UNIVERSIDADE FEDERAL DE PERNAMBUCO
CENTRO DE CIÊNCIAS DA SAÚDE
DEPARTAMENTO DE CIÊNCIAS FARMACÊUTICAS
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

EFEITO GASTROPROTETOR E SEGURANÇA DE USO DO ÓLEO ESSENCIAL DAS FOLHAS DE *Hyptis martiusii* Benth. (LAMIACEAE) E DO MONOTERPENO 1,8-CINEOL

GERMANA FREIRE ROCHA CALDAS

Recife
2013

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Orientador: Prof. Dr. Almir Gonçalves Wanderley

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Profa. Dra. Ana Cristina Lima Leite

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“Cada escolha, por menor que seja, é uma forma de semente que lançamos sobre o canteiro que somos. Um dia, tudo o que agora silenciosamente plantamos, ou deixamos plantar em nós, será plantação que poderá ser vista de longe”.

Padre Fábio de Melo

RESUMO

Hyptis martiusii Benth. (Lamiaceae) cresce em abundância no nordeste brasileiro, é utilizada pela população no tratamento de distúrbios gastrointestinais. Este estudo caracterizou os constituintes químicos do óleo essencial das folhas de *Hyptis martiusii* (OEHM), investigou o efeito gastroprotetor do OEHM e do monoterpeno 1,8-cineol (CIN), um dos compostos majoritários do óleo, e os mecanismos de ação envolvidos. Adicionalmente, descreveu o perfil de segurança pré-clínico de ambos. OEHM (100, 200 e 400 mg/kg) e CIN (50, 100 e 200 mg/kg) inibiram as lesões gástricas por etanol, etanol/HCl ou indometacina, inibiram o esvaziamento gástrico, mas não influenciaram o trânsito intestinal. OEHM (400 mg/kg) apresentou atividade antissecretória basal e interferiu nos parâmetros da secreção gástrica estimulada por histamina e pentagastrina. CIN (100 mg/kg) reduziu o volume da secreção basal, mas não interferiu na secreção ácida estimulada. O efeito gastroprotetor do OEHM e CIN envolve a participação de compostos sulfidrílicos. OEHM e CIN não apresentam atividade antioxidante *in vitro*, mas promoveram aumento nos níveis de muco, reduziram os níveis de peroxidação lipídica e preveniram a depleção dos grupamentos GSH na mucosa gástrica. OEHM e CIN aceleraram a cicatrização da úlcera crônica, reduzindo a área lesada em 70 e 43%, e também foram capazes de aumentar a expressão de PCNA. Na toxicidade aguda, OEHM não induziu nenhum sinal de toxicidade ou morte em camundongos. O tratamento oral com OEHM (100 e 500 mg/kg) por 30 dias não induziu nenhum sinal de toxicidade ou morte. Houve alterações pontuais nos valores de VCM e albumina. A análise microscópica dos orgãos apontou a presença de gordura no fígado, discreto infiltrado linfocitário no rim e pulmão e discreto aumento da atividade fagocitária no baço. Para CIN, na toxicidade aguda verificou-se que $1500<DL_{50}<1750$ mg/kg. Na toxicidade de doses repetidas, CIN (100, 500 e 1000 mg/kg) não induziu nenhum sinal de toxicidade ou morte, mas promoveu alterações no ganho de massa corporal e no consumo de água e ração em ratos. Houve redução dos valores de CHCM, VPM e fosfatase alcalina, bem como aumento de plaquetas, VCM e ureia. Nos orgãos, foi observado aumento da massa absoluta e relativa do fígado, discreto infiltrado linfocitário e eosinofílico no pulmão, fígado e útero, e aumento do espaço glomerular nos rins. Na toxicidade reprodutiva, CIN (250, 500 e 1000 mg/kg) não produziu mortes em ratas prenhes tratadas durante as fases de pré-implantação ou organogênese, mas promoveu uma redução da massa corporal. Observou-se a presença de fetos mortos em ratas tratadas com as maiores doses, indicando uma possível toxicidade materna e fetal. Os resultados indicam que OEHM possui efeito gastroprotetor que possivelmente é mediado por mecanismos citoprotetores e antioxidantes e pela sua ação antissecretória. Adicionalmente, demonstrou o efeito gastroprotetor do CIN, comprovando assim sua correlação com o OEHM. A administração de doses repetidas do OEHM e CIN induziu alterações pontuais de toxicidade que apresentam baixa relevância clínica, visto que ocorreram de modo não-generalizado. É importante investigar em maior detalhe o efeito do CIN sobre a gestação e no desenvolvimento dos fetos.

Palavras-chave: Lamiaceae. Óleos essenciais. Úlcera péptica. Toxicidade.

ABSTRACT

Hyptis martiusii Benth. (Lamiaceae) grows in abundance in the northeastern of Brazil, being used by people to treat gastric disorders. This study characterized the chemical constituents of the essential oil from the leaves of *Hyptis martiusii* (EOHM), investigated gastroprotective effect of the EOHM and monoterpene 1,8-cineole (CIN), one of the major compounds of oil, and the mechanisms of action involved. Additionally, described the preclinical safety profile of both. EOHM (100, 200 and 400 mg/kg) and CIN (50, 100 and 200 mg/kg) inhibited gastric lesions by ethanol, ethanol/HCl or indomethacin, inhibited gastric emptying, but did not affect intestinal transit. EOHM (400 mg/kg) showed basal antisecretory activity and interfered in the parameters of gastric secretion stimulated by histamine and pentagastrin. CIN reduced the volume of basal secretion but did not affect the stimulated acid secretion. The gastroprotective effect of EOHM and CIN involves the participation of sulfhydryl compounds. EOHM and CIN did not present *in vitro* antioxidant activity, but caused increase in the levels of mucus, reduced levels of lipid peroxidation and prevented the depletion of GSH groups in the gastric mucosa. EOHM and CIN accelerated the healing of chronic ulcer, reducing the lesion area by 70 and 43%, and also were able to increase the PCNA expression. In acute toxicity, EOHM did not induce any signs of toxicity or death in mice. Oral treatment with EOHM (100 and 500 mg/kg) for 30 days did not induce any signs of toxicity or death. There were occasional changes in values of VCM and albumin. Microscopic analysis of the organs showed the presence of fatty liver, discrete lymphocytic infiltrate in the kidney and lung, and discrete increase in phagocytic activity in the spleen. For CIN, the acute toxicity in mice was found used that 1500<LD50<1750 mg/kg. In repeated-doses toxicity, CIN (100, 500 and 1000 mg/kg) did not induce any signs of toxicity or death, but promoted changes in body weight gain and food and water intake in rats. There was decreased levels of MCHC, VPM and alkaline phosphatase, as well as increased platelet, VCM and urea. In the organs, it was observed increase mass absolute and relative liver, discrete lymphocytic and eosinophilic infiltrate in the lung and liver, increased glomerular space in the kidney and moderate eosinophilic and lymphocytic infiltrate in the uterus. In reproductive toxicity, CIN (250, 500 and 1000 mg/kg) produced no deaths in pregnant rats treated during the pre-implantation or organogenesis periods. It was observed the presence of dead fetuses in rats treated with higher doses, indicating a possible maternal and fetal toxicity. The results indicate that EOHM has gastroprotective effect that is possibly mediated by cytoprotective and antioxidant mechanisms and their antissecretory activity. Additionally, it was demonstrated gastroprotective effect of CIN, thus proving its correlation with EOHM. The administration of repeated-doses of EOHM and CIN induced occasional toxic changes that exhibit low clinical relevance, since it occurred in a non-generalized fashion. It is important to investigate in more detail the effect of CIN on pregnancy and the development of fetuses.

Keywords: Lamiaceae. Essentials oils. Peptic ulcer. Toxicity.

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1. INTRODUÇÃO

O uso da fitoterapia vem crescendo no Brasil, na ordem de 10 a 15% ao ano e os fitoterápicos podem alcançar a mesma eficácia dos medicamentos sintéticos com redução dos efeitos colaterais (RAMOS et al., 2005). Dos medicamentos sintéticos disponíveis, a população consome apenas 37%, dependendo quase que exclusivamente de medicamentos de origem natural (STANGARLIN et al., 1999; FUNARI; FERRO, 2005). Estima-se que 50% do total dos fármacos empregados na terapêutica utilizados mundialmente na clínica sejam derivados de produtos naturais e que 25% das drogas descobertas em estudos com produtos naturais sejam oriundas de plantas superiores (GURIB-FAKIM, 2006).

Segundo a Organização Mundial de Saúde (OMS), aproximadamente 85% da população dos países em desenvolvimento fazem uso de plantas medicinais para tratarem de problemas relacionados à atenção primária da saúde. No Brasil, mesmo não se tendo exatidão do número de pessoas que fazem uso de plantas para este fim, sua utilização como recurso terapêutico é uma tendência e inclui desde o consumo da planta fresca e seus derivados, como também de medicamentos fitoterápicos (CARVALHO et al., 2007).

Em 2008, o governo federal instituiu um grupo de trabalho interministerial para elaboração do Programa Nacional de Plantas Medicinais e Fitoterápicos que visa garantir o acesso seguro e uso racional de plantas medicinais e fitoterápicos no País, ao desenvolvimento de tecnologias e inovações, assim como ao fortalecimento das cadeias e dos arranjos produtivos, ao uso sustentável da biodiversidade brasileira e ao desenvolvimento do complexo produtivo da saúde. Essas ações transversais de melhoria do acesso da população a plantas medicinais estão em consonância com as políticas do Sistema Único de Saúde (MINISTÉRIO DA SAÚDE, 2009).

Graves problemas de saúde pública são observados pela assistência farmacêutica deficiente. A exemplo estão as lesões gastrintestinais, que vêm se tornando um dos principais focos de investigações experimentais e clínicas, adquirindo ênfase comercial para desenvolvimento de novos agentes terapêuticos (WHITTLE, 2004). A úlcera péptica constitui uma das doenças mais comuns do trato gastrointestinal, de alta incidência clínica e de recidivas. A terapêutica aponta três abordagens principais para o tratamento da úlcera péptica: 1) inibição da secreção ácida gástrica por inibidores da bomba de prótons, 2) inibição da secreção ácida gástrica por antagonistas de receptores histaminérgicos do tipo H_2 ou 3) combinação da terapia antissecretória com a antibioticoterapia para a erradicação da bactéria *Helicobacter pylori*, as quais têm sido associadas a um aumento na taxa de cura dos casos de

úlceras (ORLANDO; LENARD; ORLANDO, 2007). No entanto, além do tratamento apresentar um alto custo dificultando o uso por populações de baixa renda; o uso prolongado desses fármacos provoca muitos efeitos adversos, quase sempre culminando na falta de adesão ao tratamento (TOMA et al., 2002). Dessa forma, o desenvolvimento de novos fármacos a partir de compostos obtidos de plantas com potencial atividade antiulcerogênica pode possibilitar um conhecimento mais detalhado acerca dos mecanismos desencadeadores da úlcera, assim como alternativas de controle no processo de reparação das mesmas.

Vários estudos têm demonstrado experimentalmente que um grande número de óleos essenciais de plantas medicinais e aromáticas e seus compostos isolados possuem propriedades gastroprotetoras e de cicatrização de úlceras (ROZZA; PELLIZZON, 2013). A espécie *Hyptis martiusii* Benth., popularmente como cidreira-do-mato ou cidreira-brava, caracteriza-se por ser potencialmente fornecedora de óleos essenciais. Estudo de Araújo et al. (2003), demonstrou que a composição química do óleo essencial das folhas frescas é constituída por mono e sesquiterpenos, e tem como componentes majoritários o 1,8-cineol (24,3%), δ-3-careno (22,5%) e biciclogermacreno (6,3%). O monoterpeno 1,8-cineol, tambem denominado cineol ou eucaliptol, é um composto orgânico natural, encontrado como composto majoritário no óleo essencial de diferentes espécies dos gêneros *Eucalyptus*, *Psidium, Croton, Hyptis, Pectis,* dentre outras (ANDRADE-NETO et al., 1994; ARAUJO et al., 2003; VILELA et al., 2009; HUSSAIN et al., 2011) dentre outras.

Poucos relatos sobre as atividades biológicas e farmacológicas de *Hyptis martiusii* foram descritos na literatura. Na etnofarmacologia, a infusão ou decocção das folhas de *Hyptis martiusii* é usada no tratamento de doenças intestinais e estomáquicas, contudo, nenhum relato sobre a atividade antiulcerogênica do óleo essencial da espécie foi encontrado. Este trabalho investigou o efeito gastroprotetor do óleo essencial das folhas de *Hyptis martiusii* e do seu composto majóritário o 1,8-cineol, em modelos de lesão gástrica induzida por diferentes agentes e adicionalmente descreveu o perfil de segurança de uso por meio de testes toxicológicos pré-clínicos, visando dar suporte a futuros testes clínicos.

2. Revisão Bibliográfica

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2.1 Considerações sobre o trato gastrintestinal

O epitélio gástrico constitui uma estrutura complexa dotada de glândulas gástricas as quais contém uma variedade de tipos celulares e de neurônios. Anatomicamente, o estômago divide-se em três áreas topográficas (fundo, corpo e antro) e em duas áreas funcionais (glandular oxíntica e pilórica/antral). A área glandular oxíntica compreende 80% do órgão (fundo e corpo), e é constituida por células parietais/oxínticas produtoras de ácido clorídrico, células principais, células produtoras de somatostatina (células D) e do tipo enterocromafins (ECL). A área glandular pilórica ou antral compreende 20% do órgão (antro), contém os mesmos tipos celulares presentes nas glândulas oxínticas, exceto as células parietais. Em seu lugar encontram-se células G produtoras de gastrina (SCHUMBERT; PEURA, 2008).

As principais células responsáveis pela secreção de ácido da mucosa gástrica são as células parietais, cuja regulação é exercida por mecanismos neurais, parácrinos e hormonais em nível central e periférico (KONTUREK et al., 2005). Os principais estímulos da secreção ácida são a histamina (parácrinos), acetilcolina (neurócrinos) e a gastrina (endócrinos) que interagem em receptores específicos, presentes na membrana basolateral das células parietais onde estão acoplados a diferentes proteínas G envolvidas em duas vias importantes de transdução de sinal: adenilato ciclase e do cálcio intracelular (SCHUMBERT; MAKHLOUF, 1992).

A acetilcolina é lançada dos neurônios intramurais e se liga a receptores muscarínicos M_3, aumentando o cálcio intracelular e indiretamente estimulando a liberação de histamina pelas células ECL no fundo gástrico e a liberação de gastrina pelas células G do antro gástrico. A gastrina liga-se a receptores CCK_2 que ativam a fosfolipase C induzindo a liberação de cálcio intracelular, e também estimulam a célula parietal indiretamente, através da liberação de histamina a partir de células ECL (BRUNTON et al., 2006). A histamina liga-se aos receptores H_2 que ativam a adenilato ciclase e geram AMPc. A ativação das vias que dependem do cálcio intracelular pela gastrina e acetilcolina e/ou de AMPc pela histamina ativam diversas quinases e esse processo culmina com a translocação da bomba de prótons (H^+ K^+- ATPase) das membranas tubulovesiculares nas células parietais, e dos canais para cloro, para a superfície celular apical, resultando na secreção ácida do estômago (SCHUBERT; PEURA, 2008) (Figura 1).

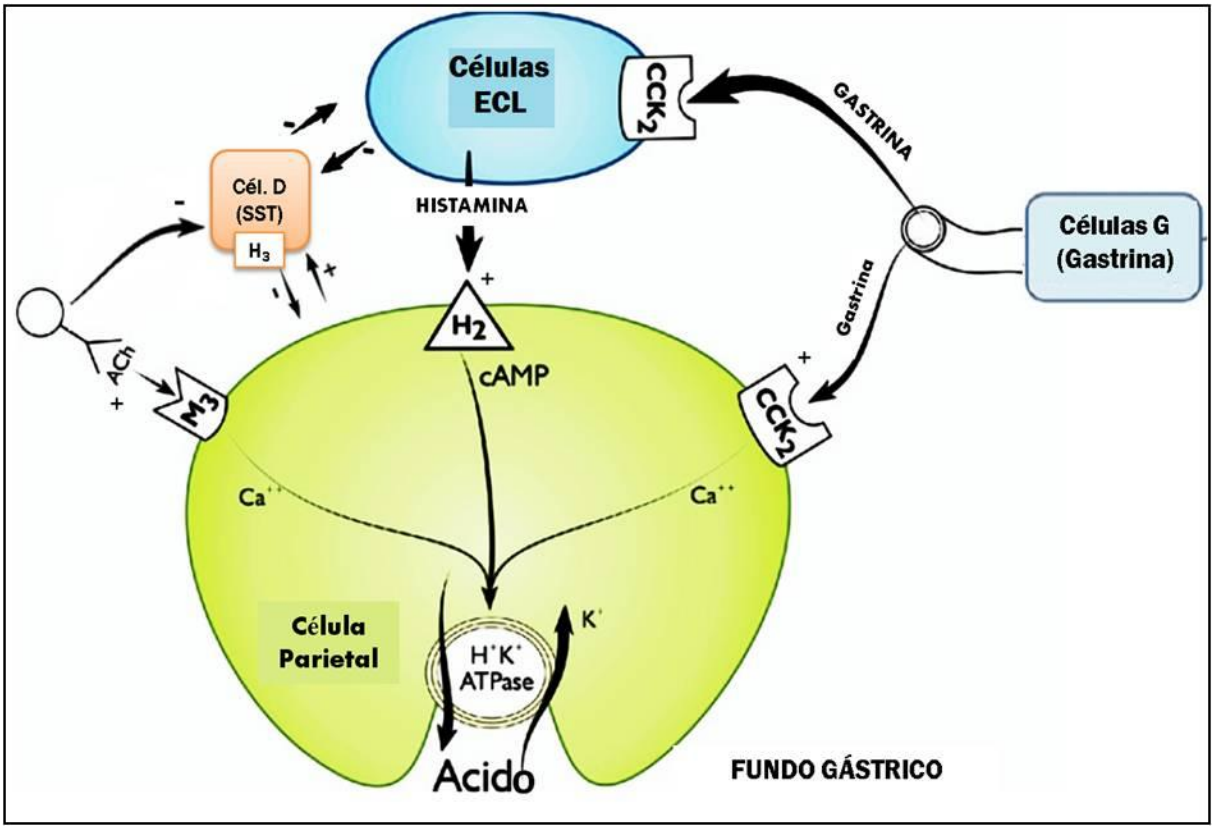

Figura 1. Modelo ilustrando a ação dos secretagogos nos receptores na célula parietal. Adaptado de Schubert e Peura (2008).

Em condições fisiológicas normais, o estômago apresenta um balanço entre os fatores agressores (ácido clorídrico, pepsina, enzimas digestivas, bile, dentre outros) e os mecanismos protetores (muco, bicarbonato, microcirculação da mucosa, motilidade, prostaglandinas, óxido nítrico, regeneração celular, dentre outros), portanto não ocorrendo nenhum dano significativo à mucosa (LAINE; TAKEUCHI; TARNAWSKI, 2008).

As diversas funções fisiológicas desempenhadas pelo estômago estão diretamente relacionadas à integridade da mucosa gástrica, que uma vez afetada, pode desencadear inúmeros distúrbios no trato gastrintestinal, dentre os quais se destacam as úlceras pépticas (úlceras duodenais e gástricas) e a doença do refluxo gastroesofágico (CHEN et al., 2006).

2.2 Úlceras pépticas – Considerações gerais

As úlceras pépticas são lesões no trato gastrointestinal que geralmente ocorrem no estômago (úlceras gástricas) e duodeno (úlcera duodenal) e se caracterizam por danos na mucosa secundários à ação agressiva dos sucos ácido-pépticos (RAMAKRISHNAN;

SALINAS, 2007). Diferentes mecanismos patogênicos podem atuar na formação da úlcera péptica, mas independente de sua etiologia, elas aparecem quando há um desequilíbrio do meio, ocasionado por aumento dos fatores agressores exógenos ou endógenos, ou por diminuição da resistência gástrica, trazendo como conseqüências irritação, ulceração e sangramento da mucosa (SAIRAM et al., 2002).

Há muito que a presença de ácido no estômago é associada como causa da úlcera péptica. No entanto, sua presença exclusiva é condição necessária, mas não suficiente para o desenvolvimento da úlcera, uma vez que a maioria das pessoas produz grandes quantidades de ácido e não desenvolvem úlceras (GUSTAFSON; WELLING, 2010). Atualmente, sabe-se que vários fatores estão envolvidos no seu aparecimento, entre eles se incluem a infecção por *Helicobacter pylori*, uso crônico de anti-inflamatório do tipo não-esteróide (AINE), uso de outros fármacos como bisfosfonatos, anticoagulantes e quimioterápicos, isquemia da mucosa gástrica, idade, hereditariedade e até o estilo de vida que engloba o estresse, uso abusivo de álcool, fumo e hábitos alimentares (STEWART; ACKROYD, 2008).

Outras causas raras específicas como o estado de hipersecreção gástrica (síndrome de Zollinger-Ellison), doença de Crohn, doença de Behcet's com envolvimento gastroduodenal, úlceras malignas não diagnosticadas, infecções virais ou infecções por outra Helicobacter (*Helicobacter heilmannii*) também podem ser responsáveis pelo surgimento das úlceras pépticas (MALFERTHEINER; CHAN; MCCOLL, 2009).

Diversos estudos têm evidenciado que a prevalência dos casos de úlceras pépticas e suas complicações como hemorragia, perfuração ou obstrução gástrica podem ocorrer em qualquer idade, mas se mostram mais acentuados na população acima de 50 anos, fato que pode estar relacionado à infecção por *H. pylori*, a ingestão de álcool, fumo e ao uso contínuo de AINE (HALLAS et al., 2006).

2.3 Mecanismos de defesa da mucosa gastrintestinal

A mucosa gástrica se utiliza de vários mecanismos defensivos como meio de manter a sua integridade contra a agressão provocada pelo ácido clorídrico, pepsina, bile, AINE, dentre outros. Constituem os principais fatores protetores da mucosa, o muco e bicarbonato, as prostaglandinas, o fluxo sanguíneo adequado, a somatostatina, o óxido nítrico e os compostos sulfidrílicos, a organização do epitélio e sua adequada reconstituição (SAIRAM et al., 2002), como ilustrado na Figura 2.

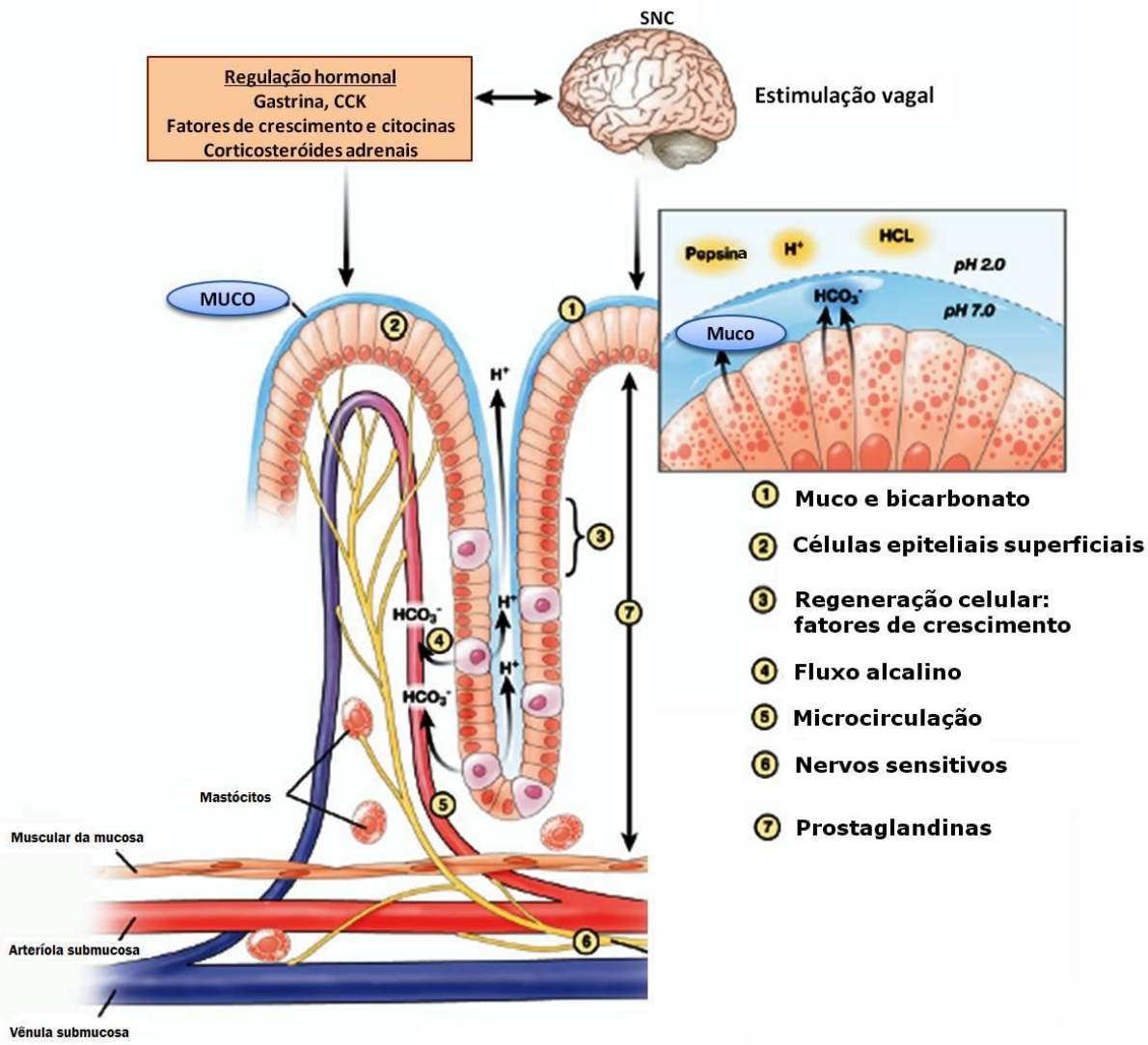

Figura 2. Mecanismos de defesa da mucosa gastrintestinal. Adaptado de Laine; Takeuchi e Tarnawski (2008).

A primeira linha de defesa contra o ácido é o muco, que juntamente com o bicarbonato, formam uma camada contínua sobre o epitélio gástrico, servindo de barreira contra autodigestão. O muco recobre toda a mucosa gástrica e a protege da colonização bacteriana e das forças mecânicas da digestão proteolítica, e atua como antioxidante e lubrificante da superfície gástrica (REPETTO; LLESUY, 2002). O bicarbonato (íon HCO_3^-) secretado no estômago e duodeno tem o papel de neutralizar os íons H^+ que se difundem na superfície da mucosa, mantendo um pH quase neutro na interface muco/mucosa da superfície gástrica. Qualquer fator que diminua a disponibilidade de muco e HCO_3^-, como anti-inflamatório do tipo não-esteróide (AINE) e sais biliares, pode aumentar a vulnerabilidade da mucosa ao ácido (ALLEN; FLEMSTRÖM, 2005).

As prostaglandinas (PGs) e o óxido nítrico (NO) representam importantes mediadores locais de defesa da mucosa gástrica (MOLLACE et al., 2005). As principais PGs sintetizadas pela mucosa gástrica são as prostaglandina E_2 (PGE_2) e prostaglandina I_2 (PGI_2). Ambas mantêm a integridade da mucosa gástrica por meio de diversos eventos, tais como a inibição da secreção ácida, estimulação da secreção de muco e bicarbonato, inibição da apoptose, aumento ou manutenção do fluxo sangüíneo e citoproteção do trato gastrointestinal (LAINE; TAKEUCHI; TARNAWSKI, 2008). O óxido nítrico é sintetizado pela NO-síntase (NOS) a partir de oxigênio (O_2) e L-arginina. No estômago, o NO está diretamente envolvido na regulação do fluxo sanguíneo, da secreção ácida e da produção de muco, além de inibir a agregação de neutrófilos (WANG et al., 2008).

A microcirculação gástrica protege a mucosa ao suprí-la com quantidades suficientes de oxigênio, nutrientes e hormônios, além de participar da remoção de íons H^+, da produção de muco, secreção de bicarbonato, remoção, diluição e neutralização dos produtos do metabolismo celular e de substâncias tóxicas que se difundem do lúmen (MARTIN; WALLACE, 2006). Vários estudos têm mostrado que a diminuição do fluxo sangüíneo da mucosa gástrica agrava as lesões induzidas por agentes ulcerogênicos, enquanto pouco ou nenhum dano ocorre depois da ação de agentes necrotizantes se o fluxo sangüíneo for mantido em níveis apropriados, juntamente com a citoproteção das prostaglandinas (ABDEL-SALAM et al, 2001).

A somatostatina é o principal inibidor da secreção ácida. Consiste em um peptídeo inibitório liberado pelas células D da mucosa antral e oxíntica do estômago. Promove a inibição de ácido gástrico por dois mecanismos, um que atua diretamente sobre as células parietais e o outro indiretamente inibindo a secreção de histamina das células enterocromafins e de gastrina das células G (KOMASAKA et al., 2002).

Os grupamentos sulfidrílicos não-protéicos (SH) endógenos encontram-se presentes no muco e em algumas enzimas do sistema antioxidante. Estão diretamente envolvidos na proteção da mucosa gástrica, visto que participam da produção do muco gástrico e que ligam-se aos radicais livres formados durante processos inflamatórios ou produzidos após exposição da mucosa a agentes nocivos, exercendo uma ação neutralizante (AVILA et al., 1996).

O processo de renovação celular do epitélio gástrico ocorre a cada 2-4 dias, e a manutenção de sua integridade depende de um balanço adequado entre perda e renovação celular (LAINE; TAKEUCHI; TARNAWSKI, 2008). Quando a mucosa é lesada, ou seja, quando há a formação de úlcera, inicia-se um processo de reparação epitelial através de crescimento e formação de glândulas gástricas, crescimento de novos vasos sanguíneos

(angiogênese), proliferação e migração rápida de células saudáveis aos locais lesionados eventos que conduzem à formação da cicatriz (TARNAWSKI, 2005, MARTIN; WALLACE, 2006).

Algumas defesas antioxidantes exercem um papel complementar na proteção da mucosa gástrica ao impedir o estresse oxidativo provocado pelo aumento nos níveis intracelulares de espécies reativas de oxigênio, tais como o ânion superóxido (O_2^-), radical hidroxila (OH) e peróxido de hidrogênio (H_2O_2) (MATÉS, 2000). Dentre essas defesas se incluem a glutationa total (GSH) e outras moléculas de baixo peso molecular, as enzimas superóxido dismutase (SOD), catalase (CAT) e a glutationa peroxidase (GPx) e outras enzimas, como a NAD(P)H quinona oxidoredutase 1 (NQO1) e a glutationa S-transferase (GST). Em condições fisiológicas normais, esses mecanismos de defesa protegem as células mantendo baixos os níveis de radicais livres (MICHIELS et al., 1994).

2.4 Terapêutica

Apesar dos avanços na compreensão da etiologia, nas modalidades de diagnóstico e na disponibilidade de modernos tratamentos dos sintomas dispépticos, a úlcera péptica e suas complicações ainda continuam sendo uma das principais causas de morbidade e mortalidade no mundo (Chow and Sung, 2009).

A doença afeta milhões de pessoas em todo mundo. Só nos Estados Unidos, aproximadamente 500 mil pessoas desenvolvem úlceras pépticas a cada ano, e cerca de 70% dos pacientes estão na faixa etária de 25 a 64 anos. Os custos diretos ou indiretos de cuidados da doença são estimados em cerca de US$ 10 bilhões (RAMAKRISHNAN; SALINAS, 2007).

Os fatores de risco predominantes na úlcera péptica permanecem sendo o a bactéria *H. pylori* e o uso de AINE. Mais de 50% da população mundial tem infecção crônica da mucosa gástrica por *H. pylori*, mas somente 5-10% dos infectados desenvolvem úlceras (MALFERTHEINER; CHAN; MCCOLL, 2009). Nesses casos a *H. pylori* é a responsável em até 95% das úlceras duodenais e 70% das úlceras gástricas (NAPOLITANO, 2009). A incidência de úlceras relacionadas a bactéria tem diminuído nos últimos anos e, em contrapartida, a proporção de pacientes com úlceras atribuídas ao uso contínuo de AINE e agentes antiplaquetários subiu (CHOW; SUNG, 2009), principalmente se esses agentes são utilizados em combinação, conduzindo a um considerável aumento da toxicidade gastrointestinal (HALLAS et al., 2006).

A terapêutica atual é baseada na inibição da secreção ácida gástrica por meio de antagonistas de receptores histaminérgicos do tipo H_2, inibidores da bomba de prótons, antimuscarínicos, bem como em terapia ácido-independente provida por antimicrobianos contra o *H. pylori*, sucralfato e bismuto (BIGHETTI et al., 2005). Outra abordagem adotada para erradicação da *H. pylori* no estômago consiste na associação de inibidores da bomba de prótons com antibióticos, que são combinados para aumentar a concentração de antibióticos no suco gástrico e a sensibilidade da bactéria, aumentando assim, a eficácia dos antibióticos (HUNT, 2005).

Dados da literatura demonstram que o tratamento da úlcera péptica enfrenta problemas como diminuição na eficácia da erradicação da *H. pylori*, ineficácia da atual terapêutica na prevenção e reincidência de úlceras em pacientes usuários de AINE, assim como nos casos de úlceras gástricas não associadas à utilização de AINE e à presença da *H. pylori* (YUAN et al., 2006). Embora as opções terapêuticas disponíveis mostrem-se eficazes, o uso prolongado dessas drogas além de produzir reações adversas como hipersensibilidade, arritmia, impotência, ginecomastia e mudanças hematopoiéticas, pode aumentar o risco de desenvolvimento de câncer (CHAN; LEUNG, 2002).

Segundo Fan et al. (2005) a úlcera péptica é uma doença comum de elevada incidência clínica e que apresenta recidivas. Enquanto a taxa de cura está em torno dos 95%, a taxa de recidivas da doença está entre 65 – 80 % dentro de um ano após o tratamento e cerca de 100% após dois anos. Sendo esse o caso, fica evidente a importância de experimentos que avaliem a atividade farmacológica de compostos que possam prevenir o seu aparecimento e/ou controlar seus efeitos agressivos ao homem.

2.5 Modelos experimentais de lesões gástricas

Diversos modelos experimentais podem levar a mucosa gástrica a um processo de ulcerogênese. Nestes são utilizados diferentes agentes indutores como os AINEs (tais como, indometacina, piroxicam e ácido acetilsalicílico) (SAIRAM et al., 2002), etanol (ROBERT et al., 1979), etanol acidificado (MIZUI; DOUTEUCHI, 1983) e ácido acético (TAKAGI et al., 1969). Estes modelos são utilizados porque incluem os agentes etiológicos mais comuns na doença da úlcera péptica humana e reproduzem em animais lesões gástricas similares às observadas em humanos.

Os AINEs, embora sejam amplamente utilizados no tratamento de diversas doenças, são responsáveis pelo aparecimento de diversas lesões na mucosa gástrica, as quais incluem

dispepsia, erosões, úlceras pépticas e complicações como sangramento e perfuração da mucosa gástrica (KIM, 2008). A indometacina e outros AINEs como o piroxicam e ácido acetilsalicílico inibem a enzima ciclooxigenase, expressa em grandes quantidades no trato gastrointestinal e responsável por manter a integridade da mucosa pela geração contínua de prostaglandinas.

O etanol absoluto atua destruindo as células epiteliais no estômago independentemente da secreção ácida seja por contato direto ou indireto (ROBERT et al., 1979), provocando a infiltração de células inflamatórias que por fim produzem vasoconstrição, edema submucoso e lesões hemorrágicas (PARK et al., 2004). A indução de úlceras gástricas por ácido acético consiste no modelo em que a úlcera e o processo de reparação mais se assemelham aos observados em humanos. O ácido acético provoca um aumento no volume de ácido, principal responsável pela necrose da mucosa e obstrução pilórica (TAKAGI et al., 1969).

Outro método utilizado na avaliação da atividade antiulcerogênica é o método da ligadura pilórica. Por meio dele, é possível avaliar o efeito sistêmico de substâncias com potencial efeito gastroprotetor e sua interferência em parâmetros da secreção ácida como volume, pH e acidez total. A obstrução promovida pela ligadura pilórica causa acúmulo de ácido no conteúdo gástrico e distensão exagerada da mucosa, consequentemente levando a uma hipersecreção gástrica e à formação de úlceras (BAGGIO et al., 2003).

2.6 Plantas medicinais na gastroproteção

O Brasil possui uma biodiversidade abundante se comparada a de outros países. De sua flora menos de 5% das espécies representam a fitoterapia mundial, significando que um verdadeiro arsenal terapêutico de princípios ativos pode ser extraído da natureza (CALIXTO, 2003). Isso é um ponto importante no que se refere à capacidade de uso e aproveitamento dos recursos biológicos que nos são oferecidos.

Os distúrbios gastrointestinais vêm se tornando um dos principais focos de investigações experimentais e clínicas, principalmente devido a sua alta prevalência na população. Uma grande variedade de substâncias químicas isoladas, mistura de ervas e extratos de plantas tiveram suas atividades terapêuticas comprovadas em modelos experimentais de indução de úlcera (ZAYACHKIVSKA et al., 2005).

De acordo com levantamento realizado por Falcão et al. (2008), o continente americano apresenta 58 espécies de plantas medicinais, distribuídas em 37 famílias, que têm sido relatados por apresentarem atividade antiulcerogênica. Os autores destacam ainda, que

dos nove países que desenvolvem pesquisas na área, o Brasil responde por 67% da atividade de busca por novos principios ativos de origem vegetal. Foram identificadas muitas espécies usadas para tratar úlceras gástricas e com atividade farmacológica já relatada na literatura, dentre elas destacam-se: *Bredemeyera floribunda* Willd (pau-rendoso), *Copaifera langsdorfii* Desf. (copaíba), *Caesalpinia ferrea* Martius (pau-ferro), *Casearia sylvestris* Swarts (guaçatonga), *Cordia verbenacea* DC. (erva baleeira), *Croton cajucara* Benth. (sacaca), *Hyptis mutabilis* (Rich.) Briq. (hortelã-brava), *Maytenus ilicifolia* Martius ex Reiss. (espinheira-santa), *Musa paradisiaca* L. (bananeira), *Quassia amara* L., (IK) (pau amargo), *Rosmarinus officinalis* L. (alecrim), *Solanum paniculatum* L. (jurubeba-roxa) e *Stryphnodendron adstringens* (Martius) Coville (barbatimão). Todas essas espécies mostraram atividade gastroprotetora, seja na forma de extratos, frações ou óleos essenciais, sobre modelos de indução de lesão gástrica em ratos ou camundongos.

Segundo Lewis e Hanson (1991), destacam-se como principais classes de compostos com atividade antiulcerogênica os terpenos, flavonóides, compostos fenólicos, taninos, alcalóides, glicosídeos, saponinas e polissacarídeos. Estes compostos apresentam atividade antiulcerogênica possivelmente por atuarem estimulando os fatores de proteção da mucosa gástrica, aumentando a produção de prostaglandinas e/ou estimulando a secreção de muco e bicarbonato (HIRUMA-LIMA et al., 2002). Isto possibilita considerar as plantas medicinais e seus princípios ativos como fontes potenciais de novas moléculas farmacologicamente ativas, que poderão vir a ser úteis na descoberta de novas terapêuticas para o tratamento das úlceras pépticas.

2.7 Família Lamiaceae – Gênero *Hyptis*

A família *Lamiaceae*, compreende cerca de 250 gêneros e 6970 espécies, incluindo ervas, arbustos ou árvores (JUDD et al., 1999). No Brasil, ocorrem 26 gêneros e cerca de 350 espécies (SOUZA; LORENZI, 2005). Dentre seus representantes, estão as espécies do gênero *Hyptis*, que vêm sendo amplamente estudadas do ponto de vista etnofarmacológico, farmacológico e químico, principalmente devido à diversidade de constituintes bioativos encontrados nos óleos essenciais aromáticos e extratos, os quais possuem efeitos biológicos interessantes tais como antimicrobiano, antineoplásico e inseticida (MCNEIL; FACEY; PORTER, 2011).

A química da família Lamiaceae, em especial a do gênero *Hyptis* é de notável variabilidade. Nestas plantas, estão presentes compostos como flavonóides, lactonas, lignanas,

alguns derivados fenólicos e ácidos graxos. Nos óleos obtidos de várias espécies de *Hyptis*, os compostos terpenoídicos parecem ser os principais responsáveis pela atividade citotóxica e inclui diferentes mono-, di-, tri- e sesquiterpenos. Outras substâncias também foram relatadas tais como hidrocarbonetos, ácidos graxos, esteróides, entre outras (FALCÃO; MENEZES, 2003).

Várias espécies do gênero *Hyptis* têm importante ação farmacológica como antiplasmódica (CHUKWUJEKWU et al., 2005), antidepressiva (BUENO et al., 2006), antinociceptivo e antiedematogênica (LISBOA et al., 2006). Estudos recentes com óleos essenciais têm mostrado atividades biológicas importantes, tais como atividades antisséptica (PEREDA-MIRANDA et al.,1993), antifúngica (DE OLIVEIRA et al., 2004), antibacteriana (SOUZA et al., 2003), anti-inflamatória (BISPO et al., 2001), antinociceptiva (ARRIGONI-BLANK et al., 2008; MENEZES et al., 2007), antiulcerogênica (BARBOSA; RAMOS, 1992; TAKAYAMA et al., 2011) dentre outras.

2.8 *Hyptis martiusii* Benth.

A espécie *Hyptis martiusii* Benth. pertence à família Lamiaceae, e é popularmente conhecida como cidreira-do-mato ou cidreira-brava. Sua ocorrência se dá no norte, sudeste e nordeste do Brasil. No nordeste cresce em abundância, encontrando-se amplamente disseminada no sul do Ceará, na Chapada do Araripe (SILVEIRA; PESSOA, 2005) e no estado do Pernambuco aonde sua ocorrência vai desde a zona da mata à zona de caatinga (ALMEIDA; ALBUQUERQUE, 2002). Segundo Martius (1864, *apud* SILVEIRA; PESSOA, 2005), esta espécie é descrita como um arbusto ereto e ramoso, de 1,6 - 2,3 m de altura, suas flores são delicadas, brancas e reunidas em inflorescências globulosas terminais (Figura 3).

Algumas espécies do gênero, tais como *Hyptis suaveolens, Hyptis pectinata, Hyptis crenata e Hyptis fruticosa*, têm seu uso medicinal amplamente difundido entre a população. Entretanto, o único relato encontrado sobre o uso medicinal da espécie *Hyptis martiusii* foi descrito por Agra et al. (2008), que cita as folhas e raízes como as partes mais utilizadas com fins medicinais pela população. A infusão ou decocção das folhas é usada contra doenças intestinais e estomáquicas, enquanto as raízes em decocto são usadas contra inflamações de ovário.

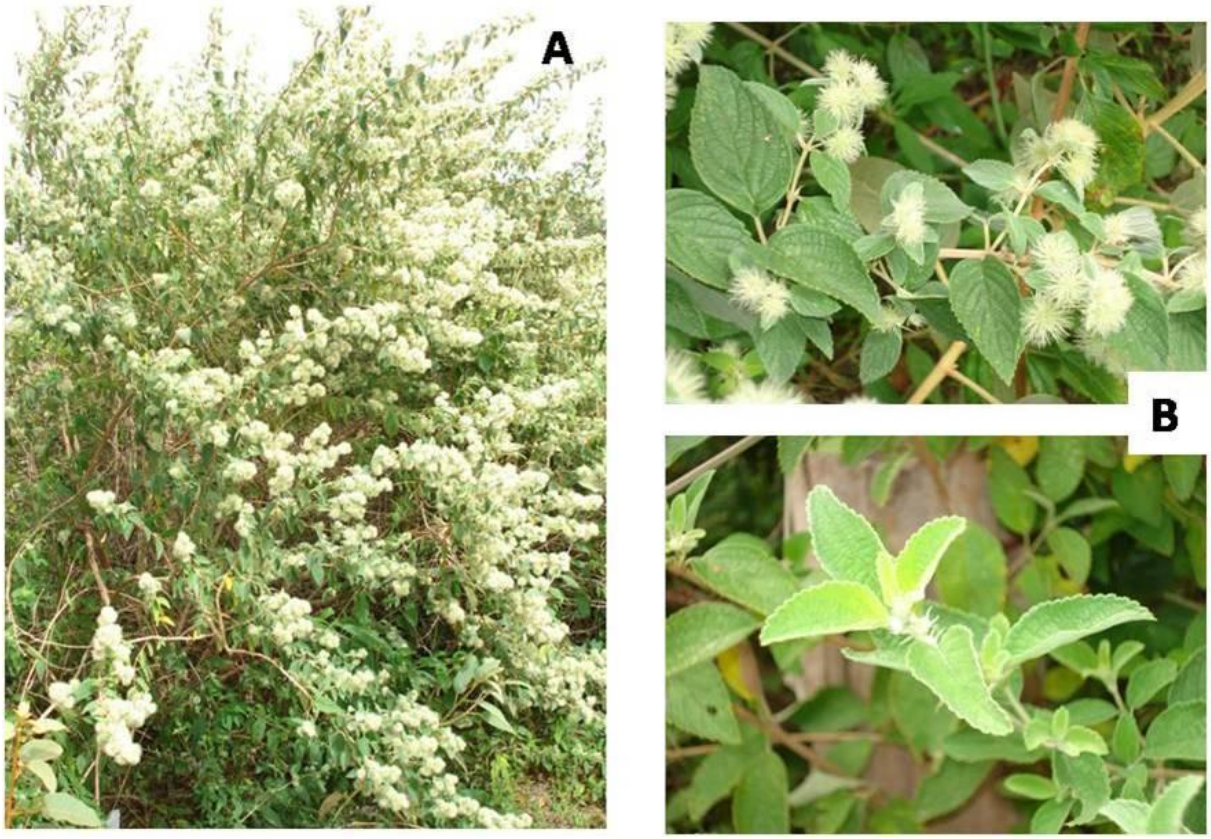

Figura 3. *Hyptis martiusii* Benth. (A), destacando capítulos florais e folhas (B). Fonte: da autora

Hyptis martiusii caracteriza-se por ser potencialmente fornecedora de óleos essenciais aromáticos, a exemplo de outras espécies de *Hyptis*. Estudo de Araújo et al. (2003), demonstrou a composição química do óleo essencial obtido das folhas e flores frescas por hidrodestilação. Foram identificados 26 constituintes nas folhas e 27 constituintes nas inflorescências o que representa 93,2% e 87,7% do óleo essencial, respectivamente. Segundo os mesmos autores, a composição do óleo essencial das folhas de *H. martiusii* é constituída por mono e sesquiterpenos, e tem como componentes majoritários o 1,8-cineol (24,3%), δ-3-careno (22,5%), biciclogermacreno (6,3%) e o β-cariofileno (6,2%); enquanto nas inflorescências tem como componentes o δ-3-careno (13,5%), viridifloreno (8,3%), β-cariofileno (6,6%), α-pineno (5,8%) e germacreno B (5,2%). Na Figura 4 estão disponíveis as estruturas químicas dos respectivos compostos.

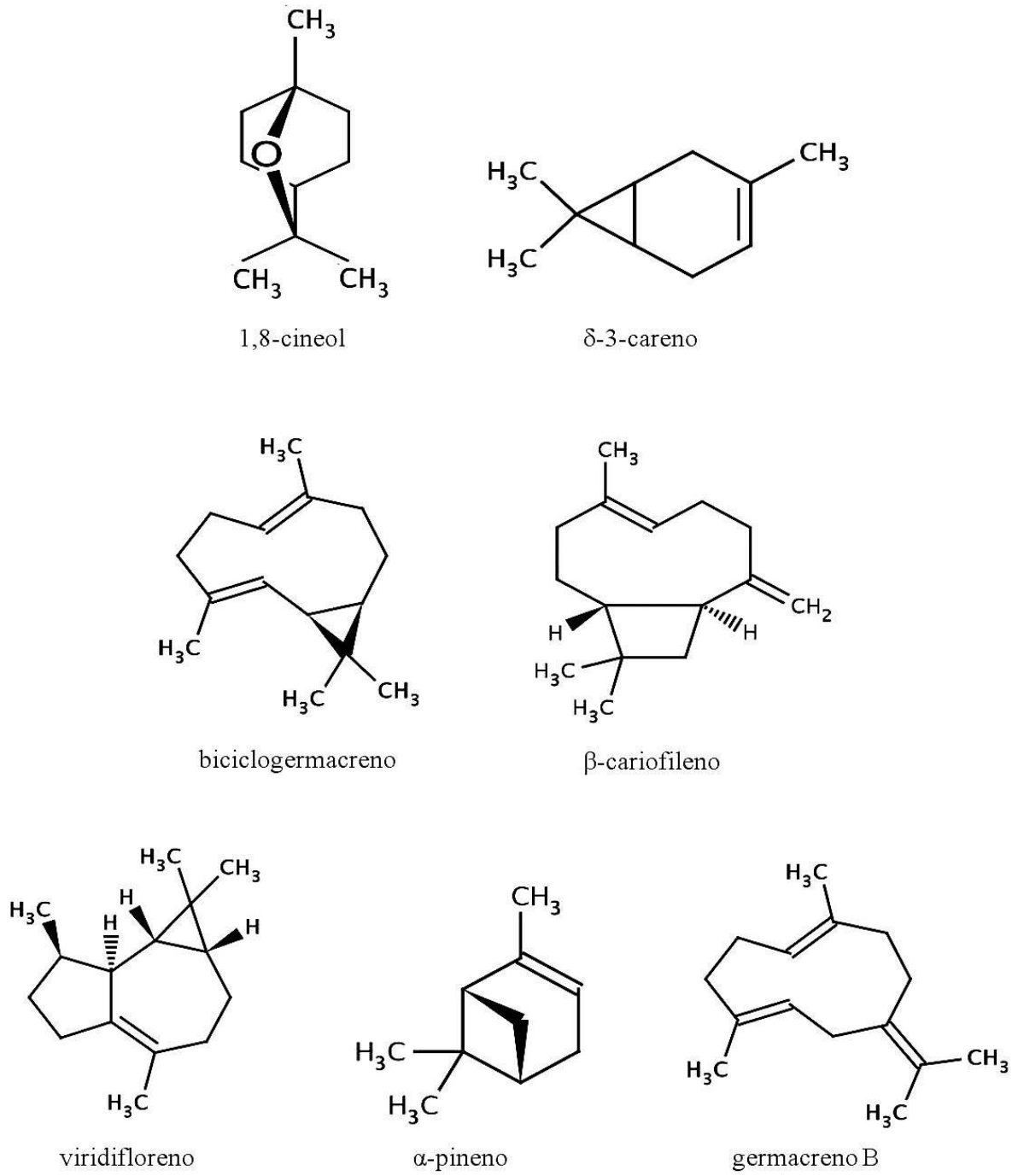

Figura 4. Estrutura química dos componentes majoritários do óleo essencial das folhas e inflorescências de *Hyptis martiusii* Benth.

O óleo essencial das folhas de *H. martiusii* e o composto isolado 1,8-cineol apresentaram efeito inseticida contra larvas de *Bemisia argentifolii* e *Aedes aegypti* (ARAÚJO et al., 2003). Essa atividade contra o *Aedes aegypti* foi confirmada posteriormente por Costa et al. (2005), que também relataram pela primeira vez a atividade do óleo essencial das folhas contra larvas de *Culex quinquefasciantus.* Outros compostos terpênicos já foram isolados de

Hyptis martiusii. Destacam-se dois diterpenos abietanos, denominados carnasol e 11,14-diidroxi-8,11,13-abietatrieno-7-ona; ambos isolados do extrato hexânico das raízes com efeito antiproliferativo em certas linhagens de células tumorais (HL-60 – células leucêmicas infantis, CEM – células leucêmicas adultas, HTC-8 – células de câncer de colo, MCF-7 – células de câncer de mama e B-16 – células de câncer de pele) e sobre o crescimento de ovos de ouriço-do-mar (COSTA-LOTUFO et al., 2004) apresentando baixa toxicidade para a célula. Das raízes também foi relatado o isolamento de dois outros diterpenos, o novo 7β-hidroxi-11,14-dioxoabieta-8,12-dieno e o já conhecido 7α-acetoxi-12-hidroxi-11,14-dioxoabieta-8,12-dieno, os quais apresentaram atividade citotóxica sobre cinco linhagens de células de tumorais. Tal atividade pode estar relacionada à inibição da síntese de DNA (ARAÚJO et al., 2006).

O extrato etanólico das folhas de *Hyptis martiusii* apresentou comprovada atividade antibacteriana *in vitro* contra cepas resistentes de *Staphylococcus aureus* e *Escherichia coli* (COUTINHO et al., 2008), assim como também foi demonstrada atividade antioxidante *in vitro* pelo método de redução do radical DPPH (SANTOS et al., 2010).

2.9 Óleos essenciais

Os óleos essenciais são princípios encontrados em várias espécies vegetais, e podem receber diferentes designações tais como óleos “voláteis”, “aromáticos” ou “etéreos”, de acordo com suas características físico-químicas. Segundo Bakkali et al. (2008), atualmente cerca de 3000 óleos essenciais são conhecidos, dos quais 300 são comercialmente importantes, especialmente para a indústria de produtos farmacêuticos, sanitários, cosméticos, agrícolas, alimentos e perfumaria.

De acordo com a família a que pertencem as espécies, os elementos voláteis podem estar concentrados em órgãos anatômicos específicos, podendo variar na sua composição de acordo com a localização em uma mesma espécie. Outros fatores podem interferir também na composição do óleo essencial de uma mesma espécie vegetal, como a época de coleta, condições climáticas e de solo, localização geográfica, ciclo vegetativo da espécie e processo de obtenção (DORMAN; DEANS, 2000).

Os óleos essenciais em geral são líquidos, no entanto, podem apresentar-se como sólidos ou semi-sólidos à temperatura ambiente. Evaporam quando expostos ao ar em temperatura ambiente, a maioria é transparente, incolor ou fracamente amarelado, e apresentam um sabor acre e picante. Na presença de luz, calor, umidade, ar e metais são

instáveis, devendo ser acondicionados em recipientes de cor âmbar, hermeticamente fechados e em lugar fresco e seco (BAKKALI et al., 2008).

Diferentes métodos podem ser aplicados para obtenção de óleo essencial a partir de plantas aromáticas, tais como a hidrodestilação, extração por solventes, extração por enfloração (*Enfleurage*), por gases supercríticos ou por prensagem. Dentre estes, o método de hidrodestilação por arraste de vapor d'água é o mais frequente. Como os óleos possuem tensão de vapor mais elevada que a da água, eles são arrastados pelo vapor e então condensados por resfriamento (SIMÕES; SPITZER, 2002).

A maior parte dos óleos essenciais consiste numa mistura complexa de princípios ativos que variam na sua composição química. Pode-se encontrar cerca de 20 - 60 componentes em concentrações muitos diferentes, bem como a presença de dois ou três destes constituintes em concentrações relativamente elevadas. Em geral, tais componentes determinam as propriedades biológicas do óleo (BAKKALI et al., 2008). Quimicamente, são constituídos de derivados de fenilpropanóides e incluem compostos aromáticos e alifáticos tais como hidrocarbonetos, alcoóis, ésteres, éteres, aldeídos, lactonas, fenóis, entre outros; ou de terpenos cujos compostos derivam da condensação de um número variável de unidades pentacarbonadas conhecidas por unidades isoprênicas (C5) e se classificam de acordo com o número dessas unidades em monoterpenóides (C10), sesquiterpenóides (C15), diterpenóides (C20), sesterpenóides (C25), triterpenóides (C30) e tetraterpenóides (C40) (DORMAN; DEANS, 2000).

Os terpenos não só compreendem o maior grupo de produtos naturais vegetais, abrangendo cerca de 30 mil compostos, como também apresentam a maior variedade de tipos estruturais (DEGENHARDT et al., 2009). Do ponto de vista biológico, os compostos terpênicos demonstram uma variedade de propriedades medicinais interessantes, dentre elas atividade gastroprotetora (PERTINO et al., 2007). Nos óleos essenciais de modo geral, os constituintes majoritários são os monoterpenos e sesquiterpenos, e isso se aplica a espécies do gênero *Hyptis* onde esses constituintes são apontados como os principais componentes (MCNEIL; FACEY; PORTER, 2011). Os monoterpenos são as moléculas mais representativas, constituindo cerca de 90% e permitem uma grande variedade de estruturas (BAKKALI et al., 2008).

Vários estudos têm demonstrado experimentalmente que um grande número de óleos essenciais de plantas medicinais e aromáticas possuem propriedades gastroprotetoras e de cicatrização de úlceras. Destacam-se os óleos essenciais obtidos de *Baccharis dracunculifolia* D.C. (partes aéreas), *Carlina acanthifolia* All. (raiz), *Casearia sylvestris* Sw. (folhas), *Citrus*

aurantium L. (casca do fruto), *Citrus lemon* Burm. (casca do fruto), *Croton cajucara* Benth (casca do caule), *Hyptis spicigera* Lam. (partes áereas), *Lippia sidoides* Cham. (folhas), *Pterodon emarginatus* Vogel (sementes), *Ocimum minimum* Linn. (folhas), *Syzygium aromaticum* (botões florais) e *Vanillosmopsis arborea* Baker (casca do caule) (ROZZA; PELLIZZON, 2013).

2.10 Cineol: um monoterpeno presente nas folhas de *Hyptis martiusii*

O 1,8-cineol, também denominado cineol, eucaliptol ou 1,8-epoxi-*p*-mentano (Figura 2), é um monoterpeno que apresenta fragrância fresca com características canforáceas e sabor picante (LANA et al., 2006). Trata-se de um composto orgânico natural, encontrado no óleo essencial de muitas plantas aromáticas, sendo considerado o composto majoritário de vários óleos essenciais em diferentes espécies. Especificamente para o gênero *Hyptis,* ele é encontrado em espécies como *Hyptis martiusii*, *Hyptis fruticosa*, *Hyptis goyazensis* e *Hyptis suaveolens* (MCNEIL; FACEY; PORTER, 2011).

Este monoterpeno é amplamente utilizado como excipiente em produtos farmacêuticos e cosméticos, tais como spray nasal ou desinfetante (MADYASTHA; CHADHA, 1986) e como um agente flavorizante em alimentos (AHMAD; MISRA, 1994). De acordo com De Vincenzi et al. (2002) plantas e/ou seus óleos essenciais contendo 1,8-cineol podem ser adicionados como aromatizantes em vários produtos alimentares processados, por exemplo, laticínios congelados, doces macios ou bebidas não-alcoólicas, proporcionando assim um potencial significativo de exposição humana ao composto. Diferentes aplicações terapêuticas são atribuídas ao 1,8-cineol, dentre elas destacam-se o tratamento de doenças respiratórias, tais como asma, bronquite, sinusite e resfriados devido a sua propriedade secretolítica (JUERGENS et al., 2003), tosse, dor muscular, neurose, reumatismo e cálculo renal (MIYAZAWA et al., 2001).

Santos e Rao (2001) relataram que o 1,8-cineol, em doses orais variando entre 50 a 200 mg/kg, reduziu as lesões gástricas induzidas por etanol e atribuiram este efeito a um aumento da quantidade de GSH, e à inibição da enzima lipoxigenase na mucosa gástrica, bloqueando a formação de leucotrienos e impedindo assim a ação prejudicial do etanol nas células do estômago. Atividades anti-alérgica, anti-inflamatória e antinociceptiva (SANTOS; RAO, 1998, 2000), hipotensiva e relaxante muscular (LAHLOU et al., 2002) e hepatoprotetora (SANTOS et al., 2001) e antitumoral (MOTEKI et al., 2002) também já foram descritas na literatura.

3. Objetivos

3. OBJETIVOS

3.1 GERAL

Investigar a atividade gastroprotetora e a segurança de uso do óleo essencial das folhas de *Hyptis martiusii* (OEHM) e do monoterpeno 1,8-cineol (CIN).

3.2 ESPECÍFICOS

- Caracterizar os constituintes químicos do OEHM*;*
- Avaliar a atividade antiulcerogênica do OEHM e CIN, em modelos agudos de úlcera gástrica induzida por etanol, HCl/etanol e indometacina;
- Verificar a influência do OEHM e CIN sobre a motilidade gastrointestinal;
- Explorar a possível atividade antissecretória gástrica basal e estimulada do OEHM e CIN, através do método da ligadura pilórica;
- Avaliar a influência da produção de muco, grupamentos sulfidrílicos e óxido nítrico no efeito gastroprotetor do OEHM e CIN;
- Analisar a atividade antioxidante *in vitro* do OEHM e CIN pelo método de captura do radical 2,2-difenil-1-picrilidrazil (DPPH);
- Investigar a atividade antioxidante *in vivo* do OEHM e CIN sobre o nível de peroxidação lipídica e sobre a produção de grupamentos sulfidrílicos não-protéicos na mucosa gástrica;
- Avaliar a ação cicatrizante do OEHM e CIN e as alterações histopatológicas promovidas no modelo de úlcera crônica induzida por ácido acético;

- Avaliar a toxicidade pré-clínica do OEHM, mediante tratamento de doses repetidas (30 dias) sobre os parâmetros bioquímicos, hematológicos e morfológicos de camundongos Swiss de ambos os sexos;

- Analisar os efeitos do tratamento agudo e de doses repetidas (50 dias), por via oral, do CIN sobre os parâmetros bioquímicos, hematológicos e morfológicos de ratos Wistar de ambos os sexos.

- Investigar o efeito da administração oral do CIN nos períodos de prenhez (pré-implantação e organogênese) em ratas Wistar.

4. Resultados

Todos os resultados obtidos nesse trabalho foram organizados na forma de artigos (publicado, submetido e em preparação)

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Antiulcerogenic activity of the essential oil of *Hyptis martiusii* Benth. (Lamiaceae)

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ABSTRACT

Ethnopharmacological relevance: Hyptis martiusii (Lamiaceae), popularly known as "cidreira-do-mato" or "cidreira-brava", grows in abundance in the Northeast region of Brazil, where its leaves are traditionally used in folk medicine in the treatment of intestinal and stomach diseases. The aim of this study was to characterize the chemical constituents and to evaluate the anti-ulcerogenic activity of the essential oil of the leaves of *Hyptis martiusii* (EOHM) in *in vivo* models of experimental ulcers in rodents.

Materials and methods: EOHM was obtained by hydrodistillation and analyzed by gas chromatography–mass spectrometry (GC–MS). Acute gastric ulcer was induced using absolute ethanol, HCl/ethanol and indomethacin. The volume, pH and total acidity of gastric secretion were determined by the pyloric ligature method and gastrointestinal motility using gastric emptying and intestinal transit.

Results: Chemical analysis revealed the presence of 24 components that account for 92.13% of the essential oil of dried leaves, indicating the occurrence of mono and sesquiterpenes. Oral administration of EOHM (100, 200 and 400 mg/kg) inhibited ethanol-, HCl/ethanol- and indomethacin-induced ulcers. In the case of pylorus ligature, the oil reduced the volume of gastric juices and total acidity, and increased gastric pH. The EOHM reduced the rate of gastric emptying with only the highest doses, but did not show any effect on intestinal transit at any of the three doses.

Conclusions: The results indicate that the essential oil of leaves of *Hyptis martiusii* has an antiulcerogenic activity, as evidenced by its significant inhibition of the formation of ulcers in various models. This effect could be related to an increase of gastric mucosal defensive factors. Further pharmacological studies are being undertaken in order to provide more precise elucidation of the action mechanism involved in this activity.

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1. Introduction

Peptic ulcers are lesions in the gastrointestinal tract that usually occur in the stomach and duodenum and are characterized by mucosal damage resulting from the aggressive action of pepsin and gastric acid secretion. The disease affects millions of people around the world and is considered a major cause of morbidity and mortality worldwide. In the United States alone, approximately 500,000 individuals develop peptic ulcers each year (Ramakrishnan and Salinas, 2007).

It is currently known that a number of factors are involved in the emergence of peptic ulcers, including *Helicobacter pylori* infection, chronic use of nonsteroidal anti-inflammatory drug (NSAID), the use of other drugs such as corticosteroids, bisphosphonates, anticoagulants and chemotherapy, gastric mucosa ischemia, age, genetic factors, a lifestyle involving stress, alcohol abuse, or smoking, and dietary habits (Stewart and Ackroyd, 2008).

The treatment of peptic ulcers is usually based on inhibition of gastric acid secretion using proton-pump inhibitors, H_2-receptor antagonists, and antimuscarinics, as well as acid-independent therapy provided by antimicrobials against *Helicobacter pylori*, sucralfate and bismuth (Bighetti et al., 2005). However, these drugs are expensive and generate numerous adverse effects (such as hypersensitivity, gynecomastia, impotence, arrhythmia and hematopoietic changes), thereby limiting their usefulness (Santin et al., 2010). It is thus important that studies be carried out to

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investigate potential antiulcerogenic herbs and their isolated compounds as alternative ways of mitigating the aggressive effects of ulcers.

The genus *Hyptis* Jacq. (Lamiaceae) consists of more than 300 species considered of great economic importance due to the content of aromatic essential oil they present (Falcão and Menezes, 2003). Various aromatic oils of different *Hyptis* species are used in folk medicine as an alternative therapy, and some properties pharmacological of these oils have already been described, such as their activity antiulcerogenic (Lee et al., 1988), antiseptic (Pereda-Miranda et al., 1993), insecticide (Araújo et al., 2003), antibacterial (Souza et al., 2003), antifungal (De Oliveira et al., 2004) and antinociceptive (Menezes et al., 2007).

Hyptis martiusii Benth. is a small shrub that belongs to the Lamiaceae family, commonly found in north, southeast and northeastern Brazil (Almeida and Albuquerque, 2002), popularly known as "cidreira-do-mato" or "cidreira-brava". According to an ethnopharmacological survey, the parts of this plant used for medicinal purposes are the leaves and roots. In the Northeast region of Brazil, the leaves of *Hyptis martiusii* is used against intestinal and stomach diseases (Agra et al., 2008). Antimicrobial, antitumoral, cytotoxic and insecticidal activities were identified (Costa-Lotufo et al., 2004; Costa et al., 2005; Araújo et al., 2006; Coutinho et al., 2008), although no antiulcerogenic activity of the essential oil this species has been reported in the literature. The aim of the present study was to evaluate the antiulcerogenic activity of the essential oil of the leaves of *Hyptis martiusii*.

2. Material and methods

2.1. Plant material and extraction of essential oil

Leaves of *Hyptis martiusii* Benth. (Lamiaceae) were colleted on the Araripe Plateau, in Crato, in the Brazilian State of Ceará (S 7°21.744′–W 39°28.691′). The entire plant was collected at the flowering stage, in June 2008 (three times) and 2009 (two times). A voucher specimen was identified by Edson P. Nunes and deposited in the Prisco Bezerra Herbarium, at the Federal University of Ceará's Department of Biology, under registration no. 43038. The leaves were dried at room temperature for 72 h prior to hydrodistillation and the essential oil was extracted immediately after this process. Five portions (700.0 ± 7.3 g) of the dried leaves were individually subjected to hydrodistillation using a Clevenger-type apparatus for 3 h. The yield of the essential oil from dried leaves of *Hyptis martiusii* was 1.30 ± 0.07% (w/w), which corresponds to 9.07 ± 0.53 g of oil, calculated according to the mean of dry weight of the leaves used in each extraction. The water/oil mixture was collected, dried over anhydrous sodium sulfate and then filtered. Essential oil was stored in an amber bottle at −20 °C until the accomplishment of the pharmacological experiments and phytochemical analysis.

2.2. Gas chromatography coupled to mass spectrometry analysis

Oil analysis was performed in a gas chromatographer coupled to a mass spectrometer (GC–MS, SHIMADZU QP5050A) equipped with a DB-5HT capillary column (30 m × 0.25 mm, 0.1 μm film thickness) with the following specifications: helium as carrier gas (1.0 mL/min flow rate); injector temperature 270 °C and detector temperature 290 °C; linear velocity of 47.3 cm/s; pressure of 107.8 kPa; column temperature programmed from 60 °C (2 min) to 180 °C (1 min) at 4 °C/min, then 180 to 260 °C at 10 °C/min (10 min). The mass spectrometer was operated using 70 eV of ionization energy. Identification of individual constituents was based on the interpretation of their mass spectral fragmentation using computer-based library MS searches standards (Wiley 229), retention indices and comparison with the mass spectral database and data from the literature (Adams, 2001).

2.3. Animals

Male Wistar rats (250–300 g) obtained from the Federal University of Pernambuco's Department of Physiology and Pharmacology and Swiss mice (35–40 g) obtained from the Aggeu Magalhães (CPqAM/Fiocruz/UFPE) Research Center, Pernambuco, Brazil, were used. These were kept under standard environmental conditions (12 h dark/light cycle) and temperature (22 ± 2 °C). Water and industrialized dry food (Labina®, Purina, Brazil) were available *ad libitum*. All the experimental protocols were submitted to and approved by the Animal Experimentation Ethics Committee of the UFPE, under license no. 007764 in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.4. Acute toxicity

"Up and down" acute toxicity studies were performed on Swiss mice of both sexes as described by OECD 420 (2001), with slight modifications. The animals were randomly divided into four groups (n = 5/group/sex) and deprived of feed for 12 h with access to water *ad libitum*. The treated groups received essential oil of *Hyptis martiusii* in a single oral dose of 5.0 g/kg and the control groups received distilled water (10 mL/kg). The observations were performed at 30, 60, 120, 180 and 240 min after the oral treatments and daily for 14 days. Behavioral changes, weight, consumption of food and water, clinical signs of toxicity, and mortality were recorded daily (Malone, 1977).

2.5. Antiulcerogenic activity

2.5.1. Ethanol-induced gastric ulcer

After 16 h of fasting, the rats (n = 6/group) were orally pretreated with the essential oil of *Hyptis martiusii* dissolved in a 1% Tween-80 aqueous solution (100, 200 and 400 mg/kg), a vehicle (1% Tween-80 aqueous solution) and pantoprazole (40 mg/kg), 1 h before administration of the ulcerogenic agent. Gastric lesions were induced using ethanol (70%, 0.5 mL/100 g, p.o.) according to the method described by Robert et al. (1979). The animals were sacrificed using thiopental (140 mg/kg, i.p.) 1 h after induction of gastric lesions. Their stomachs were removed, by opening them along the greater curvature, the contents removed and the stomach mucosa gently washed with saline (0.9%) and examined for quantification of lesions. The gastric lesion area was measured using planimetry (mm^2) in relation to the total area of the gastric corpus.

2.5.2. HCl/ethanol-induced ulcer

The experiment was performed according to the method developed by Mizui and Douteuchi (1983), with slight modifications. After 24 h of fasting, the rats (n = 6/group) were orally pretreated with the essential oil of *Hyptis martiusii* dissolved in 1% Tween-80 aqueous solution (100, 200 and 400 mg/kg), a vehicle (1% Tween-80 aqueous solution) and pantoprazole (40 mg/kg). One hour after treatment, all the animals received 0.3 M HCl/ethanol 60% solution (1 mL/150 g, p.o.) orally to induce acute gastric lesions. The animals were sacrificed 1 h after induction of gastric lesions, their stomachs were removed and examined for quantification of the lesions using the parameters described above.

2.5.3. Indomethacin-induced gastric ulcer

After 16 h of fasting, the rats (n = 6/group) were orally treated with the essential oil of *Hyptis martiusii* dissolved in a 1% Tween-80 aqueous solution (100, 200 and 400 mg/kg), a vehicle (1% Tween-80 aqueous solution) and pantoprazole (40 mg/kg). Gastric lesions

were induced by subcutaneous administration of indomethacin 30 mg/kg, 30 min after administration of treatment (Djahanguri, 1969). The animals were sacrificed 6 h after indomethacin injection, their stomachs removed and inspected under magnification to determine the gastric lesions produced. The results were expressed as lesions, ulcers and total index, which were obtained from scores determined by various alterations in the gastric mucosa, considering the color, edema and hemorrhage, loss of mucus, petechiae or damage to the mucosa folds, and the number and size of necrohemorrhagic lesions (Vela et al., 1997).

2.5.4. Evaluation of gastric juice parameters – pyloric ligature

This assay used the method described by Visscher et al. (1954), with slight modifications. The animals were randomly divided into five groups ($n = 6$) and fasted for 16 h with free access to 5% glucose solution. For pyloric ligature, the animals were anaesthetized (xylazine 6 mg/kg and ketamine 60 mg/kg, i.p), the abdomen was opened and the pylorus ligated. Immediately after ligature, essential oil of *Hyptis martiusii* dissolved in 1% Tween-80 aqueous solution (100, 200 and 400 mg/kg), a vehicle (1% Tween-80 aqueous solution) and pantoprazole (40 mg/kg) were administered intraduodenally. Four hours later, the animals were sacrificed using thiopental (140 mg/kg, i.p), their abdomens opened and their stomachs removed. The gastric secretion was collected and centrifuged at 176 × *g* for 30 min. The final volume (mL) was determined after washing the mucosal side of the stomach with 2 mL of distilled water and pH values were measured using a digital pH meter. The total acidity of the gastric juices was determined by titrating it to pH 7.0 with 0.1 N NaOH using 2% phenolphthalein as indicator. The total concentration of acid was expressed as mequiv.$[H^+]$/mL/4 h.

2.6. Evaluation of gastrointestinal motility

2.6.1. Gastric emptying

The experiment was carried out using the method described by Gupta and Brans (1978), with slight modifications. After 6 h of fasting, the rats ($n = 6$/group) were orally pretreated with the essential oil of *Hyptis martiusii* dissolved in a 1% Tween-80 aqueous solution (100, 200 and 400 mg/kg) and a vehicle (1% Tween-80 aqueous solution) and subcutaneously with atropine (3 mg/kg), which was used as a positive control. After 1 h or 30 min of the administration of the treatments, each animal received 1.5 mL of phenol red (0.5 mg/mL) orally. The zero time control group was killed immediately after the administration of the marker and the other groups were sacrificed 30 min later. The stomachs were removed, opened along the greater curvature and washed with 7 mL of distilled water. The stomach contents collected were centrifuged at 176 × *g* for 15 min. After centrifugation, 1 mL aliquots of supernatants were added to 1 mL of 1 N NaOH and the absorbance of the solution read on a spectrophotometer at 560 nm. The results were plotted on a standard curve for phenol red and expressed as the concentration (μg) of dye retained in the stomach in relation the control group.

2.6.2. Intestinal transit

After removal of the stomach of the rats in the gastric emptying model (Section 2.6.1), the small intestine was removed for the evaluation of intestinal transit. With the aid of a ruler, the total length of the small intestine of each animal (distance between the region gastropiloric to the ileocecal junction) and the distance traveled by the phenol red (until the last portion of the intestine that contained at least 1 cm continuous marker) were measured. The results were expressed as a percentage of the distance traveled by the marker in relation to the total length of the small intestine (Stickney and Northup, 1959).

2.7. Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M.). Differences between means were determined using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The analysis was performed using GraphPad Prism 5.0®. The level of significance for rejection of the null hypothesis was set at 5% ($p < 0.05$).

3. Results

3.1. Chemical analysis

The chemical characterization of the essential oil using GC–MS identified twenty-four components, accounting for 92.13% of the total oil and indicated the occurrence of monoterpenes and sesquiterpenes. Fig. 1 shows the chromatographic profile of the

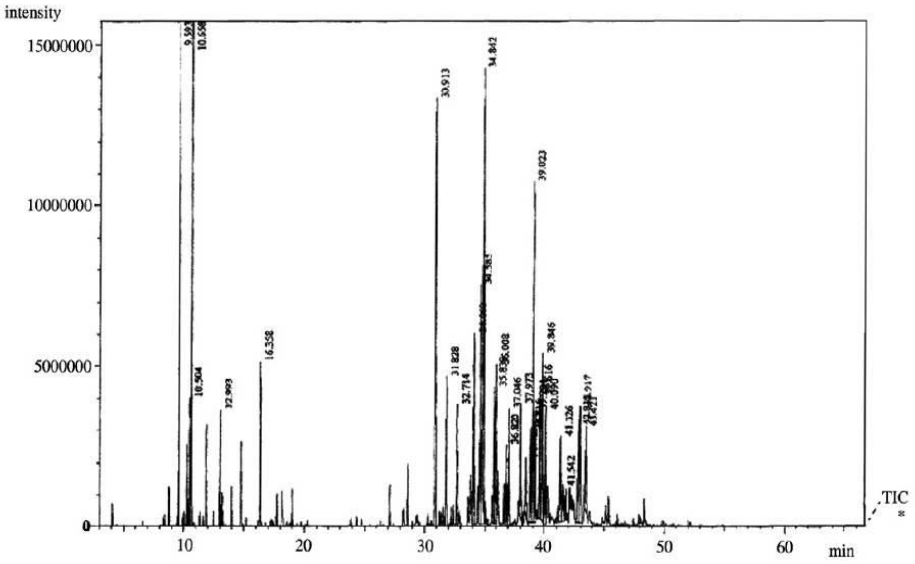

Fig. 1. Chromatographic profile of essential oil of leaves of *Hyptis martiusii* using gas chromatography coupled to a mass spectrometer.

Table 1
Chemical constituents of essential oil of leaves of *Hyptis martiusii* Benth.

Constituents	Retention Time (min)	(%)
δ-3-Carene	9.59	6.88
1,8-Cineole	10.65	7.01
Terpinolene	12.99	1.63
Camphor	16.35	2.66
trans-Caryophyllene	30.91	9.21
Aromadendrene	31.82	2.75
α-Humulene	32.71	2.21
Germacrene D	34.05	3.83
Ledene	34.58	5.41
Bicyclogermacrene	34.84	10.60
Torreiol	35.83	2.66
δ-Cadinene	36.00	3.12
Valecene	36.81	1.57
β-Guaiene	37.04	2.42
Isolongifol-8-ol	37.97	2.39
Spathulenol	38.81	1.93
Caryophyllene oxide	39.02	7.47
Globulol	39.20	2.16
Epiglobulol	39.61	2.34
Guaiol	39.85	3.35
10-Epi-α-eudesmol	41.54	1.36
α-Eudesmol	42.83	3.71
Epoxi-aromadendrene	42.91	3.02
δ-Guaiene	43.41	2.44
Total		92.13

EOHM. The major components identified were bicyclogermacrene (10.60%), trans-caryophyllene (9.21%), caryophyllene oxide (7.47%), 1,8-cineole (7.01%), δ-3-carene (6.88%) and ledene (5.41%). Table 1 shows the constituents identified, the percentage composition and retention index (RI).

3.2. Acute toxicity

It was observed that the essential oil of leaves of *Hyptis martiusii* (5.0 g/kg, v.o.) induced depression of the central nervous system (sedation) in mice of both sexes during the first 30 min and for a period of up to 4 h after administration. However, it produced no signs of acute toxicity or death in the treated animals. No significant changes in intake of food and water or body weight were observed during the 14 days of observation (data not shown). The LD_{50} could not therefore be estimated and it is possibly higher than 5.0 g/kg.

3.3. Antiulcerogenic activity

3.3.1. Ethanol-induced gastric ulcer

Administration of the essential oil of *Hyptis martiusii* inhibited the formation of ulcerative lesions induced by absolute ethanol. The percentage of ulcer inhibition was 47.1, 71.9 and 91.5% in animals pretreated with EOHM at doses of 100, 200 and 400 mg/kg, respectively, compared to the control group ($332.5 \pm 51.4\ mm^2$). The animals that received pantoprazole (40 mg/kg, p.o.) presented an inhibition of the lesions corresponding to 71.8% (Fig. 2A). In order to rule out the possibility that the protective effect of EOHM mucosa depends on local action, i.e. direct contact of the oil with the mucosa forming a protective barrier to the action of ethanol, the oil was administered intraperitoneally at the highest dose (400 mg/kg). When administered in this way, EOHM also provided significant protection of 83.0% for the gastric mucosa (data not shown).

3.3.2. HCl/ethanol-induced gastric ulcer

The essential oil of *Hyptis martiusii* provided significant gastric protection against lesions induced by acidified ethanol, the results showing that groups of animals pretreated orally with doses of 100, 200 and 400 mg/kg presented a percentage inhibition of 84.2, 88.5 and 92.2%, respectively, when compared to the control group

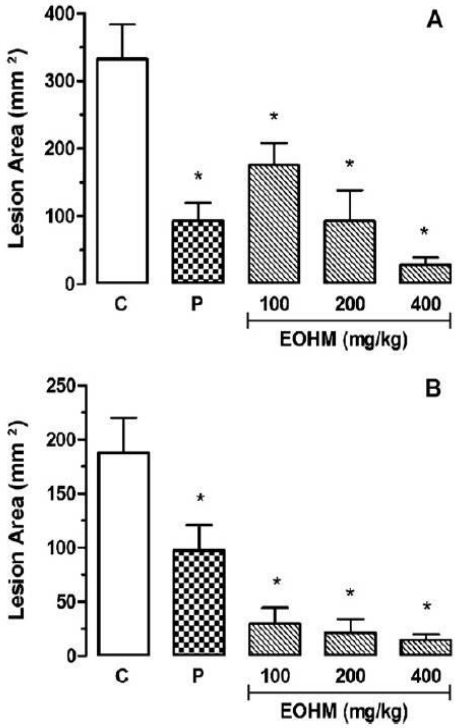

Fig. 2. Gastroprotective effect of the essential oil of *Hyptis martiusii* (EOHM) on gastric lesions induced by ethanol 70% (A) and HCl/ethanol (B) in male Wistar rats. The animals received orally a vehicle (C), pantoprazole, 40 mg/kg (P) and EOHM, 1 h before administration of ulcerogenic agent. The results are expressed as mean ± S.E.M. of 6 animals. *Statistically different from control group (ANOVA followed by Tukey's multiple comparison test, $p < 0.05$).

($187.8 \pm 32.6\ mm^2$). In the group treated with 40 mg of pantoprazole, a reduction of 48.2% in lesions was observed (Fig. 2B).

3.3.3. Indomethacin-induced gastric ulcer

Indomethacin (30 mg/kg) produced a gastric lesions index of 4.2 ± 0.4, an ulcer index of 20.8 ± 3.3 and a total index of 25.0 ± 3.5 in the control group. Pretreatment of animals with the essential oil of *Hyptis martiusii* at doses of 100, 200 and 400 mg/kg orally 1 h before subcutaneous administration of indomethacin, significantly reduced all indices as shown in Table 2. The 100 mg/kg dose reduced the incidence of lesions, ulcers and total index by 85.7, 75.5 and 77.2%, the 200 mg/kg dose by 73.8, 80.3 and 79.2%, and the 400 mg/kg dose by 88.1, 89.9 and 89.6%, respectively. Pantoprazole reduced the index of injuries, ulcers and the total index by 57.1, 84.1 and 79.6%, respectively, when compared with the control group.

3.3.4. Evaluation of the gastric secretion parameters – pylorus ligature

After 4 h of pylorus ligature it was observed that the essential oil of *Hyptis martiusii*, when administered intraduodenally, decreased the gastric volume secreted when compared the control group (3.8 ± 0.2 mL) at all doses. As for the pH of gastric juices, the doses of 200 and 400 mg/kg increased pH by 26.1 and 34.7% respectively, when compared to the control and reduced the total acidity (H^+ concentration) of acid secretion by 59.1 and 67.7%, respectively. Pantoprazole modified these three parameters, reducing the volume and gastric acidity, while increasing the pH of gastric juices (Table 3).

Table 2
Effect of oral administration of essential oil of *Hyptis martiusii* (EOHM) on gastric lesions induced by indomethacin (30 mg/kg, s.c.) in male Wistar rats.

Treatment	Dose (mg/kg)	Lesion index	Ulcer index	Total index
Control	–	4.2 ± 0.4 (–)	20.8 ± 3.3 (–)	25.0 ± 3.5 (–)
Pantoprazole	40	1.8 ± 0.2* (57.1%)	3.3 ± 1.9* (84.1%)	5.1 ± 2.0* (79.6%)
EOHM	100	0.6 ± 0.4* (85.7%)	5.1 ± 3.9* (75.5%)	5.7 ± 3.9* (77.2%)
	200	1.1 ± 0.3* (73.8%)	4.1 ± 2.6* (80.3%)	5.2 ± 2.6* (79.2%)
	400	0.5 ± 0.3* (88.1%)	2.1 ± 1.2* (89.9%)	2.6 ± 1.5* (89.6%)

Values represent the mean ± S.E.M. for 6 animals/group.
The values in parentheses represent the percentage of inhibition for each parameter observed.
*Statistically different from control group (ANOVA followed by Tukey's multiple comparison test, $p < 0.05$).

Table 3
Effect of essential oil of Hyptis martiusii (EOHM), administered intraduodenally, on gastric secretion parameters in male Wistar rats subjected to pylorus ligature.

Treatment	Dose (mg/kg)	Gastric volume (mL)	Gastric pH value	Total acidity (mequiv.[H+]/mL/4 h)
Control	–	3.8 ± 0.2	2.3 ± 0.1	9.3 ± 1.5
Pantoprazole	40	2.6 ± 0.2*	4.0 ± 0.1*	2.5 ± 0.5*
EOHM	100	2.9 ± 0.2*	2.8 ± 0.1	5.8 ± 0.8
	200	2.7 ± 0.1*	2.9 ± 0.1*	3.8 ± 0.5*
	400	2.5 ± 0.1*	3.1 ± 0.1*	3.0 ± 0.5*

Values represent the mean ± S.E.M. for 6 animals/group.
* Statistically different from control group (ANOVA followed by Tukey's multiple comparison test, $p < 0.05$).

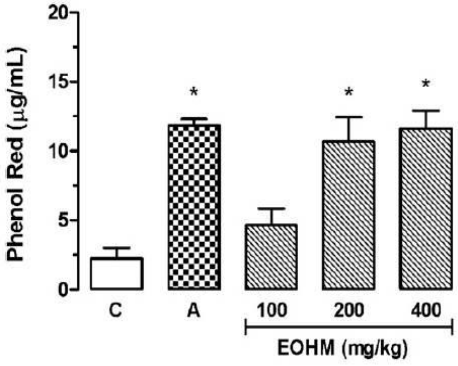

Fig. 3. Effect the essential oil of *Hyptis martiusii* (EOHM) on gastrointestinal motility in male Wistar rats. The animals received by oral route vehicle (C) and EOHM and atropine, 3 mg/kg, s.c. (A). The results expressed as mean ± S.E.M. for 6 animals. The ordinates indicate the concentration of phenol red retained in the stomach (gastric emptying) after 30 min ingestion of the marker. *Statistically different from control group (ANOVA followed by Tukey's multiple comparison test, $p < 0.05$).

3.3.5. Gastric emptying

The concentration of phenol red present in the stomach after 30 min of administration was 4.6 ± 1.2, 10.7 ± 1.7, and 11.6 ± 1.3 µg/mL in animals treated with the essential oil of *Hyptis martiusii* at doses of 100, 200 and 400 mg/kg, respectively. In the animals treated with atropine (3 mg/kg), the positive control, the concentration of phenol red increased to 11.8 ± 0.5 µg/mL, compared with animals treated with the vehicle (2.2 ± 0.8 µg/mL), as shown in Fig. 3. The results indicate that the animals treated with doses of 200 and 400 mg/kg showed a reduction in the rate of gastric emptying of 84.0 and 91.5%, while the gastric emptying rate for the group treated with atropine was 93.2% when compared to the zero time control.

3.3.6. Intestinal transit

In the control group of animals receiving only the vehicle, the intestinal transit, measured as the distance traveled by phenol red in relation to the total length of the small intestine, was 93.2 ± 1.9%. In the animals treated with the essential oil of *Hyptis martiusii* at doses of 100, 200 and 400 mg/kg had a percentage of 84.3 ± 2.5, 76.4 ± 4.4 and 76.2 ± 7.6%, respectively, indicating none of the doses showed a significant effect on intestinal transit when compared with the control group. However, the group treated with atropine reduced the percentage of intestinal transit by 50.0 ± 6.8%.

4. Discussion

This study investigated the pharmacological activity of the essential oil of dried leaves of *Hyptis martiusii* on gastric lesions induced by different necrotizing agents, as well as acid secretion parameters and gastrointestinal motility. This is the first report establishing the antiulcerogenic activity of the essential oil of this species.

The results of the acute toxicity test indicated that the essential oil of *Hyptis martiusii* when administered orally at a dose of 5.0 g/kg caused a reversible sedative effect, but did not produce any sign of toxicity or death in the treated animals, suggesting an LD_{50} of above 5.0 g/kg. Kennedy et al. (1986) reported that substances that present LD_{50} higher than 5.0 g/kg after oral administration can be considered practically non-toxic and it can therefore be suggested that acute toxicity for *Hyptis martiusii* essential oil is practically nil when administered in this way.

The results obtained demonstrate that the essential oil of dried leaves of *Hyptis martiusii* had gastroprotective effects on the various models tested. Necrotizing agents such as ethanol and HCl induce the formation of gastric mucosal injury; an effect that involves depression of gastric defense mechanisms and gastric blood flow stasis (Andreo et al., 2006). In addition, ethanol releases free radicals, increasing lipid peroxidation, decreasing production of gastric mucus and inhibiting prostaglandins (Abdel-Salam et al., 2001).

In models of ethanol and HCl/ethanol-induced ulcer, *Hyptis martiusii* displayed a significant gastroprotective dose-related effect at all doses administered orally. A similar response was observed when oil was administered intraperitoneally, which demonstrates that the antiulcer activity is not only related to an effect of local neutralization of stomach contents but is a systemic effect. These results indicate that the essential oil exhibits efficient cytoprotective activity and this probably acts to increase the protective elements of the gastric mucosa, such as mucus and/or bicarbonate, since it inhibited the formation of ulcerative lesions in both models. This hypothesis was corroborated by the action of the essential oil of *Hyptis martiusii* obtained in the model involving induction of gastric lesions by indomethacin.

It was observed that the suppression of prostaglandin synthesis induced by indomethacin caused an increased susceptibility to

gastric mucosal lesions, as verified in the control group. The essential oil of *Hyptis martiusii* significantly reduced damage to mucosa at all doses tested, when compared with the respective control, demonstrating its gastroprotective properties and suggesting the possible involvement of prostaglandins and/or mucus in antiulcerogenic activity.

In the pyloric ligature method, the digestive effect of accumulated gastric juices and interference in gastric blood flow are responsible for inducing ulceration (Patel et al., 2000). In this model, treatment with the essential oil of *Hyptis martiusii* changed all gastric secretion parameters, reduced the volume of gastric juices and total acidity, and increased gastric pH when compared with the control group. The results suggest that the pharmacological mechanism of the oil may be related to antisecretory activity, via the receptor system or mediators of the parietal cell. It also once again confirms that the antiulcer activity of essential oil is related to a systemic effect, since the treatment was also effective when administered intraduodenally.

Gastric emptying disorders may be associated with the pathophysiology of peptic ulcer diseases, gastroesophageal reflux disease and functional dyspepsia. It is known also that an increase in gastric hypermotility plays an important role in the pathogenesis of NSAID-induced lesions (Takeuchi et al., 2001). The evaluation of the effects on gastrointestinal motility demonstrated that the essential oil of *Hyptis martiusii* interfered as well as the atropine, reducing the rate of gastric emptying, but did not have an influence on intestinal transit at any of the three doses administered. In both experiments, the emptying reduction and transit response obtained with atropine, a muscarinic antagonist involved in decreasing antral contractility and gastric emptying (Chiba et al., 2002), confirm the importance of the cholinergic pathway for controlling the flow from the stomach to the duodenum. However, the inhibition of gastric emptying and the absence of a change in transit observed in animals treated with the essential oil may be related to another gastric site mediator, in addition to muscarinic receptors.

The chemical composition of the Lamiaceae family, especially the *Hyptis* genus, is remarkably variable, containing compounds such as terpenes, flavonoids, lactones, lignans, phenolic derivatives, steroids and others (Falcão and Menezes, 2003). Chemical analysis has revealed that the chemical composition of the essential oil of dried leaves consists of mono and sesquiterpenoids, its main constituents being bicyclogermacrene, trans-caryophyllene, caryophyllene oxide, 1,8-cineole, δ-3-carene and ledene. Biologically, terpene compounds exhibit a variety of interesting medicinal properties, including gastroprotective activity. The potential antiulcerogenic of several terpenoids have been evaluated, including triterpene oleanolic acid (Astudillo et al., 2002) and acetyl-aleuritolic acid (Pertino et al., 2007), sesquiterpene lactone trans-crotonin (Hiruma-Lima et al., 2002), diterpene ferruginol (Rodriguez et al., 2006), and monoterpene limonene and 1,8-cineole (Santos and Rao, 2001; Moraes et al., 2009). Some studies have pointed out that the gastroprotective activity of terpenoids may particularly involve reinforcement of defensive factors of the gastric mucosa (Ishikawa et al., 2008).

According to pharmacological studies, two species of *Hyptis* genus, *Hyptis mutabilis* and *Hyptis spicigera*, show gastroprotective activity related to terpene compounds. The essential oil obtained from leaves of *Hyptis mutabilis* reduced gastric lesions induced by indomethacin in a dose-dependent fashion, and the caryophyllene, an sesquiterpene present in this oil, also showed a similar response, suggesting that this compound would be responsible for the effect observed (Barbosa and Ramos, 1992). Takayama et al. (2011) verified that the essential oil from the aerial parts of *Hyptis spicigera* exerted gastroprotective effect in models ulcer-induced by NSAID and ethanol. Thus, it is possible that the antiulcerogenic activity observed in this study could be related to the presence of the terpene compounds present in the essential oil of the leaves of *Hyptis martiusii*; however we cannot affirm that the activity observed is limited only to this class of compounds. Other non-volatile components present in the leaves may also be involved, since in folk medicine the leaves of *Hyptis martiusii* species are used in folk medicine in the treatment of intestinal diseases and stomach in the form of decoction or infusion.

The activities proved in this study contribute to chemical, pharmacological and toxicological knowledge of little-studied species such as *Hyptis martiusii*. In conclusion, the results indicate that the essential oil of leaves of *Hyptis martiusii* has an antiulcerogenic activity, as evidenced by its significant inhibition of the formation of ulcers in various models. This effect could be related to an increase of gastric mucosal defensive factors. Further pharmacological studies are being undertaken in order to provide more precise elucidation of the action mechanism involved in this activity.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

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4.2 Artigo II: submetido ao periódico Evidence-Based Complementary and Alternative Medicine

Gastroprotective and ulcer healing effects of essential oil of *Hyptis martiusii* Benth. (Lamiaceae)

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Abstract

Hyptis martiusii Benth. is an aromatic plant found in abundance in northeastern Brazil that is used in ethnomedicine to treat gastric disorders. The aim of this study was to elucidate the mechanisms of action involved in the gastroprotection of the essential oil of *Hyptis martiusii* (EOHM) and to evaluate its healing capacity. The stimulated antisecretory activity, the involvement of nitric oxide, sulfhydryl groups (GSH), mucus and the levels of malondialdehyde (MDA) and GSH were evaluated. The acetic acid-induced gastric ulcer model and histological analysis were used to evaluate the healing capacity. EOHM (400 mg/kg) reduced the volume and acidity of gastric secretion stimulated by histamine and pentagastrin. The gastroprotective effect of EOHM involves the participation of endogenous sulfhydryl groups. EOHM increased mucus production (54.8%), reduced levels of MDA (72.5%) and prevented the depletion of GSH (73.8%) in the gastric mucosa. The treatment with OEHM reduced ulcer healing chronic (70.3%), promoting significant regeneration of the gastric mucosa, as confirmed by histological analysis. The results show that gastroprotective effect of EOHM is mediated by cytoprotective and antioxidant mechanisms and by their antisecretory activity, and suggest that the essential oil of *Hyptis martiusii* is a promising candidate for the treatment of gastric ulcers.

Keywords*: Hyptis martiusii*, essential oil, gastroprotection, healing activity

1. Introduction

Despite advances in understanding the etiology, diagnostic modalities and availability of modern treatments for dyspeptic symptoms, peptic ulcer disease and its complications remain a major cause of morbidity and mortality worldwide [1]. A variety of pathogenic mechanisms may contribute to the formation of a peptic ulcer, but, regardless of the etiology, the onset of an ulcer occurs when there is an imbalance in the environment caused by increased aggressive factors of exogenous or endogenous origin, or by decreased gastric resistance, leading to irritation, ulceration and mucosal bleeding [2].

The gastric mucosa uses various defense mechanisms to maintain its integrity against aggression caused by hydrochloric acid, pepsin, bile, NSAID, and other substances. The main mucosal protective factors are mucus, bicarbonate, the prostaglandins, adequate blood flow, somatostatin, nitric oxide, sulfhydryl compounds, motility and cell regeneration [3].

Hyptis martiusii Benth. (Lamiaceae) is an aromatic plant found in abundance in northeastern Brazil, where it is popularly known as *cidreira-do-mato* or *cidreira-brava* and is known as a potential source of essential oils, like other species of the genus Hyptis. The medical uses of some species, such as *Hyptis suaveolens, Hyptis pectinata, Hyptis crenata* and *Hyptis fruticosa* are well-known among the population. According to ethnopharmacological studies, an infusion or decoction of leaves of *Hytpis martiusii* are used against diseases of the stomach and intestine [4].

Few investigations of the pharmacological properties of *Hyptis martiusii* are described in the literature, including cytotoxic and antiproliferative effects on certain tumor cell lines [5, 6], insecticidal activity against larvae of *Aedes aegypti* and *Culex quinquefasciantus* [7], antimicrobial activity against resistant strains of *Staphylococcus aureus* and *Escherichia coli* [8] and antioxidant activity [9]. In addition, our laboratory previously reported the antiulcerogenic activity in models of acute gastric ulcers and potent antisecretory activity in the essential oil of *Hyptis martiusii* leaves [10]. We thus investigated the action mechanisms involved in the gastroprotective effects of the essential oil of *Hyptis martiusii* (EOHM) and its ulcer healing properties.

2. Material and methods

2.1 Plant material

Hyptis martiusii (Lamiaceae) leaves were colleted on the Araripe Plateau, in Crato, in the Brazilian State of Ceará (S 7°21.744’ – W 39°28.691’). Entire plants were collected during the flowering stage, in June 2011. A representative sample of this species is deposited in the Prisco Bezerra Herbarium of the Department of Biology at the Federal University of Ceará (UFC) (registration no. 43038).

2.2 Extraction of essential oil

The leaves were dried at room temperature for 72 h prior to hydrodistillation and the essential oil was extracted immediately thereafter. Three portions (780.70 ± 12.13 g) of the dried leaves were individually subjected to hydrodistillation using a Clevenger-type apparatus for 3 h. The yield of the essential oil from dried leaves of *Hyptis martiusii* was 0.97 ± 0.01% (w/w), corresponding to 7.59 ± 0.05 g of oil, calculated according to the mean dry weight of

the leaves used in each extraction. The water/oil mixture was collected, the aqueous solution was discarded, the oil was dried over anhydrous sodium sulfate and then filtered. Essential oil was stored in an amber bottle at -20 °C ready for pharmacological experiments and phytochemical analysis.

2.3 Identification of the constituents of essential oil

The EOHM analysis was performed in a gas chromatographer attached to a mass spectrometer (GC-MS, SHIMADZU QP5050A) equipped with a capillary column (DB–5HT, 30 m x 0.25 mm, 0.1 µm film thickness) with the following specifications: helium as carrier gas (1.0 mL/min flow rate); injector temperature 270 ºC and detector temperature 290 ºC; linear velocity of 47.3 cm/sec; pressure of 107.8 kPa; column temperature programmed from 60 ºC (2 min) to 180 ºC (1 min) at 4 ºC/min, then from 180 to 260 ºC at 10 ºC/min (10 min). The mass spectrometer was operated using 70 eV of ionization energy. Identification of individual constituents was based on the interpretation of their mass spectral fragmentation using computer-based library MS standard searches (Wiley 229), retention indices and comparison with the mass spectral database and data from the literature [11].

2.4 Animals

Male and female Wistar rats (200–300 g) were obtained from the Federal University of Pernambuco's Department of Physiology and Pharmacology, Pernambuco, Brazil. These were kept under standard environmental conditions (12 h dark/light cycle) and temperature (22 ± 2 °C). Water and industrialized dry food (Labina®, Purina, Brazil) were made available *ad libitum*. All the experimental protocols were submitted to and approved by the Animal Experimentation Ethics Committee of the UFPE, (license nº. 012490), in accordance with the National Institute of Health's Guide to the Care and Use of Laboratory Animals.

2.5 Reagents and Chemicals

The reagents and substances were obtained from the companies (Sigma, St. Louis, USA; Vetec, Rio de Janeiro, Brazil; FMaia and Vetbrands, São Paulo, Brazil). For the purposes of the experiment, the essential oil of *Hyptis martiusii* was emulsified in a Tween 80 at 1% before administration to the animals.

2.6 Evaluation of mucosal protective factors

Each experimental model comprised the following groups: positive control (pantoprazole – a proton pump inhibitor, carbenoxolone - a cytoprotective agent or N-acetylcysteine – the standard antioxidant drug) depending on the specificity of each model; a negative control (1% Tween-80 aqueous solution) and EOHM. Previous pharmacological studies performed by our group on the essential oil of dried leaves of *Hyptis martiusii* showed antiulcerogenic activity at doses of 100, 200 and 400 mg/kg in acute gastric ulcer models. The dose of 400 mg/kg was chosen for additional studies in order to shed light on the mechanisms underlying its gastroprotective effect, as this had been shown to be the most effective dose in previously assessed protocols.

2.6.1 Determination of gastric acid secretion stimulated with histamine, bethanechol and pentagastrin

This assay used the method described by Shay et al. [12]. The animals were divided into 10 groups (n = 6) and fasted for 16 h with free access to 5% glucose: (1) control, (2) EOHM, (3) histamine, (4) histamine plus ranitidine, (5) histamine plus EOHM (6) bethanechol, (7) bethanechol plus atropine, (8) bethanechol plus EOHM, (9) pentagatrin and (10) pentagastrin plus EOHM. For pyloric ligature, the animals were anaesthetized (xylazine, 6 mg/kg and ketamine, 60 mg/kg, i.p.) and immediately after ligature received an intraduodenal dose of EOHM (400 mg/kg), a control (1% Tween-80 aqueous solution, 0.1 mL/100 g body weight), ranitidine (60 mg/kg) or subcutaneous atropine (1 mg/kg). The abdominal wall was sutured and, 1h after pylorus ligation, the animals received a histamine (20 mg/kg s.c), bethanechol (2.5 mg/kg s.c) or pentagastrin (400 μg/kg s.c) stimulus. Four hours after pylorus ligation, the animals were sacrificed, the gastric secretion collected and centrifuged at 176 × g for 30 min. The volume (mL), pH values and the total acidity (mEquiv.[H+]/mL/4h) were determined.

2.6.2 Determination of gastric mucus

Adherence to the gastric wall mucus was quantified using the method described by Corne et al. [13] using the ethanol-induced ulcer model [14]. After fasting for 16 h, the animals (n =

6/group) were treated with 1% Tween-80 aqueous solution (CL), pantoprazole (40 mg/kg) and EOHM (400 mg/kg) 1 h before ethanol (70%, 0.5 mL/100 g, p.o) was used to induce a gastric lesion. The non-injured control group (CN) received no treatment. The animals were sacrificed 1 h after the administration of ethanol and their stomachs were removed. Each glandular segment was weighed and immediately transferred to a tube containing 10 mL of 0.1% Alcian Blue and stained for 2 h. The dye complexed to the mucus gland wall was extracted with 10 mL of magnesium chloride (0.5 mol/L) and agitated for 2 h. At 4 mL of the mixture, 4 mL of diethyl ether were added and the solution was shaken. The emulsion obtained was centrifuged at 1480 × g for 10 min. The absorbance of samples was read in a spectrophotometer at 598 nm and results were expressed as μg of Alcian Blue/g of tissue.

2.6.3 Determination of the role of nitric oxide (NO) and sulfhydryl compounds (SH) in gastroprotection

To investigate the influence of endogenous NO and SH on the gastroprotective effect, the animals fasted for 24 h and were divided into nine groups (n = 6) of which three were pretreated with saline (i.p), three with L-NAME (Nω-nitro-L-arginine methyl ester, 70 mg/kg, i.p), an inhibitor of the NO-synthase enzyme and three with NEM (N-ethylmaleimide, 10 mg/kg, i.p), a blocker of sulfhydryl compounds [15, 16]. 30 min after pretreatment, each group received an oral administration of 1% Tween-80 aqueous solution (control), carbenoxolone (100 mg/kg) and EOHM (400 mg/kg). After 1h, all the animals received 1 mL of absolute ethanol (p.o.) to induce gastric lesions. The animals were sacrificed after 1h of ethanol administration, the stomachs were removed for determination of gastric lesions as previously described.

2.7 In vitro study of radical scavenging activity – DPPH assay

The free radical scavenging ability of the EOHM and 1,8-cineole was evaluated using a modified version of the DPPH method (2,2-diphenyl-1-picryl-hydrazyl) [17]. Samples were prepared in triplicate using aliquots of 3 mL of ethanolic solution of DPPH (40 μg/mL) and 1 mL of ethanol solution containing different concentrations (0.3–3.0 mg/mL) of EOHM, 1,8-cineole or positive control (thymol). The solutions were mixed and incubated for 30 min at room temperature and the absorbance was read in a spectrophotometer at 517 nm. The IC50 (inhibitory concentration of sample required to reduce the absorbance of the negative control

by 50%) was calculated from a calibration curve obtained from the % of antioxidant activity versus concentration of EOHM, 1,8-cineole or thymol. Equation for antioxidant activity: % antioxidant activity = Abs negative control - (Abs sample - Abs blank) x 100/Abs negative control.

2.8 In vivo antioxidant activity

The antioxidant tests were performed with the homogenate of the gastric mucosa of animals with ethanol-induced ulcers [14]. The animals were divided into four groups (n = 6/group), uninjured control group (CN), control (1% Tween 80 aqueous solution), N-acetylcysteine (NAC, 750 mg/kg) as the standard antioxidant drug and EOHM (400 mg/kg). The uninjured control group consisted of untreated animals, exposed to experimental procedures, but without effective ulcer induction.

2.8.1 Determination of lipid peroxidation (LPO)

The lipid peroxidation index was determined using the method described by Ohkawa et al. [18] involving measuring malondialdehyde (MDA). The stomach tissue excised was homogenized in a cold KCl (0.15 mol/L) solution and centrifuged at 11,000 × g for 20 min at 4 °C. Aliquots of 0.2 mL of sodium lauryl sulfate (8.1%), 1.5 mL of acetic acid (20%, pH 3.5), 1.5 mL of thiobarbituric acid (0.8%, w/v) and 0.3 mL of distilled water were added to 0.5 mL of the homogenate. The samples were incubated in a water bath at a temperature of 95 ºC for 1 h. After cooling, 6 mL of an n-butanol + distilled water mixture (5:1, v/v) was added, the tubes were vortexed for 1 min, and finally centrifuged at 1073 × g for 10 min. The absorbance was measured in a spectrophotometer at 532 nm and the results were expressed as μmol of MDA/g tissue.

2.8.2 Quantification of non-protein sulfhydryl groups (GSH)

The levels of non-protein sulfhydryl groups (GSH) in the gastric mucosa were determined using the method developed by Sedlak and Lindsay [19]. The excised stomach tissue was weighed and homogenized in a cold EDTA (0.02 mol/L) solution. Aliquots of 320 μL of distilled water and 80 μL 50% aqueous solution of trichloroacetic acid were added to 400 μL of the homogenate for protein precipitation and the samples were then centrifuged at

604 × g for 15 min at 4 °C. To a total of 400 μL of supernatant was added 800 μL of 0.4 M Tris (pH 8.9) and 20 μL of 5,5-dithiobis(2-nitrobenzoic acid) to 0.01 M. The mixture was then stirred for 3 min and the absorbance was measured at 412 nm using a spectrophotometer. The concentrations of non-protein sulfhydryl groups were expressed in μg of GSH/g tissue.

2.9 Evaluation of healing properties

2.9.1 Acetic acid-induced gastric ulcer

Chronic ulcer induction was based on described by Takagi et al. [20] with some modifications. The animals were divided into 3 groups (n = 6), given a restricted solid food diet for 24 h and, after this, anesthetized in order to perform surgery to expose the stomach. 0.05 mL of 30% acetic acid was injected into the subserosal layer of the external wall of the stomach. One day after administration of acid, daily treatment began and the animals were treated orally once daily for 14 days with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and EOHM (400 mg/kg). During this period, the possible toxic effects of EOHM were evaluated using such parameters as mortality, changes in body mass and macroscopic analysis of vital organs. On day 15, all groups were sacrificed, the stomachs removed, photographed and the surface area of gastric lesion determined by computerized planimetry (Software ImageJ®) and the data expressed in mm^2.

2.9.2 Histological analysis

The stomach lesions induced by acetic acid in rats undergoing different treatments were located, sectioned, and set in 10% buffered formalin. After setting, the samples was washed with water, immersed in 70% ethyl alcohol for 3-4 days and embedded in paraffin. Five-μm thick paraffin sections were taken and stained with hematoxylin/eosin (HE) and Periodic Acid–Schiff (PAS). Histological analysis of the gastric sections was carried out using an automatic microscopy system MICRO DIP® (Kacil Inc.).

2.9.3 Immunohistochemical analysis

The immunohistochemical for PCNA was performed in samples of rat's stomach embedded in paraffin. Sections of 4 μm were obtained and incubated for 30 min with

monoclonal antibody against the anti-PCNA protein. Initially, the samples were deparaffinized in xylene and hydrated. Then antigenic retrieval was performed in microwave oven at 100 °C, the slides were cooled to room temperature and endogenous peroxidase was blocked by 7.5 min in peroxidase blocking solution. After cooling, were then incubated separately with primary antibodies for PCNA (anti-PCNA antibody [PC10] - Proliferation Marker (ab29) - Mouse monoclonal antibody, Abcan Inc), 1:100, 30 min, and with secondary antibody (Nichirei Biosciences Inc.), 1:200 for 30 min and then washed with phosphate buffered saline (PBS). After washing, slides were incubated with diaminobenzidine chromogen solution (DAB), washed in water, counter-stained with hematoxylin, dehydrated and mounted. The analysis of the reactivity was performed by the nucleus of epithelial cells using the following scores: reactivity mild, moderate and hard. Cells reactive for anti-PCNA were identified by the presence of a dark reddish-brown chromogen.

2.10 Statistical analysis

Values were expressed as mean ± standard error of mean (S.E.M.). The differences between groups were determined by analysis of variance (ANOVA) followed by Tukey's test. Statistical analysis was performed using GraphPad Prism 5.0®. The level of significance for rejection of the null hypothesis was set at 5% ($p < 0.05$).

3. Results

3.1 Chemical analysis of essential oil

Chemical characterization of the EOHM using GC-MS identified 27 components, accounting for 96.3% of the total oil. The main components identified in the essential oils of *Hytpis martiusii* were 1,8-cineole (32.8%), δ-3-carene (17.4%), camphor (6.7%), α-pinene (3.5%) and caryophyllene oxide (3.5%). Table 1 shows the constituents identified and the percentage composition according to their retention times.

Table 1. Chemical constituents of essential oil of leaves of *Hyptis martiusii* Benth.

Components	**Retention Time (min)**	**(%)**
Hexen-1-ol	4.39	1.81
α-Pinene	6.59	3.52
β-Pinene	8.18	2.28
β-Myrcene	8.69	1.81
δ-3-Carene	9.60	17.43
p-Cymene	10.01	0.87
o-Cymene	10.35	3.36
1,8-Cineole	10.76	32.80
Linalool	13.89	1.21
Camphor	16.31	6.70
Isoborneol	17.54	0.99
trans-Caryophyllene	30.69	3.37
Aromadendrene	31.63	1.96
α-Humulene	32.51	1.69
Ledene	34.32	0.99
Germacrene B	34.59	2.21
γ-Selinen	36.58	0.77
β-Panasinsene	36.81	0.97
Isolongifol	37.74	0.97
Palustrol	38.71	0.83
Spathulenol	38.62	1.85
Caryophyllene oxide	38.82	3.50
Globulol	38.98	0.91
Ledol	39.85	1.04
Rosifoliol	40.04	0.86
(*Z*)-Valerenyl acetate	42.43	0.82
Total		**96.33**

3.2.1 Effect of the EOHM on stimulated gastric acid secretion

It was found that, after 4 h of ligation of the pylorus, intraduodenal administration of EOHM reduced the volume of gastric secretion, increased the pH of gastric juices and reduced the total acidity of the acid secretion compared to controls not stimulated by secretagogues. Histamine, pentagastrin and bethanechol, when administered subcutaneously, stimulated base gastric acid secretion, increasing the volume and total acidity and decreasing the pH of gastric acid. Ranitidine (60 mg/kg) and atropine (1 mg/kg) managed to prevent the increase in volume and acidity of gastric contents, as well as decreasing the pH of gastric acid secretion stimulated by histamine and bethanechol. The EOHM was able to prevent the increase in volume and acidity of gastric acid secretion stimulated by histamine and pentagastrin, but was not able to change the gastric acid secretion parameters affected by bethanechol (Table 2).

Table 2. Effect of the essential oil of *Hyptis martiusii* (EOHM) on gastric secretion parameters basal or stimulated by histamine (20 mg/kg), bethanechol (2.5 mg/kg) and pentagastrin (400 µg/kg) in Wistar rats subjected to pylorus ligature.

Stimulus + treatment	**Gastric volume (mL)**	**pH**	**Total acidity (mequiv. H^+]/mL/4 h)**
control (not estimulated)	4.4 ± 0.7	1.6 ± 0.2	42.5 ± 11.6
EOHM (not estimulted)	1.8 ± 0.2*	2.4 ± 0.2*	9.2 ± 1.7*
histamine	6.3 ± 0.7	1.5 ± 0.0	52.8 ± 9.9
histamine + ranitidine	2.3 ± 0.3$^{\#}$	2.6 ± 0.1$^{\#}$	2.9 ± 0.6$^{\#}$
histamine + EOHM	4.2 ± 0.2$^{\#}$	1.8 ± 0.1	24.8 ± 2.9$^{\#}$
bethanechol	7.8 ± 0.5	1.5 ± 0.0	52.2 ± 5.4
bethanechol + atropine	2.8 ± 0.2$^{\#\#}$	2.5 ± 0.2$^{\#\#}$	6.6 ± 1.5$^{\#\#}$
bethanechol + EOHM	6.3 ± 0.2	1.6 ± 0.0	38.8 ± 5.3
pentagastrin	7.1 ± 1.4	1.4 ± 0.0	45.0 ± 7.8
pentagastrin + EOHM	3.4 ± 0.2$^{\#\#\#}$	1.91 ± 0.1	14.6 ± 3.4$^{\#\#\#}$

Values are expressed as mean ± S.E.M. (n = 6/group). Treatment: control (C, 1% Tween-80 aqueous solution, 0.1 mL/100 g, i.d), EOHM (400 mg/kg, i.d), ranitidine (60 mg/kg, i.d.) and atropine (1 mg/kg, s.c). *$p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. histamine group, $^{\#\#}p < 0.05$ vs. bethanechol group and $^{\#\#\#}p < 0.05$ vs. pentagastrin group (ANOVA followed by Tukey's test).

3.2 Evaluation of mucosal protective factors

3.2.2 Effect of the EOHM on the production of gastric mucus

Figure 1 shows that the animals injured with ethanol (CL) showed a significant decrease in the levels of gastric mucus (4.9 ± 0.4 μg of Alcian Blue/g tissue) compared to the non-injured control group (CN, 10.1 ± 1.0 μg of Alcian Blue/g tissue). Only treatment with EOHM at a dose of 400 mg/kg was capable of increasing mucus production to a significant degree (7.6 ± 0.5 μg of Alcian Blue/g tissue) compared to the injured group that received 1% Tween-80 aqueous solution. Pantoprazole, used as positive control, also caused a significant increase in the levels of gastric mucus (7.5 ± 0.4 μg of Alcian Blue/g tissue).

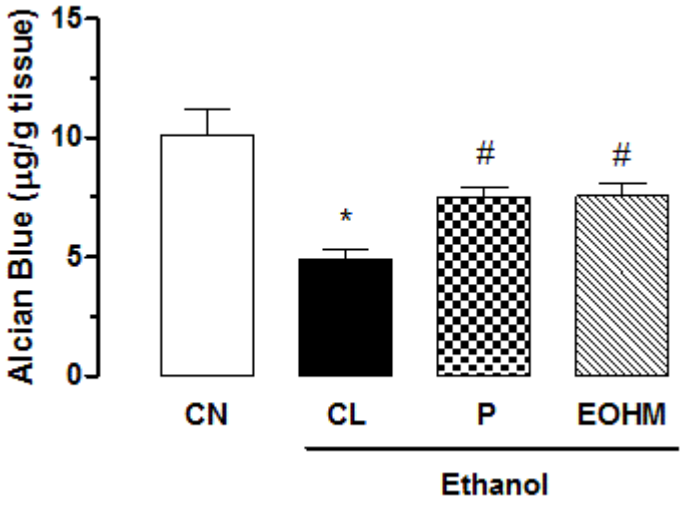

Figure 1. Quantification of adherent mucus in gastric mucosa of rats treated with essential oil of *Hyptis martiusii* (EOHM) on gastric ulcers model induced by ethanol. The non-injured control group (CN) received no treatment. 1% Tween-80 aqueous solution (CL, injured control), pantoprazole (P, 40 mg/kg) and EOHM (400 mg/kg) were administered 1 h before the induction of gastric lesions. Results are expressed as mean ± S.E.M (n = 6/group). ANOVA followed by Tukey's test (*$p < 0.05$ vs. non-injured control group-CN and $^{\#}p < 0.05$ vs. injured control group-CL).

3.2.3 Role of nitric oxide (NO) and sulfhydryl compounds (SH) in EOHM gastroprotection

Both the NO-synthase inhibitor, L-NAME (Nω-nitro-L-arginine methyl ester) and the inhibitor of sulfhydryl compounds, NEM (N-ethylmaleimide), caused an increase in gastric lesions in all groups compared to the groups pretreated with saline. In animals pretreated with saline, treatment with EOHM (400 mg/kg) had a gastroprotective effect as expected, as the oil inhibits the formation of gastric lesions induced by ethanol. In rats pretreated with L-NAME, EOHM also had a gastroprotective effect. However, the depletion of sulfhydryl groups by pretreatment with NEM was able to significantly reduce the gastroprotective effect of EOHM (Table 3).

Table 3. Effect of oral administration of essential oil of *Hyptis martiusii* (EOHM) on gastric lesions induced by ethanol in Wistar rats pretreated with L-NAME (N_ω-nitro-L-arginine methyl ester, 70 mg/kg) or NEM (N-ethylmaleimide, 10 mg/kg).

Pretreatment	**Treatment (p.o.)**	**Dose (mg/kg)**	**Lesion area (mm^2)**	**Inhibition (%)**
Saline (i.p.)	control	-	316.9 ± 49.9	-
	carbenoxolone	100	6.6 ± 2.9*	97.9
	EOHM	400	5.8 ± 5.4*	98.1
L-NAME (i.p.)	control	-	655.3 ± 51.2*	-
	carbenoxolone	100	228.5 ± 76.3$^{\#}$	65.1
	EOHM	400	6.5 ± 4.0$^{\#}$	99.0
NEM (i.p.)	control	-	563.0 ± 1.3*	-
	carbenoxolone	100	380.0 ± 42.4	32.5
	EOHM	400	427.6 ± 59.3	24.0

Results are expressed as mean ± S.E.M (n = 6/group). *$p < 0.05$ compared to saline + control, $^{\#}p < 0.05$ compared to L-NAME + control (ANOVA followed by Tukey's test).

3.3 DPPH free-radical scavenging assay

The results show that the essential oil of *Hyptis martiusii* (EOHM) exhibited no significant relative ability to promote the capture of the DPPH radical at any of the concentrations tested (0.3-3.0 mg/mL), since the IC50 (15.78 ± 0.99 mg/mL) was very high.

The 1,8-cineole, a major component of the essential oil, was likewise unable to promote the capture of DPPH. It was not possible to calculate the IC50. Thymol, a monoterpene common in essential oils, was used as a reference compound since it has known antioxidant properties and an IC50 of 0.67 ± 0.02 mg/mL [21].

3.4 Effects of the EOHM on the antioxidant activity system

The rate of lipid peroxidation (LPO) in gastric mucosa of rats subjected to ethanol-induced gastric ulcer was determined by quantifying malondialdehyde, which reacts with thiobarbituric acid. Animals in the control group showed an increase in gastric levels of malondialdehyde in injured rats (15.3 ± 1.5 μmol MDA/g of tissue) compared to the uninjured control group that received only 1% Tween-80 aqueous solution (9.4 ± 1.5 μmol of MDA/g of tissue). Treatment with EOHM (400 mg/kg) decreased the rate of lipid peroxidation by significantly diminishing the production of malondialdehyde produced by ethanol (4.2 ± 0.8 μmol MDA/g of tissue). Oral treatment with N-acetylcysteine (NAC, 750 mg/kg) also inhibited the increase in the levels of malonadildehyde (2.7 ± 0.6 μmol of MDA/g of tissue) (Figure 2).

The levels of sulfhydryl groups in the gastric mucosa of the uninjured animals of the control group were 75.1 ± 5.7 μg GSH/g of tissue, but in animals with an ethanol-induced gastric lesion (injured control) a reduction in the levels of GSH in the glandular region of the gastric mucosa (45.0 ± 8.7 μg GSH/g of tissue) was observed, compared to baseline levels in the uninjured control group. Treatment with N-acetylcysteine (NAC, 750 mg/kg) and EOHM (400 mg/kg) showed that both were capable of reversing the reduction in levels of sulfhydryl groups in the mucosa, returning the antioxidant system to base levels (85.3 ± 9.2 and 78.2 ± 6.0 μg GSH/g of tissue), respectively (Figure 2).

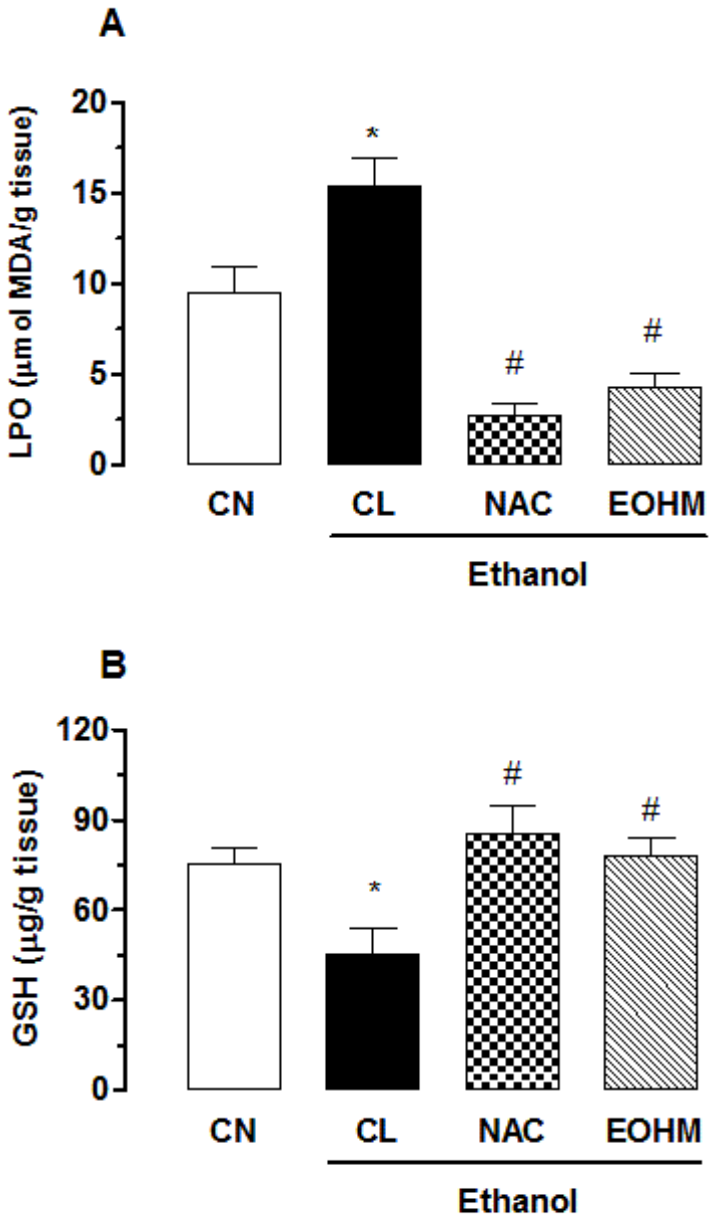

Figure 2. Effect of the essential oil of *Hyptis martiusii* (EOHM) on the levels of malondialdehyde (A) and non-protein sulfhydryl groups (B) in the gastric ulcers model induced by ethanol (70%, 0.5 mL/100 g, p.o). The non-injured control group (CN) received no treatment. 1% Tween-80 aqueous solution (CL, injured control), N-acetylcysteine (NAC, 750 mg/kg) and EOHM (400 mg/kg) were administered 1 h before the induction of gastric lesions. Results are expressed as mean ± S. E. M. (n = 6/group). ANOVA followed by Tukey's test (*$p < 0.05$ vs. non-injured control group-CN and $^{\#}p < 0.05$ vs. injured control group-CL).

3.5 Evaluation of the healing properties of EOHM

3.5.1 Effect of EOHM on acetic acid-induced gastric ulcer

Oral administration of EOHM (400 mg/kg) for 14 consecutive days of treatment, significantly decreased (70.3%) the area of chronic ulcer to 9.9 ± 2.2 mm² compared to the control group treated with 1% Tween-80 aqueous solution (control), in which the injured area corresponded to 33.3 ± 7.8 mm² (Figure 3). Pantoprazole (40 mg/kg) speeded up the healing of gastric ulcer, reducing the area of the lesion to a statistically significant extent by 5.2 ± 1.0 mm² (82.7%) compared to the control group. There were no visible signs of toxicity (diarrhea or changes in behavior or locomotor activity) in animals treated with EOHM and pantoprazole for 14 days, the animals treated with the essential oil showed body weight gain (Figure 4) and organ weight (data not shown) similar to those of animals in the control group.

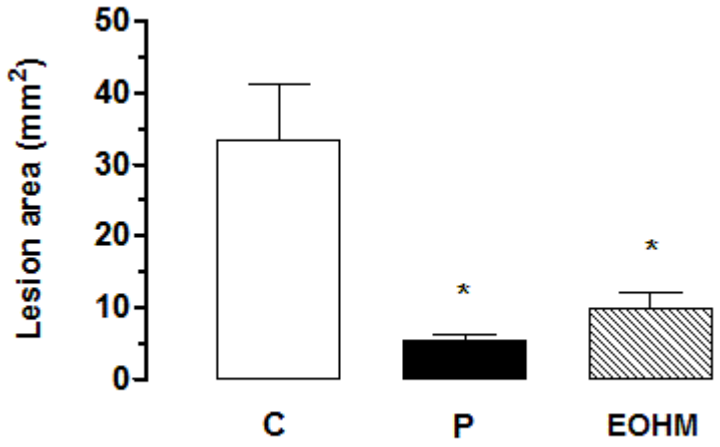

Figure 3. Effect of the essential oil of *Hyptis martiusii* (EOHM) in the healing of chronic ulcer induced by 30% acetic acid. 1% Tween-80 aqueous solution (C) pantoprazole (P, 40 mg/kg) and EOHM (400 mg/kg) were administered for 14 days. Values represent the mean ± S. E. M. (n = 6/group). *Statistically different from control group, $p < 0.05$ (ANOVA followed by Tukey's test).

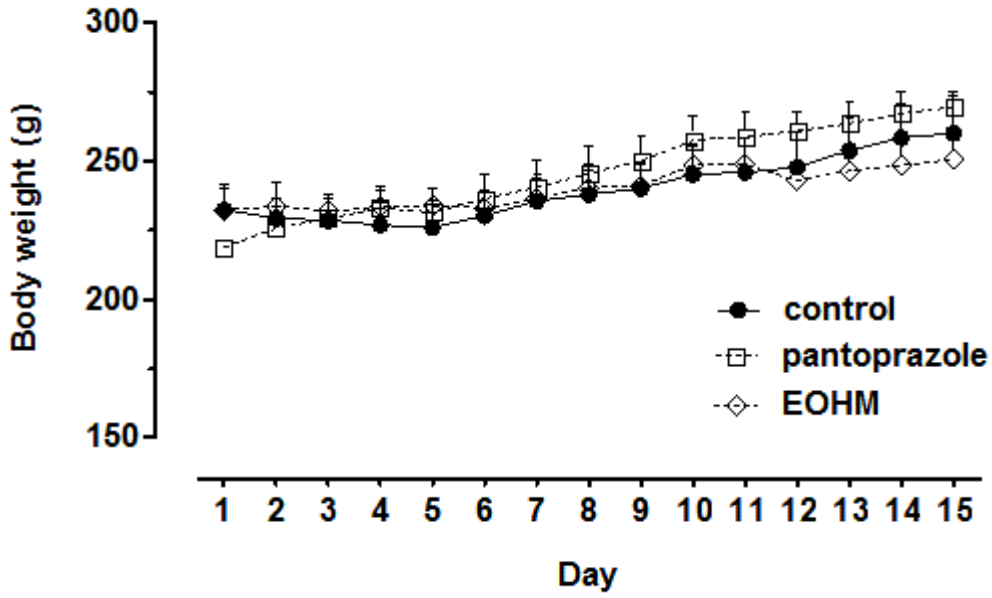

Figure 4. Body weight of rats treated orally with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and essential oil of *Hyptis martiusii* (EOHM, 400 mg/kg) for 14 days after formation of chronic ulcer induced by 30% acetic acid. Values represent the mean ± S. E. M. (n = 6/group).

3.5.2 Histological analysis

The HE staining revealed the presence of an ulcer penetrating the wall caused by gastric mucosa damage induced by 30% acetic acid and confirmed the gastroprotective action of EOHM (400 mg/kg) or pantoprazole (40 mg/kg) after 14 days of treatment, with significant regeneration of the gastric mucosa. PAS staining also revealed the presence of gastric mucus and the arrangement of intact glands in the groups treated with pantoprazole or EOHM, as shown in Figure 5.

3.5.3 Immunohistochemical analysis

The gastric tissues obtained in the acetic acid-induced gastric ulcer model were used for immunohistochemical localization of PCNA antibody. In Figure 6, notice that the PCNA-positive nuclei are marked by reaction with the color brown. In the control group, it was observed the absence of reactivity due to the destruction of the epithelial layer, but it is possible to observe a moderate activity in the layer adjacent to the ulcer. In animals treated

with pantoprazole or OEHM intense reactivity was observed, with a greater number of labeled cells to the antibody. The OEHM group showed a higher percentage of labeled nuclei, suggesting that the treatment promotes an expressive increase in cell proliferation in the area of gastric mucosal healing.

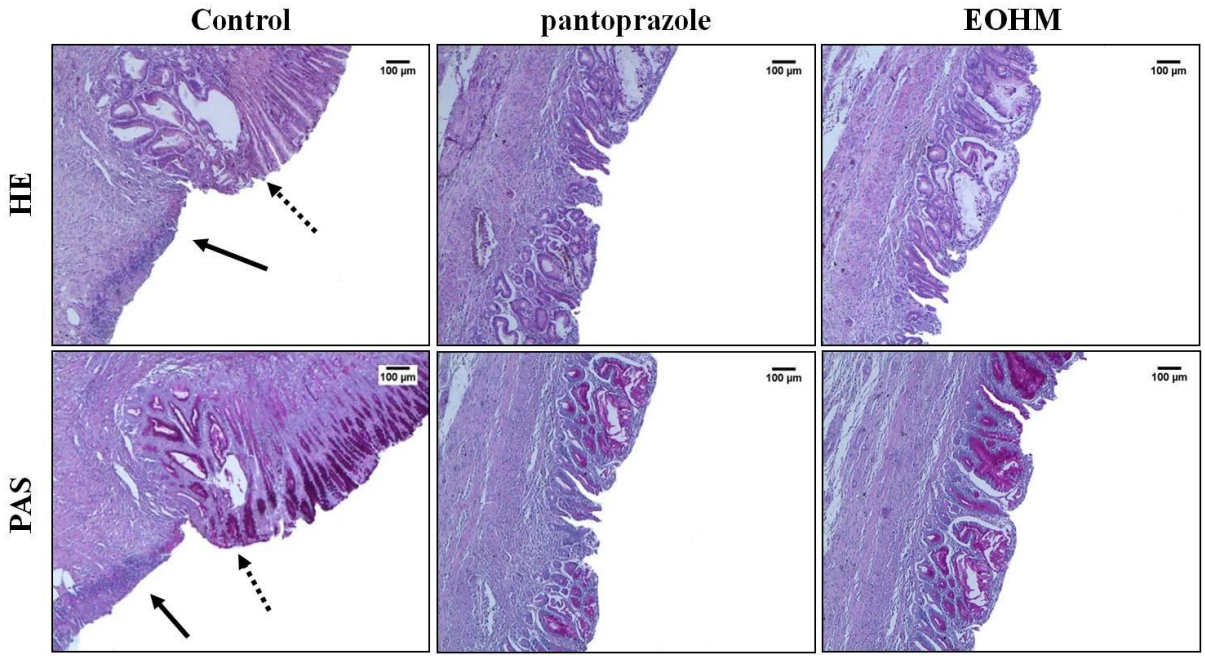

Figure 5. Histological analysis of rats' stomachs treated with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and essential oil of *Hyptis martiusii* (EOHM, 400 mg/kg) for 14 days after injury induced by 30% acetic acid on the haematoxylin/eosin staining (HE) and Periodic Acid–Schiff staining (PAS). The arrows indicate the area internal (filled arrow) and edge (dashed arrow) of the ulcer. Notice in the pantoprazole and EOHM groups, lesion regeneration area (HE) and the great amount of mucus secretion (PAS) evidenced by the intense tone of pink. Magnification 40x.

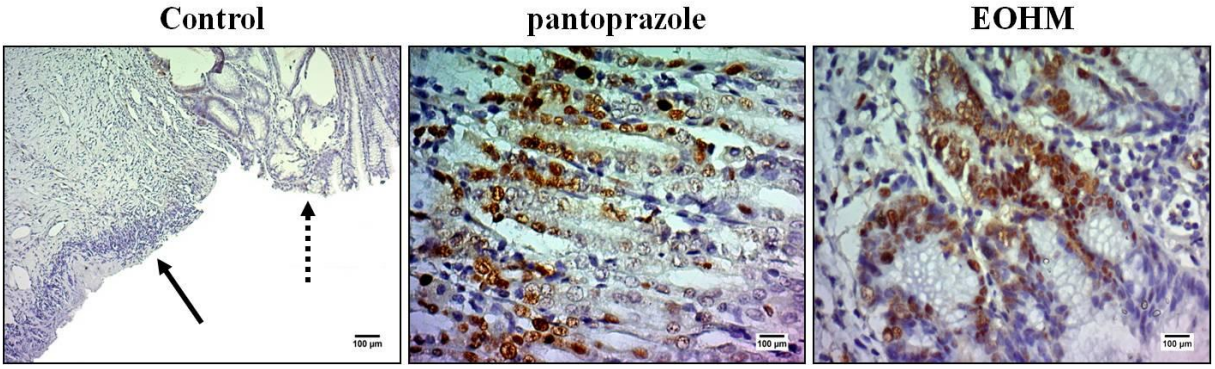

Figure 6. Immunohistochemical analysis for PCNA (proliferating cell nuclear antigen) of rat's stomachs treated with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and essential oil of *Hyptis martiusii* (EOHM, 400 mg/kg) for 14 days after injury induced by 30% acetic acid. The filled arrow indicates the absence of the epithelial layer (ulcer area internal). The dashed arrow indicates epithelial layer remaining (ulcer edge). Photomicrographs depict absence of PCNA immunoreactivity in the control group. Notice in pantoprazole and EOHM intense reactivity with PCNA-positive nuclei marked (brown color). Magnification, 200x (control) and 500x (pantoprazole or EOHM).

4. Discussion

In this studies we investigated the cytoprotective mechanisms that may be involved in the gastroprotective effect of the essential oil of *Hyptis martiusii*, as an influence on mucus production, the role of nitric oxide and sulfhydryl groups, possible antioxidant activity and its healing action regarding acetic acid-induced chronic ulcer. We also tried to clarify the possible mechanism of action responsible for the antisecretory acid effect of EOHM.

Data from the literature have demonstrated experimentally that a large number of essential oils of aromatic and medicinal plants possess gastroprotective and ulcer healing properties [22]. In essential oils in general, the major constituents are monoterpenes and sesquiterpenes, and this applies to species of the genus *Hyptis* where these constituents are identified as the main components [23]. Confirming these data the chemical characterization of the essential oil of *Hyptis martiusii* identified 27 components and between monoterpenes and sesquiterpenes the main components were 1,8-cineole, δ-3-carene, camphor, α-pinene and caryophyllene oxide.

Recently, data from our laboratory showed that the essential oil of *Hyptis martiusii* leaves displayed a significant gastroprotective effect on different models of gastric lesions in Wistar rats [10]. In this same study, the intraduodenal administration of the essential oil was able to decrease basal acid secretion in the gastric mucosa in rats with the pyloric ligation model, providing significant antisecretory activity.

In order to identify the effect of the EOHM on the receptors/mediators of the parietal cell in the gastric mucosa, we used the same technique of pyloric ligature, but the gastric acid secretion was stimulated with the agonists (secretagogues) of receptors of histamine (H2), acetylcholine (M3) and gastrin (CCK2). EOHM exhibited inhibitory action regarding gastric acid secretion, interfering with the volume and acidity of secretion induced by stimulation with histamine in the H2 receptor and with pentagastrin in the CCK2 receptor, but with no effect on gastric acid secretion induced by bethanechol in muscarinic receptors. These results suggest that the decrease in the volume and acidity of secretion in animals treated with EOHM is through interactions of compounds in connection with oil/signaling mediated by the histamine H2 and gastrin CCK2 receptors.

The first line of defense against acid is the mucus, which together with bicarbonate, covers the entire gastric mucosa and protects against bacterial colonization and mechanical forces of proteolytic digestion. The mucus acts as an antioxidant, reducing the damage caused by free radicals and lubricant gastric surface [24]. The results show a statistically significant increase in the amount of mucus adhering to the gastric mucosa in animals treated with OEHM, thereby explaining the gastroprotective action observed previously.

Nitric oxide (NO) is synthesized by NO-synthase (NOS) from oxygen (O2) and L-arginine and, owing to its ability to increase blood flow in gastric mucosa, to regulate mucus production and inhibit the attachment of neutrophils to endothelial cells, has been described as an important modulator of the integrity of the gastric mucosa, along with endogenous PGs [25]. In order to establish the involvement of NO in the gastroprotective effect of EOHM an NO synthase inhibitor (L-NAME) was used and it was found that in animals pretreated with L-NAME, EOHM continued to exert a gastroprotective effect, possibly suggesting that this effect is not dependent on NO release/synthesis.

The endogenous non-protein sulfhydryl groups (SH) present in the mucus and some enzymes of the antioxidant system are directly involved in protection of the gastric mucosa, since they participate in the production of gastric mucus and bind to the free radicals formed during inflammation or produced after exposure of the mucosa to harmful agents, performing a neutralizing function [26]. The participation of sulfhydryl compounds in gastric protection

provided by EOHM was assessed by pretreatment of animals with an inhibitor of SH compounds (NEM). The decrease in sulfhydryl compounds caused by NEM was able to reduce the gastroprotective effect of EOHM to a statistically significant extent, suggesting the participation of sulfhydryl compounds and that the protective effect is dependent on the presence of these compounds.

Although some terpene compounds present in essential oils have been described as antioxidants and antioxidant activity (IC50 = 0.13 mg/mL) has already been reported for the ethanol extract of this species [9], in the present study, the DPPH method revealed no such activity with the essential oil of *Hyptis martiusii*. A similar response was observed for other species of the same genus, Rebelo et al. [27] reported that the methanol extract of *Hyptis crenata* showed significant antioxidant activity (IC50 = 0.01 mg/mL) while the essential oil of leaves did not (IC50 = 6.88 mg/mL). It is possible that the absence of antioxidant activity of the oil is related to the fact that its major compound 1,8-cineole, did not show such activity. These data corroborate the results obtained by Amakura et al. [28] and Ojeda-Sana et al. [29] in which it was not possible to infer an IC50 value for 1,8-cineole.

The fact that EOHM does not exhibit in vitro antioxidant activity when using the DPPH method, does not mean that it will not show an antioxidant profile in other types of tests of the same activity. We thus evaluated the action of EOHM on oxidative stress caused by ethanol-induced gastric damage in rats, to show possible antioxidant activity. Ethanol destroys cells by causing mucosal disturbances in the microcirculation of free radicals in the mucosa, increased lipid peroxidation, a decrease of non-protein sulfhydryl groups (GSH) and mucus production and inhibition of gastric prostaglandins [30]. Furthermore, ethanol also destroys epithelial cells in the stomach, causing infiltration of inflammatory cells such as neutrophils and macrophages, which eventually produce vasoconstriction, submucosal edema and hemorrhagic lesions [31].

Confirming data already reported in the literature, we found increased levels of malondialdehyde (MDA) and a decrease in non-protein sulfhydryl groups (GSH) in stomachs with ethanol-induced gastric damage, compared to the levels found in non-injured animals. As expected, N-acetylcysteine, an antioxidant, inhibited the increase in MDA levels and reduced levels of GSH. Lipid peroxidation occurs when reactive oxygen species attack cell membranes, allowing them to enter intracellular structures. Malondialdehyde (MDA) appears as an end product of fatty acid oxidation and the higher the concentration, the higher the level of substances that react with thiobarbituric acid, which is indicative of increased lipid peroxidation [32]. Pretreatment with EOHM (400 mg/kg) significantly reduced lipid

peroxidation, as evidenced by reduced levels of malondialdehyde. In view of previous reports regarding sulfhydryl groups, we can say that they are directly associated with maintaining the integrity of the gastric mucosa. Quantification of mucosal GSH revealed that EOHM significantly increased basal levels of GSH, confirming the involvement of these groups in the gastroprotective effect.

The administration of acetic acid to the gastric mucosa of rats is capable of producing a well-defined lesion and delaying the healing of wounds, similar to peptic ulcers in humans [33]. Changes in the levels of prostaglandins, growth factors, nitric oxide, cytokines and the amount of mucus may be involved in this type of lesion [34]. The results showed that, besides protecting the gastric mucosa against acute gastric lesions, treatment with EOHM at a dose of 400 mg/kg also speeded up healing of chronic ulcers in a manner comparable to pantoprazole. Moreover, according to histological analysis, rats treated with EOHM demonstrated the ability to regenerate the gastric mucosa (HE staining) and restore mucus production in glandular cells (PAS staining), as evidenced by the accumulation of pink in the layer of mucus cells not being observed in abundance in the internal area of the ulcer in animals from the control group. These results were confirmed by immunohistochemical analysis for PCNA, an important factor for healing of gastric mucosa, in which EOHM promoted an increase in cell proliferation in the region of regeneration.

This is the first report establishing the action mechanisms involved in the gastroprotective effects of the essential oil of *Hyptis martiusii* and its ulcer healing properties.

5. Conclusions

The results suggest that the mechanism of action by which the essential oil of *Hyptis martiusii* protects the gastric mucosa can be partly attributed to its antisecretory properties, and partly to cytoprotective and antioxidant mechanisms, given their interaction with sulfhydryl compounds, resulting in an increase in gastric mucus, preventing depletion of non-protein sulfhydryl groups and reducing the levels of lipid peroxidation in the gastric mucosa. They also suggest that this essential oil is a promising candidate for the treatment of gastric disorders, in view its potential gastroprotective properties.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

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4.3 Artigo III: submetido ao periódico Evidence-Based Complementary and Alternative Medicine

Repeated-doses toxicity study of the essential oil of *Hyptis martiusii* Benth. (Lamiceae) in Swiss mice

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Abstract

Hyptis martiusii Benth. (Lamiaceae) is found in abundance in northeastern Brazil where is used in traditional medicine to treat gastric disorders. Since that there are no pharmacological studies reporting the toxicity and safety profile of this species, we investigated repeated-doses toxicity of essential oil of *Hyptis martiusii* (EOHM). Swiss mice of both sexes were orally treated with EOHM (100 and 500 mg/kg) for 30 days, and biochemical, hematological and morphological parameters were determined. No toxicity signs or deaths were recorded during the treatment with EOHM. The body weight gain was not affected, but there was an occasional variation in water consumption and food on mice of both sexes treated with both doses. The hematological and biochemical profiles did not show significant differences, except for a decrease in the MCV and an increase in albumin, these variations are within the limits described for the species. The microscopic analysis showed changes in liver, kidneys, lungs and spleen, however, these changes do not have clinical relevance, since varied among groups, including the treated groups and the control group. The results indicate that the treatment of repeated-doses with the essential oil of Hyptis martiusii showed low toxicity in mice.

Keywords: *Hyptis martiusii*, Lamiaceae, essential oil, repeated-doses toxicity

1. Introduction

The genus Hyptis (Lamiaceae), comprising approximately 400 species distributed across a wide area, from the southern United States to Argentina, has been widely studied from an ethnopharmacological, pharmacological and chemical point of view, mainly owing to the diversity of bioactive constituents found in essential oils and extracts, which have interesting biological effects, such as antimicrobial, anticancer and insecticidal properties [1].

Some species of the Hyptis genus are characterized by the presence of essential oils, such as *Hyptis suaveolens*, *Hyptis pectinata*, *Hyptis crenata* and *Hyptis fruticosa*, with important biological activity, such as antiseptic [2], antifungal [3], antibacterial [4], antiinflammatory [5], antinociceptive [6], and antiulcer properties [7, 8] among others.

Hyptis martiusii Benth., commonly known as "*cidreira-do-campo*" or "*cidreira-brava*", is an aromatic plant found in abundance in northeastern Brazil and characterized as a potential source of essential oils, like other species of the Hyptis genus. In folk medicine, the

infusion or decoction of *Hyptis martiusii* leaves is used to combat intestinal and stomach diseases, while a decoction of the roots is used to counter inflammation of the ovaries [9]. Few studies have been carried out into the biological and pharmacological properties of *Hyptis martiusii*. Cytotoxic and antiproliferative effects on certain tumor cell lines [10, 11], insecticidal activity against the larvae of *Aedes aegypti* and *Culex quinquefasciantus* [12], antimicrobial activity against resistant strains of *Staphylococcus aureus* and *Escherichia coli* [13] and antioxidant activity [14] have been reported for *Hyptis martiusii.*

Recently, our research group first reported that the essential oil of the leaves of *Hyptis martiusii* has an antiulcerogenic and antisecretory activity in acute gastric ulcer models [15]. Given that there are no studies regarding the toxicological profile of this species. A repeated-doses (30 days) toxicity study was conducted to evaluate the safety of oral administration of essential oil of *Hyptis martiusii.*

2. Material and methods

2.1 Plant material and extraction of essential oil

Hyptis martiusii (Lamiaceae) leaves were collected on the Araripe Plateau, in Crato, in the Brazilian State of Ceará (S 7°21.744' – W 39°28.691'). Entire plants were collected during the flowering stage, in June 2011. A representative sample of this species is deposited in the Prisco Bezerra Herbarium of the Department of Biology at the Federal University of Ceará (UFC) (registration no. 43038). The leaves were dried at room temperature for 72 h prior to hydrodistillation and the essential oil was extracted immediately thereafter. Two portions (830.00 ± 30.00 g) of the dried leaves were individually subjected to hydrodistillation using a Clevenger-type apparatus for 3 h. The yield of the essential oil from dried leaves of *Hyptis martiusii* (EOHM) was 0.95 ± 0.03% (w/w), corresponding to 7.95 ± 0.55 g of oil, calculated according to the mean dry weight of the leaves used in each extraction. The water/oil mixture was collected, the aqueous solution was discarded, the oil was dried over anhydrous sodium sulfate and then filtered. Essential oil was stored in an amber bottle at -20 °C ready for toxicological experiments and phytochemical analysis had been carried out.

2.2 Chemical analysis of the essential oil

Analysis of the EOHM was performed using a gas chromatograph attached to a mass spectrometer (GC-MS, SHIMADZU QP5050A) equipped with a capillary column (DB-5HT, 30 m x 0.25 mm, 0.1 μm-thick film) with the following specifications: helium as carrier gas (1.0 mL/min flow rate); injector temperature 270 ºC and detector temperature 290 ºC; a linear velocity of 47.3 cm/sec; a pressure of 107.8 kPa; a column temperature programmed to increase from 60 ºC (2 min) to 180 ºC (1 min) at 4 ºC/min, and from then 180 to 260 ºC at 10 ºC/min (10 min). The mass spectrometer was operated using 70 eV of ionization energy. Identification of individual constituents was based on the interpretation of their mass spectral fragmentation using computer-based library MS search standards (Wiley 229), retention indices and comparison with the mass spectra database and data from the literature [16].

2.3 Animals

All the experiments were conducted in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals and were submitted to and approved by the Animal Experimentation Ethics Committee of the UFPE, (license no. 012490). Male and female Swiss mice (35–45 g) obtained from the Keizo Asami Immunopathology Laboratory (LIKA/UFPE), Pernambuco (Brazil) were used in experiments after a one-week adaptation period in the laboratory. These were kept under standard environmental conditions (12 h dark/light cycle) and temperature (22 ± 2 °C). Water and industrialized dry food (Labina®, Purina, Brazil) were made available *ad libitum*.

2.4 Repeated-doses toxicity study

Healthy male and female Swiss mice were randomly divided into three groups (n = 10/group/sex). Animals received 1% Tween-80 aqueous solution (control group) or EOHM orally at doses of 100 and 500 mg/kg for 30 consecutive days. During treatment, the body-weight was recorded weekly, and food consumption and water intake of the animals were recorded every two days. Animals were observed twice daily for signs of toxicity, such as piloerection, diarrhea and changes in locomotor activity, and mortality throughout the experimental period. At the end of the 30-day experiment, the animals fasted overnight, although water was made available *ad libitum*. They were then anesthetized with thiopental (35 mg/kg, i.p.) and blood samples were obtained by retro-orbital puncture using capillary tubes and collected in two tubes: tube 1 containing anticoagulant ethylenediaminetetraacetic

acid (EDTA) for tube 2 without additions for hematological and biochemical parameters, respectively.

2.5 Hematological and biochemical analyses

Hematological analyses were carried out immediately after collection using an automatic hematological analyzer (Coulter STKS, Beckman). Parameters included red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count, mean platelet volume (MPV) and differential leukocyte count (lymphocytes, monocytes, neuthrophils, eosinophils and basophils). For biochemical analysis, blood was centrifuged at 1480 × g for 10 min to obtain serum, which was stored at -20 °C, and the following parameters were determined: glucose, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, triglycerides, total protein, albumin and lactate dehydrogenase (HDL) [17]. Dosages were made using Architect (Abbott®) automation with Boehringer Ingelheim® biochemical kits.

2.6 Morphological study

After blood collection, the animals (n = 5/group/sex) were euthanized with an excess of thiopental (140 mg/kg, i.p.) and a necropsy was performed for macroscopic external evaluation of the heart, lungs, liver, kidneys, adrenal glands, spleen, stomach, intestine, pancreas, brain and reproductive organs, testicles and prostate (male) or uterus and ovaries (female). These organs were carefully removed and weighed individually. Organ weights were expressed in absolute and relative terms (g and g/10 g of body weight, respectively).

For microscopic analysis, the remaining animals (n = 5/group/sex) were anesthetized, perfused with saline (to remove blood), then the organs described above were removed and fixed "*in totum*" in 10% buffered formalin for 48h at room temperature, after fixing each sample was washed with water and immersed in 70% ethyl alcohol for 3 to 4 days, then were embedded in paraffin. Paraffin sections of 5 μm were obtained and stained with hematoxylin/eosin (HE) [18]. Histological analyses of ogans were made using a automatic microscopy system MICRO DIP® (Kacil Inc).

2.7 Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M). The differences between groups were determined by analysis of variance (ANOVA) followed by Dunnett's test. Statistical analysis was performed using GraphPad Prism 5.0®. The level of significance for rejection of the null hypothesis was set at 5% ($p < 0.05$).

3. Results

3.1 Chemical analysis of essential oil

The chemical characterization of the essential oil using GC-MS identified 27 components, accounting for 96.3% of the total oil. The major components identified were 1,8-cineole (32.8%), δ-3-carene (17.4%), camphor (6.7%), α-pinene (3.5%) and caryophyllene oxide (3.5%). Table 1 shows the constituents identified, the percentage composition and retention index (RI).

3.2 Repeated-doses toxicity study

No signs of toxicity such as piloerection, diarrhea, sedation, abdominal contortions, alterations in locomotor activity or deaths were recorded during the 30 consecutive days of treatment by oral route with essential oil of *Hyptis martiusii* at doses of 100 and 500 mg/kg. The body weight gain in mice of both sexes was not affected during treatment with EOHM when compared to mice in the control group (Figure 1). Occasional alterations in the food and water intake were observed in mice of both sexes during the treatment period in relation with control group. There was a decrease in food and water intake in male mice treated with both doses (Figure 2) and a decrease in food intake and an increase in water intake in female mice treated likewise (Figure 3).

Table 1. Chemical constituents of essential oil of leaves of *Hyptis martiusii* Benth.

Components	**Retention Time (min)**	**(%)**
Hexen-1-ol	4.39	1.81
α-Pinene	6.59	3.52
β-Pinene	8.18	2.28
β-Myrcene	8.69	1.81
δ-3-Carene	9.60	17.43
p-Cymene	10.01	0.87
o-Cymene	10.35	3.36
1,8-Cineole	10.76	32.80
Linalool	13.89	1.21
Camphor	16.31	6.70
Isoborneol	17.54	0.99
trans-Caryophyllene	30.69	3.37
Aromadendrene	31.63	1.96
α-Humulene	32.51	1.69
Ledene	34.32	0.99
Germacrene B	34.59	2.21
γ-Selinen	36.58	0.77
β-Panasinsene	36.81	0.97
Isolongifol	37.74	0.97
Palustrol	38.71	0.83
Spathulenol	38.62	1.85
Caryophyllene oxide	38.82	3.50
Globulol	38.98	0.91
Ledol	39.85	1.04
Rosifoliol	40.04	0.86
(*Z*)-Valerenyl acetate	42.43	0.82
Total		**96.33**

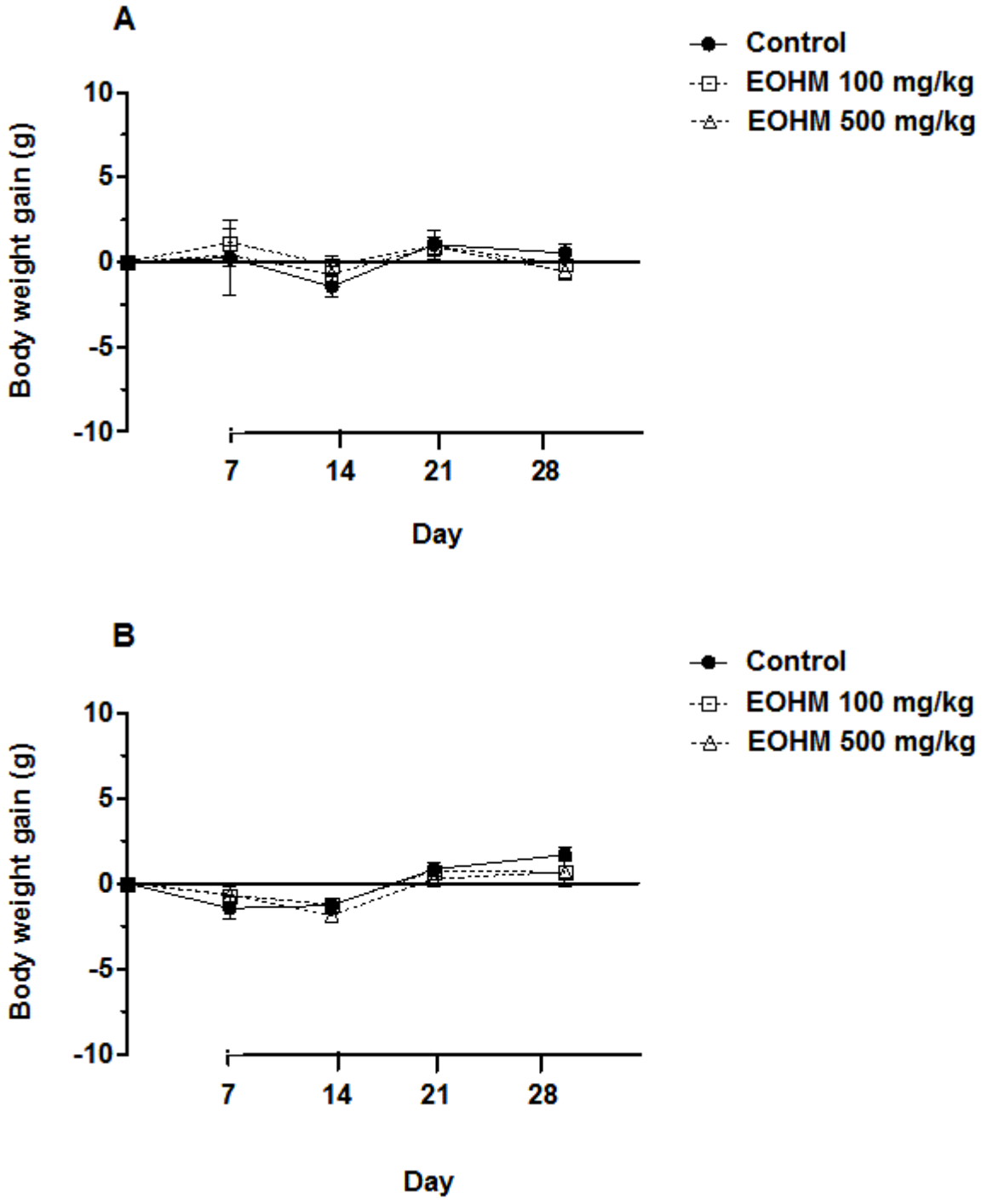

Figure 1. Effect of the essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg, p.o.) on body weight gain (g) from male (A) and female (B) Swiss mice treated orally for 30 days. Values are expressed as mean ± S. E. M. (n = 10/group).

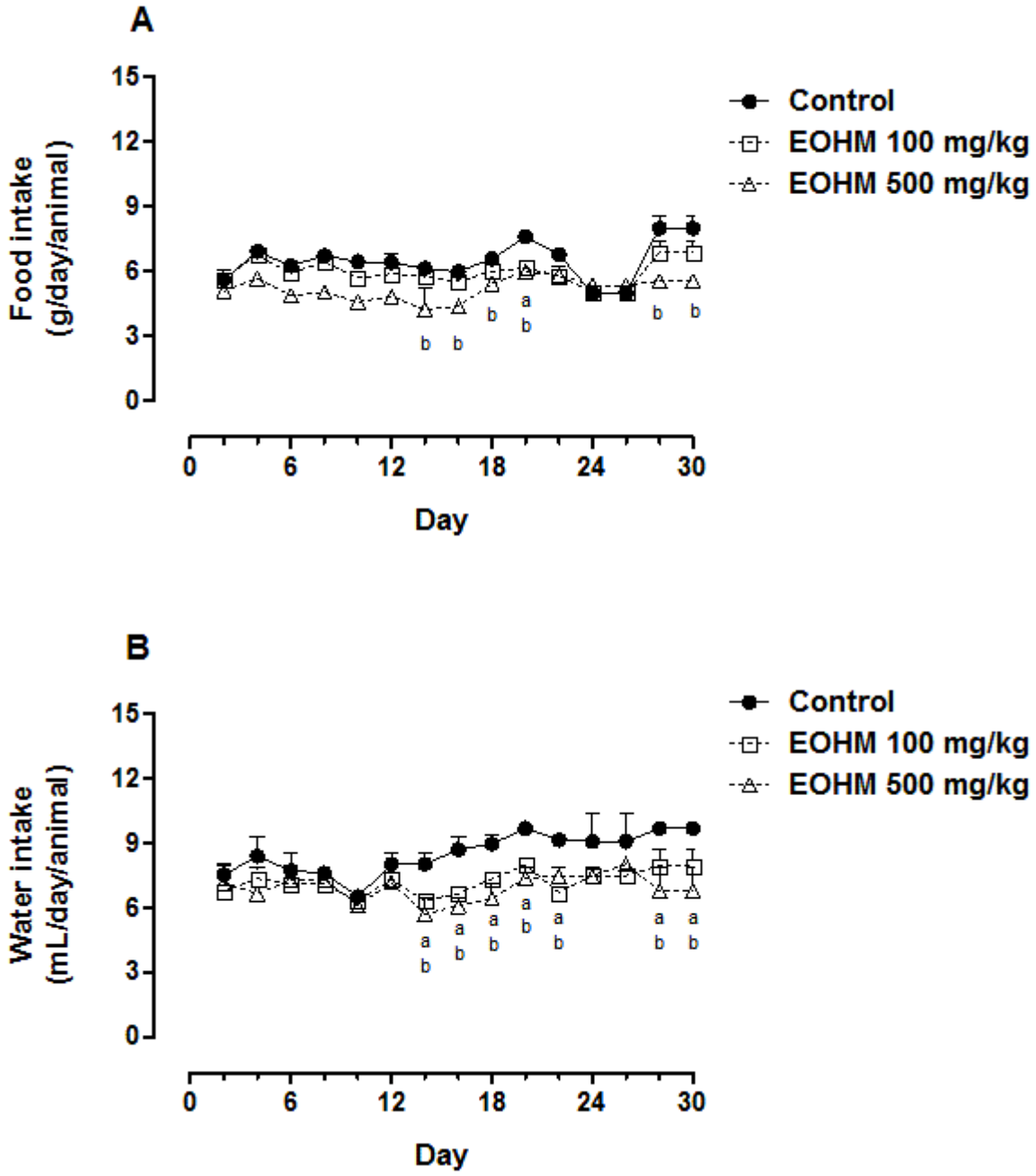

Figure 2. Effect of the essential oil of *Hyptis martiusii* (EOHM) on food (A) and water (B) intake in male Swiss mice treated orally for 30 days. Data are mean ± S.E.M. (n = 10/group) and letters represent differences in relation to the control group (a: EOHM 100 mg/kg and b: EOHM 500 mg/kg) at the same day (ANOVA followed by Dunnett's test, $p < 0.05$).

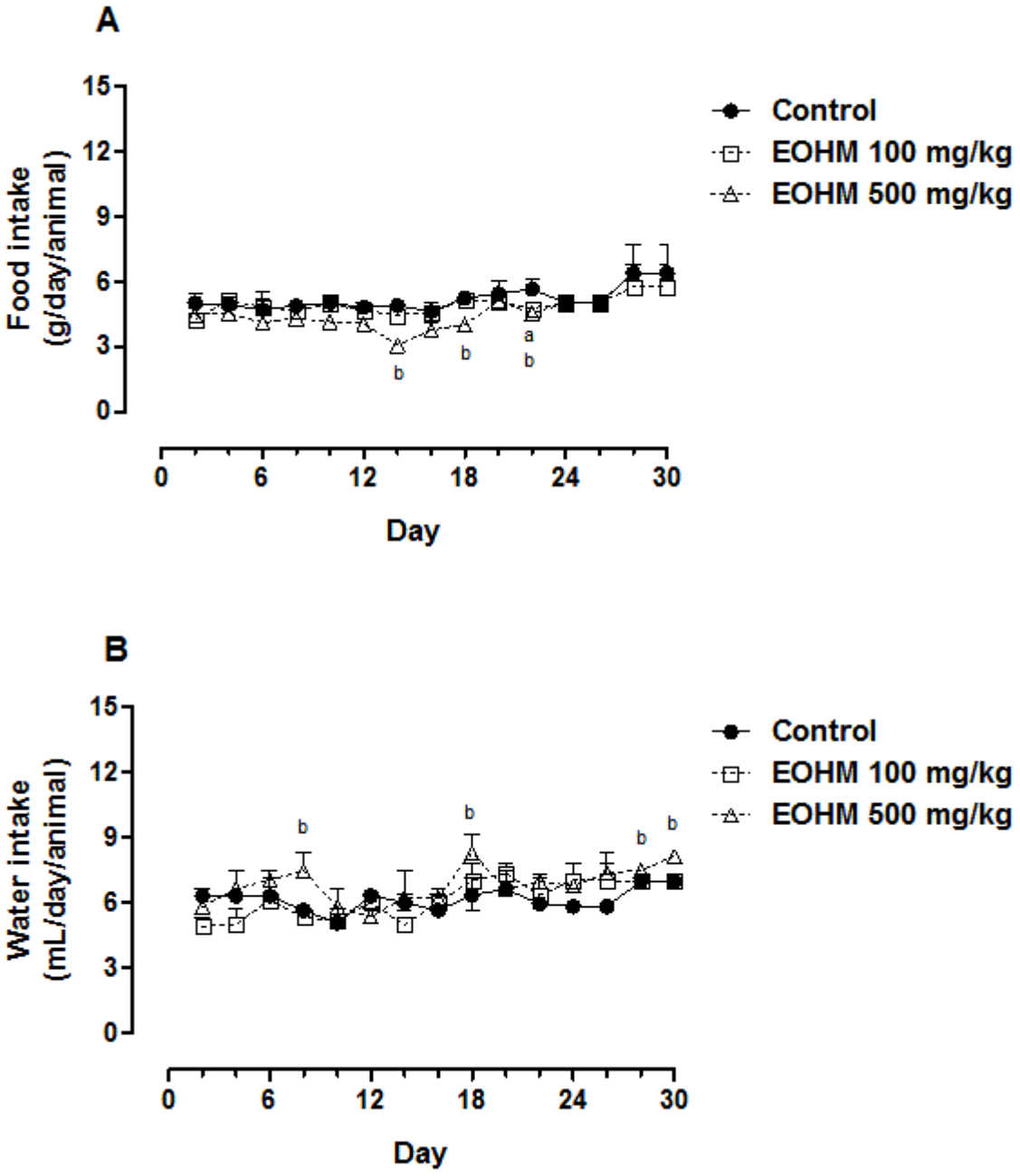

Figure 3. Effect of the essential oil of *Hyptis martiusii* (EOHM) on food (A) and water (B) intake in female Swiss mice treated orally for 30 days. Data are mean ± S.E.M. (n = 10/group) and letters represent differences in relation to the control group (a: EOHM 100 mg/kg and b: EOHM 500 mg/kg) at the same day (ANOVA followed by Dunnett's test, $p < 0.05$).

3.3 Hematological and biochemical parameters

The hematological profiles of the experimental and control groups are shown in Tables 2 and 3. There was no change in the clinical hematological profile of groups treated with essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg) in either sex, except, for a statistical decrease of 4.7% in the mean corpuscular volume (MCV) of male mice (EOHM 500 mg/kg) in relation to control group. Regarding the biochemical parameters in the group of male mice, no statistically significant differences were recorded for any of the parameters examined. In female mice, however, treatment with EOHM 500 mg/kg showed a significant increase of 24.1 % in albumin when compared to the control group. The biochemical profile is shown in Tables 4 and 5, respectively.

Table 2. Effect of the essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg) on hematological parameters in male Swiss mice treated orally for 30 days.

Parameter	**Control**	**EOHM 100 mg/kg**	**EOHM 500 mg/kg**
Erythrocytes (10^6/µL)	9.59 ± 0.18	9.40 ± 0.58	8.96 ± 0.57
Hemoglobin (g/dL)	16.40 ± 0.48	16.51 ± 0.68	14.64 ± 1.28
Hematocrit (%)	45.34 ± 1.15	44.26 ± 2.61	40.06 ± 2.96
MCV (fL)	47.71 ± 0.28	47.57 ± 0.36	45.43 ± 0.86*
MCH (pg)	17.21 ± 0.09	17.14 ± 0.25	16.27 ± 0.54
MCHC (g/dL)	36.17 ± 0.22	36.60 ± 0.28	35.93 ± 0.58
RDW (%)	18.17 ± 0.48	17.19 ± 0.40	18.76 ± 1.08
WBC (10^3/µL)	10.70 ± 1.03	14.41 ± 1.26	14.83 ± 2.04
Platelets (10^3/µL)	1061.00 ± 98.70	839.50 ± 91.68	1140.00 ± 58.77
MPV (fL)	6.07 ± 0.12	6.00 ± 0.13	6.31 ± 0.18
Lymphocytes (%)	92.59 ± 0.70	91.41 ± 0.6	91.33 ± 0.80
Monocytes (%)	0.87 ± 0.08	0.75 ± 0.12	1.61 ± 0.48
Neuthrophils (%)	6.32 ± 0.60	7.05 ± 0.40	6.45 ± 0.30
Eosinophils (%)	0.05 ± 0.02	0.04 ± 0.02	0.04 ± 0.04
Basophils (%)	0.42 ± 0.06	0.50 ± 0.03	0.52 ± 0.16

MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, WBC: white blood cell, MPV: mean platelet volume. Values represent the mean ± SEM (n = 10/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 3. Effect of the essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg) on hematological parameters in female Swiss mice treated orally for 30 days.

Parameter	**Control**	**EOHM 100 mg/kg**	**EOHM 500 mg/kg**
Erythrocytes (10^6/µL)	9.90 ± 0.10	9.69 ± 0.17	10.02 ± 0.17
Hemoglobin (g/dL)	16.71 ± 0.33	16.89 ± 0.15	17.50 ± 0.20
Hematocrit (%)	46.61 ± 0.28	46.43 ± 0.45	47.16 ± 0.90
MCV (fL)	47.29 ± 0.42	48.29 ± 0.35	47.71 ± 0.52
MCH (pg)	17.24 ± 0.09	17.44 ± 0.17	17.30 ± 0.15
MCHC (g/dL)	36.60 ± 0.10	36.33 ± 0.14	36.74 ± 0.10
RDW (%)	15.53 ± 0.29	15.47 ± 0.21	15.59 ± 0.34
WBC (10^3/µL)	12.36 ± 1.55	11.70 ± 1.21	13.67 ± 1.26
Platelets (10^3/µL)	872.60 ± 17.88	761.70 ± 31.97	975.30 ± 73.71
MPV (fL)	5.58 ± 0.13	5.71 ± 0.09	5.71 ± 0.09
Lymphocytes (%)	94.53 ± 0.43	92.83 ± 0.46	92.85 ± 0.80
Monocytes (%)	0.42 ± 0.06	0.58 ± 0.09	0.50 ± 0.06
Neuthrophils (%)	4.58 ± 0.41	5.21 ± 0.46	5.25 ± 0.64
Eosinophils (%)	0	0.07 ± 0.02	0.04 ± 0.02
Basophils (%)	0.35 ± 0.08	0.45 ± 0.03	0.41 ± 0.05

MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, WBC: white blood cell, MPV: mean platelet volume. Values represent the mean ± SEM (n = 10/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 4. Effect of the essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg) on biochemical parameters in male Swiss mice treated orally for 30 days.

Parameter	**Control**	**EOHM 100 mg/kg**	**EOHM 500 mg/kg**
Glucose (mg/dL)	105.20 ± 4.70	88.57 ± 6.62	107.40 ± 4.62
BUN (mg/dL)	47.17 ± 4.44	49.17 ± 5.52	48.87 ± 3.41
Creatinine (mg/dL)	0.22 ± 0.03	0.24 ± 0.04	0.28 ± 0.02
AST (U/L)	75.67 ± 3.46	84.00 ± 7.31	74.40 ± 5.73
ALT (U/L)	10.67 ± 0.88	12.71 ± 1.94	15.29 ± 3.91
Total cholesterol (mg/dL)	179.80 ± 11.70	171.30 ± 13.30	155.50 ± 12.33
Triglycerides (mg/dL)	136.20 ± 8.54	156.90 ± 8.56	109.70 ± 7.68
Total protein (g/dL)	6.00 ± 0.13	5.88 ± 0.21	5.83 ± 0.21
Albumin (g/dL)	3.83 ± 0.13	3.64 ± 0.18	3.53 ± 0.18
HDL (U/L)	211.80 ± 10.89	207.70 ± 13.22	255.92 ± 28.87

BUN: blood urea nitrogen, AST: aspartate aminotransferase, ALT: alanine aminotransferase and HDL: lactate dehydrogenase. Values represent the mean ± SEM (n = 10/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 5. Effect of the essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg) on biochemical parameters in female Swiss mice treated orally for 30 days.

Parameter	**Control**	**EOHM 100 mg/kg**	**EOHM 500 mg/kg**
Glucose (mg/dL)	104.40 ± 5.46	98.00 ± 13.95	91.33 ± 9.46
BUN (mg/dL)	41.41 ± 4.99	49.68 ± 2.36	34.83 ± 3.53
Creatinine (mg/dL)	0.21 ± 0.02	0.22 ± 0.03	0.22 ± 0.02
AST (U/L)	89.30 ± 19.05	126.2 ± 39.83	107.20 ± 15.45
ALT (U/L)	14.71 ± 0.92	20.50 ± 6.46	22.67 ± 2.03
Total cholesterol (mg/dL)	102.10 ± 13.86	131.20 ± 20.78	121.10 ± 8.48
Triglycerides (mg/dL)	161.10 ± 18.62	159.80 ± 10.08	112.60 ± 8.15
Total protein (g/dL)	4.97 ± 0.27	4.98 ± 0.23	5.70 ± 0.09
Albumin (g/dL)	3.24 ± 0.13	3.40 ± 0.14	4.02 ± 0.14*
HDL (U/L)	193.40 ± 30.46	279.80 ± 81.40	260.80 ± 27.01

BUN: blood urea nitrogen, AST: aspartate aminotransferase, ALT: alanine aminotransferase and HDL: lactate dehydrogenase. Values represent the mean ± SEM (n = 10/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

3.4 Morphological parameters

The The absolute and relative weight of the tissues were not changed by treatment with *Hyptis martiusii*, except for a increased in relative weight of the kidneys (26.0 %) of female mice treated with dose 500 mg/kg and a decrease in relative weight of the spleen (26.4 %) of female mice treated with doses of 100 mg/kg (Table 7). The macroscopic analysis of target organs of the animals treated with essential oil of *Hyptis martiusii* did not show significant changes in color or texture when compared to the control group.

Microscopic examination of organs showed the presence of fat in the liver of females from the control group and treated with both doses of EOHM, as was not observed in the livers of male mice (Figure 4). A slight lymphocytic infiltrate was observed in the kidneys of females treated with EOHM (500 mg/kg) (Figure 5) and in the lungs of males and females treated with both doses of EOHM (Figure 6), as well as a slight increase in phagocytic activity (increase in number of macrophages) in the spleens of males and females treated with both doses of EOHM (Figure 7). The other organs of male and female mice in the experimental and control groups exhibited no histological alteration.

Table 6. Effect of the essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg, p.o.) on absolute (g) and relative organ weight (g/10g of body weight animal) in male Swiss mice treated orally for 30 days.

Organs	**Control**	**EOHM 100 mg/kg**	**EOHM 500 mg/kg**
Heart (g)	0.251 ± 0.010	0.234 ± 0.013	0.225 ± 0.010
(g/10 g)	0.056 ± 0.001	0.051 ± 0.003	0.054 ± 0.003
Lung (g)	0.238 ± 0.016	0.265 ± 0.018	0.234 ± 0.021
(g/10g)	0.052 ± 0.002	0.058 ± 0.004	0.056 ± 0.005
Liver (g)	2.211 ± 0.310	2.036 ± 0.190	2.219 ± 0.141
(g/10g)	0.482 ± 0.060	0.443 ± 0.037	0.532 ± 0.034
Kidney (g)	0.332 ± 0.022	0.339 ± 0.019	0.304 ± 0.013
(g/10g)	0.072 ± 0.003	0.074 ± 0.004	0.073 ± 0.003
Adrenal (g)	0.018 ± 0.005	0.013 ± 0.004	0.010 ± 0.001
(g/10g)	0.004 ± 0.001	0.003 ± 0.001	0.002 ± 0.000
Spleen (g)	0.287 ± 0.079	0.210 ± 0.041	0.205 ± 0.031
(g/10g)	0.062 ± 0.016	0.046 ± 0.009	0.049 ± 0.007
Stomach (g)	0.259 ± 0.036	0.306 ± 0.034	0.255 ± 0.019
(g/10g)	0.056 ± 0.006	0.066 ± 0.006	0.061 ± 0.004
Intestine (g)	0.258 ± 0.021	0.236 ± 0.018	0.221 ± 0.041
(g/10g)	0.057 ± 0.006	0.051 ± 0.004	0.053 ± 0.010
Pancreas (g)	0.293 ± 0.071	0.344 ± 0.074	0.325 ± 0.068
(g/10g)	0.064 ± 0.015	0.075 ± 0.016	0.078 ± 0.017
Brain (g)	0.399 ± 0.007	0.441 ± 0.016	0.398 ± 0.012
(g/10g)	0.088 ± 0.002	0.096 ± 0.002	0.095 ± 0.003
Testicle (g)	0.092 ± 0.021	0.111 ± 0.009	0.104 ± 0.007
(g/10g)	0.020 ± 0.005	0.024 ± 0.002	0.025 ± 0.002
Prostate (g)	0.011 ± 0.001	0.008 ± 0.002	0.007 ± 0.001
(g/10g)	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000

Values represent the mean ± SEM (n = 5/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 7. Effect of the essential oil of *Hyptis martiusii* (EOHM, 100 and 500 mg/kg, p.o.) on absolute (g) and relative organ weight (g/10g of body weight animal) in female Swiss mice treated orally for 30 days.

Organs	**Control**	**EOHM 100mg/kg**	**EOHM 500mg/kg**
Heart (g)	0.181 ± 0.007	0.181 ± 0.015	0.156 ± 0.004
(g/10 g)	0.113 ± 0.074	0.036 ± 0.002	0.044 ± 0.008
Lung (g)	0.246 ± 0.036	0.280 ± 0.027	0.218 ± 0.009
(g/10g)	0.052± 0.005	0.050 ± 0.002	0.062 ± 0.023
Liver (g)	2.114 ± 0.103	2.002 ± 0.051	1.918 ± 0.060
(g/10g)	0.457 ± 0.026	0.422 ± 0.036	0.546 ± 0.010
Kidney (g)	0.233 ± 0.011	0.253 ± 0.015	0.220 ± 0.007
(g/10g)	0.050 ± 0.003	0.054 ± 0.003	0.063 ± 0.024*
Adrenal (g)	0.009 ± 0.001	0.015 ± 0.004	0.007 ± 0.001
(g/10g)	0.005± 0.003	0.002 ± 0.000	0.002 ± 0.004
Spleen (g)	0.335 ± 0.028	0.271 ± 0.041	0.253 ± 0.019
(g/10g)	0.072 ± 0.004	0.053 ± 0.008*	0.072 ± 0.001
Stomach (g)	0.284 ± 0.020	0.298 ± 0.021	0.301 ± 0.019
(g/10g)	0.170 ± 0.109	0.068 ± 0.005	0.086 ± 0.046
Intestine (g)	0.214 ± 0.015	0.214 ± 0.013	0.200 ± 0.024
(g/10g)	0.140 ± 0.096	0.048 ± 0.004	0.057 ± 0.006
Pancreas (g)	0.433 ± 0.083	0.382 ± 0.044	0.431 ± 0.031
(g/10g)	0.226 ± 0.129	0.086 ± 0.013	0.123 ± 0.009
Brain (g)	0.449 ± 0.013	0.458 ± 0.011	0.430 ± 0.010
(g/10g)	0.290 ± 0.193	0.106 ± 0.004	0.123 ± 0.004
Uterus(g)	0.089 ± 0.022	0.152 ± 0.029	0.103 ± 0.016
(g/10g)	0.039 ± 0.025	0.036 ± 0.007	0.029 ± 0.043
Ovary (g)	0.019 ± 0.001	0.078 ± 0.056	0.013 ± 0.001
(g/10g)	0.010 ± 0.006	0.018 ± 0.012	0.003 ± 0.004

Values represent the mean ± SEM (n = 5/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

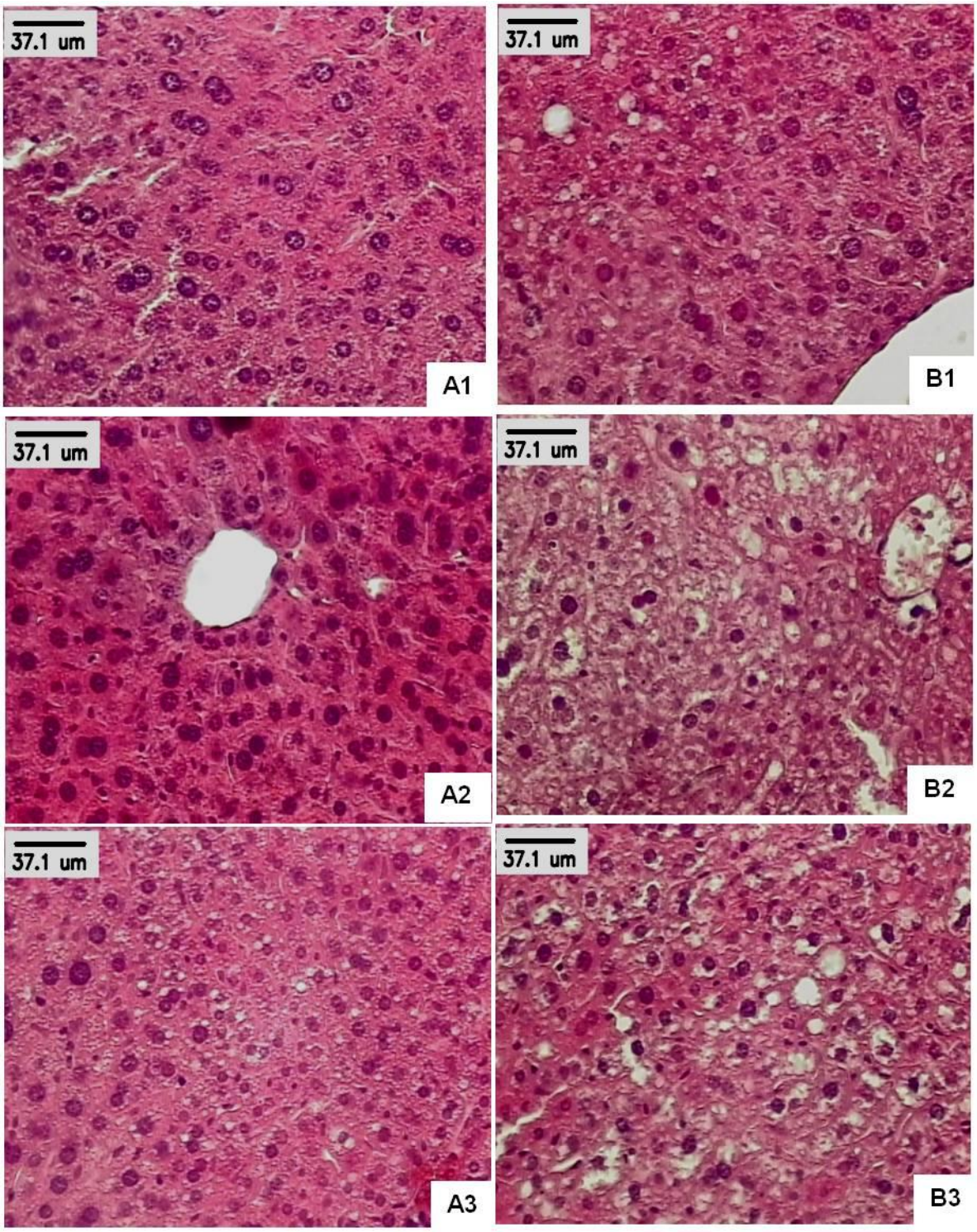

Fig. 4. Paraffin sections of liver (HE) male (A) and female (B) Swiss mice treated orally for 30 days with 1% Tween-80 aqueous solution (Control, A1 and B1) and essential oil of *Hyptis martiusii*, EOHM 100 mg/kg (A2, B2) and 500 mg/kg (A3, B3).

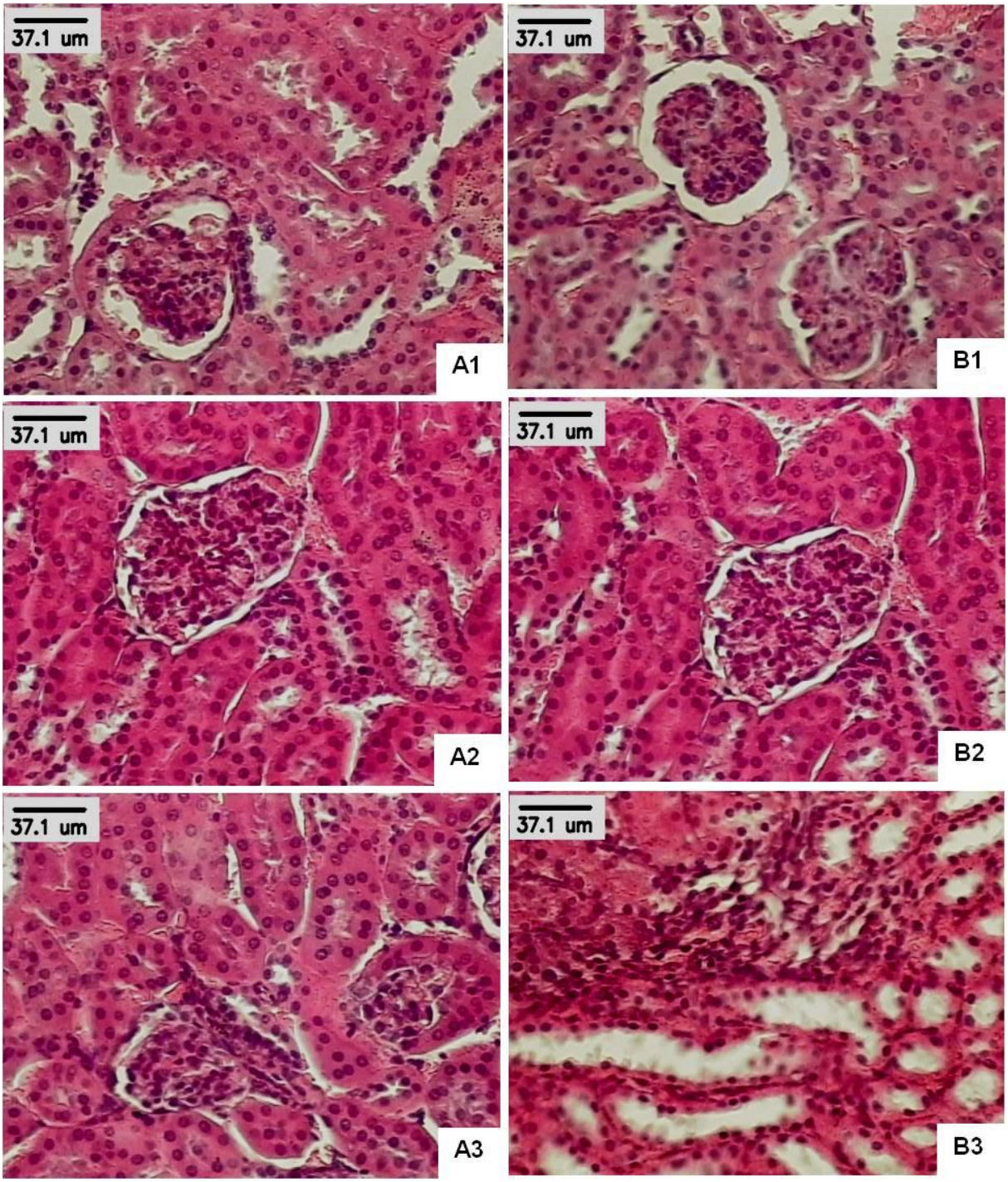

Fig. 5. Paraffin sections of kidney (HE) male (A) and female (B) Swiss mice treated orally for 30 days with 1% Tween-80 aqueous solution (Control, A1 and B1) and essential oil of *Hyptis martiusii*, EOHM 100 mg/kg (A2, B2) and 500 mg/kg (A3, B3).

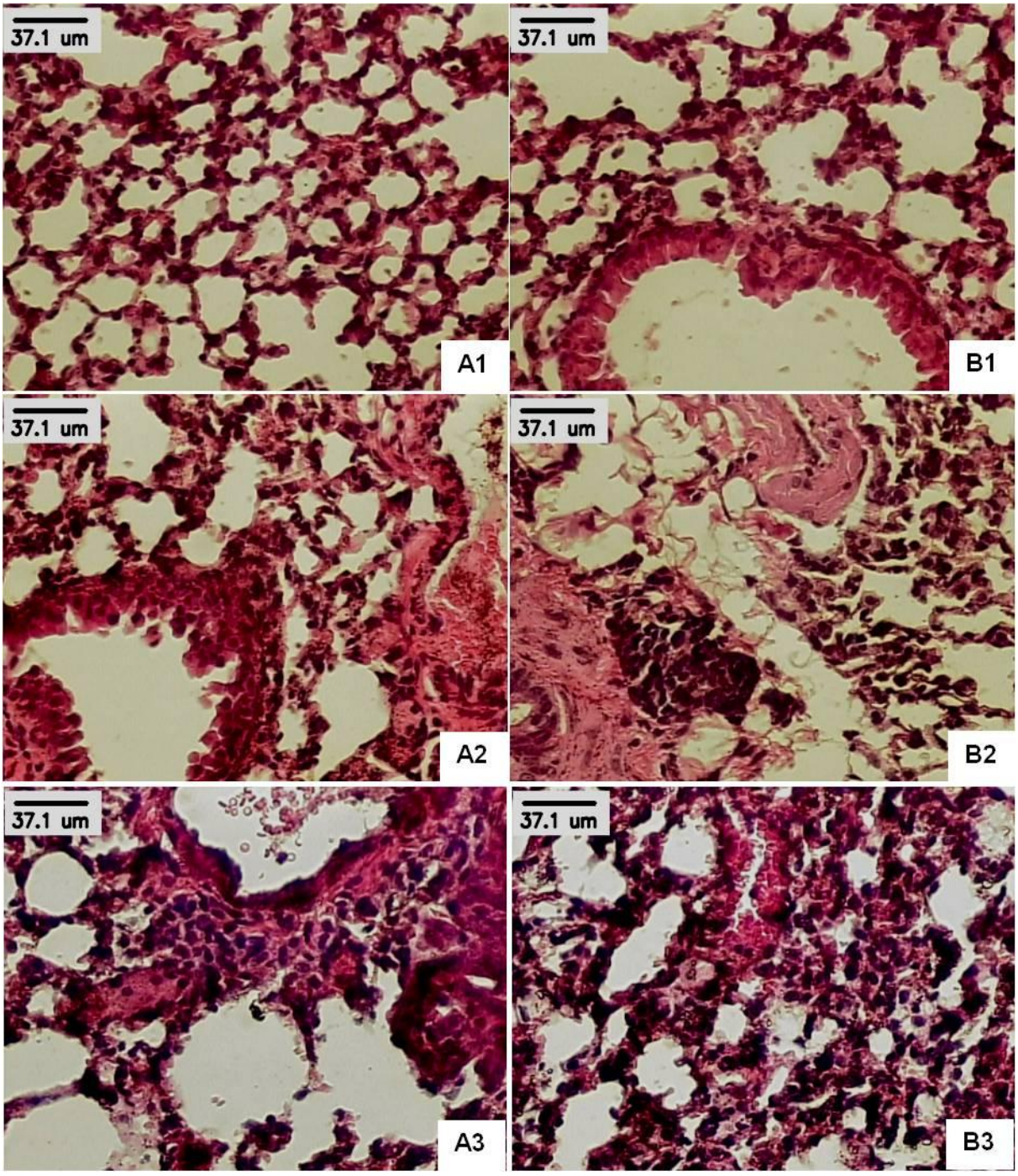

Fig. 6. Paraffin sections of lung (HE) male (A) and female (B) Swiss mice treated orally for 30 days with 1% Tween-80 aqueous solution (Control, A1 and B1) and essential oil of *Hyptis martiusii*, EOHM 100 mg/kg (A2, B2) and 500 mg/kg (A3, B3).

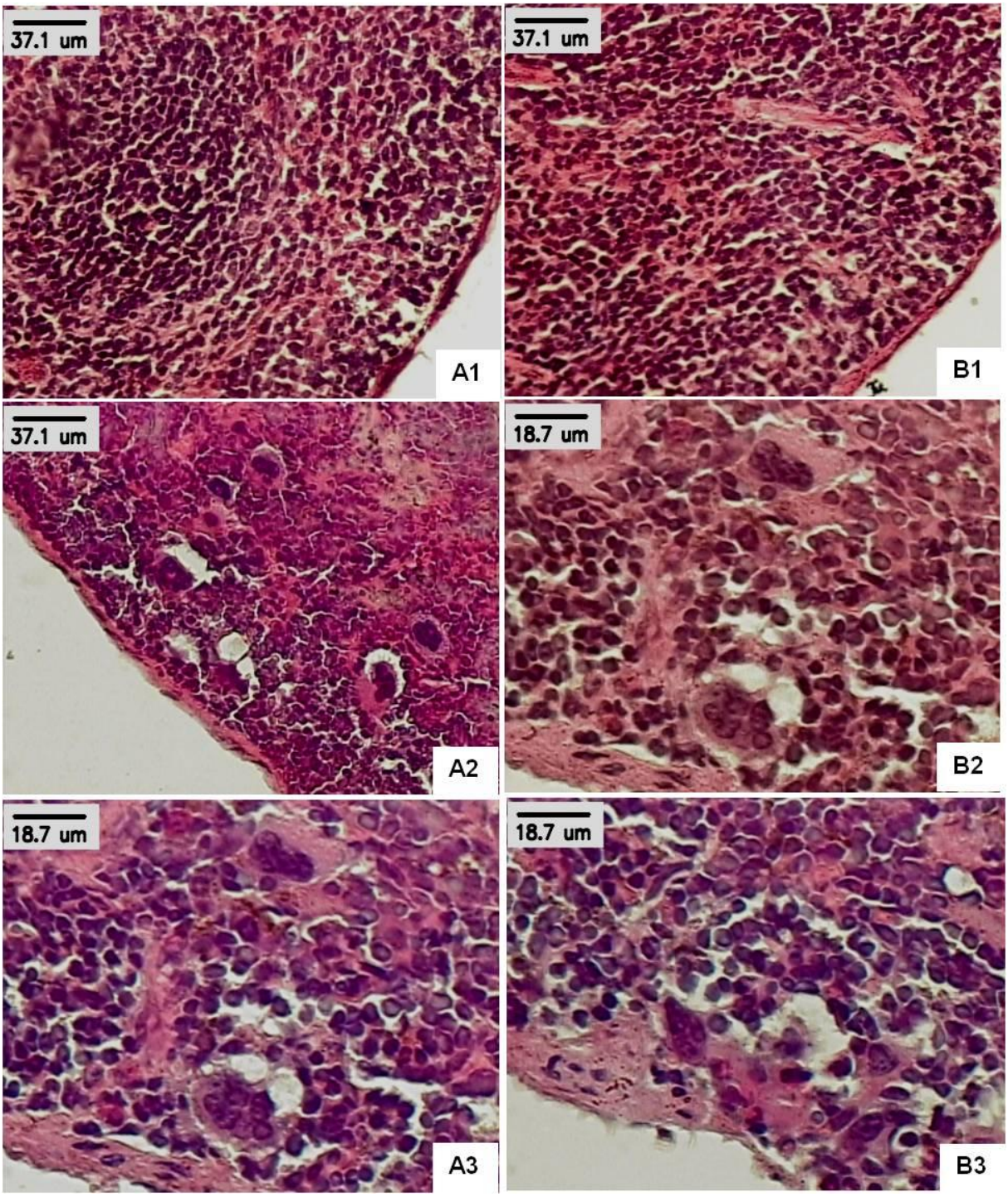

Fig. 7. Paraffin sections of spleen (HE) male (A) and female (B) Swiss mice treated orally for 30 days with 1% Tween-80 aqueous solution (Control, A1 and B1) and essential oil of *Hyptis martiusii*, EOHM 100 mg/kg (A2, B2) and 500 mg/kg (A3, B3).

4. Discussion

The gastroprotective activity of the essential oil from the leaves of *Hyptis martiusii* it was shown recently by our research group [15], confirming its use etnomedicinal. In this study, we have assessed for the first time the repeated doses toxicity essential oil of the leaves of *Hyptis martiusii* (EOHM).

Few studies have reported the toxicity profile of the essential oils of the Hyptis genus. Raymundo et al. [19] and Menezes et al. [6] described the low acute toxicity of the essential oil of the leaves of *H. pectinata* and *H. fruticosa* respectively, finding no deaths or any signs of toxicity up to a dose of 3 or 5 g/kg. Considering this scenery of little toxicological information available in the literature, this study was designed to obtain information regarding the safety profile of this species.

Our research group has recently demonstrated that, when administered orally at a dose of 5 g/kg, the essential oil of *Hyptis martiusii* brought on depression of the central nervous system (sedation) in mice of both sexes during the first 30 min and for a period of up to 4 h after administration. However, it produced no signs of acute toxicity or death in the treated animals, and no significant changes in consumption of food and water or body weight were observed during the 14 days of observation, suggesting an LD50 above 5000 mg/kg, which is characteristic of low toxicity [15].

Generally-speaking, the main constituents of essential oils are monoterpenes and sesquiterpenes, and this is also true of species of the Hyptis genus, in which these constituents have been identified as the main components [1]. GC–MS analysis of the essential oil of *Hyptis martiusii* identified 27 components and, the main monoterpene and sesquiterpene components were 1,8-cineole, δ-3-carene, camphor, α-pinene and caryophyllene oxide.

Oral administration at repeated-doses (30 days) of the essential oil of *Hyptis martiusii* in mice of both sexes did not cause death or any clinical signs of toxicity. There was an occasional variation in water consumption and food on mice of both sexes, but these variations did not influence the body weight of the animals during the treatment period, suggesting an absence of toxic effect. The doses used in this study represent the effective dose (100 mg/kg) and one dose five times higher (500 mg/kg). In studies of repeated dose toxicity, body weight gain and organ weight are considered important parameters and changes in these can indicate a toxic effect of the drug [20].

With the exception of the decrease in mean corpuscular volume (MCV) in male animals treated with EOHM (500 mg/kg), where levels are in the physiological limits

described for the specie [21], no other hematological parameter was changed. These data indicate that the essential oil of *Hyptis martiusii* had no effects on the circulating blood cells nor on their production. The analysis of blood parameters is important for risk evaluation, as any changes in the hematological system have a higher predictive value for human toxicity, when data are translated from animal studies [22].

In the analysis of biochemical parameters, no significant differences were found in serum levels of urea, creatinine, AST, ALT, glucose, cholesterol, triglycerides, total protein, between groups of both sexes treated with *Hyptis martiusii*. Only among females in the group treated with EOHM (500 mg/kg) was there a slight increase in the level of albumin when compared to the control group. However, the changed value to this parameter it was within the physiological limits described for the species [23]. The increase of plasmatic levels of BUN and creatinine are indicative of renal overload, acute renal failure or increase in proteic catabolism [24]. On the other hand, the increase of serum transaminase enzymes (ALT e AST) is a good indicative of hepatocyte damage because both enzymes are found in higher concentrations in those cells and could be due to transmembrane permeability modifications or cellular damage [25]. Since the enzyme AST is also found in a large number of tissues, such as heart, lung, skeletal muscle and kidney, whereas ALT is primarily limited to hepatocytes, the later is considered a highly sensitive indicator of hepatotoxicity [26]. Thus the fact of the administration of *Hyptis martiusii* did not produce changes in these biomarkers suggests absence of renal and hepatic toxicity.

No abnormal signs were found in internal organs on macroscopic examination in animals treated with EOHM. Only the females presented an increase in kidney relative weight and a decrease in spleen relative weight. These changes were not found in males and there was no dose-dependent response, we attribute this change to a floating point not correlated to treatment with essential oil of *Hyptis martiusii* and therefore do not have clinical relevance.

Although fat was found in the liver and discrete lymphocytic infiltrate in the kidneys of females treated with EOHM, no adverse effect on the usual biomarkers of liver and kidney toxicity (liver enzymes, ALT, AST and creatinine) was observed, suggesting that EOHM did not do significant damage to these organs. As a slight lymphocytic infiltrate was observed in the kidneys of females treated with EOHM (500 mg/kg), is possible that the kidney relative weight increase observed could be due to an inflammatory edema. The occurrence of lymphocytic infiltrate observed in the lungs could be related to possible oil aspiration at the time of orogastric gavage.

Histopathological findings from the liver, kidneys, lungs and spleen varied between mice of both sexes, even in the control group and showed no correlation with the dose employed. Furthermore, the morphology of all other organs analyzed maintained remained unchanged. Similar results have been found by Attawish et al. [27], who described the toxicity profile of repeated doses of *Hyptis suaveolens* in rats. The authors showed that animals treated with a dose of 500 mg/kg showed some alterations in tissue samples, such as fatty liver, myocarditis, nephrocalcinosis, pyelonephritis, splenomegaly and granuloma sperm. However, this result was not widespread, and such changes were observed even in the control group.

5. Conclusions

The data suggest that oral administration of the essential oil of *Hyptis martiusii* showed low toxicity in mice. The histopathological changes showed no clinically relevant changes, since varied among in animals of both sexes in treated groups and in the control group. However, it should be noted that further studies involving chronic toxicity, reproductive toxicity, genotoxicity and carcinogenicity in other species (rodent and non-rodent) are needed to better assess the safety profile of *Hyptis martiusii*.

Conflicts of interest

There is no conflict of interest.

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4.4 Artigo IV: a ser submetido ao periódico Phytomedicine

Título: Gastroprotective effect of the monoterpene 1,8-cineole (eucalyptol) in Wistar rats

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Abstract

Monoterpene 1,8-cineole is found naturally in many aromatic plants of several genera including species of the Hyptis genus. Recent data obtained by our research group has identified the presence of 1,8-cineole as one of the main compounds in the essential oil of dried leaves of *Hyptis martiusii* and shown the gastroprotective effect of this oil in different models of gastric lesions in rats. The aim of this study was to investigate the gastroprotective and healing activity of this compound to confirm its correlation with the gastroprotective effect of EOHM. The gastroprotective effect of 1,8-cineole (CIN) was evaluated using different experimental models (ethanol, HCl/ethanol, indomethacin and acetic acid-induced gastric ulcer). Gastric secretion parameters (volume, pH and total acidity) using the pyloric ligature method and gastrointestinal motility (gastric emptying and intestinal transit) were determined. The mechanisms of action involved in gastroprotection were investigated by assessing the involvement of NO, sulfhydryl, mucus and levels of malondialdehyde (MDA) and non-protein sulfhydril groups (GSH). The results show that CIN (50, 100 and 200 mg/kg) inhibited ethanol-, ethanol/HCl- and indomethacin-induced gastric lesions. With regard to gastrointestinal motility, the highest doses of CIN inhibited gastric emptying but did not affect intestinal transit. CIN (100 mg/kg) reduced the volume of base acid secretion but did not affect stimulated acid secretion. The gastroprotective effect of CIN involves the participation of endogenous sulfhydryl compounds. CIN increased levels of mucus (89.2%), reduced levels of lipid peroxidation (55.3%) and prevented depletion of GSH (62.6%) in the gastric mucosa. Furthermore, treatment with CIN speeded up the healing of an acetic acid-induced gastric ulcer, reducing the area of the lesion (43.1%), as confirmed by histological analysis of the lesions. The results of this study suggest that 1,8-cineole presents gastroprotective activity against gastric damage induced by various agents, mediated in part by a cytoprotective mechanism, since it leads to an increase in gastric mucus, and in part by antioxidant activity preventing depletion of sulfhydryl groups and reducing the levels of lipid peroxidation in the gastric mucosa. The study also provides evidence that 1,8-cineole is related to the gastroprotective effect of EOHM.

Keywords: 1,8-cineole, monoterpene, gastroprotection, healing activity

Introduction

A wide variety of chemical substances, mixtures of herbs and plant extracts have been proved to possess therapeutic properties in experimental models of ulcer induction. The gastroprotective effect of these compounds and extracts (combined or in isolation) has been attributed to three main functions, including antisecretory, cytoprotective and antioxidant properties (Al Mofleh, 2010).

1.8-cineole, also known as eucalyptol, is a monoterpene found naturally in many aromatic plants of the *Eucalyptus, Croton, Hyptis*, *Pectis, Rosamarinus and Salvia* genera (Araújo et al., 2003; Vilela et al., 2009; Hussain et al., 2011), among others as the main compound or in smaller quantities. Previous studies show that 1,8-cineole has been examined for a number of biological and pharmacology activities, including insecticidal and antimicrobial (Balacs, 1997), antiallergic and anti-inflammatory (Santos and Rao, 1998), hepatoprotective (Santos et al., 2001a), antitumoral (Moteki et al., 2002) and gastroprotective activities (Santos and Rao, 2001b).

In the specific case of the Hyptis genus, cineole is found to be the main compound in species such as *H. fruticosa, H. goyazensis, H. suaveolens* and *H. martiusii* (McNeil et al., 2011). *Hyptis martiusii* Benth. (Lamiaceae), popularly known as *cidreira-do-mato*, and, characterized as a potential source of essential oils, like other species of the genus. A study conducted by Araújo et al. (2003) showed that the essential oil of fresh leaves of *H. martiusii* consists of mono- and sesquiterpenes, and its major components are 1,8-cineole, δ-3-carene, bicyclogermacrene and β-caryophyllene. Recent data obtained by our research group have shown that the chemical composition of the essential oil of the dried leaves of *H. martiusii* also contains 1,8-cineole as one of the major compounds and that this essential oil has a gastroprotective effect in various gastric lesion models in rats (Caldas et al., 2011).

As phytochemical analysis of the oil has shown 1,8-cineole (CIN) to be one of the main compounds and previous results described in the literature have demonstrated that this compound inhibits ethanol-induced gastric lesions, we conducted a detailed investigation of the antiulcerogenic activity of this compound and the mechanisms of action involved, as a way of confirming its correlation with the gastroprotective effect of EOHM.

Material and methods

Animals

Male and female Wistar rats (200-300 g) obtained from the Federal University of Pernambuco's Department of Physiology and Pharmacology, Pernambuco, Brazil, were used. These were kept under standard environmental conditions (12 h dark/light cycle) and temperature (22 ± 2 °C). Water and industrialized dry food (Labina®, Purina, Brazil) were made available *ad libitum*. All the experimental protocols were submitted to and approved by the Animal Experimentation Ethics Committee of the UFPE, under license no. 037544 in accordance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals.

Drugs and doses

The reagents and substances were obtained from the companies (Sigma, St. Louis, USA; Vetec, Rio de Janeiro, Brazil; FMaia and Vetbrands, São Paulo, Brazil). For the purposes of the experiment, the 1,8-cineole (CIN) was emulsified in a Tween 80 at 1% before administration to the animals. The animals were divided into five groups (n = 3 female and 3 male/group) consisting of the standard (pantoprazole - a proton pump inhibitor, ranitidine - an antagonist of the histamine H2 receptor, atropine - a cholinergic antagonist, carbenoxolone - a cytoprotective agent or N-acetylcysteine - an antioxidant) depending on the specificity of each model; 1% Tween-80 aqueous solution (control group) and 1,8-cineole (CIN) at doses of 50, 100 and 200 mg/kg. A dose of 100 mg/kg was chosen for additional studies aiming to shed light on the mechanisms underlying gastroprotective activity, since this dose was the most effective of the protocols previously evaluated.

Antiulcerogenic activity

Ethanol-induced gastric ulcer

After 16 h of fasting, the rats (n = 6/group) were orally pretreated with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and CIN (50, 100 and 200 mg/kg), 1 h before administration of the ulcerogenic agent. Gastric lesions were induced using ethanol (70%, 0.5 mL/100 g, p.o.) according to the method described by Robert et al. (1979). The animals were sacrificed with thiopental (140 mg/kg, i.p.) 1 h after induction of gastric lesions. Their stomachs were removed by opening them along the greater curvature and photographed.

The area of gastric lesion was determined by computerized planimetry (Software ImageJ®) and data expressed in mm².

HCl/ethanol-induced ulcer

After 24 h of fasting, the rats (n = 6/group) were orally pretreated with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and CIN (50, 100 and 200 mg/kg). One hour after treatment, all the animals received 0.3 M HCl/etanol 60% solution (1 mL/150 g, p.o.) orally to induce acute gastric lesions (Mizui and Douteuchi, 1983). The animals were sacrificed 1 h after induction of gastric lesions and their stomachs were removed and examined for quantification of the lesions using the parameters described above.

Indomethacin-induced gastric ulcer

After 16 h of fasting, the rats (n = 6/group) were orally treated with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and CIN (50, 100 and 200 mg/kg). Gastric lesions were induced by subcutaneous administration of indomethacin 30 mg/kg, 30 min after administration of treatment (Djahanguri, 1969). The animals were sacrificed 6 h after indomethacin injection, their stomachs removed and inspected under magnification to determine the gastric lesions produced. The results were expressed as lesions, ulcers and total index, which were obtained from scores determined by various alterations in the gastric mucosa, considering the color, edema and hemorrhage, loss of mucus, petechiae or damage to the folds of the mucosa, and the number and size of necro-hemorrhagic lesions.

Evaluation of gastrointestinal motility

Gastric emptying

The experiment was carried out using the method described by Gupta and Brans (1978), with slight modifications. After 6 h of fasting, the rats (n = 6/group) were orally pretreated with 1% Tween-80 aqueous solution (control) and CIN (50, 100 and 200 mg/kg), and subcutaneously with atropine (3 mg/kg), as a positive control. After 1 h or 30 min of the treatments, each animal received 1.5 mL of phenol red (0.5 mg/mL) orally. The zero time control group was euthanized immediately after administration of the marker and the other

groups were sacrificed 30 min later. The stomachs were removed, opened along the greater curvature and washed with 7 mL of distilled water. The stomach contents collected were centrifuged at 176 × g for 15 min. After centrifugation, 1 mL aliquots of supernatants were added to 1 mL of 1 N NaOH and the absorbance of the solution read on a spectrophotometer at 560 nm. The results were plotted on a standard curve for phenol red and expressed as the concentration (µg) of dye retained in the stomach in relation to the control group.

Intestinal transit

After removal of the rats' stomachs in the gastric emptying model, the small intestine was removed for evaluation of intestinal transit. With the aid of a ruler, the total length of the small intestine of each animal (distance between the gastropyloric region and the ileocecal junction) and the distance traveled by the phenol red (until the last portion of the intestine containing at least 1 cm of continuous marker) was measured. The results were expressed as a percentage of the distance traveled by the marker in relation to the total length of the small intestine (Stickney and Northup, 1959).

Evaluation of mucosal protective factors

Determination of gastric acid secretion basal and stimulated – piloric ligature method

This assay used the method described by Shay et al. (1945). The animals were divided into 11 groups (n = 6) and fasted for 16 h with free access to 5% glucose: (1) control, (2) CIN, (3) pantoprazole, (4) histamine, (5) histamine plus ranitidine, (6) histamine plus CIN, (7) bethanechol, (8) bethanechol plus atropine, (9) bethanechol plus CIN, (10) pentagastrin and (11) pentagastrin plus CIN. For pyloric ligature, the animals were anaesthetized (xylazine, 6 mg/kg and ketamine, 60 mg/kg, i.p.) and immediately after ligature received an intraduodenal dose of CIN (100 mg/kg), a control (1% Tween-80 aqueous solution, 0.1 mL/100 g body weight), ranitidine (60 mg/kg) or subcutaneous atropine (1 mg/kg). The abdominal wall was sutured and, 1h after pylorus ligation, the animals received a histamine (20 mg/kg s.c), bethanechol (2.5 mg/kg s.c) or pentagastrin (400 µg/kg s.c) stimulus. Four hours after pylorus ligation, the animals were sacrificed, the gastric secretion collected and centrifuged at 176 × g for 30 min. The volume (mL), pH values and the total acidity (mEquiv.[H+]/mL/4h) were determined.

Determination of gastric mucus

Quantification of adherence to gastric wall mucus was determined using the method devised by Corne et al. (1974) with modifications for the ethanol-induced ulcer model. After fasting for 16 h, the animals (n = 6/group) were subjected to treatment with 1% Tween-80 aqueous solution (CL), pantoprazole (40 mg/kg) and CIN (100 mg/kg) 1 h before induction of gastric lesions using ethanol. The non-injured control group (CN) received no treatment. The animals were sacrificed 1 h after the administration of ethanol and their stomachs were removed. Each glandular segment was weighed and immediately transferred to a tube containing 10 mL of 0.1% Alcian Blue and stained for 2 h. The dye complexed to the mucus gland wall was extracted with 10 mL of magnesium chloride (0.5 mol/L) and agitated for 2 h. At 4 mL of the mixture, 4 mL of diethyl ether were added and the solution was shaken. The emulsion obtained was centrifuged at 1480 × g for 10 min. The absorbance of samples was read in a spectrophotometer at 598 nm and results were expressed as μg of Alcian Blue/g of tissue.

Determination of the role of nitric oxide (NO) and sulfhydryl compounds (SH) in gastroprotection

To investigate the influence of endogenous NO and SH on the gastroprotective effect, the animals fasted for 24 h and were divided into nine groups (n = 6), of which three were pretreated with saline (i.p), three with L-NAME (N_{ω}-nitro-L-arginine methyl ester, 70 mg/kg, i.p), an inhibitor of the NO-synthase enzyme and three with NEM (N-ethylmaleimide, 10 mg/kg, i.p), a sulfhydryl compound blocker (Matsuda and Yoshikawa, 1999; Arrieta et al., 2003). Thirty min after pretreatment, each group was treated orally with 1% Tween-80 aqueous solution, carbenoxolone (100 mg/kg) and CIN (100 mg/kg). After 60 min, all the animals received 1 mL of absolute ethanol (p.o.) to induce gastric lesions. After 60 min of ethanol administration, the stomachs were removed for determination of gastric lesions as previously described.

In vivo antioxidant activity

The antioxidant tests were performed with the homogenate of the gastric mucosa of animals with ethanol-induced ulcers as described previously. The animals were divided into

four groups (n = 6/group): an uninjured control group (CN), a control (1% Tween 80 aqueous solution), N-acetylcysteine (NAC, 750 mg/kg) as standard antioxidant drug, and CIN (100 mg/kg). The uninjured control group consisted of untreated animals exposed to experimental procedures but without effective ulcer induction.

Determination of lipid peroxidation (LPO)

The lipid peroxidation index was determined using the method described by Ohkawa et al. (1989) involving measuring malondialdehyde (MDA). The stomach tissue excised was homogenized in a cold KCl (0.15 mol/L) solution and centrifuged at 11,000 × g for 20 min at 4 °C. Aliquots of 0.2 mL of sodium lauryl sulfate (8.1%), 1.5 mL of acetic acid (20%, pH 3.5), 1.5 mL of thiobarbituric acid (0.8%, w/v) and 0.3 mL of distilled water were added to 0.5 mL of the homogenate. The samples were incubated in a water bath at a temperature of 95 ºC for 1 h. After cooling, 6 mL of an n-butanol + distilled water mixture (5:1, v/v) was added, the tubes were vortexed for 1 min, and finally centrifuged at 1073 × g for 10 min. The absorbance was measured in a spectrophotometer at 532 nm and the results were expressed as μmol of MDA/g tissue.

Quantification of non-protein sulfhydryl groups (GSH)

The levels of non-protein sulfhydryl groups (GSH) in the gastric mucosa were determined using the method developed by Sedlak and Lindsay (1968). The excised stomach tissue was weighed and homogenized in a cold EDTA (0.02 mol/L) solution. Aliquots of 320 μL of distilled water and 80 μL 50% aqueous solution of trichloroacetic acid were added to 400 μL of the homogenate for protein precipitation and the samples were then centrifuged at 604 × g for 15 min at 4 °C. To a total of 400 μL of supernatant was added 800 μL of 0.4 M Tris (pH 8.9) and 20 μL of 5,5-dithiobis(2-nitrobenzoic acid) to 0.01 M. The mixture was then stirred for 3 min and the absorbance was measured at 412 nm using a spectrophotometer. The concentrations of non-protein sulfhydryl groups were expressed in μg of GSH/g tissue.

Evaluation of healing properties

Acetic acid-induced gastric ulcer

The experiment was conducted using the method described by Takagi et al (1969) with some modifications. The animals were divided into 3 groups (n = 6), given a restricted solid food diet for 24 h and, after this, anesthetized in order to perform surgery to expose the stomach. 0.05 mL of 30% acetic acid was injected into the subserosal layer of the external wall of the stomach. One day after administration of acid, daily treatment began and the animals were treated orally once daily for 14 days with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and CIN (100 mg/kg). During this period, the possible toxic effects of CIN were evaluated using such parameters as mortality, changes in body mass and macroscopic analysis of vital organs. On day 15, all groups were sacrificed, the stomachs removed, photographed and the surface area of gastric lesion determined by computerized planimetry (Software ImageJ®) and the data expressed in mm^2.

Histological and immunohistochemical analyses

The stomach lesions induced by acetic acid in rats undergoing different treatments were located, sectioned, and set in 10% buffered formalin. After setting, the samples was washed with water, immersed in 70% ethyl alcohol for 3-4 days and embedded in paraffin. Five-µm thick paraffin sections were taken and stained with hematoxylin/eosin (HE) and Periodic Acid–Schiff (PAS). Histological analysis of the gastric sections was carried out using an automatic microscopy system MICRO DIP® (Kacil Inc.).

The immunohistochemical for PCNA was performed in samples of rat's stomach embedded in paraffin. Sections of 4 µm were obtained and incubated for 30 min with monoclonal antibody against the anti-PCNA protein. Initially, the samples were deparaffinized in xylene and hydrated. Then antigenic retrieval was performed in microwave oven at 100 °C, the slides were cooled to room temperature and endogenous peroxidase was blocked by 7.5 min in peroxidase blocking solution. After cooling, were then incubated separately with primary antibodies for PCNA (anti-PCNA antibody [PC10] - Proliferation Marker (ab29) - Mouse monoclonal antibody, Abcan Inc), 1:100, 30 min, and with secondary antibody (Nichirei Biosciences Inc.), 1:200 for 30 min and then washed with phosphate buffered saline (PBS). After washing, slides were incubated with diaminobenzidine chromogen solution (DAB), washed in water, counter-stained with hematoxylin, dehydrated and mounted. The analysis of the reactivity was performed by the nucleus of epithelial cells using the following scores: reactivity mild, moderate and hard. Cells reactive for anti-PCNA

were identified by the presence of a dark reddish-brown chromogen. The positive nuclear staining cells were observed under a microscope (× 500).

Statistical analysis

The results were expressed as mean ± standard error of mean (S.E.M). Differences between means were determined using one way analysis of variance (ANOVA) followed by Tukey's test. The statistical analysis was performed using GraphPad Prism 5.0®. The level of significance for rejection of the null hypothesis was set at 5% ($p < 0.05$).

Results

Antiulcerogenic activity of CIN

The administration of ethanol caused extensive damage to the gastric mucosa with hemorrhagic erosions in the control. CIN provided significant gastric protection, decreasing the area of the lesion caused by ethanol and HCl/ethanol in all doses tested. Oral administration of CIN (50, 100 and 200 mg/kg), 1 h before the induction of gastric lesions with ethanol, significantly reduced the lesion area to 40.3 ± 11.6, 4.9 ± 2.2 and 2.3 ± 1.8 mm^2, respectively, when compared to the control group (339.8 ± 49.3 mm^2), which corresponds to a percentage inhibition of 88.1, 98.5 and 99.2%, respectively (Fig. 1A).

In HCl/ethanol-induced gastric ulcer model, CIN (50, 100 and 200 mg/kg) also caused a significant level of gastroprotection (28.2 ± 12.8; 11.8 ± 5.4 and 1.3 ± 0.8 mm^2, respectively) in comparison to the control group (245.5 ± 43.0 mm^2), corresponding to 88.5, 95.2 and 99.4% of inhibition of the lesion area, respectively (Fig. 1B). Pantoprazole (40 mg/kg, p.o.) significantly inhibited the gastric lesions induced by ethanol and HCl/ethanol in 53.7% and 91.5%, respectively, in relation to control group

Oral administration of indomethacin (30 mg/kg) produced a gastric mucosal lesions index of 3.5 ± 0.5, an ulcer index of 22.8 ± 2.3 and a total index of 26.3 ± 2.6 in the control group. Pretreatment of animals with CIN at doses of 50, 100 and 200 mg/kg orally 1 h before subcutaneous administration of indomethacin produced significant inhibition with all indices as shown in Table 1. The 50 mg/kg dose reduced the incidence of lesions, ulcers and the total index by 66.8, 62.0 and 85.7%, the 100 mg/kg dose reduced these by 56.8, 61.0 and 72.5%, and the 200 mg/kg dose by 81.7, 61.1 and 74.2%, respectively.

Effect of CIN on gastrointestinal motility

The concentration of phenol red present in the stomach after 30 min of administration was 4.4 ± 1.7, 11.7 ± 0.4 and 11.0 ± 0.7 µg/mL in animals treated with CIN (50, 100 and 200 mg/kg) respectively. The results indicate that CIN at a dose of 50 mg/kg did not affect gastric emptying, however the animals treated with doses of 100 and 200 mg/kg showed a reduction in the rate of gastric emptying of 92.1 and 86.8% when compared to the control group. The muscarinic antagonist atropine (3 mg/kg), used as positive control, increased the concentration of phenol red to 12.6 ± 0.1 µg/mL, compared to the control group (2.1 ± 0.9 µg/mL) The rate of gastric emptying in the group treated with atropine was 99.1%.

The intestinal transit, as measured by the distance traveled by the phenol red in relation to the overall length of the small intestine in the control group was 72.7 ± 3.4%. CIN at doses of 100 and 200 mg/kg, whose transit percentage was 83.5 ± 2.8 and 80.2 ± 1.9%, respectively, showed no effect on intestinal transit compared with the control group. In animals treated with a dose of 50 mg/kg, the percentage of transit increased to 96.0 ± 2.5%, corroborating the decrease in concentration of phenol red present in the stomach of animals treated with this dose. The percentage of intestinal transit in animals treated with atropine (3 mg/kg) fell to 55.9 ± 5.2%.

Effect of CIN on basal and stimulated gastric acid secretion

After 4 h of pylorus ligation, it was observed that the only one of the base gastric antisecretory parameters altered by CIN was gastric volume, with pH and total acidity remaining unchanged. Histamine, pentagastrin and bethanechol, administered subcutaneously, stimulated gastric acid secretion, increasing volume and pH, and decreasing the total acidity of basal gastric acid secretion. Ranitidine (60 mg/kg) and atropine (1 mg/kg) prevented the increase in volume and acidity of gastric contents, as well as decreasing the pH of gastric acid secretion stimulated by histamine and bethanechol. CIN showed no inhibitory action on gastric acid secretion stimulated by histamine, bethanechol and pentagastrin (Table 2).

Effect of CIN on the production of gastric mucus

As shown in Fig. 2, the animals with ethanol-induced lesions (CL) showed a significant decrease in the levels of gastric mucus (2.8 ± 0.4 µg of Alcian Blue/g tissue) compared to the non-injured control group (CN, 6.6 ± 0.4 µg of Alcian Blue/g tissue). Treatment with CIN at a dose of 100 mg/kg was able to increase mucus production significantly (5.3 ± 0.5 µg of Alcian Blue/g tissue) compared to the injured control group. Pantoprazole also caused a significant increase in the levels of gastric mucus (6.4 ± 0.5 µg of Alcian Blue/g tissue).

The role of nitric oxide (NO) and sulfhydryl compounds (SH) in CIN gastroprotection

Both the NO synthase inhibitor, L-NAME (N_{ω}-nitro-L-arginine methyl ester) and the inhibitor of sulfhydryl compounds, NEM (N-ethylmaleimide), exacerbated ethanol-induced gastric lesions in all groups compared to the groups pretreated with saline. Table 3 shows that, in rats pretreated with L-NAME, CIN continued to exert its gastroprotective effect without the action of NO-synthase, thereby showing that its activity does not depend on NO. However, depletion of sulfhydryl groups by pretreatment with NEM, was able to significantly reduce the gastroprotective effect of CIN in relation to the group pretreated with saline, suggesting that SH compounds are involved in protection of the gastric mucosa and that the gastroprotective effect of CIN is dependent on the presence/production of these compounds.

Effects of CIN on the antioxidant activity system

The lipid peroxidation (LPO) index in the gastric mucosa of rats subjected to an ethanol-induced gastric ulcer model was determined by quantification of malondialdehyde, which reacts with thiobarbituric acid. Animals in the injured control group (CL) showed an increase in gastric levels of malondialdehyde (29.8 ± 4.4 µmol MDA/g tissue) compared to the uninjured control group (CN, 12.6 ± 2.9 µmol MDA/g tissue). Oral treatment with CIN (100 mg/kg) decreased the rate of lipid peroxidation by diminishing the production of malondialdehyde by ethanol (13.3 ± 3.8 MDA µmol/g tissue). N-acetylcysteine (NAC, 750 mg/kg) also inhibited the increase in levels of malondialdehyde (15.0 ± 3.6 MDA µmol/g tissue) as shown in Fig. 3.

The level of GSH found in the gastric mucosa of the uninjured control group (CN) animals was 147.2 ± 7.5 µg GSH/g tissue, but in animals submitted to ethanol-induced gastric damage (CL) a reduction in levels of GSH (64.2 ± 6.8 µg GSH/g tissue) was observed compared to the uninjured control group. Oral treatment with N-acetylcysteine (NAC, 750 mg/kg) and CIN (100 mg/kg) showed that both were capable of reversing the reduction in levels of mucosa in the sulfhydryl groups, restoring the antioxidant system to base levels (147.9 ± 13.8 and 104.4 ± 7.3 µg GSH/g tissue, respectively) (Fig. 3).

Acetic acid-induced gastric ulcer

In the acetic acid model, the results show that oral administration of CIN (100 mg/kg) for 14 consecutive days decreased (43.1%) the area of chronic ulcer to 27.3 ± 3.2 mm^2, as can be seen in Fig. 4. Pantoprazole (40 mg/kg) speeded up the healing of gastric ulcers in rats, significantly reducing the area of the injury to 20.09 ± 6.21 mm^2 (58.1%) when compared to the control group (48.0 ± 7.5 mm^2). During the 14 days of treatment with CIN and pantoprazole, no signs of toxicity were observed and animals treated with the essential oil showed an increase in body weight similar to that of the control animals (Fig. 5). No significant changes were detected on macroscopic analysis of the vital organs (data not shown).

Histological and immunohistochemical analyses

Histological analysis was performed by HE and PAS staining. HE slices revealed well-defined ulcers with complete destruction of the mucosal and submucosal layer caused by acetic acid in animals in the control group. In the stomachs of rats treated orally with CIN (100 mg/kg) and pantoprazole (40 mg/kg, p.o.) slices from ulcers demonstrated a regeneration of gastric mucosa. PAS staining also showed increased mucus production (Fig. 6). These results were confirmed by immunohistochemical analysis for PCNA (Proliferating Cell Nuclear Antigen), a cell division marker used to evaluate healing properties. Immunohistochemical localization showed intense reactivity and a great quantity of PCNA-positive nuclei (marked with brown color), in the gastric mucosa of animals treated with pantoprazole or CIN, when compared to control group, where it was observed the absence of reactivity due to the destruction of the epithelial layer, as shown in Fig. 7.

Discussion

1,8-cineole is a monoterpene present in many essential oils of aromatic plants used in folk medicine. This study investigated the antiulcerogenic activity of 1,8-cineole, a major compound of the essential oil of *Hyptis martiusii* leaves on acute and chronic gastric lesions induced by necrotizing agents and the mechanisms of action involved.

A previous study conducted by Santos and Rao (2001b) showed that 1,8-cineole, at oral doses ranging from 50 to 200 mg/kg, reduced ethanol-induced gastric lesions and attributed this to the increased amount of GSH, involving antioxidant action, and partly to inhibition of the gastric mucosa enzyme, lipooxygenase, by blocking the formation of leukotrienes and thus preventing the harmful action of ethanol on stomach cells. It has also been reported that the gastroprotective effect of the compound is not mediated by endogenous nitric oxide. The present study has confirmed these results and also examined other mechanisms of action involved in the gastroprotection provided by the compound.

Agents such as ethanol and hydrochloric acid produce lesions in the gastric mucosa by way of an effect which involves the depression of defense mechanisms, such as prostaglandins, and stasis in gastric blood flow, which contributes to the development of lesions and of seemingly necrotic hemorrhagic tissue (Singh et al., 2008). Pretreatment with CIN protected the rats' gastric mucosa against ethanol- and acidified ethanol-induced ulcer, suggesting cytoprotective action on the part of the extract.

It has been reported that non-steroidal anti-inflammatory drugs (NSAID) may cause damage to the mucus of the gastrointestinal tract, inhibiting cyclooxygenase and thereby reducing final prostaglandin production and diminishing the resistance of the gastric mucosa (Chiba et al., 2008). In the model involving NSAID, indomethacin-induced gastric ulcer, CIN significantly reduced damage to mucosal at all doses tested, demonstrating its gastroprotective properties and suggesting the possible involvement of prostaglandins and/or mucus in antiulcer activity.

Several studies have correlated the formation of gastric ulcers with gastric emptying and gastric motility, although the data are still unclear (Fülöp et al., 2005). The evaluation of the effects of 1,8-cineole on gastrointestinal motility showed that higher doses of CIN interfered as atropine, reducing the gastric emptying rate, but did not alter intestinal transit, while the lowest dose increased the percentage of intestinal transit, as corroborated by the decreased concentration of phenol red present in the stomach of animals treated with this dose. Data from the literature show that intravenous administration of 1,8-cineole delays

gastric emptying and gastrointestinal transit of liquid in rats as demonstrated by Magalhães et al. (1998).

The antiulcer activity of 1,8-cineol observed in the models described above led us to conduct experiments to evaluate the possible influence of mucosal protection factors, such as antisecretory activity, mucus production, nitric oxide and sulfhydryl groups, on its gastroprotective effect. As it was observed that CIN was able to protect the gastric mucosa and that different protective factors may be involved in this, we verified their influence on the parameters (volume, pH and total acidity) of basal and stimulated gastric secretion with the agonists (secretagogues) of receptors of histamine (H2), acetylcholine (M3) and gastrin (CCK2). The results demonstrate that CIN reduced the volume of basal acid secretion but did not reveal any changes in the parameters for gastric acid secretion stimulated by histamine, pentagastrin and bethanechol, suggesting possible lack of antisecretory activity.

Gastric mucus is an important protective factor for the gastric mucosa and consists of a viscous, elastic, adherent and transparent gel formed by water and glycoproteins that covers the entire gastrointestinal mucosa (Laine et al., 2008). The results revealed a significant increase in the amount of mucus adhering to the gastric mucosa in animals treated with CIN, thereby explaining the gastroprotective action observed previously.

Nitric oxide (NO) and non-protein sulfhydryl groups (GSH) is acknowledged to be an important mediator in gastric defense mechanisms. The nitric oxide increases blood flow in the gastric mucosa, regulate mucus production and inhibit the attachment of neutrophils to endothelial cells (Tanaka et al., 2001). The non-protein sulfhydryl groups (GSH) are directly associated with maintaining the integrity of the gastric mucosa, because they limit the production of free radicals and enable the production and maintenance of mucus units (Avila et al., 1996). In animals pretreated with L-NAME, an inhibitor of NO synthase, CIN continued to exert a gastroprotective effect, suggesting that nitric oxide is not involved in this. Inhibition promoted by NEM, an inhibitor of sulfhydryl groups, was able to reduce the gastroprotective effect of CIN to a statistically significant extent, suggesting that sulfhydryl groups are involved in the protection of the gastric mucosa and that the protective effect of CIN is dependent on the presence/production of these groups.

Besides the previously mentioned mechanisms, the consumption of ethanol-induced gastric lesions is associated with increased lipid peroxidation and decreased non-protein sulfhydryl groups (GSH), leading to increased ROS (Glavin and Szabo, 1992). Confirming data already reported in the literature, stomachs undergoing ethanol-induced damage exhibited increased levels of malondialdehyde (MDA) and a decrease in non-protein

sulfhydryl groups (GSH) compared to the levels found in non-injured animals. Pretreatment with CIN (100 mg/kg) decreased lipid peroxidation as evidenced by reduced levels of malondialdehyde and increased basal levels of GSH, confirming the involvement of these groups in its gastroprotective effect. These results suggest the involvement of the antioxidation mechanism of CIN in the oxidative stress induced by ethanol in gastric mucosa.

We further investigated the healing action of the compound on chronic acetic acid-induced ulcers. This ulcer model closely resembles human ulcers in terms of pathological features and the healing process (Takagi et al., 1969). The results of this study suggest that oral treatment with CIN at a dose of 100 mg/kg speeds up the healing of chronic ulcers and this was demonstrated by the reduction in the area of lesion, as confirmed by histological analysis (HE staining) and the large quantity of mucus secreted (PAS staining). The immunohistochemical analysis for PCNA, a cell proliferating marker, confirm that there was a significant increase in cell proliferation in the region of healing of gastric mucosa of animals subjected to treatment with CIN.

In conclusion, the results of this study show that 1,8-cineole has gastroprotective properties regarding gastric damage induced by different agents. This is mediated in part by a cytoprotective mechanism, since it causes an increase in gastric mucus, and in part by antioxidant activity, preventing depletion of sulfhydryl groups and reducing levels of lipid peroxidation in the gastric mucosa. The study also provides evidence that 1,8-cineole is related to the gastroprotective effect of EOHM.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

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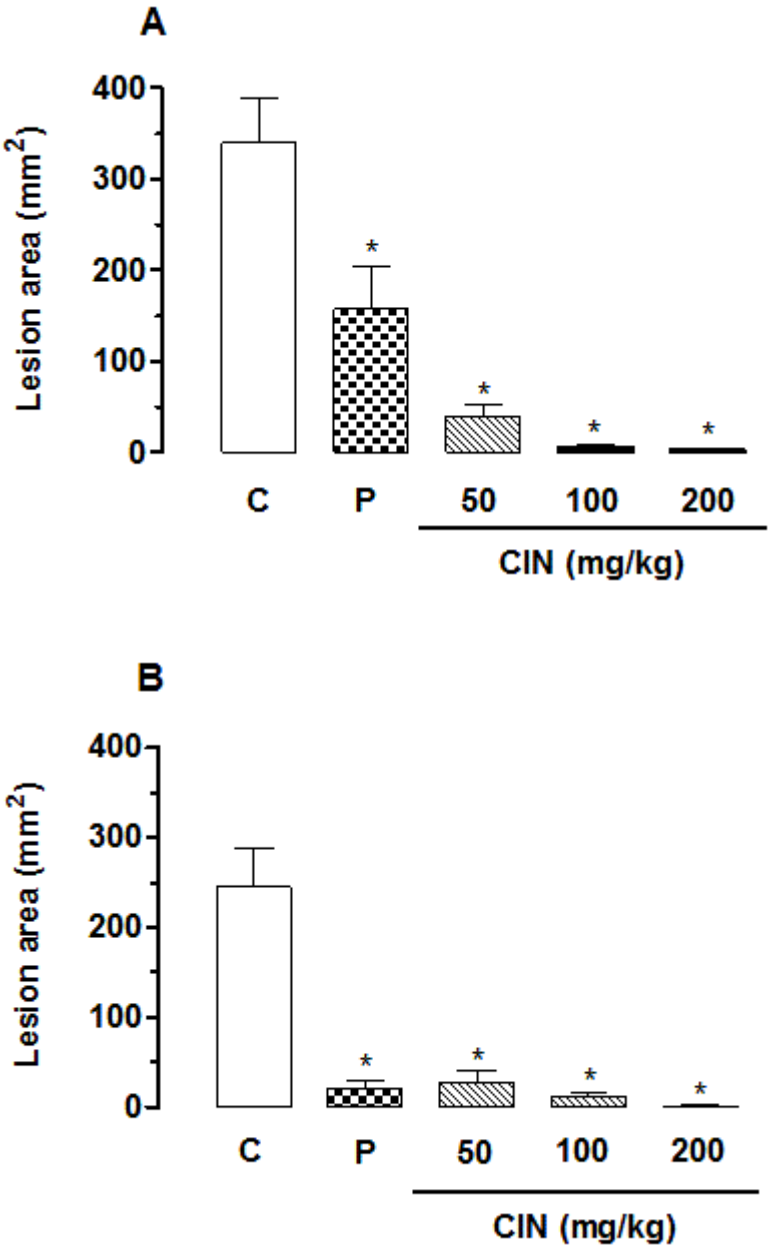

Fig. 1. Gastroprotective effect of 1,8-cineole (CIN) on gastric lesions induced by ethanol 70% (A) and HCl/ethanol (B) in Wistar rats. The animals were treated orally with 1% Tween-80 aqueous solution (C, control group), pantoprazole (P, 40 mg/kg) and CIN, 1 h before administration of ulcerogenic agent. The results are expressed as mean ± S.E.M. (n = 6/group). *Statistically different from control group (ANOVA followed by Tukeys's test, $p < 0.05$).

Table 1. Effect of oral administration of 1,8-cineole (CIN) on gastric lesions induced by indomethacin (30 mg/kg, s.c.) in Wistar rats.

Treatment	**Dose (mg/kg)**	**Lesion index**	**Ulcer index**	**Total index**
control	-	3.5 ± 0.5	22.8 ± 2.3	26.3 ± 2.6
pantoprazole	40	2.3 ± 0.4	0*	2.3 ± 0.4*
CIN	50	1.2 ± 0.3*	9.8 ± 2.1*	11.0 ± 2.1*
	100	1.3 ± 0.4*	8.9 ± 4.5*	10.2 ± 4.7*
	200	0.5 ± 0.2*	6.3 ± 3.5*	6.8 ± 3.6*

Values represent the mean ± S.E.M (n = 6/group). *Statistically different from control group (ANOVA followed by Tukeys's test, $p < 0.05$).

Table 2. Effect of 1,8-cineole (CIN), administered intraduodenally, on gastric secretion parameters basal or stimulated by histamine (20 mg/kg), bethanechol (2.5 mg/kg) and pentagastrin (400 µg/kg) in Wistar rats subjected to pylorus ligature.

Stimulus + treatment	**Gastric volume (mL)**	**pH**	**Total acidity (mequiv. H^+]/mL/4 h)**
control (not estimulated)	3.3 ± 0.2	2.0 ± 0.2	8.6 ± 2.2
pantoprazole (not estimulated)	2.1 ± 0.2*	3.7 ± 0.5*	3.2 ± 0.6*
CIN (not estimulated)	2.5 ± 0.2*	2.5 ± 0.1	4.8 ± 1.0
histamine	6.3 ± 0.7	1.5 ± 0.1	52.8 ± 9.9
histamine + ranitidine	2.3 ± 0.3$^{\#}$	2.6 ± 0.1$^{\#}$	2.9 ± 0.6$^{\#}$
histamine + CIN	4.6 ± 0.7	1.5 ± 0.1	36.0 ± 10.2
bethanechol	7.8 ± 0.5	1.5 ± 0.0	52.2 ± 5.4
bethanechol + atropine	2.8 ± 0.2$^{\#\#}$	2.5 ± 0.2$^{\#\#}$	6.6 ± 1.5$^{\#\#}$
bethanechol + CIN	6.6 ± 0.3	1.6 ± 0.0	46.8 ± 4.5
pentagastrin	7.2 ± 1.4	1.4 ± 0.0	45.0 ± 7.8
pentagastrin + CIN	4.9 ± 0.6	1.4 ± 0.0	41.0 ± 9.4

Treatment: control (1% Tween-80 aqueous solution, 0.1 mL/100 g, i.d), pantoprazole (40 mg/kg), CIN (100 mg/kg, i.d), ranitidine (60 mg/kg, i.d.) and atropine (1 mg/kg, s.c). *$p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. histamine group and $^{\#\#}p < 0.05$ vs. bethanechol group (ANOVA followed by Tukeys's test, $p < 0.05$).

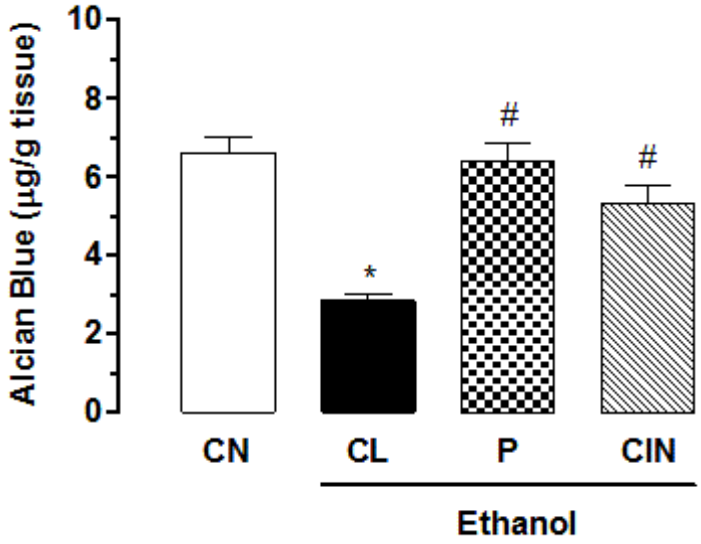

Fig. 2. Quantification of adherent mucus in gastric mucosa of rats treated with 1,8-cineole (CIN) on gastric ulcers model induced by ethanol. The non-injured control group (CN) received no treatment. 1% Tween-80 aqueous solution (CL, injured control), pantoprazole (P, 40 mg/kg) and CIN (100 mg/kg) were administered 1 h before the induction of gastric lesions. Results are expressed as mean ± S. E. M. (n = 6/group). ANOVA followed by Tukeys's test (*$p < 0.05$ vs. non-injured control group and $^{\#}p < 0.05$ vs. injured control group).

Table 3. Effect of oral administration of 1,8-cineole (CIN) on gastric lesions induced by ethanol in Wistar rats pretreated with L-NAME (N_{ω}-nitro-L-arginine methyl ester, 70 mg/kg) or NEM (N-ethylmaleimide, 10 mg/kg).

Pretreatment	**Treatment (p.o.)**	**Dose (mg/kg)**	**Lesion area (mm^2)**	**Inhibition (%)**
Saline (i.p.)	control	-	298.0 ± 57.2	-
	carbenoxolone	100	42.8 ± 16.6*	85.6
	CIN	100	2.5 ± 1.2*	99.1
L-NAME (i.p.)	control	-	440.7 ± 6.98*	-
	carbenoxolone	100	43.5 ± 15.2$^{\#}$	90.12
	CIN	100	4.0 ± 2.4$^{\#}$	99.0
NEM (i.p.)	control	-	739.8 ± 10.2*	-
	carbenoxolone	100	623.3 ± 154.9	15.7
	CIN	100	709.0 ± 43.7	4.2

Results are expressed as mean ± S.E.M (n = 6/group). *$p < 0.05$ compared to saline + control, $^{\#}p < 0.05$ compared to L-NAME + control (ANOVA followed by Tukey's test).

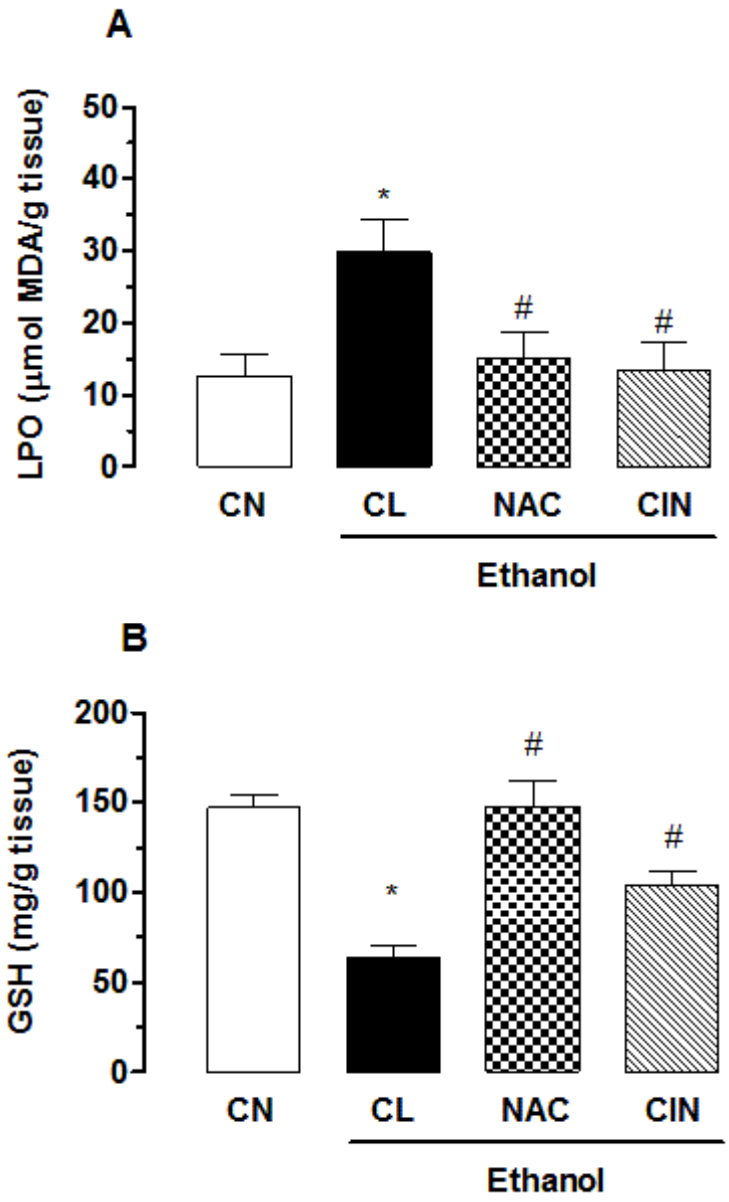

Fig. 3 Effect of 1,8-cineole (CIN) on the levels of malondialdehyde (A) and non-protein sulfhydryl groups (B) in the gastric ulcers model induced by ethanol (70%, 0.5 mL/100 g, p.o). The non-injured control group (CN) received no treatment. 1% Tween-80 aqueous solution (CL, injured control), N-acetylcysteine (NAC, 750 mg/kg) and CIN (100 mg/kg) were administered 1 h before the induction of gastric lesions. Results are expressed as mean ± S. E. M. (n = 6/group). ANOVA followed by Tukey's test (*$p < 0.05$ vs. non-injured control group-CN and $^{\#}p < 0.05$ vs. injured control group-CL).

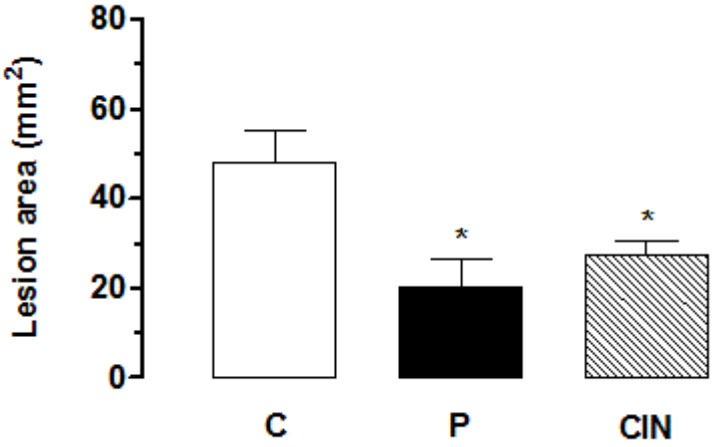

Fig. 4. Effect of 1,8-cineole (CIN, 100 mg/kg) in the healing of chronic ulcer induced by 30% acetic acid. 1% Tween-80 aqueous solution (C) pantoprazole (P, 40 mg/kg) and CIN (100 mg/kg) were administered for 14 days. Values represent the mean ± S. E. M. (n = 6/group). *Statistically different from control group, $p < 0.05$ (ANOVA followed by Tukey's test).

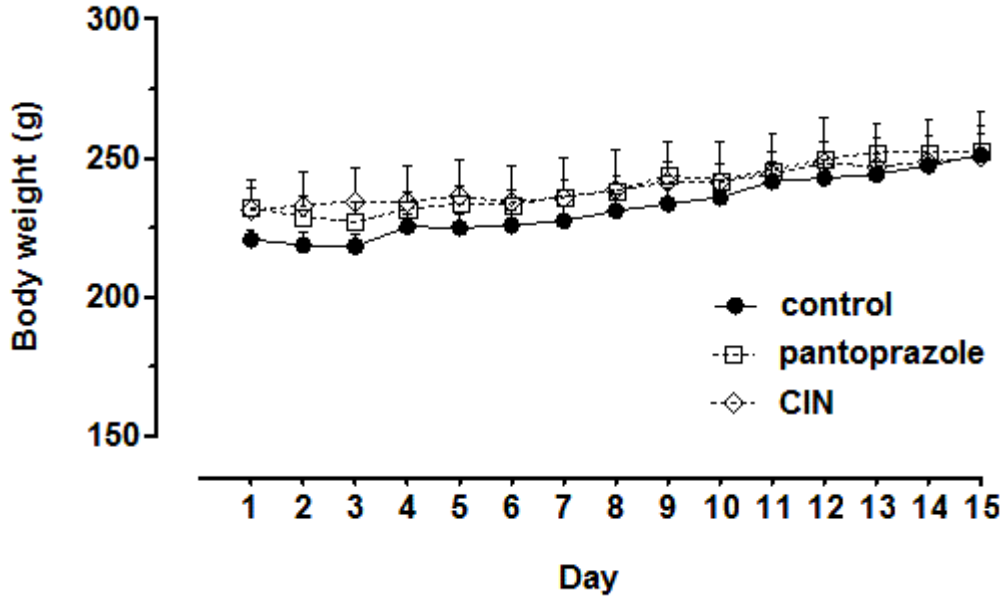

Fig. 5. Body weight of rats treated orally with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and 1,8-cineole (CIN, 100 mg/kg) for 14 days after formation of chronic ulcer induced by 30% acetic acid. Values represent the mean ± S. E. M. (n = 6/group).

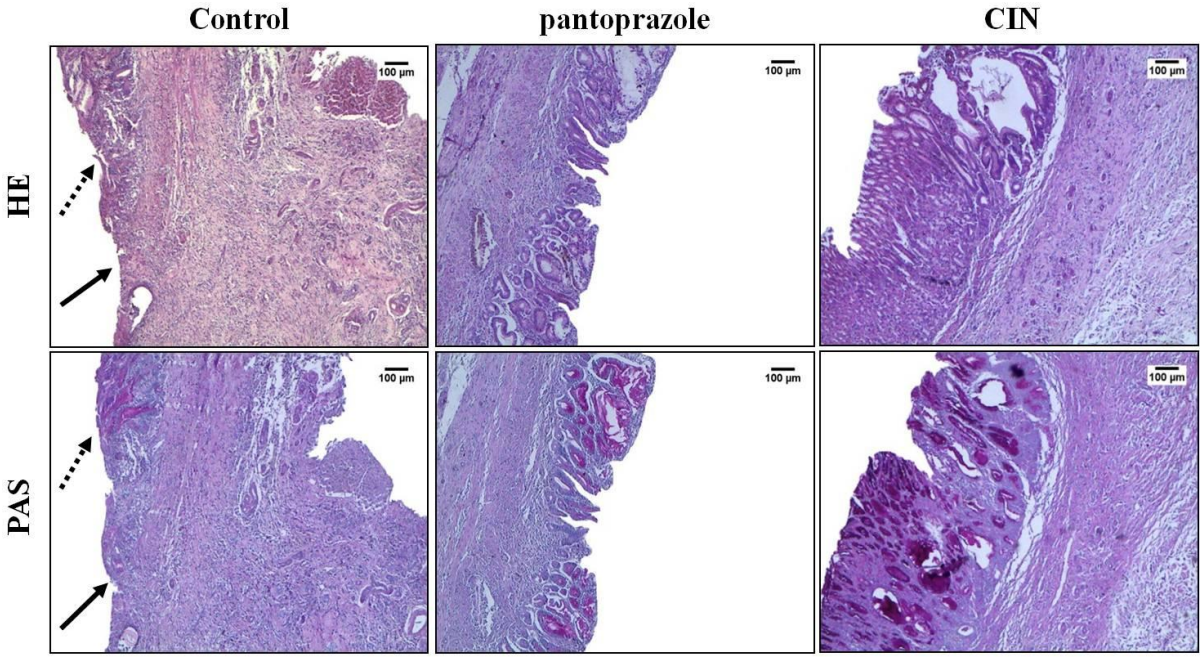

Fig. 6. Histological analysis of rats' stomachs treated with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and 1,8-cineole (CIN, 100 mg/kg) for 14 days after injury induced by 30% acetic acid on the haematoxylin/eosin staining (HE) and Periodic Acid–Schiff staining (PAS). The arrows indicate the area internal (filled arrow) and edge (dashed arrow) of the ulcer. Notice in the pantoprazole and EOHM groups, lesion regeneration area (HE) and the great amount of mucus secretion (PAS) evidenced by the intense tone of pink. Magnification 40x.

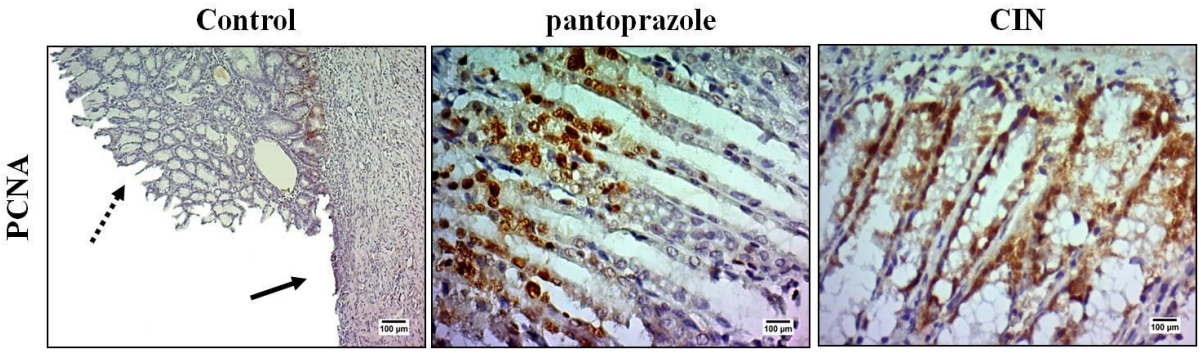

Fig. 7. Immunohistochemical analysis for PCNA (proliferating cell nuclear antigen) of rats' stomachs treated with 1% Tween-80 aqueous solution (control), pantoprazole (40 mg/kg) and 1,8-cineole (CIN, 100 mg/kg) after 14 days injury induced by 30% acetic acid. The filled arrow indicate the area internal of the ulcer and the absence of the epithelial layer. The dashed arrow indicate the ulcer edge with epithelial layer remaining. In the control group, it was observed the absence of reactivity in the area of the ulcer. Notice in pantoprazole and CIN intense reactivity with nuclei positive PCNA marked (brown color). Magnification 200x (control) and 500x (pantoprazole or EOHM).

4.5 ArtigoV: a ser submetido ao periódico Food and Chemical Toxicology

Título: Repeated-doses and reproductive toxicity studies of the monoterpene 1,8-cineole (eucalyptol) in Wistar rats

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Abstract

The monoterpene 1,8-cineole (eucalyptol) is an organic constituent naturally occurring in the essential oil of many herbs and widely used as an excipient in the pharmaceutical industry and as a food flavoring agent, thus providing significant potential for human exposure to the compound. The aim of this study was to evaluate the preclinical toxicity (acute and repeated-doses toxicity) as well as reproductive toxicity of CIN. For the acute toxicity, Swiss mice received a single dose of CIN (2000 mg/kg) and for the repeated-doses toxicity, Wistar rats were treated with CIN (100, 500 and 1000 mg/kg) for 50 days. For reproductive toxicity, pregnant rats were treated with CIN (250, 500 and 1000 mg/kg) during pre-implantation or organogenic periods. The results for acute toxicity test showed that mice tolerate CIN well up to a dose of 1500 mg/kg. It is thus estimated that 1500 mg/kg<LD_{50} <1750 mg/kg. In the repeated-doses toxicity study, CIN did not produce any signs of toxicity or deaths, but affected body weight gain and caused variations in the food and water intake. CIN increased VCM, platelet and urea levels, and reduced levels of MCHC, VPM and alkaline phosphatase, however, the changed values lie within the physiological limits described for the species. Histopathological analysis showed changes in the lungs, liver, kidneys and uterus in animals treated with all doses of CIN, although, the morphology of all other organs remained unchanged. In reproductive toxicity, CIN produced a reduction in body weight in pregnant rats treated during the pre-implantation or organogenesis periods. There was a reduction in fetus mass during pre-implantation and dead fetuses were registered among animals in the control group and those treated with high doses during both periods. The results indicate that the treatment with CIN for 50 days, showed occasional alterations in hematological, biochemical parameters and histopathological evaluation, but these changes showed low clinically relevant changes, since these occurred in a non-generalized manner among rats of both sexes. However, the alterations observed of the reproductive parameters provide evidence that CIN presents maternal and fetal toxicity, this requires more detailed investigation to better characterize the toxic effects of this compound.

Keywords: 1,8-cineole, monoterpene, repeated dose toxicity, reproductive toxicity

1. Introduction

Essential oils are complex mixtures of volatile organic compounds produced as secondary metabolites in plants whose chemical composition varies (Nerio et al., 2010). Around 3000 essential oils are currently known, of which 300 are commercially important, especially for the pharmaceutical, health, cosmetics, agricultural, food and fragrances industries (Bakkali et al., 2008).

Chemically, essential oils are composed primarily of terpenoids, in particular mono- and sesquiterpenes. Derivatives of phenylpropanoids may also be present along with compounds such as aromatic and aliphatic hydrocarbons, alcohols, esters, ethers, aldehydes, lactones, phenols, and others (Dorman and Deans, 2000). The monoterpenes are the most common molecules, making up about 90% and enabling a wide variety of structures (Bakkali et al., 2008).

Terpenoids not only comprise the largest group of natural plant products, with around 30,000 compounds, but also exhibit the greatest variety of structural types (Degenhardt et al., 2009). From a biological perspective, they have a variety of interesting medicinal properties, such as antinociceptive activity – *limonene* (Amaral et al., 2007), gastroprotective activity – *linalool* (Barocelli et al., 2004), antimicrobial activity – *carvacrol* (Burt, 2004), anticonvulsive activity – *citronellol* (De Sousa et al., 2006) and antimalarial activity – *artemisinin* (Rodriguez-Concepcion, 2004).

1,8-cineole, also called cineole, eucalyptol or 1,8-epoxy-p-methane is a monoterpene oxide with a fresh fragrance, camphoraceous features , and a spicy flavor (Lana et al., 2006). It is an organic compound naturally found in the essential oil of many herbs and is considered the main compound in various species and their essential oils. This monoterpene is widely used as an excipient in the pharmaceutical and cosmetics industry, for example, in nasal sprays or as a disinfectant (Madyastha and Chadha, 1986) and as a food flavoring agent (Ahmad and Misra, 1994). According to De Vincenzi et al. (2002) plants and/or their essential oils containing 1,8-cineole may be added as flavorings to various processed foodstuffs, such as frozen dairy products, soft candy or non-alcoholic beverages, and there is thus significant potential for human exposure to this compound.

Various therapeutic applications have been attributed to 1,8-cineole, in particular in the treatment of respiratory diseases such as asthma, bronchitis, sinusitis, and colds, owing to its secretolytic properties (Juergens et al., 2003), as well as muscle pain, neurosis, rheumatism and kidney stones (Miyazawa et al., 2001). Despite reports of various biological properties,

there is insufficient evidence regarding the safety profile of oral administration of this monoterpene. As 1,8-cineole is the main component of many essential oils from aromatic plants used in folk medicine, the aim of this study was to evaluate the preclinical toxicity of 1,8-cineole for acute, repeated-doses and reproductive toxicity in rodents.

2. Material and methods

2.1 Animals

Male and female Wistar rats (200-350 g) obtained from the Federal University of Pernambuco's Department of Physiology and Pharmacology and female Swiss mice (35-45 g) obtained from the Keizo Asami Immunopathology Laboratory (LIKA/UFPE), Pernambuco, Brazil, were used. These were kept under standard environmental conditions (12 h dark/light cycle) and temperature (22 ± 2 °C). Water and industrialized dry food (Labina®, Purina, Brazil) were made available *ad libitum*. All experimental protocols were submitted to and approved by the UFPE Animal Experimentation Ethics Committee, under license no. 037544 in accordance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals.

2.2. Drugs and doses

1,8-cineole (CIN) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and for experimental use was emulsified in a 1% Tween-80 aqueous solution before administration to the animals. Animals were treated with the substance and doses used for each protocol were based on pilot experiments previously conducted in our laboratory. The control group received a 1% Tween-80 aqueous solution.

2.3 Acute toxicity study in mice

Acute toxicity studies were performed on Swiss female mice, as described by OECD 420 (2001), with slight modifications. The animals were randomly divided into two groups (n = 5/group) and fasted overnight with free access to water. The group control received a 1% Tween-80 aqueous solution (0.1 mL/10 g) orally and the group treated with CIN a single 2000 mg/kg dose. The animals were observed at 30, 60, 120, 180 and 240 minutes after oral

treatment and daily for 14 days. Behavioral changes, weight, food and water consumption, clinical signs of toxicity, and mortality were recorded daily (Malone, 1977).

2.4 Repeated oral dose toxicity in rats

This protocol was performed according to the Organization for Economic Cooperation and Development Test Guidelines (OECD 452, 2008) with slight modifications. Healthy male and female Wistar rats were randomly divided into four groups by sex (n = 10/group/sex). The group control received a 1% Tween-80 aqueous solution (0.1 mL/10 g) orally and the group treated with CIN doses of 100, 500 and 1000 mg/kg for 50 consecutive days. During treatment, the body-weight and food and water intake of the animals were recorded weekly. The animals were observed for signs of toxicity, such as piloerection, diarrhea and changes in locomotor activity, and for mortality for the duration of the experiment. At the end of the 50-day experiment, the animals fasted overnight (12 h), but were allowed water *ad libitum*. They were then anesthetized with thiopental (35 mg/kg, i.p.) and blood samples were obtained by retro-orbital puncture using capillary tubes and collected in two tubes, tube 1 containing anticoagulant ethylenediaminetetraacetic acid (EDTA) and tube 2 without addition, for hematological and biochemical parameters, respectively.

2.4.1 Hematological and biochemical analysis

Hematological analyses were conducted immediately after collection and performed using an automatic hematological analyzer (Coulter STKS, Beckman). Parameters included: red blood cell (RBC) count, white blood cell (WBC) count, hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count, mean platelet volume (MPV) and differential leukocyte count (lymphocytes, monocytes and granulocytes). For biochemical analysis, blood was centrifuged at 1480 × g for 10 min to obtain serum, which was stored at −20 °C. The following parameters were determined: glucose; blood urea nitrogen (BUN); creatinine; uric acid, sodium, potassium, aspartate aminotransferase (AST); alanine aminotransferase (ALT); gamma glutamyltranspeptidase (GGT), total cholesterol; triglycerides; alkaline phosphatase (ALP); total and direct bilirubin. Samples were analyzed using Architect (Abott®) automation with Boehringer Ingelheim® biochemical kits (Costa-Silva et al., 2008).

2.4.2 Morphological study

After blood collection, the animals (n = 5/group/sex) were euthanized with an excess of thiopental (140 mg/kg, i.p.) and a necropsy was performed for macroscopic external evaluation of the heart, lungs, liver, kidneys, adrenal glands, spleen, stomach, intestine, pancreas, brain and reproductive organs, testicles and prostate (male) or uterus and ovaries (female). These organs were carefully removed and weighed individually. Organ weights were expressed in absolute and relative terms (g and g/100 g of body weight, respectively).

For microscopic analysis, the remaining animals (n = 5/group/sex) were anesthetized, perfused with saline (to remove blood), then the organs described above were removed and fixed "*in totum*" in 10% buffered formalin for 48h at room temperature, after fixing each sample was washed with water and immersed in 70% ethyl alcohol for 3 to 4 days, then were embedded in paraffin. Paraffin sections of 5 μm were obtained and stained with hematoxylin/eosin (HE) [18]. Histological analyses of ogans were made using a automatic microscopy system MICRO DIP® (Kacil Inc).

2.5 Reproductive toxicity

The observation of the presence of sperm in the vaginal smear was used to define the 1st day of pregnancy. The pregnant rats were randomly divided into four groups (n = 7-10 animals/group). Control group received a 1% Tween-80 aqueous solution and groups treated received CIN at doses of 250, 500 and 1000 mg/kg, respectively. Treatment was administered during two different periods of pregnancy: pre-implantation (1st to 6th day of pregnancy) and organogenesis (7th to 14th day of pregnancy). During pregnancy, the rats were evaluated for survival, altered appearance and any clinical signs of toxicity, such as changes in food and water intake, piloerection, diarrhea, changes in locomotor activity and vaginal bleeding. Maternal weight gain was also recorded daily during pregnancy.

On the 20th day of pregnancy the rats were anesthetized with thiopental (35 mg/kg, i.p.) and sacrificed by cervical dislocation, laparotomised and their uterine horns removed. The number of implantations, resorptions and live and dead fetuses were then recorded. Ovaries (right and left) were weighed and the number of corpora lutea counted. The absolute mass of fetuses and placentae were weighed and observed for any visible abnormalities. From these data, the implantation index (total number of implantation sites/total number of corpora

lutea × 100), the resorption index (total number of resorption sites/total number of implantation sites × 100, the pre-implantation loss rate (number of corpora lutea − number of viable implantations/number of corpora lutea × 100) and post-implantation loss rate (number of implantations − number of live fetuses/number of implantations × 100) were calculated. Macroscopic features were observed to detect any abnormalities.

2.7 Statistical analysis

Values were expressed as mean ± standard error of mean (S.E.M.) and the differences are analyzed by variance analysis (ANOVA) followed by Dunnett's test or Student's T test for unpaired samples. The implantation and resorption indexes, as well as pre-implantation and post-implantation loss rates, were analyzed using the Kruskal–Wallis test followed by the Dunn test, when necessary. The level of significance for rejection of the null hypothesis was set at 5% ($p < 0.05$). Statistical analyses were performed using GraphPad Prism 5.0®.

3. Results

3.1 Acute toxicity study in mice

The effects of oral administration of 1,8-cineole (CIN) in female mice are summarized in Table 1. Oral administration of CIN (2000 mg/kg) initially produced signs of sedation, tremors, and diarrhea, intensifying over time. Furthermore, after 180 min of observation progressive difficulty breathing and seizures were observed, leading to the death of all animals within a period of less than 24 h. In view of these effects, the oral dose of CIN was reduced to 1750 mg/kg, but, even with this lower dose, the effects were similar to those obtained with the higher dose, culminating in the death of animals within less than 24 h. The dose of CIN was again reduced to 1500 mg/kg. At this dose sedation and tremors were detected in the animals in the initial stages of observation, but there were no deaths within a 24 h period or during the 14 days of observation. It is thus estimated that 1500 mg/kg < LD_{50} < 1750 mg/kg. Furthermore, a significant increase in the consumption of food (10.14 ± 0.13 g vs. 4.09 ± 0.07 g) and water (12.50 ± 0.33 mL vs. 7.88 ± 0.34 mL), as well as body weight (37.09 ± 0.45 g vs. 34.16 ± 0.17 g) was noted when compared to the control group, at the end of the 14 days of observation.

3.2 Repeated oral dose toxicity

No signs of toxicity (such as piloerection or alteration in locomotor activity) or deaths were recorded during the 50 consecutive days of treatment by oral route with CIN at doses of 100, 500 and 1000 mg/kg. However, animals treated with CIN at doses of 500 and 1000 mg/kg presented diarrhea during the first week of treatment although this ceased from the second week onwards. Body weight gain slowed in males (CIN 500 and 1000 mg/kg) and females (CIN 1000 mg/kg) only in the first week of treatment (Fig. 1). Changes were observed in the consumption of water and food during the entire treatment in animals treated with all doses of CIN (Fig. 2).

3.2.1 Hematological and biochemical parameters

The hematology profiles for treated and control groups are shown in Tables 2 and 3. For male rats, there was a significant increase of 6.9% in MCV (CIN 1000 mg/kg) and of 43.5 and 38.9% in the platelet count (CIN 500 and 1000 mg/kg, respectively) and a decrease of 6.7% in MCHC (CIN 500 and 1000 mg/kg) and MPV of 10.6 and 15.7% (CIN 500 and 1000 mg/kg, respectively), when compared to the control group. In the female groups, no statistically significant clinical findings were recorded for any of the parameters examined.

With regard to the biochemical parameters, no significant differences were observed in the serum levels of glucose, creatinine, uric acid, sodium, potassium, AST, ALT, total cholesterol, triglycerides, gamma glutamyltranspeptidase (GGT), total bilirubin, or direct and indirect bilirubin. However, a decrease was observed in the level of alkaline phosphatase (30.0%) in male rats treated with CIN 100 mg/kg and an increase of 29.0% and 25.0% in the level of urea (BUN) in the female groups treated with CIN 500 and 1000 mg/kg, respectively, in relation to the control group. The biochemical profiles are presented in Tables 4 and 5, respectively.

3.2.2 Morphological parameters

The macroscopic analysis of the target organs of the animals treated with 1,8-cineole did not show significant changes in weight, color or texture when compared with the control group, although there was a decrease in absolute weight of the lungs (15.1%) and spleen (20.2%) in males rats treated with CIN at doses of 500 and 1000 mg/kg, respectively, as well

as an increase in absolute (32.5%) and relative (39.3%) weight of the liver in females treated with CIN at a dose of 1000 mg/kg compared to control group. No changes were observed in any other organs of male and female animals in the control or treatment groups (Tables 6 and 7).

Microscopic examination of the organs showed slight eosinophilic infiltration of lymphocytes in the lungs of males and females treated with all doses of CIN (Fig. 3), a small lymphocytic infiltrate in the liver of males treated with all doses and females (CIN, 500 and 1000 mg/kg) (Fig. 4), slightly increased glomerular space in the kidneys of females (CIN, 500 and 1000 mg/kg) and males (CIN 1000 mg/kg) (Fig. 5), and moderate eosinophilic infiltration of lymphocytes into the uterus of all females treated with doses of CIN (Fig. 6). The remaining organs of male and female animals in the treatment and control groups exhibited no changes, with histological findings all within normal limits.

3.3 Reproductive toxicity

Treatment with CIN at doses of 250, 500 and 1000 mg/kg produced no deaths in pregnant rats treated during pre-implantation or organogenesis. No changes in the intake of food and water were observed during pregnancy in either period. There were no signs of toxicity, such as salivation, piloerection, diarrhea, changes in locomotor activity or changes in behavior at any of the doses administered, but there was a significant decrease in maternal weight gain during pre-implantation (1st to 6th day) and organogenesis (7th to 14th day) in females treated with all doses of CIN, and during pregnancy (1st to 20th day) in females treated with CIN (1000 mg/kg) during the pre-implantation period (Tables 8 and 9).

With regard to reproductive parameters, the ovary and placental mass, the implantation or resorption index and the loss rate of pre-and post-implantation were similar in all the experimental groups treated during the pre-implantation or organogenesis periods. However, during the pre-implantation period, dead fetuses and reduction in the mass of fetuses were observed in females treated with CIN (1000 mg/kg). During the organogenesis period, a rat treated with CIN at a dose of 1000 mg/kg during, presented vaginal bleeding on the 13th day of gestation and laparotomy did not reveal either live or dead fetuses. There was a reduction in the number of corpora lutea in females treated with CIN (250 mg/kg) during the organogenesis, when compared to the control group (Table 9).

4. Discussion

1,8-cineole is an organic constituent naturally occurring in the essential oils of many herbs and is considered the main compound of several essential oils of species of the genera *Eucalyptus, Psidium, Croton, Hyptis, Pectis, Rosamarinus* and *Salvia* (Andrade-Neto et al., 1994; Araújo et al., 2003; Hussain et al., 2011; Vilela et al., 2009) among others. Many studies have reported that cineole displays a variety of biological properties, such as antiallergic, antinociceptive and anti-inflammatory (Santos and Rao, 1998, 2000), hypotensive and muscle relaxant (Lahlou et al., 2002), hepatoprotective (Santos et al., 2001) and antitumoral activities (Moteki et al., 2002).

Despite reports of the various pharmacological properties of cineole, there are insufficient data on the safety profile of oral administration of the compound. Given that 1,8-cineole is a major constituent of many essential oils from aromatic plants used in folk medicine, we evaluated the preclinical toxicity of 1,8-cineole in acute and repeated oral doses (50 days) with regard to biochemical and hematological and morphological parameters in Wistar rats.

The acute toxicity test results show that CIN was tolerated well by mice up to a dose of 1500 mg/kg. Deaths were observed and some symptoms of toxicity in mice receiving a dose of up 1750 mg/kg. Santos and Rao (2000) reported an LD_{50} of 3.5 g/kg for mice and this finding differs from the results observed in our study, since lower doses produced signs of toxicity and death in all animals.

The administration of a repeated dose of CIN (100, 500 and 1000 mg/kg/day) in rats of both sexes did not cause any deaths or significant clinical signs of toxicity. Oscillations in the consumption of water and food were observed during the whole period of treatment in animals that received CIN at doses of 100, 500 and 1000 mg/kg. However, these changes did not prevent an increase in body weight in animals of both sexes. Though there was reduced body mass gain in animals treated with CIN at doses of 500 and 1000 mg/kg during the first week, this decrease was not affected by treatment, indicating that this response may be associated with the occurrence diarrhea in rats treated during this period. De Vincenzi et al. (2002) reported that administration of 1,8-cineole for 28 days at doses equal to or higher than 600 mg/kg lowered the increase in body weight, especially after prolonged administration and also found an LD_{50} of 2400 mg/kg for rats.

No reports were found for hematological or biochemical parameters. In the present study, with the exception of an increase in MCV and platelet count and a decrease in MCHC

and MPV in male animals treated with CIN 500 and 1000 mg/kg, no other significant alterations in the hematological parameters were found. A similar absence of toxic effects was observed for biochemical parameters: there was no effect on the levels of serum glucose, creatinine, uric acid, sodium, potassium, AST, ALT, total cholesterol, triglycerides, GGT or total bilirubin, direct or indirect; except for a decreased level of alkaline phosphatase in male rats treated with CIN 100 mg/kg and a slight increase in the level of urea in the group of females treated with CIN 500 and 1000 mg/kg in relation to the control group. However, these values lie within the physiological limits described for the species (Harkness and Wagner, 2010).

Macroscopically no changes were observed in the color or texture of organs in rats of both sexes, although there was a decrease in the absolute mass of the spleen and lungs in males (CIN 500 and 1000 mg/kg, respectively) and an increase in the absolute and relative mass of the liver in the females (CIN 1000 mg/kg). Microscopic analysis showed animals treated with all doses of CIN to have slight eosinophilic infiltration of lymphocytes in the lungs and liver of animals of both sexes, a slight increase in glomerular space in the kidneys of females (CIN 500 and 1000 mg/kg) and males (CIN 1000 mg/kg) and moderate eosinophilic infiltration of lymphocytes in the uterus of females. These results are in accordance with previous data described by Kristiansen and Madsen (1995), who reported that treatment for 28 days with 1,8-cineole at these same doses also produced an increase in liver mass in male rats. The same study reported the presence of accumulation of eosinophilic protein in the cytoplasm of the proximal tubular cells of the kidney of male rats treated with doses of 500 and 1000 mg/kg. According to De Vincenzi et al. (2002), administration of cineole at doses equal to or higher than 600 mg/kg also caused histological changes in the liver and kidneys of Fischer rats. Taken together, these results for repeated oral dose treatment (50 days) suggest that the compound brought about specific changes in the different organs examined.

With regard to reproductive toxicity, no reports were found in the literature regarding the effect of 1,8-cineole during the pre-implantation and organogenenic stages of pregnancy in rats. We thus evaluated the effect of oral administration of this compound, in order to investigate possible toxic effects capable of altering the maternal-embryonic development.

Variables such as mortality, reduced bodyweight and food or water intake, behavioral changes, piloerection and diarrhea are the tools commonly used to identify maternal toxicity (Manson and Kang, 1994). CIN at doses of 250, 500 and 1000 mg/kg produced no deaths or signs of toxicity, such as salivation, piloerection, diarrhea, changes in locomotor activity or

changes in behavior in pregnant rats treated during the pre-implantation or organogenesis periods. No alterations were observed in maternal food and water intake during the course of pregnancy in both periods, although, during the pre-implantation period, a reduction in body mass gain was observed in females concomitant with oral administration of CIN at all doses. After discontinuation of this treatment, the situation was reversed, as evidenced by the lack of difference in body mass gain during pregnancy (1st to 20th day). A similar response was observed during organogenesis.

With regard to reproductive parameters, the data showed no significant differences in the number of fetuses, placentae and ovary masses, implantation or resorption index, or pre-implantation and post-implantation losses. The rate of implantation and pre-implantation loss was evaluated using the implantation of the blastocyst in the uterus (Chang et al., 2002), while the rate of resorption and post-implantation loss established correlations between the number of blastocysts implanted and those that did not develop (Almeida and Lemonica, 2000). These parameters were similar in the groups treated with CIN and the control group, suggesting a normal capacity for reproduction and normal development of the blastocysts implanted.

Dead fetuses were observed after treatment during the pre-implantation and organogenesis periods at doses of 1000 and 500 mg/kg, and also in the control group. Since there was no reduction in the mass of fetuses in animals treated with CIN at a dose of 1000 mg/kg during the organogenesis period, it can be inferred that there is a direct relation between reduced maternal body mass gain and a reduction in the mass of rat fetuses treated with this dose during the pre-implantation period.

In conclusion, the preclinical toxicity assay for oral treatment with a repeated dose of 1,8-cineole showed occasional alterations in rats of both sexes, as evidenced by histological changes in the liver, lungs, kidneys and uterus. However, these changes showed low clinically relevant, since they occurred in a non-generalized fashion. The results also provide evidence that CIN presents maternal and fetal toxicity, and further tests are now being conducted into treatment during the fetal period and the development of offspring, in order to better characterize the toxic effects of this compound.

Conflicts of interest

There is no conflict of interest.

Acknowledgements

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Table 1. Acute oral toxicity of 1,8-cineole (CIN) in female mice.

Groups	Mice	Symptoms of toxicity
	T/D	
Control	5/0	none
CIN (1500 mg/kg)	5/0	sedation, trembling
CIN (1750 mg/kg)	5/5	sedation, breathing difficulty, diarrhea, death
CIN (2000 mg/kg)	5/5	sedation, trembling, spasms, contortions, breathing difficulty, seizures, immobility, diarrhea, death

T/D: treated/dead mice

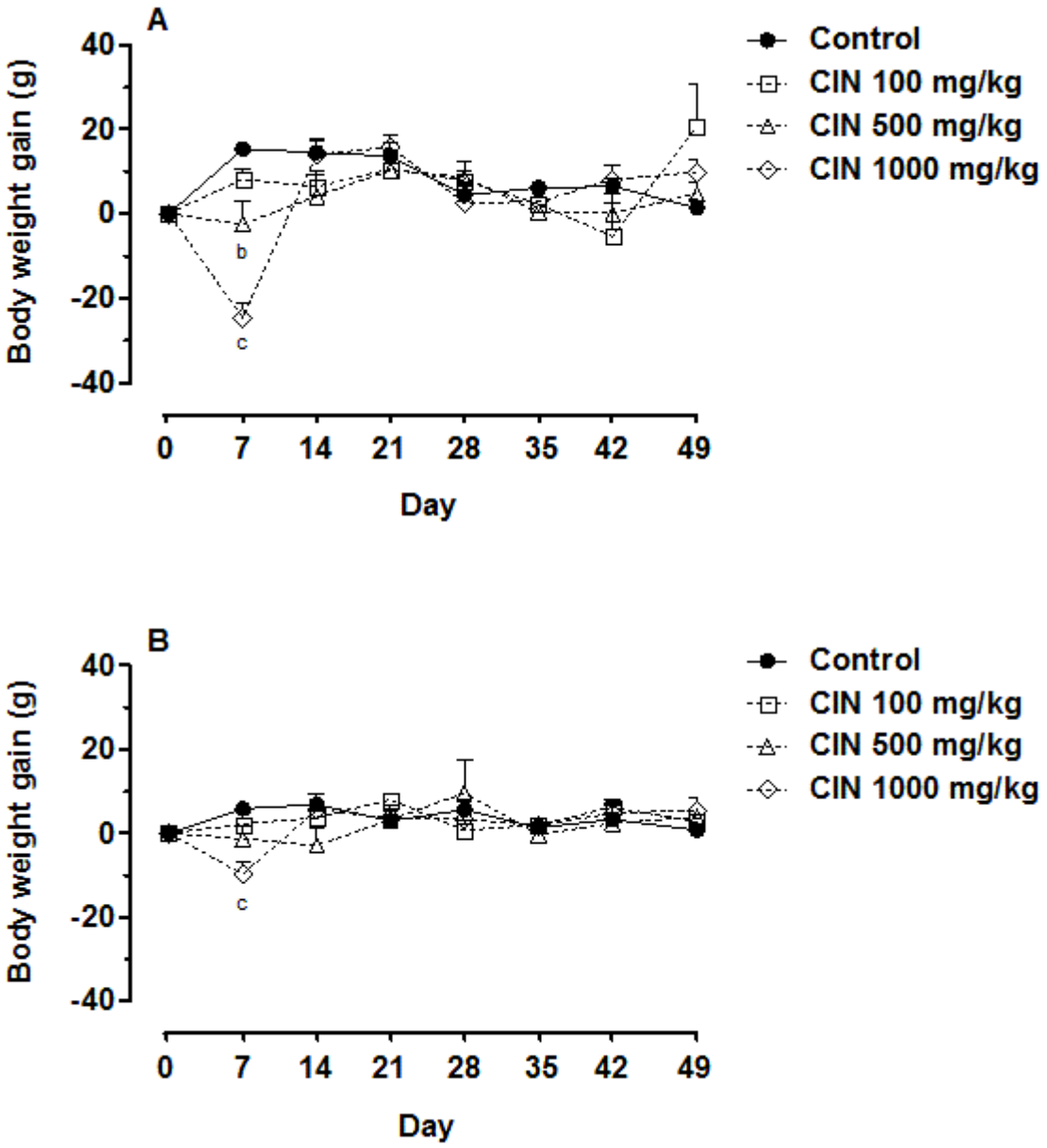

Fig. 1. Effect of 1,8-cineole (CIN) of body weight gain (g) from male (A) and female (B) Wistar rats treated orally for 50 days. Data are mean ± S.E.M. (n = 10/group) and letters represent differences in relation to the control group (a: CIN 100 mg/kg, b: CIN 500 mg/kg and c: CIN 1000 mg/kg) at the same day (ANOVA followed by Dunnett's test, $p < 0.05$).

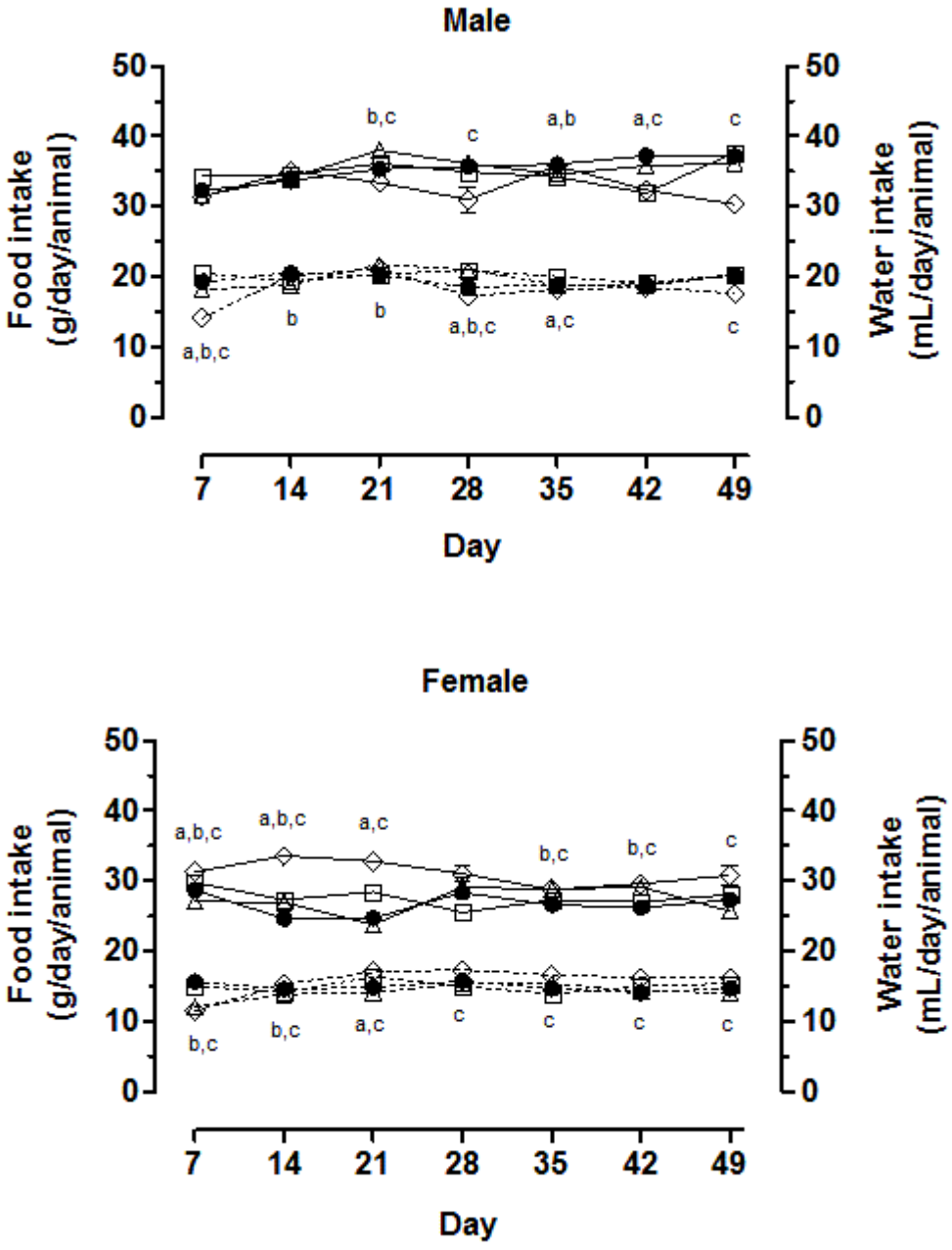

Fig. 2. Effect of 1,8-cineole (CIN) on water (solid lines) and food (dotted lines) consumption in male and female Wistar rats treated orally for 50 days. The groups are represented by symbols: ● (Control), □ (CIN 100 mg/kg), △ (CIN 500 mg/kg) and ◇ (CIN 1000 mg/kg). Data are mean ± S.E.M. (n = 10/group) and letters represent differences in relation to the control group (a: CIN 100 mg/kg, b: CIN 500 mg/kg and c: CIN 1000 mg/kg) at the same day (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 2. Effect of 1,8-cineole (CIN, 100, 500 and 1000 mg/kg) on hematological parameters in male Wistar rats treated orally for 50 days.

Parameters	**Control**	**CIN 100 mg/kg**	**CIN 500 mg/kg**	**CIN 1000 mg/kg**
Erythrocytes (10^6/µL)	5.31 ± 0.20	5.79 ± 0.31	6.33 ± 0.49	6.16 ± 0.39
Hemoglobin (g/dL)	11.30 ± 0.41	12.29 ± 0.48	12.76 ± 0.99	13.06 ± 0.67
Hematocrit (%)	27.95 ± 1.10	31.72 ± 1.80	34.04 ± 2.90	34.69 ± 2.16
MCV (fL)	52.50 ± 1.07	54.80 ± 0.20	53.71 ± 0.77	56.14 ± 0.40*
MCH (pg)	21.36 ± 0.57	21.37 ± 0.35	20.24 ± 0.79	21.26 ± 0.32
MCHC (g/dL)	40.49 ± 0.37	39.12 ± 0.74	37.76 ± 1.25*	37.77 ± 0.49*
RDW (%)	13.81 ± 0.09	13.67 ± 0.27	14.57 ± 0.24	14.56 ± 0.23
WBC (10^3/µL)	12.99 ± 0.74	11.06 ± 1.06	11.11 ± 1.72	12.69 ± 0.65
Platelets (10^3/µL)	479.50 ± 30.50	490.40 ± 28.68	688.30 ± 28.13*	666.40 ± 38.02*
MPV (fL)	7.50 ± 0.27	6.72 ± 0.08*	6.70 ± 0.17*	6.32 ± 0.06*
Lymphocytes (%)	57.32 ± 1.23	47.43 ± 6.10	61.66 ± 3.49	59.56 ± 1.57
Monocytes (%)	11.98 ± 0.40	15.53 ± 1.54	15.40 ± 3.07	13.51 ± 0.78
Granulocytes (%)	30.70 ± 0.96	37.04 ± 4.62	22.94 ± 1.83	26.93 ± 1.30

MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, WBC: white blood cell, MPV: mean platelet volume. Values represent the mean ± S.E.M (n = 10/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 3. Effect of 1,8-cineole (CIN, 100, 500 and 1000 mg/kg) on hematological parameters in female Wistar rats treated orally for 50 days.

Parameters	**Control**	**CIN 100 mg/kg**	**CIN 500 mg/kg**	**CIN 1000 mg/kg**
Erythrocytes (10^6/µL)	5.63 ± 0.33	5.14 ± 0.13	5.61 ± 0.32	5.89 ± 0.42
Hemoglobin (g/dL)	12.39 ± 0.59	11.88 ± 0.22	12.39 ± 0.46	13.20 ± 0.78
Hematocrit (%)	31.78 ± 2.16	28.91 ± 0.77	31.57 ± 1.72	33.62 ± 2.54
MCV (fL)	56.00 ± 0.68	56.33 ± 0.33	56.11 ± 0.58	56.90 ± 0.56
MCH (pg)	22.12 ± 0.37	23.13 ± 0.28	22.26 ± 0.40	22.56 ± 0.35
MCHC (g/dL)	39.42 ± 0.75	41.13 ± 0.46	39.50 ± 0.69	39.69 ± 0.76
RDW (%)	13.84 ± 0.22	13.77 ± 0.10	14.04 ± 0.21	13.73 ± 0.11
WBC (10^3/µL)	9.93 ± 0.65	8.73 ± 0.96	12.28 ± 1.05	8.81 ± 0.49
Platelets (10^3/µL)	540.00 ± 30.42	472.60 ± 18.24	609.00 ± 43.58	623.10 ± 29.29
MPV (fL)	6.32 ± 0.13	6.35 ± 0.07	6.56 ± 0.26	6.22 ± 0.06
Lymphocytes (%)	67.23 ± 2.16	65.70 ± 1.53	63.91 ± 1.66	63.26 ± 1.77
Monocytes (%)	10.59 ± 0.73	10.73 ± 0.49	11.70 ± 0.83	11.96 ± 1.12
Granulocytes (%)	22.18 ± 1.67	23.57 ± 1.16	24.39 ± 1.00	24.78 ± 0.90

MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, WBC: white blood cell, MPV: mean platelet volume. Values represent the mean ± S.E.M (n = 10/group).

Table 4. Effect of 1,8-cineole (CIN, 100, 500 and 1000 mg/kg) on biochemical parameters in male Wistar rats treated orally for 50 days.

Parameters	**Control**	**CIN 100 mg/kg**	**CIN 500 mg/kg**	**CIN 1000 mg/kg**
Glucose (mg/dL)	106.80 ± 12.19	125.20 ± 6.44	118.00 ± 5.53	116.60 ± 8.75
BUN (mg/dL)	34.20 ± 2.32	31.20 ± 1.21	30.29 ± 2.09	31.86 ± 1.87
Creatinine (mg/dL)	0.76 ± 0.10	0.58 ± 0.06	0.64 ± 0.03	0.70 ± 0.06
Uric acid (mg/dL)	2.17 ± 0.12	1.87 ± 0.15	1.61 ± 0.16	1.86 ± 0.27
Sodium (mEq/L)	139.30 ± 1.45	146.10 ± 5.05	140.90 ± 0.62	141.20 ± 0.45
Potassium (mEq/L)	4.28 ± 0.12	4.34 ± 0.07	4.61 ± 0.10	4.56 ± 0.13
AST (U/L)	101.40 ± 5.51	101.80 ± 5.62	77.14 ± 11.99	77.57 ± 8.32
ALT (U/L)	57.40 ± 3.48	63.40 ± 3.17	53.86 ± 1.95	60.68 ± 2.64
Total cholesterol (mg/dL)	82.40 ± 10.81	83.90 ± 5.28	87.29 ± 4.99	102.90 ± 5.60
Triglycerides (mg/dL)	73.90 ± 7.11	66.60 ± 8.20	51.71 ± 3.95	51.43 ± 4.08
GGT (U/L)	6.16 ± 0.75	8.55 ± 1.45	7.14 ± 0.96	7.57 ± 1.23
Alkaline phosphatase (U/L)	204.00 ± 19.41	142.70 ± 12.72*	189.30 ± 23.78	230.40 ± 20.33
Total bilirrubin (mg/dL)	0.43 ± 0.04	0.42 ± 0.07	0.46 ± 0.05	0.44 ± 0.08
Direct bilirrubin (mg/dL)	0.22 ± 0.03	0.18 ± 0.03	0.18 ± 0.03	0.22 ± 0.05
Indirect bilirrubin (mg/dL)	0.21 ± 0.03	0.24 ± 0.04	0.27 ± 0.04	0.24 ± 0.03

BUN: blood urea nitrogen, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma-glytamyl transpeptidase. Values represent the mean ± S.E.M (n = 10/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 5. Effect of 1,8-cineole (CIN, 100, 500 and 1000 mg/kg) on biochemical parameters in female Wistar rats treated orally for 50 days.

Parameters	**Control**	**CIN 100 mg/kg**	**CIN 500 mg/kg**	**CIN 1000 mg/kg**
Glucose (mg/dL)	112.60 ± 6.71	101.00 ± 5.03	94.11 ± 6.05	94.40 ± 8.11
BUN (mg/dL)	27.33 ± 1.25	29.00 ± 2.03	35.33 ± 1.98*	34.40 ± 1.33*
Creatinine (mg/dL)	0.73 ± 0.40	0.84 ± 0.09	0.80 ± 0.08	0.70 ± 0.04
Uric acid (mg/dL)	2.11 ± 0.32	1.46 ± 0.22	1.59 ± 0.25	1.90 ± 0.25
Sodium (mEq/L)	140.20 ± 0.51	140.70 ± 0.35	139.40 ± 0.36	139.00 ± 0.73
Potassium (mEq/L)	4.33 ± 0.11	4.24 ± 0.08	4.27 ± 0.08	4.56 ± 0.11
AST (U/L)	118.90 ± 5.57	128.00 ± 8.36	134.30 ± 9.78	122.20 ± 3.91
ALT (U/L)	52.89 ± 4.86	50.11 ± 4.49	49.78 ± 5.49	52.00 ± 2.25
Total cholesterol (mg/dL)	98.11 ± 4.32	99.22 ± 5.25	104.20 ± 6.65	119.00 ± 7.29
Triglycerides (mg/dL)	69.89 ± 6.33	72.78 ± 6.80	81.33 ± 4.52	85.40 ± 7.82
GGT (U/L)	13.94 ± 4.48	10.56 ± 2.53	6.28 ± 0.96	8.80 ± 1.05
Alkaline phosphatase (U/L)	201.40 ± 16.86	178.20 ± 23.99	188.60 ± 19.96	212.30 ± 15.03
Total bilirrubin (mg/dL)	0.37 ± 0.02	0.32 ± 0.03	0.38 ± 0.03	0.39 ± 0.05
Direct bilirrubin (mg/dL)	0.16 ± 0.02	0.16 ± 0.02	0.20 ± 0.04	0.22 ± 0.04
Indirect bilirrubin (mg/dL)	0.20 ± 0.03	0.15 ± 0.03	0.18 ± 0.03	0.17 ± 0.04

BUN: blood urea nitrogen, AST: aspartate aminotransferase, ALT: alanine aminotransferase, GGT: gamma-glytamyl transpeptidase. Values represent the mean ± S.E.M (n = 10/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 6. Effect of 1,8-cineole (CIN, 100, 500 and 1000 mg/kg) by oral route on absolute (g) and relative organ weight (g/100g of body weight animal) in male Wistar rats treated orally for 50 days.

Organs	**Control**	**CIN 100 mg/kg**	**CIN 500 mg/kg**	**CIN 1000 mg/kg**
Heart (g)	1.24 ± 0.03	1.30 ± 0.05	1.22 ± 0.01	1.23 ± 0.18
(g/100g)	0.34 ± 0.02	0.38 ± 0.01	0.42 ± 0.04	0.39 ± 0.06
Lung (g)	1.72 ± 0.04	1.56 ± 0.04	1.46 ± 0.05*	1.66 ± 0.10
(g/100g)	0.47 ± 0.01	0.45 ± 0.01	0.50 ± 0.05	0.52 ± 0.03
Liver (g)	10.51 ± 0.73	10.65 ± 0.37	10.20 ± 0.64	11.35 ± 0.53
(g/100g)	2.86 ± 0.13	3.09 ± 0.11	3.54 ± 0.05	3.56 ± 0.06
Kidney (g)	1.32 ± 0.05	1.23 ± 0.07	1.26 ± 0.05	1.16 ± 0.06
(g/100g)	0.36 ± 0.10	0.35 ± 0.01	0.43 ± 0.05	0.36 ± 0.03
Adrenal (g)	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00
(g/100g)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Spleen (g)	0.79 ± 0.01	0.74 ± 0.03	0.72 ± 0.04	0.63 ± 0.05*
(g/100g)	0.21 ± 0.00	0.21 ± 0.01	0.24 ± 0.02	0.20 ± 0.02
Stomach (g)	1.83 ± 0.07	1.63 ± 0.05	1.65 ± 0.10	1.56 ± 0.13
(g/100g)	0.49 ± 0.03	0.47 ± 0.02	0.57 ± 0.08	0.49 ± 0.03
Intestine (g)	0.55 ± 0.03	0.48 ± 0.04	0.62 ± 0.07	0.60 ± 0.04
(g/100g)	0.15 ± 0.00	0.14 ± 0.01	0.22 ± 0.03	0.15 ± 0.04
Pancreas (g)	1.03 ± 0.08	1.25 ± 0.09	1.29 ± 0.24	0.75 ± 0.15
(g/100g)	0.28 ± 0.03	0.36 ± 0.02	0.43 ± 0.08	0.24 ± 0.05
Brain (g)	1.81 ± 0.08	1.77 ± 0.05	1.88 ± 0.06	1.80 ± 0.20
(g/100g)	0.49 ± 0.02	0.51 ± 0.00	0.65 ± 0.08	0.56 ± 0.06
Testicle (g)	1.51 ± 0.06	1.49 ± 0.07	1.50 ± 0.03	1.49 ± 0.06
(g/100g)	0.41 ± 0.01	0.43 ± 0.00	0.52 ± 0.05	0.47 ± 0.02
Prostate (g)	0.44 ± 0.05	0.63 ± 0.05	0.52 ± 0.03	0.41 ±0.06
(g/100g)	0.11± 0.01	0.18 ± 0.01	0.13 ± 0.03	0.13 ± 0.02

Values represent the mean ± S.E.M (n = 5/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 7. Effect of 1,8-cineole (CIN, 100, 500 and 1000 mg/kg) by oral route on absolute (g) and relative organ weight (g/100g of body weight animal) in female Wistar rats treated orally for 50 days.

Organs	**Control**	**CIN 100 mg/kg**	**CIN 500 mg/kg**	**CIN 1000 mg/kg**
Heart (g)	0.85 ± 0.03	0.86 ± 0.11	0.82 ± 0.03	0.85 ± 0.02
(g/100g)	0.34 ± 0.00	0.36 ± 0.04	0.32 ± 0.04	0.36 ± 0.01
Lung (g)	1.24 ± 0.06	1.32 ± 0.06	1.34 ± 0.14	1.36 ± 0.15
(g/100g)	0.49 ± 0.02	0.56 ± 0.03	0.52 ± 0.09	0.57 ± 0.07
Liver (g)	6.82 ± 0.50	7.22 ± 0.12	7.70 ± 0.46	9.04 ± 0.19*
(g/100g)	2.72 ± 0.14	3.08 ± 0.07	2.97 ± 0.36	3.79 ± 0.14*
Kidney (g)	0.81 ± 0.05	0.81 ± 0.03	0.77 ± 0.01	0.84 ± 0.02
(g/100g)	0.32 ± 0.01	0.34 ± 0.01	0.29 ± 0.03	0.35 ± 0.02
Adrenal (g)	0.02 ± 0.00	0.08 ± 0.04	0.04 ± 0.00	0.05 ± 0.00
(g/100g)	0.01 ± 0.00	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
Spleen (g)	0.49 ± 0.03	0.53 ± 0.02	0.54 ± 0.04	0.54 ± 0.04
(g/100g)	0.19 ± 0.01	0.18 ± 0.04	0.21 ± 0.03	0.22 ± 0.07
Stomach (g)	1.37 ± 0.02	1.33 ± 0.03	1.38 ± 0.06	1.42 ± 0.05
(g/100g)	0.55 ± 0.01	0.57 ± 0.01	0.53 ± 0.06	0.60 ± 0.04
Intestine (g)	0.50 ± 0.02	0.42 ± 0.02	0.52 ± 0.02	0.44 ± 0.04
(g/100g)	0.20 ± 0.01	0.18 ± 0.01	0.20 ± 0.02	0.18 ± 0.01
Pancreas (g)	0.63 ± 0.08	0.68 ± 0.08	0.74 ± 0.04	0.76 ± 0.06
(g/100g)	0.25 ± 0.03	0.29 ± 0.04	0.29 ± 0.03	0.32 ± 0.06
Brain (g)	1.79 ± 0.02	1.80 ± 0.03	1.73 ± 0.06	1.79 ± 0.04
(g/100g)	0.72 ± 0.01	0.77 ± 0.02	0.67 ± 0.07	0.75 ± 0.02
Uterus (g)	0.45 ± 0.03	0.38 ± 0.02	0.49 ± 0.05	0.48 ± 0.08
(g/100g)	0.18 ± 0.01	0.16 ± 0.01	0.19 ± 0.03	0.20 ± 0.04
Ovary (g)	0.08 ± 0.01	0.06 ± 0.00	0.07 ± 0.00	0.06 ± 0.01
(g/100g)	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00

Values represent the mean ± S.E.M (n = 5/group). *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

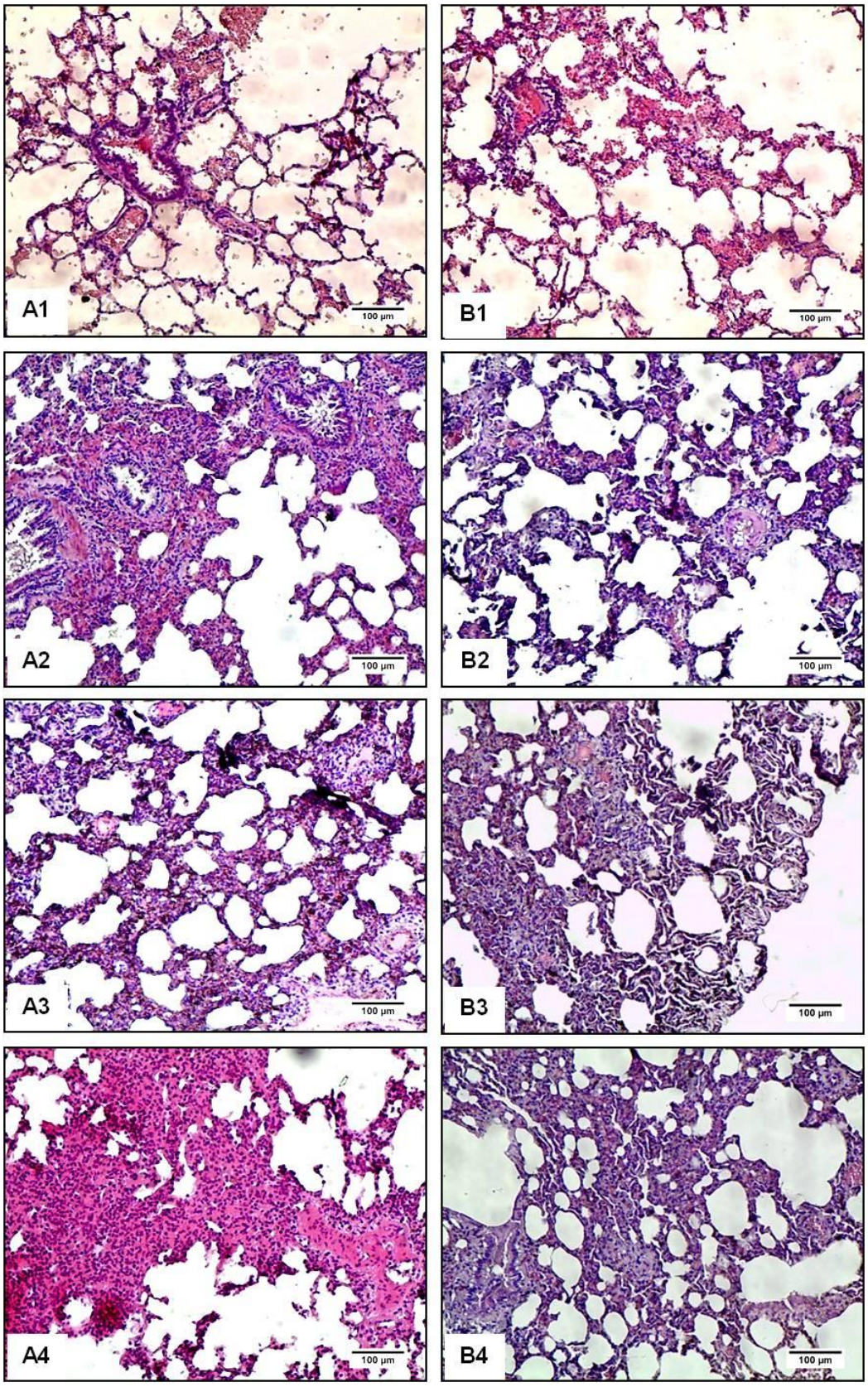

Fig. 3. Paraffin sections of lung (HE, magnification 100x) male (A) and female (B) Wistar rats treated orally for 50 days with 1% Tween-80 aqueous solution (Control, A1 and B1), CIN 100 mg/kg (A2, B2), 500 mg/kg (A3, B3) and 1000 mg/kg (A4, B4).

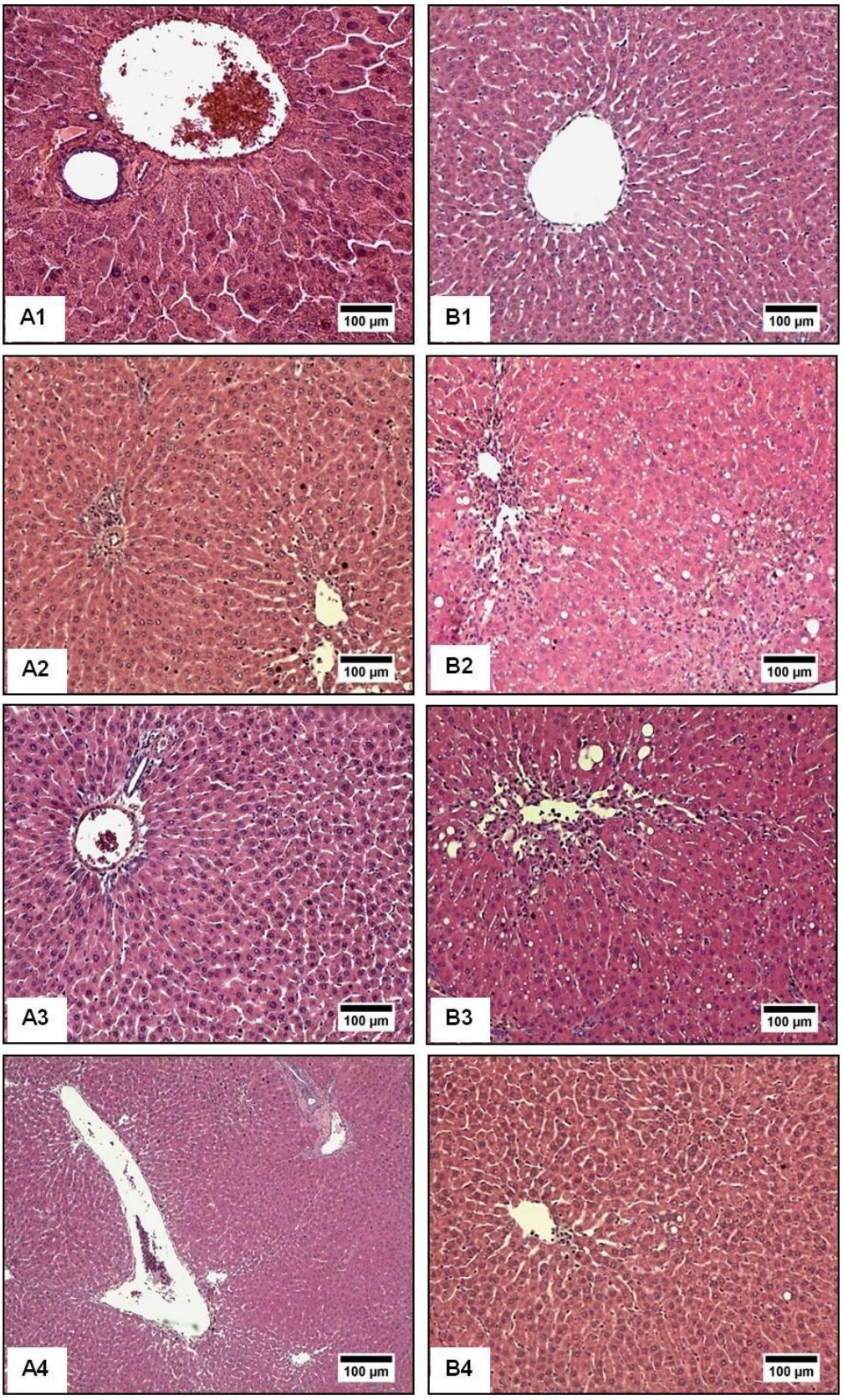

Fig. 4. Paraffin sections of liver (HE, magnification 100x) male (A) and female (B) Wistar rats treated orally for 50 days with 1% Tween-80 aqueous solution (Control, A1 and B1), CIN 100 mg/kg (A2, B2), 500 mg/kg (A3, B3) and 1000 mg/kg (A4, B4).

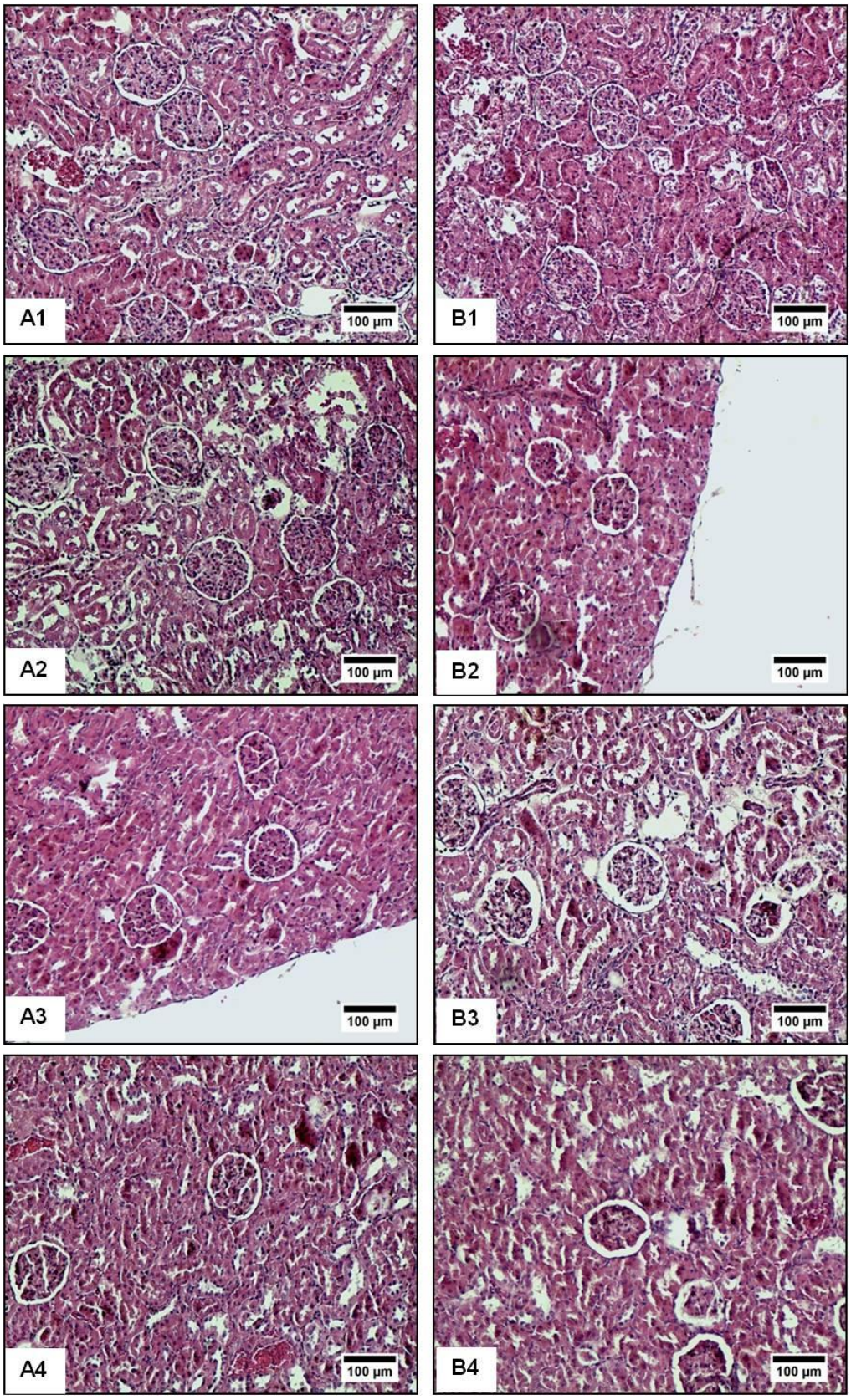

Fig. 5. Paraffin sections of kidney (HE, magnification 100x) male (A) and female (B) Wistar rats treated orally for 50 days with 1% Tween-80 aqueous solution (Control, A1 and B1), CIN 100 mg/kg (A2, B2), 500 mg/kg (A3, B3) and 1000 mg/kg (A4, B4).

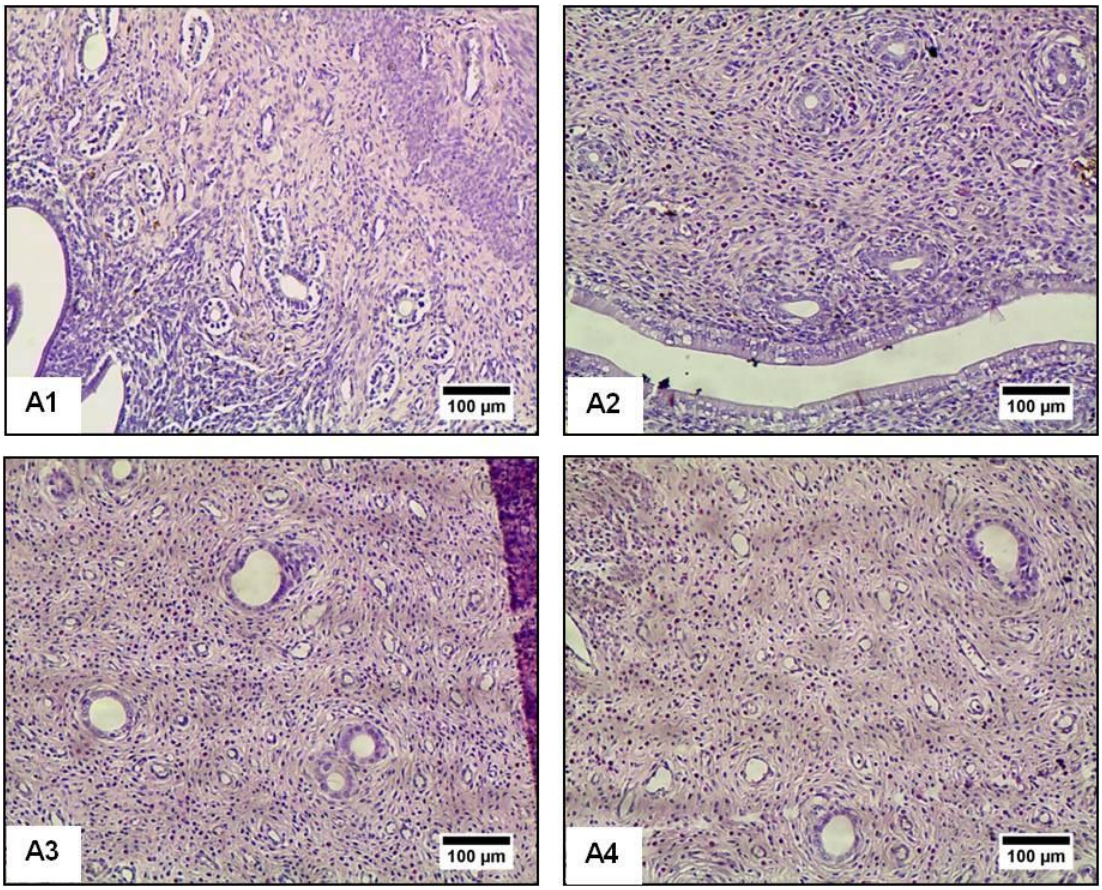

Fig. 6. Paraffin sections of uterus (HE, magnification 100x) female Wistar rats (A) treated orally for 50 days with 1% Tween-80 aqueous solution (Control, A1), CIN 100 mg/kg (A2), 500 mg/kg (A3) and 1000 mg/kg (A4).

Table 8. Reproductive parameters of female Wistar rats treated with 1,8-cineole (CIN, 250, 500 and 1000 mg/kg, p.o.) from 1st to the 6th day of pregnancy (pre-implantation period).

Reproductive parameters	**Control**	**CIN 250 mg/kg**	**CIN 500mg/kg**	**CIN 1000 mg/kg**
Pregnant rats	9	10	10	10
Mass gain in the pre-implantation period (g)[a]	15.29 ± 1.51	-1.98 ± 2.93*	0.66 ± 3.53*	-6.12 ± 3.73*
Mass gain in the pregnancy period (g)[a]	73.82 ± 7.53	73.04 ± 6.53	71.14 ± 3.64	50.12 ± 7.57*
Number of live fetuses	96	116	117	84
Number of dead fetuses	1	0	0	4
Offspring/dam relationship[a]	10.67 ± 0.88	11.60 ± 1.05	11.60 ± 0.52	8.80 ± 1.45
Fetuses mass (g)[a]	2.35 ± 0.06	2.32 ± 0.10	2.29± 0.07	1.60 ± 0.28*
Placentae mass (g)[a]	0.51 ± 0.02	0.47 ± 0.01	0.45 ± 0.01	0.42 ± 0.05
Ovary mass (mg/100g)[a]	22.87 ± 0.87	18.70 ± 1.25	22.16 ± 1.83	20.96 ± 1.63
Number of implantation sites	107	123	121	94
Number of resorption sites	10	7	4	6
Number of corpora lutea[a]	12.89 ± 0.54	12.70 ± 1.11	12.10 ± 0.43	11.80 ± 0.99
Implantation index (%)[b]	100	100	100	89.96
Resorption index (%)[b]	0	0	0	6.67
Pre-implantation loss (%)[b]	0	0	0	10.05
Post-implantation loss (%)[b]	0	0	0	3.33

Implantation index (total number of implantation sites/total number of corpora lutea × 100), resorption index (total number of resorption sites/total number of implantation sites × 100, pre-implantation loss rate (number of corpora lutea − number of viable implantations/number of corpora lutea × 100) and post-implantation loss rate (number of implantations − number of live fetuses/number of implantations × 100). The values are expressed as mean ± S.E.M.[a] or median[b]. *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

Table 9. Reproductive parameters of female Wistar rats treated with 1,8-cineole (CIN, 250, 500 and 1000 mg/kg, p.o.) from 7th to the 14th day of pregnancy (organogenic period).

Reproductive parameters	**Control**	**CIN 250 mg/kg**	**CIN 500mg/kg**	**CIN 1000 mg/kg**
Pregnant rats	8	9	8	7
Weight gain in the organogenic period (g)[a]	20.05 ± 1.60	-3.00 ± 3.22*	-1.60 ± 4.20*	0.37 ± 8.89*
Weight gain in the pregnancy period (g)[a]	74.79 ± 5.13	56.67 ± 4.72	54.91 ± 9.38	57.17 ± 10.34
Number of live fetuses	85	90	81	62
Number of dead fetuses	1	0	1	0
Offspring/dam relationship[a]	10.63 ± 0.84	10.00 ± 0.60	10.13 ± 1.10	8.85 ± 1.33
Fetuses mass (g)[a]	2.33 ± 0.01	2.42 ± 0.12	2.34 ± 0.18	2.34 ± 0.23
Placentae mass (g)[a]	0.47± 0.03	0.47 ± 0.01	0.49 ± 0.02	0.50 ± 0.03
Ovary mass (mg/100g)[a]	24.69 ± 1.87	23.45 ± 1.47	24.77 ± 2.36	25.99 ± 2.38
Number of implantation sites	91	90	80	64
Number of resorption sites	7	10	3	12
Number of corpora lutea[a]	13.75 ± 0.83	11.11 ± 0.48*	11.88 ± 0.74	12.14 ± 0.96
Implantation index (%)[b]	78.73	100	88.31	78.57
Resorption index (%)[b]	6.25	8.33	0	0
Pre-implantation loss (%)[b]	21.27	0	11.69	21.43
Post-implantation loss (%)[b]	0	8.33	0	0

Implantation index (total number of implantation sites/total number of corpora lutea × 100), resorption index (total number of resorption sites/total number of implantation sites × 100, pre-implantation loss rate (number of corpora lutea − number of viable implantations/number of corpora lutea × 100) and post-implantation loss rate (number of implantations − number of live fetuses/number of implantations × 100). The values are expressed as mean ± S.E.M.[a] or median[b]. *Statistically different from control group (ANOVA followed by Dunnett's test, $p < 0.05$).

5. Conclusão

5. CONCLUSÃO

- A análise química do óleo essencial das folhas de *Hyptis martiusii* revelou a presença do 1,8-cineol como composto majoritário, corroborando com resultados já descritos na literatura.

- Ambos, óleo essencial de *Hyptis martiusii* e 1,8-cineol, apresentaram atividade gastroprotetora em modelos experimentais de lesão gástrica aguda induzida por etanol, etanol acidificado e indometacina, demonstrando eficácia por via oral.

- Na avaliação da motilidade gastrointestinal tanto o óleo essencial de *Hyptis martiusii* e quanto o 1,8-cineol inibiram o esvaziamento gástrico, mas não exerceram qualquer influência sobre o trânsito intestinal.

- O óleo essencial de *Hyptis martiusii* diminuiu o volume gástrico secretado e a acidez, e aumentou o pH da secreção ácida basal, excercendo assim um efeito antissecretório. O mecanismo pelo qual o óleo essencial reduz a secreção ácida parece envolver a inibição dos receptores H_2 de histamina e receptores CCK_2 de gastrina da célula parietal. O 1,8-cineol reduziu o volume da secreção ácida basal, mas não interferiu em nenhum dos parâmetros na secreção ácida estimulada.

- O efeito gastroprotetor do óleo essencial de *Hyptis martiusii* e do 1,8-cineol, observado no modelo de lesão gástrica induzida por etanol, pode ser atribuído a um aumento na produção de muco, tendo como base sua interação com grupamentos sulfidrílicos.

- Ambos, óleo essencial de *Hyptis martiusii* e 1,8-cineol, não apresentaram atividade antioxidante *in vitro*, mas preveniram a depleção dos grupamentos sulfidrílicos não-proteícos e reduziram os níveis de peroxidação lipídica na mucosa gástrica de ratos, indicando uma ação antioxidante como parte de seu mecanismo gastroprotetor.

- O efeito gastroprotetor do óleo essencial de *Hyptis martiusii* e do 1,8-cineol foram confirmados pela atividade cicatrizante observada em modelo experimental de úlcera

crônica induzida pelo ácido acético, promovendo significante regeneração do epitélio da mucosa gástrica.

- As atividades comprovadas neste estudo indicam que o óleo essencial das folhas de *Hyptis martiusii* constitui um agente promissor para o tratamento de úlceras pépticas por apresentar potencial atividade gastroprotetora. Adicionalmente, demonstram o efeito gastroprotetor do monoterpeno 1,8-cineol, comprovando assim sua correlação com o efeito observado no óleo essencial de *Hyptis martiusii*.

- A administração aguda do óleo essencial de *Hyptis martiusii* não produziu efeitos tóxicos em camundongos Swiss de ambos os sexos, enquanto o 1,8-cineol só foi bem tolerado em camundongos até a dose de 1500 mg/kg. O ensaio de toxicidade pré-clínica (doses repetidas) não demonstrou sinais de toxicidade, excetuando-se pelas alterações histopatológicos observados no fígado, rim, pulmão e baço (OEHM) e fígado, pulmão, rins e útero (1,8-cineol), contudo tais alterações apresentam baixa relevância clínica, uma vez que ocorreram de modo não generalizado em animais do grupo controle e tratados.

- Os resultados obtidos no ensaio de toxicidade reprodutiva fornecem evidências de que 1,8-cineol apresenta toxicidade materna e fetal, entretanto, novos testes devem realizados, incluindo o tratamento durante o período fetal e sobre o desenvolvimento da prole, com a finalidade de melhor caracterizar os possíveis efeitos tóxicos deste composto.

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