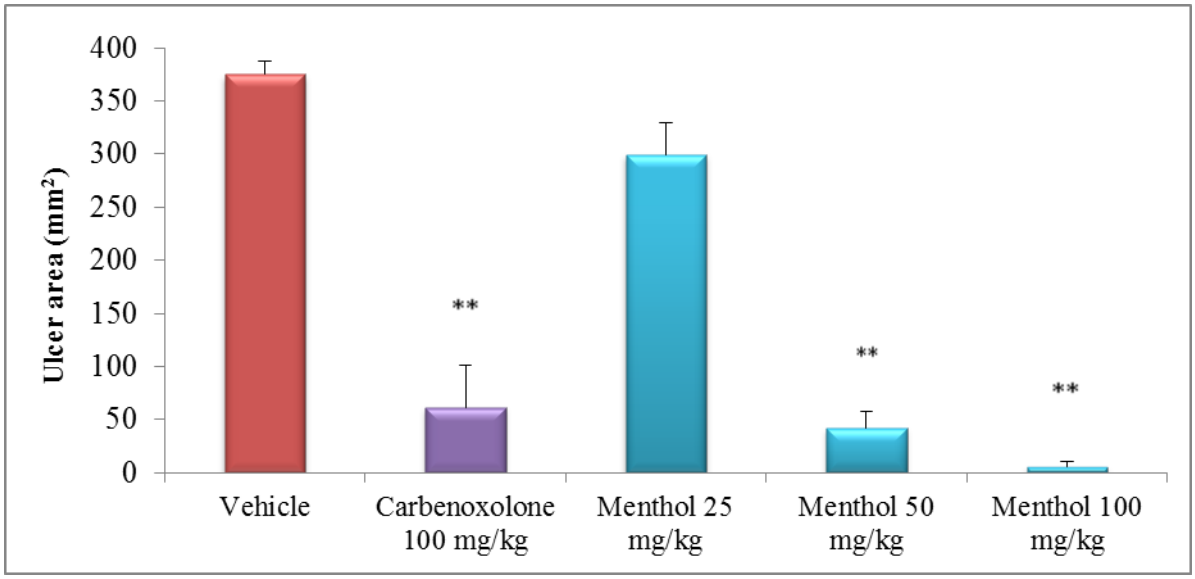

Figure 2: Gastric ulcer area (mm^2) of rat stomachs with ethanol-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (25, 50 or 75 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett´s test, $p<0.01$.

3.1.2. Microscopic analyses

Microscopically, ME presented moderate epithelial desquamation, mild hemorrhage and glandular damage as well as an absence of eosinophilic infiltration, presenting a score of 3(1-3). Carbenoxolone showed moderate epithelial desquamation and glandular damage as well as mild hemorrhage and eosiniphilic infiltration, presenting a score of 4(2-5). The score of the vehicle group was 12(11-12). The mucus polysaccharides were evidenced after the PAS staining, which conferred a purple barrier covering the gastric pits in the ME-treated rats. The HE and PAS staining of the ulcers are displayed in figure 3.

3.2. Involvement of the NO/cGMP/K^+_{ATP} pathway in gastroprotection

In rats that were pre-treated with L-NAME (a NO-synthase inhibitor) or ODQ (a guanylate cyclase inhibitor), the gastroprotective effect of ME (50 mg/kg) was maintained (62.65% and 97.71% gastroprotection, respectively, compared to the vehicle-treated group, $p<0.01$), showing that the NO synthesis or cGMP were not involved in the protective mechanism of ME. However, in rats pretreated with glibenclamide (a K^+_{ATP} channel blocker), the gastroprotective effect of ME (50 mg/kg) was reversed, indicating the involvement of K^+_{ATP} channels in gastroprotection (table 1).

3.3. Involvement of NP-SH compounds or calcium channels in gastroprotection

In rats pretreated with NEM (an NP-SH compound reagent), the gastroprotective effect of ME (50 mg/kg) was reversed, indicating the involvement of SH compounds in gastroprotection. However, in rats that were pre-treated with verapamil (a calcium ion channel blocker), the gastroprotective effect of ME (50 mg/kg) was maintained (a protective effect of 98.31% compared to the vehicle-treated group, $p<0.001$), indicating that the calcium ion channels are not involved in the gastroprotective mechanism of ME (table 2).

Figure 3: Photomicrography of rat stomachs with ethanol-induced gastric ulcers after treatment with (A, D) vehicle, (B, E) carbenoxolone (100 mg/kg) or (C, F) menthol (50 mg/kg). In the HE staining (A, B, C), * indicates epithelial desquamation and # indicates glandular damage. In the PAS staining (D, E, F), + indicates the mucus secretion in the gastric glands.

Table 1: Effect of menthol (50 mg/kg) on ethanol-induced gastric ulcer area (mm^2) in rats that were pretreated with L-NAME (NO synthase inhibitor), ODQ (guanylate cyclase inhibitor) or glibenclamide (K^+_{ATP} channels blocker)

Pretreatment (i.p)	**Treatment (p.o)**	**Ulcer area (mm^2)**	**Gastroprotection (%)**
Vehicle	Vehicle	501.70 ± 35.00	-
	Menthol	50.52 ± 10.99 ***	89.93
L-NAME 70 mg/kg	Vehicle	476.44 ± 89.17	-
	Menthol	177.94 ± 25.29 **	62.65
ODQ 10 mg/kg	Vehicle	652.54 ± 144.21	-
	Menthol	14.94 ± 6.10 **	97.71
Glibenclamide 3 mg/kg	Vehicle	134.13 ± 16.03	-
	Menthol	154.41 ± 28.50	0

The results are reported as the mean ± SEM. Unpaired t test, **p<0.01, ***p<0.001.

Table 2: Effect of menthol (50 mg/kg) on ethanol-induced gastric ulcer area (mm^2) in rats that were pretreated with NEM (SH blocker) or verapamil (calcium channel blocker)

Pretreatment (i.p)	**Treatment (p.o)**	**Ulcer area (mm^2)**	**Gastroprotection (%)**
Vehicle	Vehicle	1825.62 ± 192.76	-
	Menthol	17.86 ± 11.46 ***	99.02
NEM 5 mg/kg	Vehicle	1799.24 ± 250.73	-
	Menthol	1574.03 ± 346.59	8.34
Verapamil 5 mg/kg	Vehicle	1155.17 ± 336.88	-
	Menthol	19.53 ± 12.38 **	98.31

The results are reported as the mean ± SEM. Unpaired t test, **p<0.01, ***p<0.001.

3.4. Evaluation of the gastric juice parameters

A comparison of the gastric juice parameters of rats treated with oral or intra-duodenal ME (50 mg/kg) demonstrated that the oral treatment was able to diminish the H^+ concentration ($p<0.01$) in the gastric juice without modifying its volume. The intra-duodenal administration was not able to decrease the H^+ concentration, but it did diminish the volume of the gastric juice (table 3).

Table 3: Effect of menthol (50 mg/kg) on gastric juice parameters in rats with pyloric ligation

Route	**Treatment**	**Gastric Juice Volume (mL)**	**[H^+] mequiv/mL/4h**
Oral	Vehicle	2.93 ± 0.36	9.60 ± 0.95
	Cimetidine 100 mg/Kg	1.9 ± 0.13	2.23 ± 0.18 **
	Menthol 50 mg/Kg	3.39 ± 0.63	5.90 ± 0.67 **
Intraduodenal	Vehicle	5.08 ± 0.21	10.23 ± 0.44
	Cimetidine 100 mg/Kg	2.42 ± 0.17 **	4.62 ± 0.50 **
	Menthol 50 mg/Kg	2.95 ± 0.15 **	9.05 ± 0.32

The results are reported as the mean ± SEM. ANOVA followed by Dunnett‟s test, $p<0.01$.

3.5. Determination of mucus adherence to the gastric wall

There was a 2.2-fold increase in the amount of gastric mucus adhering to the stomach wall in the ME-treated (50 mg/kg) group (3632.00 ± 66.06 µg/g, $p<0.01$) compared to the vehicle-treated group (1633.43 ± 43.73 µg/g) (figure 4).

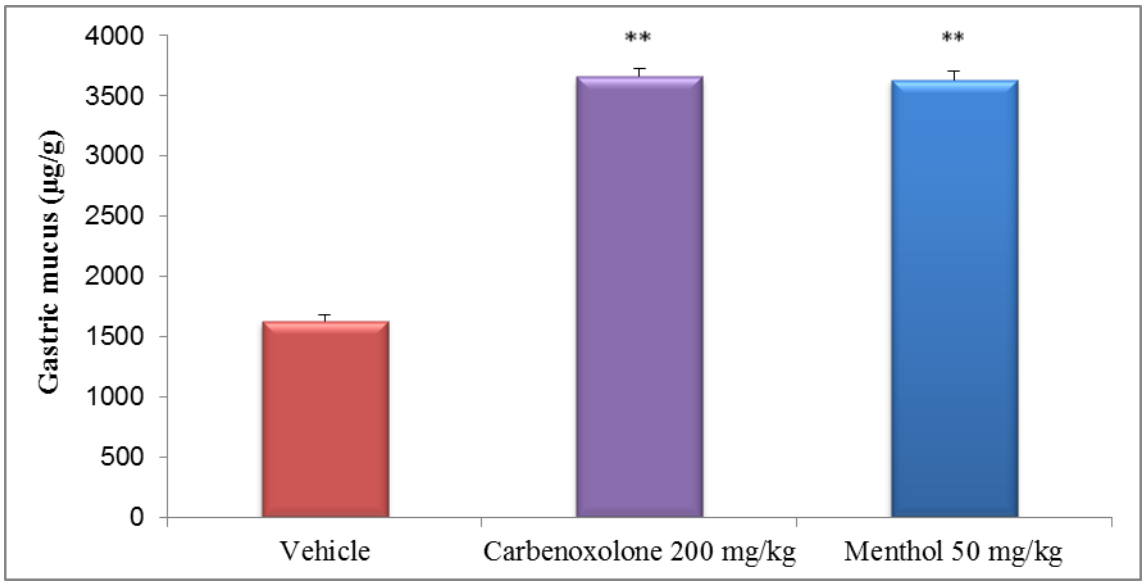

Figure 4: Quantification of adherent mucus (µg/g of tissue) in the gastric mucosa of rats treated with vehicle, carbenoxolone (200 mg/kg) or menthol (50 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett´s test, $p<0.01$.

3.6. Indomethacin-induced gastric ulcers

The vehicle-treated group had a large quantity of small petechiae in the stomach and a mean ulcer area of 38.06 ± 8.02 mm^2. In this model, ME presented a gastroprotective effect of 72.62% (ulcer area 10.42 ± 2.71 mm^2, $p<0.01$) in comparison to the vehicle group (figure 5).

3.7. PGE_2 assay

The decreased level ($p<0.01$) of PGE_2 in the vehicle + indomethacin group, compared to that of the sham group, proves that indomethacin induces decrease of PGE_2 production. The ME groups maintained PGE_2 levels near that of the sham group, even with indomethacin administration (figure 6).

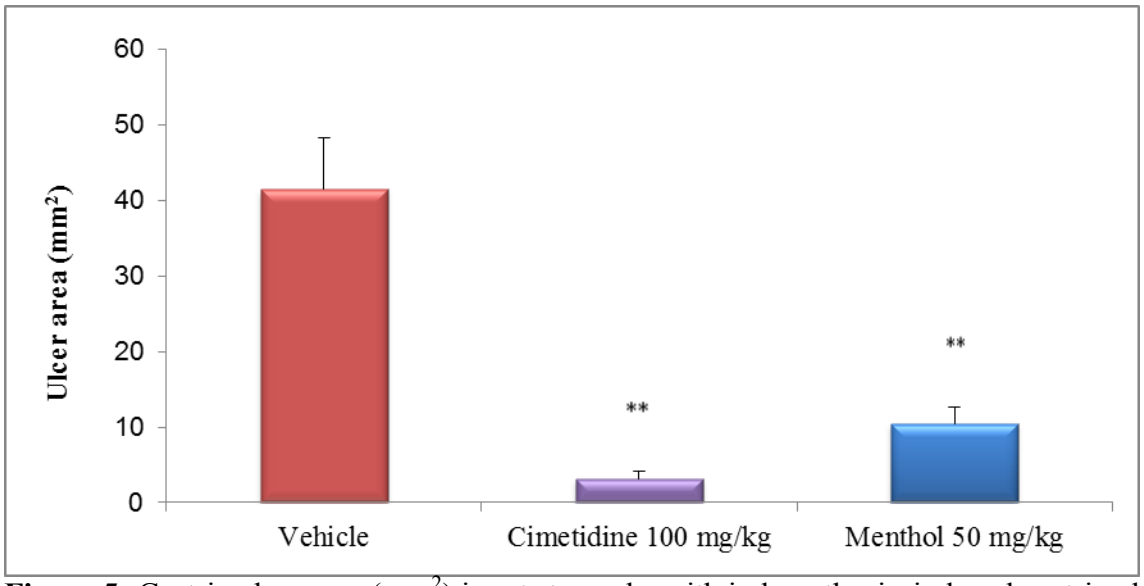

Figure 5: Gastric ulcer area (mm^2) in rat stomachs with indomethacin-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (50 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett´s test, compared to vehicle group, $p<0.01$.

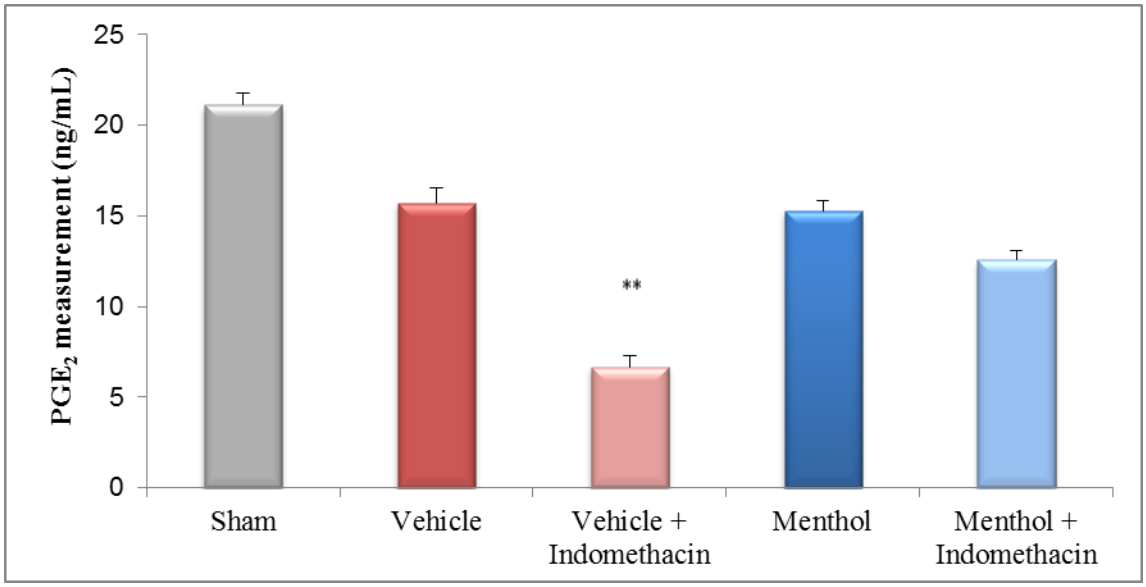

Figure 6: PGE_2 measurement (ng/mL) in the gastric mucosa of rats treated with vehicle or menthol (50 mg/kg) alone or in association with nonselective COX inhibitor (indomethacin). The results are reported as the mean ± SEM. ANOVA followed by Dunnett"s test, compared to sham group, $p<0.01$.

3.9. Effect of menthol on castor oil-induced diarrhea and gastrointestinal motility

ME inhibited evacuation in 36.15% of the treated group compared to the vehicle group ($p<0.01$), indicating an antidiarrheal activity comparable to the effect of the standard drug loperamide hydrochloride (38.50%, table 4). The antidiarrheal effect of ME was connected to an antiperistaltic activity, decreasing the intestinal motility in 56.18% of the treated animals compared to the vehicle group ($p<0.01$, table 5).

Table 4: Evacuation index and % of evacuation inhibition after oral treatment with vehicle, loperamide hydrochloride (3 mg/kg) or menthol (50 mg/kg)

Treatment	Evacuation index	% of evacuation inhibition
Vehicle	13.0 ± 0.84	-
Loperamide 3 mg/Kg	8.0 ± 1.0 **	38.50
Menthol 50 mg/Kg	8.3 ± 0.42 **	36.15

The results are reported as the mean ± SEM. ANOVA followed by Dunnett‟s test, $p<0.01$.

Table 5: Evaluation of intestinal motility ($cm^{1/2}$) after oral treatment with vehicle, loperamide hydrochloride (5 mg/kg) or menthol (50 mg/kg)

Treatment	Intestinal motility	% of motility inhibition
Vehicle	4.15 ± 0.31	-
Loperamide 5 mg/Kg	1.91 ± 0.23 **	53.97
Menthol 50 mg/Kg	1.81 ± 0.32 **	56.38

The results are reported as the mean ± SEM. ANOVA followed by Dunnett‟s test, $p<0.01$.

3.10. Acute toxicity

No signs or symptoms associated with toxicity were observed on the day of oral administration or over the subsequent 14 days. Body weights progressed normally. After the rats were euthanized, there were no alterations in the macroscopic appearance of the kidney or liver or in their weights. There were no alterations in the biochemical parameters analyzed in the rats‟ serum between the groups, indicating that there was no hepatotoxicity or nephrotoxicity associated with the ME oral treatment (table 6).

Table 6: Effect of orally administered menthol (500 mg/kg) on rats‟ body weight, organ weight, and serum biochemical parameters

	Vehicle	**Menthol**
Initial body weight (day 0)	196.70 ± 5.31	190.45 ± 4.54
Final body weight (day 14)	290.60 ± 8.29	272.67 ± 8.11
Kidneys weight	0.15 ± 0.00	0.15 ± 0.00
Liver weight	0.72 ± 0.02	0.70 ± 0.02
AST	105.80 ± 6.24	115.70 ± 7.44
ALT	46.90 ± 0.99	47.90 ± 1.00
Gama-GT	5.36 ± 0.46	5.09 ± 0.58
Alkaline phosphatase	295.00 ± 10.71	277.37 ± 14.30
Creatinine	0.28 ± 0.02	0.30 ± 0.03
Urea	45.30 ± 1.51	42.44 ± 1.20

Body weights are expressed in g. Organ weights are expressed in comparison to the body weight ($g^{1/2}$). AST, ALT, γ-GT and alkaline phosphatase are expressed in U/L, creatinine and urea are expressed in mg/dL. The results are reported as the mean ± SEM. Unpaired t test.

4. DISCUSSION

Gastrointestinal diseases are major public health issues throughout the world and are estimated to affect 70% of the general population (Ouyang & Chen, 2004). Although the exact ulcer etiology is complex and multifactorial, it is generally accepted that an imbalance between acid production and mucosal integrity is one of the main causative factors (AlRashdi et al., 2012). The present study investigated the gastroprotective activity of (-)-menthol (ME) in experimental models of gastric ulcer and its possible mechanisms of action.

The oral administration of ethanol is widely used to induce experimental gastric ulcer because it is easily reproducible and rapidly penetrates into the gastric mucosa. Ethanol causes necrotic lesions of the gastric mucosa in a multifactorial way. Ethanol is known to induce ulcers in the glandular part of stomach due to the secretion of mast cell secretory products and reactive oxygen species (Rao et al., 2004). Ethanol also acts by reducing the secretion of bicarbonate and the production of mucus, thus resulting in an increased flow of Na^+ and K^+, increased pepsin secretion, and a loss of H^+ ions into the lumen (Szabo, 1987; Mahmood et al., 2005), which leads to cell necrosis and ulcer formation. HCl secretion further deepens necrosis and increases tissue injury. Because of these factors, ethanol-induced ulcers can be inhibited by agents that enhance mucosal defensive factors (Morimoto et al., 1991; Sener et al., 2004). In the ethanol-induced gastric ulcer model, ME exhibited a dose-dependent effect and exerted substantial protective action on the gastric mucosa. Considering the multifactorial ways in which ethanol exerts its ulcerogenic action, the possible mechanisms of gastroprotection conferred by ME were investigated as follows.

Several studies have shown the importance of mucus secretion in gastroprotection (Alrashdi et al., 2012; Laloo et al., 2013). The mucus barrier is considered the first line of mucosal defense because it decreases physical damage to the epithelium by ingested foods. It is an important barrier against self-digestion (Wallace, 2008) and acts as an antioxidant,

scavenging free radicals in the mucosa (Repetto & Llesuy, 2002). Ulcerogenic substances cause disruptions in this barrier and allow contact between the gastric juice and epithelial cells, leading to mucosal injury (Darling et al., 2004). Thus, the increased stimulation of mucus production by 2.2-fold over the control group is a relevant part of the hypothesized mechanism of gastric mucosal protection by ME. The increase in mucus secretion was easily evidenced in the photomicrographies of the PAS-stained gastric ulcer.

Endogenous NP-SH compounds help maintain the integrity of the mucus barrier by uniting its subunits by disulfide bridges, preventing the mucus from becoming soluble and easily withdrawn by ulcerogenic agents, including ethanol (Avila et al., 1996). NP-SH compounds also prevent the production of free radicals by ethanol and act as recycling antioxidants (Banerjee et al., 2008). The rats that were pretreated with an inhibitor of NP-SH compounds presented ulcer areas similar to those of the vehicle-treated rats, indicating the importance of an intact NP-SH barrier to the maintenance of the ME gastroprotective effect.

Mucus secretion also can be regulated by nitric oxide (NO) (Brown et al., 1993), an endogenous gaseous mediator synthesized by the enzyme NO-synthase (NOs). NO also plays key roles in enhancing blood flow (Wallace & Miller, 2000), regulating acid secretion and inhibiting neutrophil aggregation (Wallace et al., 1997) and leukocyte adherence to the vascular endothelium (Zanardo et al., 2006). Our results showed that despite the inhibition of NO synthesis by the action of the NO-synthase inhibitor (L-NAME), ME exerted a gastroprotection similar to that observed in the group receiving ME treatment without NO blocking, indicating that maintaining NO synthesis is not crucial to gastroprotection by ME.

In the gastric mucosa, NO interacts with neuropeptides and prostaglandins to maintain mucosal integrity. NO activates guanylyl cyclase to increase cyclic guanosine monophosphate (cGMP) levels and subsequently activates the ATP sensitive potassium channels (K^+_{ATP}). The activation of this NO/cGMP/K^+_{ATP} pathway leads to gastroprotection (Medeiros et al., 2008).

K^+_{ATP} channels mediate gastroprotection by enhancing gastric microcirculation and inhibiting neutrophil activation and the subsequent superoxide production (Campos et al., 2008). Despite this mediation, there was no loss of gastroprotection after the inhibition of NO synthesis or the cGMP blockade, but the blockade of the K^+_{ATP} channels reversed the gastroprotective action of ME, thus indicating the involvement of these channels in the mechanism of action of ME.

K^+_{ATP} channels are not exclusively activated by the NO pathway but can also be activated by PGE_2 activity (Lira et al., 2009). The oral treatment with ME was able to maintain PGE_2 levels, even after the administration of the COX-inhibitor indomethacin, indicating the importance of PGE_2 in the mechanism of action of ME. This result can explain the activation of the K^+_{ATP} channels as well as the cytoprotective activity observed in the indomethacin-induced gastric ulcer. Indomethacin, as a non-steroidal anti-inflammatory drug (NSAID), induces gastric damage mainly by inhibiting prostaglandin production through inhibiting the activity of COX-1 and COX-2 isozymes (Wallace et al., 2000; Takeuchi et al., 2010).

The physiological functions of PGE_2 in the gastrointestinal tract include stimulating bicarbonate and mucus secretion, maintaining mucosal integrity, and inducing a trophic effect in gastric and intestinal mucosa by triggering mitogenic signaling in mucosal cells (Pai et al., 2002). It is also known that PGE_2 presents a strong anti-secretory activity (Peskar & Maricic, 1998), which can, at least in part, explain the anti-secretory effect observed after the oral administration of ME. The H^+ concentration was decreased, but the gastric juice volume was not altered. This effect did not occur in the intra-duodenally treated rats, leading to the conclusion that ME acts via a local rather than a systemic mechanism. In addition to the increasing effect on PGE_2 production, this antisecretory effect may also be due to increased mucus secretion, as the mucus barrier is able to neutralize secreted H^+.

The cellular perturbation caused by a damaging agent such as ethanol disrupts the normal Ca^{2+} homeostasis. Ethanol administration leads to an intracellular Ca^{2+} accumulation, providing an injurious action in the gastric mucosa (Kokoska et al., 1998; Miller et al., 2001). The oral administration of the calcium ion channel blocker verapamil was not able to reverse the ME gastroprotective effect, indicating that the blockade of the calcium ions efflux did not interfere with the protective mechanism and, therefore, that this gastroprotective pathway is not involved in ME activity.

The incidence of gastric ulcers also can be influenced by changes in gastric motility. Natural compounds that affect gastrointestinal motility usually present effects on the ulcer formation (Abdulla et al., 2010). Constipation and diarrhea are common and prevailing disorders that affect the intestinal tract. Constipation is known to affect approximately 27% of the population and is more prevalent in women than in men (Cheng et al., 2009). Diarrhea is the passage of watery stool three or more times in a day or the passage of unformed bowel contents at a daily rate twice that of a person‟s usual rate (Feldman & Pickering, 1981). In developing countries, the death rate due to diarrhea is high (approximately 4 million deaths/year), and it is one of the leading causes of mortality (WHO, 2009). Antidiarrheal therapy is used to achieve increased resistance to flow (e.g., segmental contraction, decreased propulsion and peristalsis), increased mucosal absorption or decreased secretion (Burks, 1991).

Medicinal plants are commonly employed to treat constipation and diarrhea (Gilani & Rahman, 2005). This study also evaluated ME activity in castor oil-induced diarrhea and gastrointestinal motility. Experimentally, castor oil induces diarrhea by increasing the secretion of fluids and electrolytes in the intestinal lumen, thus resulting in fluid accumulation and in an aqueous content that flows rapidly in the small and large intestines (Burks, 1991). In this assay, ME displayed significant activity against the diarrhea induced by castor oil, an

effect that can be compared to the standard drug loperamide. The antidiarrheal effect of ME was accompanied by an antiperistaltic effect.

The acute toxicity test did not show any signs of toxicity or mortality. Behavioral changes such as irritation, restlessness, respiratory distress, abnormal locomotion, and catalepsy were not observed over a period of 14 days. There were no alterations in the dosage of renal and hepatic enzymes or in body weight evolution, revealing that ME has no toxicity when administered orally at a dose of 500 mg/kg, which is ten times higher than the therapeutic dose employed in this study.

5. CONCLUSION

The results described here suggest that menthol presents antiulcer activities against ethanol and indomethacin. The gastroprotective activity of menthol is associated mainly with mucus secretion, which is related to the maintenance of NP-SH compounds, PGE_2 production and K^+_{ATP} channel activation and to an anti-secretory effect. Menthol also presents an antidiarrheal and antiperistaltic effect. No signs of acute toxicity have been associated with the administration of a high dose of menthol. However, further clinical and toxicological studies must be conducted to support the use of menthol as a potential antiulcerogenic drug.

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Capítulo 2

Artigo a ser submetido para

Evidence-Based Complementary and Alternative Medicine (eCAM)

THE GASTROPROTECTIVE EFFECT OF MENTHOL: INVOLVEMENT OF ANTI-APOPTOTIC, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITIES

ABSTRACT

The aim of this research was to investigate the anti-apoptotic, antioxidant and anti-inflammatory activities of menthol (ME) against ethanol-induced gastric ulcers in rats. Rats were orally treated with vehicle, carbenoxolone (100 mg/kg) or ME (50 mg/kg) and then were treated with ethanol to induce gastric ulcers. Histological slices were prepared of the rats‟ stomachs and biochemical analyzes were performed. Immunohistochemical reactions for the cytoprotective HSP-70 and the apoptotic Bax protein were performed. The neutrophils were manually counted. The activity of myeloperoxidase (MPO) was measured. To determine the antioxidant activity, the levels of glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione reductase (GR) and superoxide dismutase (SOD) were measured using ELISA. The levels of the pro-inflammatory cytokines tumor necrosis factor- α (TNF-α) and interleukin-6 (IL-6) and the anti-inflammatory cytokine interleukin-10 (IL-10) were assessed using ELISA kits. The ME-treated group presented 92.21% of gastroprotection when compared to the vehicle-treated rats. An increased immunolabeled area was observed for the HSP-70, and a decreased immunolabeled area was observed for the Bax protein in the ME-treated group. ME treatment induced a decrease in the activity of the MPO and the SOD, whereas the levels of the GSH, the GSH-Px and the GR were increased. There was also a decrease in the levels of the TNF-α and the IL-6 and an increase in the IL-10 levels. In

conclusion, oral treatment with ME displayed a gastroprotective activity through anti-apoptotic, anti-oxidant and anti-inflammatory mechanisms.

Keywords: anti-inflammatory, antioxidant, Bax, gastroprotection, HSP-70, menthol, MPO.

1. INTRODUCTION

Peptic ulcer is a heterogeneous disease of multifactorial etiology. Gastric ulcer, the most common gastric disease, is considered a global health problem, affecting approximately 14.5 million people worldwide (Maity and Chattopadhyay, 2008).

Current treatments for patients suffering from gastric ulcers include antacids, histamine H_2 receptor antagonists and proton pump inhibitors. However, the long-term use of these drugs is responsible for the development of side effects such as hematopoietic disturbances, arrhythmia, and hypersensitivity (Chan & Leung, 2002). Furthermore, their use does not necessarily prevent the recurrence of the disease, as patients can develop tolerances (Takahashi & Katayama, 2010).

The main therapeutic target of these classes of medications is to control gastric acid secretion. However, the concept of management of ulcer disease is fast changing. There is growing evidence that the ideal antiulcer compound should act not only in the gastric secretion, but against multiple targets. In addition to an antisecretory effect, it has to possess antioxidant, anti-apoptotic and anti-inflammatory activities (Maity et al., 2009).

Previous research by our group demonstrated the gastroprotective effect of the cyclic terpene (-)-menthol (ME, unpublished data). The present study was undertaken to investigate the possible gastroprotective anti-apoptotic, antioxidant and anti-inflammatory effects of ME against ethanol-induced gastric ulcer in rats.

2. MATERIAL AND METHODS

2.1. Menthol

(-)-Menthol (catalog #63660) was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Animals

Male Wistar rats (200–250 g) from the Central Animal House of UNESP were fed a certified diet with free access to tap water under standard light–dark cycles (12 h dark–12 h light), humidity (60 ± 1%) and temperature (21 ± 2^{o}C). Prior to experimentation, all rats were fasted for 16 hours and housed in cages with raised floors of a wide mesh to prevent coprophagy. All experimental protocols followed the recommendations of the Canadian Council on Animal Care and were approved by the UNESP Institutional Animal Care and Use Committee.

2.3. Ethanol-induced gastric ulcers

Male Wistar rats that had been fasted for 24 hours were distributed into three groups (n=7). Animals were then orally dosed with the vehicle (10 mL/kg), carbenoxolone (100 mg/kg) or ME (50 mg/kg). The dose of the ME was determined by a previous dose-effect assay in which the dose of 50 mg/kg was shown to be the lowest effective dose (unpublished data). After 1 hour, the animals received an oral dose of 1 mL of absolute ethanol. One hour after ethanol treatment, the rats were euthanized, and their stomachs were removed (Robert et al., 1979). The stomachs were then opened along the greater curvature and washed. The flattened stomach samples were scanned, and the ulcer area (mm^2) was measured using AVSoft BioView software.

After the scanning, stomach samples were scraped and frozen at −80°C until biochemical analyses were performed. Additionally, histological slides were prepared from intact samples.

2.3.1. Histological analysis

Samples of the stomach of each rat were fixed in Alfac solution (85% alcohol 80, 10% formalin and 5% acetic acid) and processed in a paraffin tissue processing machine. Sections of the stomach were cut to a thickness of 5 μm and stained with hematoxylin and eosin (HE) for histological evaluation. The HE-stained slides were submitted to a count of the neutrophils. Slides that were not HE-stained were submitted to immunohistochemical analysis.

2.3.2. Immunohistochemical analysis

Tissue section slides were deparaffinized, rehydrated and immunostained with peroxidase. Non-specific reactions were blocked with H_2O_2 and goat serum prior to incubation with the specific antibody. After rinsing in a phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.4), the sections were incubated in a secondary antibody (ABC kit, Easypath Erviegas). The sections were then washed in the PBS buffer, the ABC complex was applied, and the reaction was carried out in a DAB (3,3‟-diaminobenzidine tetrahydrochloride) solution containing 0.01% H_2O_2 in PBS buffer. After immunostaining, the sections were lightly counterstained with hematoxylin and the immunolabeled cells were observed and photographed under a Leica microscope using Leica QWin Software (Leica, England). In the control reaction, the slides were processed without the primary or the secondary antibody. Positive findings of the immunohistochemical staining were seen as brown stains under light microscopy. The slides were stained with antibodies for heat-shock protein 70 (HSP-70,

dilution 1:500) and Bax (dilution 1:200) (Santa Cruz Biotechnology). Ten fields in each slide were photographed and the marked areas (μm^2) measured using AVSoft BioView software.

2.4. Preparation of samples for biochemical assays

Immediately after the euthanasia, the mucosa of each stomach was gently scraped using two glass slices, homogenized in a phosphate buffer (0.1 M, pH 7.4), and frozen at −80°C until biochemically assayed. The protein concentration of the samples was determined by the method described by Bradford (1976).

2.4.1. Effect of menthol in the myeloperoxidase activity (MPO)

The MPO activity in the gastric mucosa was measured to evaluate neutrophil accumulation. The samples were centrifuged at 3,000 g for 15 minutes at 4°C. Aliquots of the supernatant were then mixed with a reaction buffer of 50 mM phosphate buffer containing 0.005% H_2O_2 and 1.25 mg/mL O-dianisidine dihydrochloride, at pH 6.8, and measured at 460 nm (Krawisz et al., 1984).

Assays of antioxidant activity

2.4.2. Determination of total glutathione levels (GSH)

The GSH levels in the gastric tissue were determined with the Ellman‟s reaction using 5‟5‟-dithio-bis-2-nitrobenzoic acid (DTNB) (Faure & Lafond, 1995). The intensity of the yellow color was read using spectrophotometry at 412 nm.

2.4.3. Glutathione peroxidase activity (GSH-Px)

The GPx activity was measured by monitoring the decrease in absorbance every minute for 10 minutes at 365 nm induced by 0.25 mM H_2O_2 in the presence of 10 mM of

reduced glutathione, 4 mM of NADPH, and 1 U enzymatic activity of GSH-Px (Yoshikawa et al., 1993).

2.4.4. Glutathione reductase activity (GR)

The GR activity was measured by monitoring the decrease in absorbance at 340 nm induced by oxidized glutathione in the presence of NADPH in phosphate buffer, pH 7.8. The absorbance was read every minute for 10 minutes (Carlberg & Mannervick, 1985).

2.4.5. Superoxide dismutase activity (SOD)

The SOD activity was analyzed by the reduction of nitroblue tetrazolium using a xanthine-xanthine oxidase system (superoxide generation). The absorbance was read every minute for 10 minutes at 560 nm (Winterbourn, 1975).

Assays of anti-inflammatory activity

2.4.6. Determination of the gastric mucosal levels of TNF-α, IL-6 and IL-10

The tissue homogenate was centrifuged and the cytokines were detected in the supernatant with an enzyme-linked immunosorbent assay (ELISA) using commercial kits for TNF-α, IL-6 and IL-10 (BioLegend, catalog numbers 438204, 431304 and 431404, respectively).

2.5. Statistical analysis

The results were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett‟s test and compared to the vehicle group. The results are presented as the mean ± the standard error of the mean (SEM). All analyses were performed using GraphPad InStat or Prism software. A value of $p<0.05$ was considered significant.

3. RESULTS

3.1. Ethanol-induced gastric ulcers

The vehicle-treated group presented the characteristic necrotic bands in the gastric mucosa, with an average ulcer area of 389.79 ± 6.46 mm^2. The carbenoxolone-treated group presented an average ulcer area of 66.82 ± 23.98 mm^2, representing 82.86% of gastroprotection. The ME-treated group presented a significant decrease ($p<0.01$) in the average ulcer area (30.34 ± 8.04 mm^2), with a gastroprotection of 92.21% (figure 1).

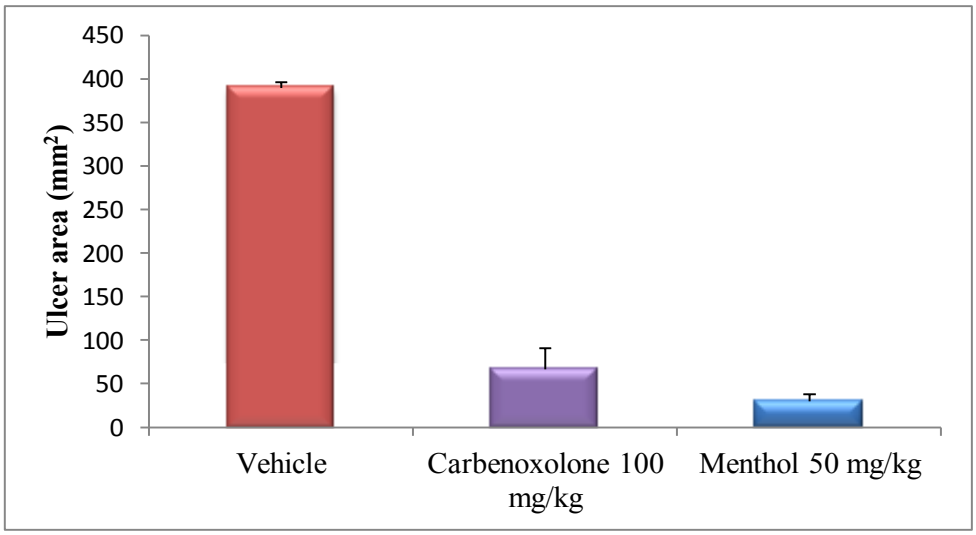

Figure 1: Gastric ulcer area (mm^2) of rat stomachs with ethanol-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (50 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett´s test, $p<0.01$.

3.1.1. Histological analysis

The neutrophil infiltration in the gastric submucosa was significantly decreased in the carbenoxolone- and ME-treated groups ($p<0.01$) compared to the vehicle-treated group (table 1).

3.1.2. Immunohistochemical analysis

The treatment with ME caused an over-expression of the HSP-70 in comparison to the vehicle-treated group, confirmed by the analyses of the immunolabeled area for this protein. Treatment with ME also reduced the expression of the apoptotic protein Bax, with a decrease in the immunolabeled area. These results are presented in the table 1 and figure 2.

Table 1: Neutrophils count and immunolabeled area (μm^2) for HSP-70 and Bax in rats stomachs submitted to ethanol-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (50 mg/kg)

	Vehicle	**Carbenoxolone**	**Menthol**
Neutrophils count	23.40 ± 0.62	13.60 ± 1.65 **	9.60 ± 1.68 **
Immunolabeled area for HSP-70	659.32 ± 88.47	6547.01 ± 984.22***	9620.86 ± 544.65***
Immunolabeled area for Bax	11443.28 ± 486.13	821.36 ± 95.80**	199.36 ± 26.84***

The results are reported as the mean ± SEM. ANOVA followed by Dunnett´s test, **p<0.01 or ***p<0.001.

Figure 2: Photomicrography of immunohistochemical analysis of expression of HSP-70 (A, B, C) and Bax protein (D, E, F) in the rat stomachs submitted to ethanol-induced gastric ulcers after treatment with vehicle (A, D), carbenoxolone 100 mg/kg (B, E) or menthol 50 mg/kg (C, F). The * in the brown stains indicate in situ expression for the proteins. Notice the upregulation of HSP-70 expression and downregulation of Bax expression in the menthol-treated group.

3.2. Effect of menthol in the MPO activity

The ME treatment induced a significant decrease in the activity of the MPO enzyme in comparison to that of the vehicle-treated group (figure 3), which confirmed the infiltration and activation of neutrophils in the gastric mucosa induced by the ethanol administration.

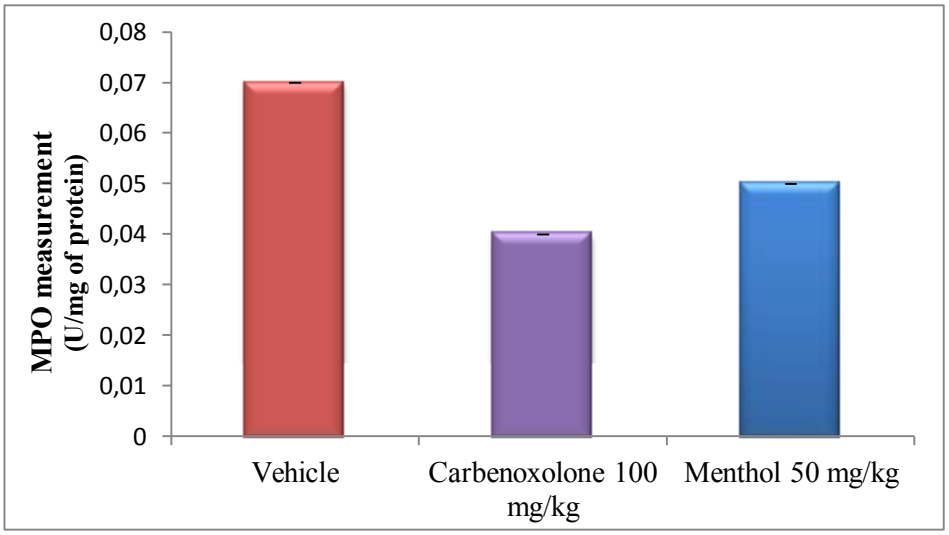

Figure 3: Myeloperoxidase (MPO) activity (U/mg of protein) in rat stomachs with ethanol-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (50 mg/kg). The results are reported as the mean ± SEM. ANOVA followed by Dunnett´s test, $p<0.01$.

3.3. Antioxidant assays

The ME presented an antioxidant activity, demonstrated by increases in the activities of the antioxidant compound GSH and the enzymes GSH-Px and GR in comparison to those of vehicle-treated group. However, the level of the SOD enzyme in the gastric tissue was decreased in the ME-treated group (table 2).

Table 2: Effect of menthol (50 mg/kg) on the levels of antioxidant enzymes and compound in rat stomachs with ethanol-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (50 mg/kg)

	Vehicle	**Carbenoxolone**	**Menthol**
GSH	27.62 ± 2.85	50.78 ± 6.82 *	60.87 ± 6.00 **
GPx	96.07 ± 7.21	108.30 ± 14.67	171.30 ± 13.15 ***
GR	32.64 ± 1.99	40.95 ± 3.12	48.80 ± 3.34 **
SOD	3.33 ± 0.50	1.65 ± 0.38 *	1.97 ± 0.26 *
MPO	0.07 ± 0.00	0.04 ± 0.00 **	0.05 ± 0.00 **

GSH level is expressed in nmol/mg of protein; GPx and GR are expressed in nmol/min/ mg of protein; SOD and MPO are expressed in U/mg of protein. The results are reported as the mean ± SEM. ANOVA followed by Dunnett‟s test, *p<0.05, **p<0.01, ***p<0.001.

3.4. Anti-inflammatory activity

The ME demonstrated an immunomodulatory effect, modulating the levels of cytokines in the gastric tissue, diminishing the levels of the pro-inflammatory cytokines TNF-α and IL-6 and augmenting the level of the anti-inflammatory IL-10 (table 3).

Table 3: Effect of menthol (50 mg/kg) on the levels of TNF-α, IL-6 and IL-10 cytokines in rat stomachs with ethanol-induced gastric ulcers after treatment with vehicle, carbenoxolone (100 mg/kg) or menthol (50 mg/kg).

	Vehicle	**Carbenoxolone**	**Menthol**
TNF-α	2076.00 ± 122.80	1307.00 ± 283.30*	144.70 ± 59.04***
IL-6	1239.00 ± 83.47	871.30 ± 173.10	143.50 ± 50.95***
IL-10	1718.00 ± 344.10	4772.00 ± 581.50***	3776.00 ± 122.40**

The results are expressed as pg/mg of protein and reported as the mean ± SEM. ANOVA followed by Dunnett‟s test, *p<0.05, **p<0.01, ***p<0.001.

4. DISCUSSION

Ethanol is commonly used for inducing ulcers in experimental rats, leading to intense gastric mucosal damage, directly and indirectly through such mediators as oxygen-derived free radicals and cytokines (Abdel-Salam et al., 2001). In this study, the HSP-70-inducing, anti-apoptotic, antioxidant and anti-inflammatory activities of menthol (ME) in an ethanol-induced gastric ulcer model were investigated in rats. The dose of the ME used in this study (50 mg/kg) was based on previous results reported by our group on the gastroprotective effect of ME (unpublished data). It was observed that the ME treatment presented 92.21% of gastroprotection in comparison to vehicle-treated group, a gastroprotection that is similar to the effect of the standard drug carbenoxolone (82.86%). The mechanisms responsible for the gastroprotective effect were investigated.

HSPs (heat-shock proteins) are a type of protective protein involved in diverse biological activities, including apoptosis, carcinogenesis, and protection of various cells from cytotoxic damage. HSP-70 is a molecular chaperone that is rapidly induced by stresses such as heat, oxidative stress, and drug exposure (Hartl & Hayer-Hartl, 2002). Therefore, drugs or treatments that induce HSP-70 expression may positively contribute to gastric mucosal defense and cytoprotection (Yeo et al., 2004; Hirata et al., 2009).

In addition to its cytoprotective effect, HSP-70 has anti-apoptotic activity (Beere, 2004; Konturek et al., 2010) and can decrease oxidative stress and cell injury (Hirata et al., 2009). Standard antiulcer drugs such as omeprazole are known to possess antioxidant and anti-apoptotic potential (Itoh & Tashima, 1991), demonstrating the importance of this mechanism in gastroprotection. The HSP-70-induction is part of the gastroprotective mechanism of ME. Furthermore, the inhibition of apoptosis observed through inhibition of Bax expression in the gastric mucosa of rats treated with ME can be partially explained by ME‟s HSP-70-inducing effect. It has been suggested that interaction in the expression of the

HSP-70 and the proapoptotic Bax genes may occur under stress conditions, with suppression of Bax activation in cells with high HSP-70 levels (Stankiewicz et al., 2005).

A decrease in the number of the neutrophils in the stomach submucosa of ME-treated rats is also important to clarify the gastroprotective action mechanism. There is growing evidence that the migration and activation of neutrophils is the major source of reactive oxygen species (ROS), which are responsible for tissue injury in many cases. Ethanol administration promotes oxidative stress by increased formation of ROS and depletion of oxidative defenses in the cell in a process triggered by the neutrophil activation, causing a sequential ROS-mediated induction of lipid peroxidation and protein oxidation. Recent studies have linked the genesis of ethanol-induced gastric ulcers to the number of infiltrated neutrophils (Rocha et al., 2011).

The myeloperoxidase (MPO) enzyme is the main marker of neutrophil infiltration in ulcerogenic lesions. This enzyme is found within the neutrophils and catalyzes the oxidation of the chloride ion (Cl^-) by hydrogen peroxide (H_2O_2) to form hypochlorous acid (HClO), which is toxic to pathogenic microorganisms but is also harmful to host tissues (Halliwell and Gutteridge, 2006). This process is responsible for the generation of free radicals, resulting in an acute inflammation in the gastric tissue (Fialkow et al., 2007). The gastroprotection induced by ME can in part be explained by the inhibition of neutrophil infiltration and subsequent MPO generation.

Neutrophils also produce the superoxide radical anion (O_2^-), the product of the reaction of oxygen molecules and electrons from the transport chain in mitochondria. The superoxide radical anion reacts with cellular lipids, leading to the formation of lipid peroxides. The cells of the gastrointestinal tract have an antioxidant defense system capable of preventing the cytotoxicity of ROS through mechanisms that involve the action of enzymes and compounds with the potential to scavenge free radicals and prevent their destructive

action. The major antioxidative enzyme is superoxide dismutase (SOD) (Brzozowski et al., 2001). SOD catalyzes the dismutation of O_2^- into less noxious hydrogen peroxide (H_2O_2), which is further degraded by catalase or glutathione peroxidase (GSH-Px). Catalase is an enzyme that accelerates the degradation of H_2O_2 into water and oxygen in lysosomes (Halliwell, 1990). The second pathway of H_2O_2 metabolism depends on the activity of GSH-Px and cooperating glutathione reductase (GR) in mitochondria.

The measurement of the SOD activity demonstrated a decrease in the stomachs of rats treated with ME or in positive control. We propose that this finding might result from the consumption of the SOD in the decomposition of the O_2^- generated by lipid peroxidation, because a high SOD activity in vehicle-treated rats indicates increased production of O_2^- (Fridovich, 1986). However, increases in the amounts of the GSH-Px and GR enzymes and the antioxidant compound glutathione (GSH), associated with the inhibition of MPO production, confirms an antioxidant activity of the ME in the gastric ulcer and supports an important role for oxidative stress in the pathogenesis of ethanol-induced gastric ulcer. There are several reports in the literature indicating that the administration of potential antioxidant natural products can prevent the gastric damage caused by the ethanol action (Ismail et al., 2012; AlRasdi et al., 2012; Ahmad et al., 2013).

In generating ROS, the administration of absolute ethanol provokes an inflammatory response that is the result of a complex chain of events involving the immune response, which releases a great number of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Salga et al., 2012). Cytokines play a decisive role in the communication between cells and are key players in physiological and pathological processes (Sabat, 2010).

In general, inflammation is considered a harmful process that should be avoided. However, in the gastrointestinal tract, an adequate inflammatory response is a key component

of mucosal defense against exogenous and endogenous factors. The inflammatory response is coordinated by an array of mediators that are released from the epithelium and from cells of the lamina propria (mast cells, lymphocytes, fibroblasts) (Martin & Wallace, 2006).

TNF-α is a pro-inflammatory cytokine increasingly secreted by macrophages during the gastric ulcer induction (Hamaguchi et al., 2001). It is a potent stimulator of neutrophil infiltration into gastric mucosa (Wei et al., 2003). Suppression of TNF-α production and neutrophil infiltration are closely associated (Taha et al., 2012). IL-6 is a pleiotropic cytokine that plays a crucial role in acute inflammation and immune regulation (Kishimoto, 2005). Elevated levels of IL-6 activate neutrophils, lymphocytes and monocytes/macrophages at the inflammatory site, triggering oxidative pathways responsible for local tissue damage in gastric ulcer disease (Mei et al., 2012).

It was suggested that the pro-inflammatory cytokines TNF-α and IL-6, which play key roles in the inflammation process, are important in the regulation of the severity of gastric ulcers (Augusto et al., 2007). TNF-α and IL-6 mRNA levels in gastric mucosa correlate with the level of gastric mucosal inflammation (Mei et al., 2012). The secretion of both cytokines enhances the effects of oxidative stress by inducing mitochondrial ROS generation and cytotoxicity (Wassmann et al., 2004).

IL-10 is one of the most important anti-inflammatory and immunosuppressive cytokines (Sabat et al., 2010). IL-10 suppresses the inflammatory activities by influencing important functions of the monocytes/macrophages: the antigen presentation, the release of immune mediators, and the phagocytosis. Furthermore, IL-10 inhibits the production of TNF-α (Basak & Hoffmann, 2008; Kruglov et al., 2008). The primary sources of IL-10 are monocytes, macrophages, and various T-cell subsets; minor sources include dendritic cells, mast cells, and neutrophilic and eosinophilic granulocytes (Sabat et al., 2010). The results reported here demonstrated that the treatment with ME induced a decrease in the levels of

pro-inflammatory mediators TNF-α and IL-6 and increased the level of the anti-inflammatory cytokine IL-10, demonstrating an anti-inflammatory activity of ME.

5. CONCLUSION

The treatment of ethanol-induced gastric ulcer in rats with ME demonstrated that ME exerts gastroprotection through an HSP-70-inducing effect, which leads to an anti-apoptotic effect through inhibition of Bax production. Furthermore, ME induces a decrease in the neutrophil migration and activation as demonstrated by the decrease in MPO activity, which results in an antioxidant activity, increasing the activities of GSH-Px, GR and the levels of GSH and decreasing SOD activity. ME also induced an immunomodulatory and anti-inflammatory activity, as the treatment decreased the levels of the pro-inflammatory cytokines TNF-α and IL-6 and augmented the levels of the anti-inflammatory cytokine IL-10.

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Conclusões

CONCLUSÕES

Os resultados expostos neste trabalho sugerem que o mentol apresenta atividade gastroprotetora contra os agentes lesivos etanol e indometacina. O mecanismo de ação gastroprotetor do mentol está associado principalmente à secreção de muco pelas glândulas gástricas e a produção de PGE_2, que culmina num efeito antissecretório. Além disso, o tratamento oral com mentol estimula a produção de proteínas *heat-shock* e inibe a produção de proteínas pró-apoptóticas, além de inibir a infiltração de neutrófilos ativados que desencadeariam reações oxidativas e inflamatórias na mucosa gástrica, sobre as quais o mentol também apresenta efeito inibitório. O efeito gastroprotetor do mentol também envolve a diminuição da motilidade intestinal, resultando num efeito antidiarreico. Além disso, o mentol não induz efeitos tóxicos para os rins ou fígado após administração oral de uma alta dose em ratos.