UNIVERSIDADE ESTADUAL DE MARINGÁ

CENTRO DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

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Efeito hepatoprotetor do óleo essencial de alecrim e gengibre com pré-tratamento em modelo experimental de lesão induzida por paracetamol.

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências da Saúde.

Área de concentração: Saúde Humana

Orientador: Prof. Dr. Roberto Kenji Nakamura Cuman

Maringá

2013

Dados Internacionais de Catalogação da Publicação (CIP)

FAZER NA BIBLIOTECA UEM

Impressa no verso da segunda folha

DEDICATÓRIA

Dedico este humilde trabalho a todos aqueles que de maneira direta ou indireta colaboraram para sua finalização.

AGRADECIMENTO(S)

A todos os camundongos eutanaziados/sacrificados para a realização deste humilde trabalho.

Ao excepcional orientador, Prof. Dr. Roberto Kenji Nakamura Cuman, por seu inquestionável profissionalismo, competência, integridade moral e paciência, para o desenvolvimento e finalização deste trabalho. A quem devo inúmeras ajudas na condição de discente, para que este trabalho pudesse ser concluido. Que Deus continue a abençoar infinitamente esta pessoa, por tudo aquilo que fez e tem feito por mim.

À Prof.ª Maria Angélica R. C. P. da Silva e Profª Silvana M. C. Assef, pela gentileza de terem participado da banca de defesa do mestrado e feitas considerações importantes na conclusão desta dissertação.

Aos funcionários do Laboratório de Inflamação e que se tornaram meus queridos amigos, por todos os momentos de alegrias e conversas que tivemos: Célia Regina Miranda e Jailson Araujo Dantas (uma das pessoas mais “iluminadas” que já conheci).

À Prof.ª Dr. Renata Grespan, por todo o apoio intelectual e técnico, paciência e competência prestada a mim. Pessoa que tenho grande carinho e admiração, uma pessoa integra e dedicada em tudo àquilo que faz.

E aquele que tornou se meu grande amigo, meu irmão, Dr. Márcio Damião, pessoa que pude e ainda posso contar para todos os momentos em minha vida.

E a todos aqueles que de maneira direta ou indireta, agradável ou desagradável, fizeram parte do meu convívio no Laboratório de Inflamação.

Efeito hepatoprotetor do óleo essencial de alecrim e gengibre com pré-tratamento em modelo experimental de lesão induzida por paracetamol.

RESUMO

O objetivo deste estudo foi investigar e avaliar o efeitos hepatoprotetor e anti-oxidante do óleo essencial de gengibre (GEO) e alecrim (REO), contra os danos hepáticos em camundongos machos da linhagem Balb/c induzidos pelo modelo experimental de lesão hepática induzida por paracetamol. Os animais experimentais foram divididos em oito grupos com n=5. Cada grupo recebeu por gavagem, durante sete dias o seguinte tratamento: Grupo (1): grupo controle, não receberam tratamento. Grupo (2) receberam somente o veículo com solução salina que continha 0,1% de Tween 80, GEO ou REO. Grupos (3, 4 e 5): receberam pré-tratamento com GEO nas doses de 125, 250 e 500 mg/kg. Grupos (6, 7 e 8) receberam pré-tratamento com REO nas doses de 125, 250, e 500 mg/kg. No sétimo dia após receberem o pré-tratamento com os óleos, os animais foram colocados em jejum por um período de oito horas. Após esse período os animais do grupo segundo ao oitavo receberam por gavagem paracetamol na dose de 250 mg/kg. Depois de 12 h, os camundongos foram anestesiados com halotano, o sangue foi coletado para determinar os marcadores plasmáticos alanina (ALT) e aspartato aminotransferase (AST) no soro. Secções de fígados foram utilizados por amostras para determinar a atividade da enzima mieloperoxidase (MPO). Ensaios de peroxidação lipídica foram realizados, utilizou-se homogeneizados de gema de ovo como meio ricos em lipídios. O óleo GEO e REO foram avaliados nas concentrações de 0,5, 5,0, 50,0 e $500x10^{-3}$ mg/ml. O ácido ascórbico foi utilizado como controle positivo. Ensaios de DPPH foram feitos com ambos os óleos GEO e REO nas concentrações de 3,21 – 100 mg/ml. O ácido ascórbico foi utilizado como controle positivo. Os dados foram expressos como a média ± SEM para cada grupo . Os resultados foram analisados estatisticamente por análise de variância (ANOVA One-way) seguido pelo teste de Tukey. As diferenças foram consideradas significativas para $p < 0,05$. A lesão hepática induzida por paracetamol, elevou os níveis das enzimas AST e ALT no soro quando comparados com os animais controles normais. O pré-tratamento com doses de (500 mg/kg) de GEO e REO durante 7 dias, reduziram significativamente os níveis séricos de ALT e AST, quando comparados com os controles normais, mas não nas doses de (125 e 250 mg/kg) para ambos os óleos. A atividade da MPO, em camundongos pré-tratados

com GEO nas doses de (250 e 500 mg/kg), foi significativamente diminuída (0,046 ± 0,020 e 0,036 ± 0,022 UI/L), quando comparado com o grupo de paracetamol (0,2800 ± 0,600). Considerando-se o pré-tratamento com REO, as doses de (250 e 500 mg/kg) foram também eficazes na redução da atividade de MPO (0,051 ± 0,056 e 0,01 ± 0,02 UI/L). A RSC de GEO em concentrações de (12,5-100 mg/ml) mostrou atividade antioxidante in vitro, com IC_{50}: (32 mg/ml) . Em contrapartida, a RSC de REO mostrou esta atividade em concentrações de (3,12-100mg/ml) com IC_{50}: (40 mg/ml). Na avaliação da peroxidação lipídica GEO não mostrou nenhum resultado significativo nas concentrações testadas sobre a peroxidação não enzimática. No entanto REO na concentração de (0,5 mg/ml) mostrou 15% de inibição da peroxidação lipídica. Os dados, em conjunto, sugerem que o pré-tratamento com REO e GEO podem proteger contra os danos hepáticos promovidos por paracetamol, assim como, contra a ação dos danos causados por radicais livres.

Palavras-chave: Óleo essencial, gengibre, alecrim, hepatoproteção, antioxidante, paracetamol.

Hepatoprotective effect of pretreatment with rosemary and ginger essential oil in experimental model of acetaminophen-induced injury.

ABSTRACT

The objective of this study went to investigate and evaluate the hepatic protection and antioxidant effects of the essential oil of ginger (GEO) and rosemary (REO), against hepatic damage in male mice of BALB/c induced by the experimental of liver injury acetaminophen-induced model. The experimental animals have been divided into eight groups with n=5. Each group received by gavage for seven days following treatment: Group (1) control group received no treatment. Group (2) received only vehicle saline containing 0.1% Tween 80, REO or GEO. Groups (3, 4 and 5) received pre-treatment with GEO at doses of 125, 250 and 500 mg/kg. Groups (6, 7 and 8) received pre-treated with REO at doses of 125, 250, and 500 mg/kg. On the seventh day after receiving pre-treatment with oil, the animals were fasted for a period of eight hours. After this period animals of Group second to eighth acetaminophen received by gavage at the dose of 250 mg/kg. After 12 h, the mice were anesthetized with halothane, blood was collected to determine plasma markers alanine (ALT) and aspartate aminotransferase (AST) in serum. Sections of liver samples were used to determine the activity of the enzyme myeloperoxidase (MPO). Lipid peroxidation assays were performed in homogenates was used as egg yolk lipid rich medium. The oil GEO and REO were evaluated at concentrations of 0.5, 5.0, 50.0 and $500x10^{-3}$ mg/ml. Ascorbic acid was used as positive control. DPPH assays were made with both GEO and REO oils at concentrations from 3.21 to 100 mg/ml. Ascorbic acid was used as positive control. Data were expressed as mean ± SEM for each group. The results were statistically analyzed by analysis of variance (One-way ANOVA) followed by Tukey test. Differences were considered significant at $p < 0.05$. Liver injury induced by acetaminophen, elevated levels of AST and ALT in serum compared to normal control animals. The pretreatment with doses of (500 mg/kg) and GEO and REO for 7 days significantly reduced serum ALT and AST levels as compared to normal controls, but not in doses (125 and 250 mg/kg) for both oils. MPO activity in mice pretreated with GEO at doses (250 and 500 mg/kg) was significantly decreased (0.046 ± 0.020 and 0.036 ± 0.022 IU/L) when compared with the group of acetaminophen (0.2800 ± 0.600 IU/L). Considering the pretreatment with REO, the doses of (250 and 500 mg/kg) were also effective in reducing MPO activity

(0.051 ± 0.056 and 0.01 ± 0.02 IU/L). The RSC GEO at concentrations (12.5 to 100 mg/ml) showed antioxidant activity in vitro with IC50 (32 mg/ml). On the other hand, the RSC REO showed this activity at concentrations (3.12-100 mg/ml) and IC50 (40 mg/ml). In the evaluation of lipid peroxidation GEO showed no significant result in concentrations on the non-enzymatic peroxidation. In the evaluation of lipid peroxidation GEO showed no significant result in concentrations on the non-enzymatic peroxidation. However the concentration of REO (0.5 mg/ml) showed 15% inhibition of lipid peroxidation. The data together suggest that pretreatment with GEO REO and can protect against liver damage promoted by acetaminophen, as well as action against the damage caused by free radicals.

Keywords: Essential oil, ginger, rosemary, hepatoprotection, antioxidant, acetaminophen.

LISTA DE ILUSTRAÇÕES

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Dissertação elaborada conforme as normas da ABNT (Capítulo I) e das publicações científicas (Capítulo II): a ser submetido á Revista *African Journal of Tradicional Complementary and Alternative Medicine*, ISSN: 0189-6016, *Qualis Medicina II* (FI: 0.71) (JCR).

SUMÁRIO

CAPÍTULO I

PRODUTOS NATURAIS

A utilização de plantas com fins medicinais, para tratamento, cura e prevenção de doenças, é uma das mais antigas formas de prática medicinal da humanidade [1]. Plantas medicinais simbolizam muitas vezes o único recurso terapêutico em muitas comunidades e grupos étnicos [2]. O uso de plantas medicinais no tratamento e na cura de enfermidade é tão antigo quanto à espécie humana [3]. Ao longo do tempo têm sido registrados variados procedimentos clínicos tradicionais utilizando plantas medicinais [4]. A fácil obtenção e a grande tradição do uso de plantas medicinais, contribuem para sua utilização pelas populações dos países em desenvolvimento [1].

As supostas propriedades farmacológicas anunciadas não possuem validade científica, por não terem sido investigadas, ou por não terem tido suas ações farmacológicas comprovadas em testes científicos pré-clínicos ou clínicos [5]. A elucidação dos componentes ativos presentes nas plantas, bem como seus mecanismos de ação, vem sendo um dos maiores desafios para a química farmacêutica, farmacologia e a bioquímica [6].

Várias substâncias têm sido estudadas em protocolos experimentais e clínicos para reduzir ou prevenir a hepatotoxicidade induzida por paracetamol. Algumas plantas medicinais têm recebido considerável atenção devido as suas diversificadas propriedades farmacológicas, efeitos antioxidantes e hepatoprotetoras [7 - 8].

GENGIBRE

A espécie vegetal Zingiber officinale Roscoe, pertence à família Zingibiraceae, e é popularmente conhecida como gengibre [3]. É utilizado como especiaria e erva medicinal, sendo há milênios, empregado pela população oriental como digestivo, anti-emético, no tratamento de hemorragias e doenças respiratórias [9].

A destilação a vapor do rizoma fresco produz o óleo que contém os maiores ingredientes ativos [9]. Estes são constituídos principalmente pelos mono e sesquiterpenos: canfeno, beta-felandreno, alfa-zingibereno, cineol, terfineol, borneol, geraniol, limoneno entre outros [10].

Os gingeróis são os maiores componentes ativos, sendo o gingerol [5-hidroxi-1-(4-hidroxi-3-methoxifenil)] decan-3-ona, o mais abundante [10]. O gingerol pode ser convertido nos derivados shogalos, zingirone e paradol [11]. O 6-gingerol, possui substancial atividade antioxidante, determinada pela inibição da peroxidação fosfolipídica induzida pelo sistema $FeCl_3$-ascorbato [12, 13] Aeschabach/proj). Foi também demonstrado efeito inibitório da xantina-oxidase que é responsável pela produção de espécies reativas de oxigênio (ROS), tais como o ânion superóxido [14].

ALECRIM

O *Rosmarinus officinalis* L., pertence à família *Lamiaceae*, e é conhecida popularmente como alecrim.[15]. O uso popular das partes desta planta e suas atividades compreende desde, estimulantes, carminativa, enemagoga, as essências de qualidade superior são utilizadas como aromatizantes em produtos alimentares [5].

Nas folhas e flores, foram identificados terpenóides, saponinas, flavonoides, nicotinamida, colina, pectina, taninos, rosmaricina [15]. No óleo essencial foram identificado os seguintes constituintes: alfa-pineno, nopineno, canfeno, limoneno, cariofileno, cineol, cânfora, borneol, verbenona e outros terpenos [16].

Na literatura há poucos relatos sobre as propriedades farmacológicas do óleo essencial de *Rosmarinus officinalis* L., sabe se que é encontrado o ácido rosmarínico, o qual aumenta a produção de prostaglandina E_2 [17].

PARACETAMOL

O paracetamol é um medicamento de venda livre, de ação antipirética e analgésica, com fraca ação antinflamatória.. É considerada a principal causa de insuficiência hepática na Grã-Bretanha e EUA, seja de forma acidental ou uso abusivo, e principal causa de morte por medicamentos relatada à Academia Americana de Pediatria (AAP).(22) Encontra-se dentre os medicamentos mais consumidos no Brasil. A dose recomendada pelo Food Drug Administration (FDA) para adultos é de 325-650mg e para crianças menores que 12 anos de 10-15mg/kg/dose ambas com intervalos de 4 - 6h. A dose diária limite para adultos é 4 gramas, ao passo que para crianças é 75mg/Kg [18,19, 20].

O paracetamol (N-acetil-p-aminofenol), metabólito ativo da fenacetina, analgésico derivado do alcatrão, é um dos fármacos mais extensamente utilizados como analgésicos e antipiréticos [21]. O paracetamol apresenta rápida absorção pelo trato gastrointestinal e sua concentração plasmática atinge o pico máximo em 30 e 60 minutos. A excreção ocorre após o fármaco ser sulfatado e glicuronizado a nível hepático. Uma pequena percentagem sofre N- hidroxilação através do citocromo P-450 formando o metabólito tóxico N-acetil-p-benzoquinoneimina (NAPQI) que inicialmente é ligado a glutationa e excretado. Na administração de doses tóxicas de paracetamol, as vias de glicuronização e sulfatação se saturam e a via do citocromo P-450 adquire importância para a biotransformação da droga, ocasionando uma maior formação de NAPQI. Com isto as reservas de glutationa hepática se esgotam e a reação com os grupos sulfidrílicos das proteínas hepáticas é aumentada, interrompendo o fluxo de cálcio mitocondrial e conduzindo à necrose dos hepatócitos [22]. A cascata é amplificada pela ativação das células de Kupffer e pela produção de citocinas e radicais livres que conduzem a apoptose e necrose centrolobular na zona 3 [22, 23]. A necrose ocorre nesta zona porque é onde se localizam os hepatócitos que apresentam maior quantidade do citocromo P-450, convertendo a droga em metabólito ativo [24].

ANTIOXIDANTES

Muitos agentes antioxidantes têm sido citados em estudos experimentais e clínicos para reduzir ou evitar a hepatotoxicidade induzida por paracetamol [25, 26]. Os antioxidantes mais populares de hepatotoxicidade por paracetamol, é a N-acetil-L-cisteína (NAC) [26, 22, 24]. Protecção por NAC pode ser atribuível a sua habilidade de regenerar grupos GSH devido à sua capacidade de fornecer resíduos de cisteínas [14, 22, 24]. Os óleos de *Zingiber officinalis Roscoe* e *Rosmarinus officinalis* L. apresentam atividades antioxidantes, que podem ajudar a reverter danos como no caso das lesões causada por infusão de altas doses de paracetamol, porém não se tem muitos estudos sobre esse efeito hepatoprotetor [22, 25]. Deste modo, neste estudo investigou se essa possível capacidade dos óleos, visto que não há dados suficientes na literatura demonstrando a sua atividade hepatoprotetora.

FÍGADO

Muitos medicamentos empregados na prática clínica podem induzir ou progredir a lesão hepática, levando a limitação e aos benefícios esperados para a terapêutica.

O fígado é o principal órgão de biotransformação de fármacos e outras substâncias, sendo frequentemente associados a efeitos adversos de drogas, devido às ações diretas agudas e crônicas sobre este órgão [27]. As hepatopatias podem ser caracterizadas por lesão e necrose celular, resposta imunológica e regeneração nodular que comprometem a estrutura hepática e a capacidade funcional dos hepatócitos. Nas hepatopatias, há uma elevação dos níveis séricos de marcadores bioquímicos tais como: ALT, AST, ALP, Gama-GT, e na atividade da MPO e do NO [28].

HEPATOTOXICIDADE

A insuficiência hepática está relacionada com o metabolismo do paracetamol pela via citocromo P-450. Sabe-se que a glutationa é a responsável pela neutralização do potencial tóxico do paracetamol [29]. Como as crianças normalmente apresentam altos estoques de glutationa esperaria-se que estas fossem menos vulneráveis ao paracetamol; entretanto, estados de febre prolongada, diarréia, vômitos, ou subnutrição baixam os níveis de glutationa o que torna as crianças tão suscetíveis quanto os adultos [30]. O paracetamol pode causar danos hepáticos severos além de icterícia. A insuficiência hepática está relacionada com o metabolismo do paracetamol pela via citocromo P-450 [20]. Sabe-se que a glutationa é a responsável pela neutralização do potencial tóxico do paracetamol [18]. Como as crianças normalmente apresentam altos estoques de glutationa esperaria-se que estas fossem menos vulneráveis ao paracetamol; entretanto, estados de febre prolongada, diarréia, vômitos, ou subnutrição baixam os níveis de glutationa o que torna as crianças tão suscetíveis quanto os adultos [31]. A hepatotoxicidade, que pode levar à falência hepática fulminante, constitui-se uma manifestação tardia de difícil tratamento para recuperação do fígado e é observada principalmente na interação entre o paracetamol e o uso de álcool. A indução enzimática mediada pelo álcool da citocromo P-450 hepática em combinação com a depleção de glutationa é particularmente importante neste caso. O consumo crônico ao nível de 3 ou mais drinques/dia eleva o risco, devido ao nível tóxico ou terapêutico elevado do paracetamol. O comprometimento irreversível desse órgão é resultado do acúmulo de

benzoquinonaimina (NAPBQI), metabólito extremamente tóxico do paracetamol às células hepáticas [32]. Uma atenção adicional deve ser dada às numerosas associações que contenham paracetamol já que o usuário/paciente geralmente não observa ou desconhece a formulação fazendo uso concomitante com o paracetamol em formulação única, aumentando ainda mais o risco hepático com sobrecarga do fármaco no organismo [33].

JUSTIFICATIVA

As doenças do fígado têm se tornado uma das principais causas de morbidade e mortalidade em todo o mundo [34]. Paracetamol em grandes doses causa lesão hepática grave que pode evoluir para insuficiência hepática, degeneração e necrose hepatocelular, também estão associados com elevados níveis de marcadores bioquímicos enzimáticos [34]. Nos últimos anos, os produtos naturais derivados das plantas têm recebido considerável atenção, devido às suas diferentes propriedades farmacológicas [35]. Com base nessas noções, a investigação do efeito hepatoprotetor e antioxidante dos óleos no referido estudo, torna-se necessário e conveniente, visto que na literatura existem poucas descrições entre o pré-tratamento com alecrim e gengibre frente ao possível efeito de hepatoprotetor por intoxicação causada por doses elevadas de paracetamol.

OBJETIVOS

GERAIS

Avaliar a possível atividade hepatoprotetora e antioxidante dos óleos essenciais de alecrim (REO) e gengibre (GEO), utilizando o modelo experimental de hepatotoxicidade induzida por paracetamol em camundongos machos da linhagem Balb/c.

ESPECÍFICOS

Induzir em modelo experimental de hepatotoxicidade induzida por paracetamol em camundongos machos da linhagem Balb/c.

Verificar as alterações dos marcadores bioquímicos funcionais (AST e ALT) na intoxicação por paracetamol.

Avaliar o efeito hepatoprtetor do pré-tratamento com o óleo essencial de alecrim e gengibre frente as alterações promovidas pela intoxicação induzida pelo paracetamol.

Analisar a migração leucocitária (MPO) na intoxicação por paracetamol.

Testar a atividade antioxidante do óleo essencial de alecrim e gengibre nos efeitos de eliminação de radical livre pelo método de DPPH.

Demonstrar a atividade antioxidante do óleo essencial de alecrim e gengibre no ensaio de peroxidação lipídica.

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CAPÍTULO II

ARTIGO I: “Hepatoprotective effect of pretreatment with rosemary and ginger essential oil in experimental model of acetaminophen-induced injury”.

Hepatoprotective effect of pretreatment with rosemary and ginger essential oil in experimental model of acetaminophen-induced injury

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ABSTRACT

Liver diseases have become one of the major causes of morbidity and mortality all over the world. This study investigated the hepatoprotective and antioxidant effect of rosemary essential oil (REO) and ginger essential oil (GEO), against paracetamol-induced liver damage. The hepatoprotective effects of REO and GEO at doses of 125, 250 and 500 mg/kg, respectively, orally for 7 days were determined by assessing serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in mice. The liver samples were then to determine myeloperoxidase (MPO) enzyme activity. *In vitro* antioxidant activity of REO and GEO were evaluated by assessing the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•)-scavenging activity and lipid peroxidation. REO and GEO reduced the levels of AST, ALT, and also MPO activity. Both essential oils also exhibited antioxidant activity, reflected by its DPPH radical-scavenging effects and in the lipid peroxidation assay. These results indicated that REO and GEO have hepatoprotective effects on acetaminophen-induced hepatic damage in mice, probably due to their antioxidant effect.

Keywords: Essential oil, ginger, rosemary, hepatoprotection, antioxidant, acetaminophen.

1. Introduction

Acetaminophen (APAP) at large doses causes serious liver injury that may develop into liver failure[1]. Hepatocellular degeneration and necrosis are also associated with elevated enzyme markers, such as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)[2]. Liver injury induced by acetaminophen in mice is a commonly used as experimental model for screening substances with potential hepatoprotective activity[3].

Many aromatic plants are considered important sources for the extraction of essential oils and have many applications in ethno-medicine[4]. Biological activity of essential oils depends on its composition, beeing natural mixtures of terpenes, mainly monoterpenes and sesquiterpenes, which it have been used increasingly in the practice of complementary therapies, such as aromatherapy[2,5].

Zingiber officinale Roscoe essential oil (GEO) has been showed anti-inflammatory, antinociceptive and immunomodulatory effects of ginger essential oil (GEO) in animal models[1,6]. Studies have been focused in various biological activities of secondary metabolites of *Rosmarinus officinalis* L essential oil (REO) such as phenolic compounds, which are powerful antioxidants, hepatoprotective, antimicrobial, antinociceptive and anti-inflammatory agents[1,6,7,8,9].

Therefore, the present study investigated the hepatoprotective effect of GEO and REO on acetaminophen-induced hepatic damage in mice, and also the anti-oxidant effects of these oils in hepatic lesion.

2. Methods

2.1 Extraction of essential oil

Fresh rizhoms of *Zingiber officinale* and the fresh leaves of *Rosmarinus officinalis*. were collected from the Profa Irenice Silva Medicinal Plant Garden in the State University of Maringá, Paraná, Brazil. The vegetal materials were identified and authenticated by botanist Maria Aparecida Sert. The voucher specimens of each vegetal were deposited in the Herbarium of the Department of Botany, State University of Maringá. The essential oil was obtained by hydrodistillation using a Clevenger-type

apparatus for 2 h. The oils were dried over sodium sulfate and stored in an amber flask at 4°C.

2.2 Animals

Male Balb/c mice, weighing 24 ± 2 g, were provided by the Central Animal House of the State University of Maringá. The animals were housed at 22 ± 2ºC under a 12/12 h light/dark cycle. Prior to the experiments, the animals were fasted overnight, with water provided *ad libitum*. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (CEAE/UEM 126/2010).

2.3 Treatment of animals

The experimental animals were divided into eight groups of five animals each. Firstly, each group received orally during seven days the following treatment: Group I, the mice did not receive any treatment. In Group II, the mice received GEO or REO vehicle (saline that contained 0.1% Tween 80). In Groups III-VIII, the mice were pretreated with GEO at doses of 125, 250, and 500 mg/kg, or REO at doses of 125, 250, and 500 mg/kg, respectively. After this time, the animals were fasted for 8 h and then received oral acetaminophen on the seventh day at a dose of 250 mg/kg in Groups II-VIII. The group I orally received saline that contained 0.1% Tween 80 (APAP vehicle). After 12 h, the mice were anesthetized with halothane, and blood was collected for the determination of serum AST and ALT, using the Analyze Gold Kit®. The livers were then used to determine myeloperoxidase (MPO) enzyme activity.

2.4 Determination of serum ALT and AST

Blood samples were collected and centrifuged at 3000 xg for 15 min at 4°C. Serum ALT and AST levels were then measured using the Analyze Gold® enzymatic test kit.

2.5 Determination of MPO activity

The livers were used to determine MPO enzyme activity in the homogenate supernatant of the liver sections, which were placed in potassium phosphate buffer that contained hexadecyltrimethylammonium bromide in a Potter homogenizer. 10 µl of the supernatant was added to each well in triplicate in a 96-well microplate. Two hundred milliliters of the buffer solution containing 16.7 mg *O*-dianisidine dihydrochloride (Sigma), 90 ml double-distilled water, 10 ml potassium phosphate buffer, and 50 µl of 1% H_2O_2 was added. The enzymatic reaction was stopped by the sodium acetate addition. Enzyme activity was determined by absorbance measured at 460 nm using a Spectra Max Plus microplate spectrophotometer.

2.6 Lipid peroxidation assay

A lipid peroxidation assay was performed as previously reported with a minor modification [7]. Egg yolk homogenates were prepared as lipid-rich media. Briefly, 0.1 ml of REO and GEO (0.5; 5.0, 50.0 and $500x10^{-3}$ mg/ml) dissolved in methanol was thoroughly mixed with 0.5 ml of egg yolk homogenate (10%, v/v, diluted with pure water) and made up to 1 ml with pure water. Ferrous sulfate (50 µl, 70 mM) was added to induce lipid peroxidation, and the mixture was incubated for 30 min at 37.5°C. Afterward, 1.5 ml of 20% acetic acid (v/v, pH 3.5, diluted with pure water) and 1.5 ml of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulfate (w/v, diluted with pure water) were added, and the resulting mixture was vortexed and heated at 95°C for 60 min. After cooling, 5 ml of 1-butanol was added to each tube and centrifuged at 5000 rotations per minute for 15 min. The organic upper layer was collected and measured spectrophotometrically at 532 nm using a Beckman DU-65 spectrophotometer. The essential oil was diluted in methanol (the solvent expressed no antioxidant activity). Ascorbic acid was used as a positive control. The inhibition of lipid peroxidation was calculated as: Inhibition (%) = $(1 - A_{sample} / A_{control}) \times 100$. $A_{control}$ was considered the absorbance of the control (i.e., methanol, instead of the sample). The IC_{50} value, representing the concentration of the essential oil that caused 50% inhibition of lipid peroxidation in the Fe^{2+}/ascorbate system, was determined by linear regression analysis from the obtained inhibition (%) values.

2.7 DPPH assay

Free radical scavenging capacity (RSC) was evaluated by measuring the 2,2-diphenyl-1-picrylhydrazil (DPPH)-scavenging activity of REO and GEO. The DPPH assay was performed as previously described [8], with minor modifications. The samples 3.12 - 100 mg/ml were mixed with 1 ml of 25 mM of DPPH• solution (Sigma, St. Louis, MO, USA), with the addition of 95% methanol to a final volume of 4 ml. The absorbance of the resulting solutions and blank (i.e., with the same chemicals, with the exception of the sample) were recorded against ascorbic acid (Chem Cruz; used as a positive control) after 30 min at room temperature. For each sample, four replicates were recorded. The disappearance of DPPH• was measured spectrophotometrically at 515 nm using a Beckman DU-65 spectrophotometer. The percentage of RSC was calculated using the following equation: RSC (%) = 100 × (A_{blank} - A_{sample} / A_{blank}). The IC_{50} value, representing the concentration of the essential oil that caused 50% RSC inhibition, was determined by linear regression analysis from the obtained RSC values.

2.8 Statistical analysis

The data are expressed as the mean ± SEM for each group. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered significant at $p < 0.05$.

3. Results

We evaluated the effects of GEO and REO on serum enzyme markers. As shown in Figure 1, the hepatic damage induced by acetaminophen, elevated the serum ALT and AST enzyme levels when compared with the normal animals. Pretreatment with 500 mg/kg GEO and REO but not 125 and 250 mg/kg for both oils during 7 days prior to acetaminophen administration markedly reduced serum ALT and AST levels when compared with vehicle-treated controls.

The activity of MPO in mice treated with GEO (250 and 500 mg/kg), was significantly decreased (0.046 ± 0.020 and 0.036 ± 0.022 IU/L) when compared with acetaminophen group (0.2800 ± 0.600) (Figure 2A). Considering the REO treatment,

doses of 250 and 500 mg/kg (Figure 2B) also were effective in reduce the MPO activity (0.051 ± 0.01 and 0.056 ± 0.02 IU/L, respectively).

In the DPPH test, the ability of GEO and REO to act as a donor for hydrogen atoms or electrons in the transformation of DPPH• to its reduced form (DPPH-H) was measured spectrophotometrically. The RSC of GEO at concentrations of 12.5-100 mg/ml showed antioxidant activity *in vitro, with* IC_{50}: 32 mg/ml ($y = 1.8889x – 10.996$; $R^2 = 0.9901$). On the other hand, REO showed this activity at concentrations of 3.12-100mg/ml with IC_{50}: 40 mg/ml ($y = 1.1456x + 4.0215$; $R^2 = 0.9932$). (Table 1)

Egg yolk lipids undergo rapid lipid peroxidation when incubated in the presence of ferrous sulfate. GEO did not showed inhibition of lipid peroxidation effect at all concentrations tested on non-enzymatic peroxidation. However REO at concentration of 0.5 mg/ml showed 15 % of inhibition in the lipid peroxidation (Table 2).

4. Discussion

In this study, it was used hepatotoxicity acetaminophen-induced to evaluate the hepatoprotective of REO and GEO, because an acetaminophen overdose (i.e., at doses that are different from analgesic doses that are safely and effectively used therapeutically) can induce severe hepatotoxicity in experimental animals and humans[9,10,11]. A single dose of acetaminophen caused severe hepatic damage. It was reflected by a marked elevation of the levels of hepatic marker enzymes (i.e., AST and ALT), and an increased MPO activity. These serum enzymes are useful quantitative markers of the extent and type of hepatocellular damage. High levels of AST indicate a loss of the functional integrity of the liver, similar to the effects seen in viral hepatitis, cardiac infraction, and muscle injury. The ALT enzyme catalyzes the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore, ALT is more specific to the liver and thus, a better parameter for detecting liver injury[12,13,14]. Furthermore, the free radical-initiated oxidation of cellular membrane lipids can lead to cellular necrosis and is now accepted to be important in various pathological conditions[15]. Acetaminophen treatment significantly elevated reactive oxygen species levels, ultimately depleting the levels of superoxide dismutase (SOD) and GSH in liver tissue, whereas oxidative stress contributes to the initiation and progression of liver damage[16]. We assessed the hepatoprotective effect of GEO and REO on acetaminophen-

induced hepatic damage in mice, and the results suggested that these essential oils likely acts to preserve the functional integrity of the cell plasma membrane of hepatocytes in the liver and protect the membrane from damage by toxic reactive metabolites produced by acetaminophen biotransformation[15,16].

Various essential oils have been shown to have antioxidant activity[17,18,19]. Therefore, we evaluated the *in vitro* antioxidant activity of GEO and REO. These natural compounds are generally considered biologically active components that could donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step, so our date suggest that the hepatoprotective effect of these essential oils could be due pontially to their antioxidant properties.

Inflammation also plays a central role during drug-induced acute hepatitis and products of arachidonic acid metabolism have been extensively involved in inflammatory processes[20]. Previous results shown by our research group confirmed the anti-inflamatory activity of GEO and REO[6,21,22]. Therefore, these data suggest that these two essential oils are partially involved in the hepatoprotective effect in this experimental model.

Thus, it has been demonstrated that extracts of plants protect the liver from acetaminophen overdose, suggesting that the hepatoprotective effect can be considered an expression of the functional improvement of hepatocytes, that results from accelerated cellular regeneration[23,24]. Furthermore, previous studies have also shown that some essential oils have properties of scavenging free radicals and antioxidants hepatoprotective activity[17]. GEO and REO could interact directly with components of the cell membrane to prevent abnormalities in the content of the lipid fraction which is responsible for maintaining normal fluidity these lipid fraction.

5. Conclusion

Our data suggest that GEO and REO pretreatment improves hepatic status in mice against acetaminophen-induced damage. The effects could involve the antioxidative effect of these essential oils, similar to that effects observed in other medicinal plants. However, further detailed studies are required to investigate the mechanism by which GEO and REO exerts its effects and determine the specific constituents that are responsible for this action.

6. Conflict of interest

The authors declare that there are no conflicts of interest.

7. Aknowledgments

This study was supported by grants from the CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior, Fundação Araucária, and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), Brazil. We thank Mr. Jailson Araujo Dantas and Mrs. Celia Regina Miranda for technical assistance.

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Figures/Table Legend

Figure 1 . Ginger Essencial Oil (GEO) and Rosemary Essencial Oil (REO) reduced the serum ALT and AST on acetaminophen-induced hepatotoxicity in mice - The animals were pretreated with GEO and REO (125, 250 and 500 mg/kg, orally) daily for 7 days. After, the mice were treated with APAP (250 mg/kg, orally) and serum parameter of ALT **(A)** and AST **(B)** of GEO and ALT **(C)** and AST **(D)** of REO were determined 12 h after APAP intoxication. The control group (C) was given vehicle of APAP. Results represent mean ± SEM of 5 mice per group. *$p<0.05$ versus control group (C), #$p<0.05$ versus APAP group.

Figure 2 . Effect of the Ginger Essential Oil (GEO) and Rosemary Essential Oil (REO) on MPO enzyme activity in liver of mice – The animals were pretreated with GEO and REO (125, 250 and 500 mg/kg, orally) daily for 7 days. After, the mice were treated with APAP (250 mg/kg, orally) and parameter of MPO (A) GEO and (B) REO was quantified 12 h after APAP intoxication. The control group (C) was given vehicle of APAP. Results represent mean of MPO activity ± SEM of 5 mice per group. *$p<0.05$ versus control group (C), #$p<0.05$ versus APAP group.

Table 1 . Assessment of DPPH free radical scavenging activity of Rosemary Essential Oil (REO) and Ginger Essential Oil (GEO).

Table 2 . Inhibition of lipid peroxidation in Fe^{2+} system of induction by the essential oils Rosemary Essential Oil (REO) and Ginger Essential Oil (GEO) measured by TBARS Assay.

Figure 1

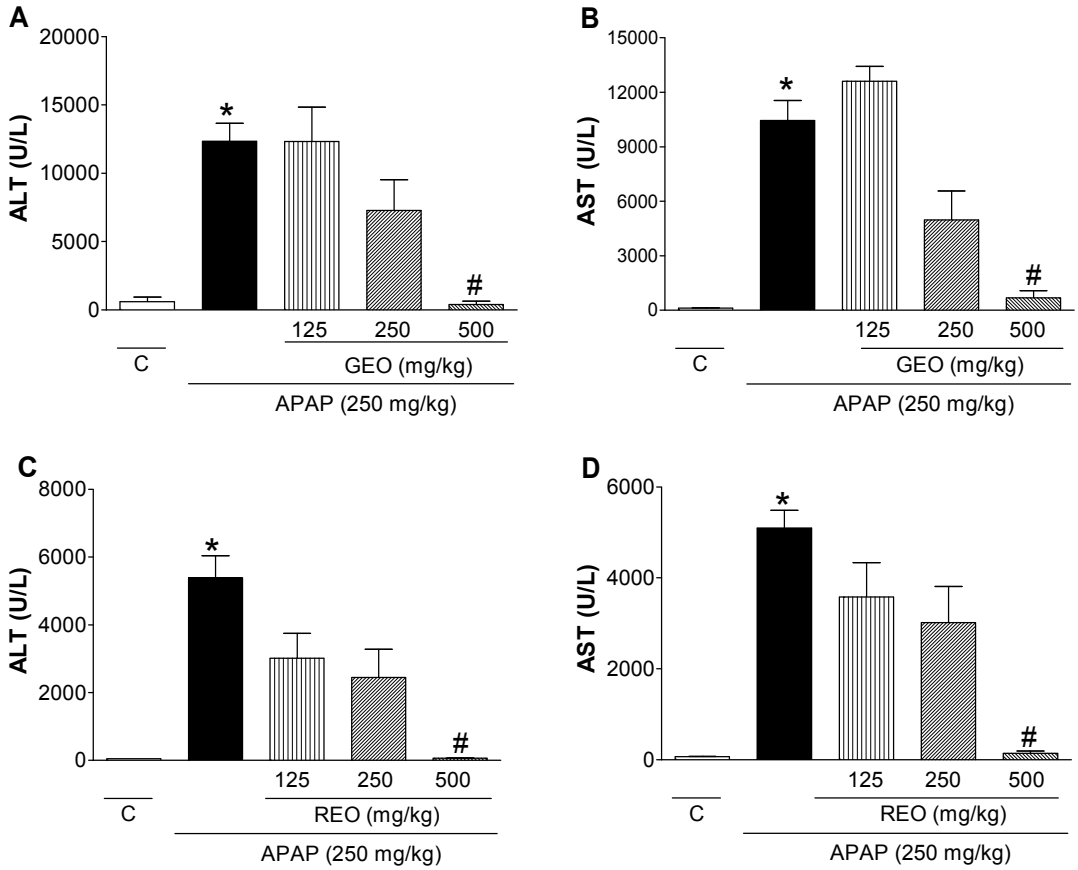
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Figure 2

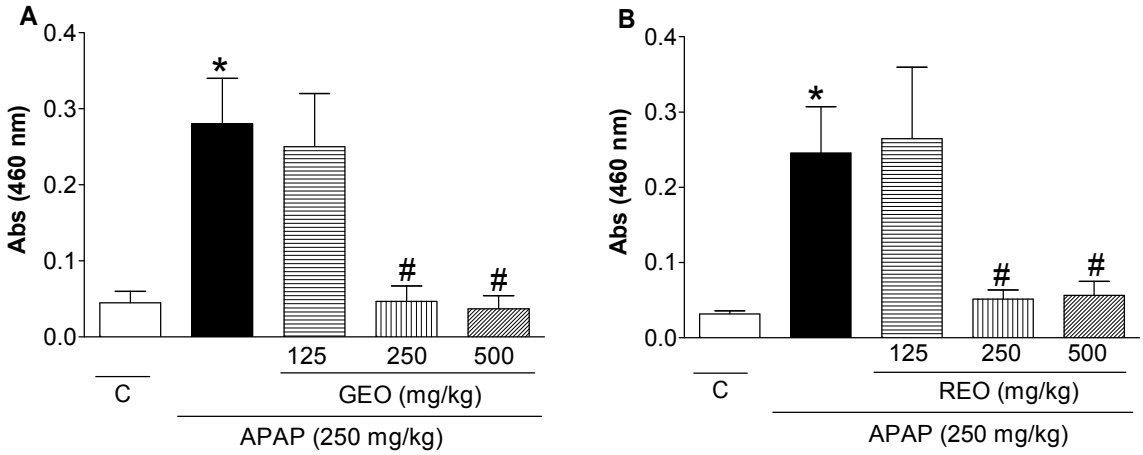
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Table 1

	Activity (%)		
Concentration (mg/ml)	REO	GEO	Ascorbic Acid
0.2	-	-	96
3.12	7	0	-
6.25	9	0	-
12.5	19	8	-
25	35	35	-
50	60	85	-

Table 2

% Inhibition of Radical Scavenging			
Concentration (mg/ml)	REO	GEO	Ascorbic Acid
$0.5x10^{-3}$	1	3	-
$5x10^{-3}$	2	3	-
$50x10^{-3}$	10	3	-
$500x10^{-3}$	15	4	-
0.2	-	-	63

CAPÍTULO III

CONCLUSÕES

Este estudo sugere a hepatoproteção pelos óleos REO e GEO no dano hepático induzido por paracetamol em camundongos, provavelmente devido ao seu efeito antioxidante.

Os óleos REO e GEO sugerem redução nos níveis de AST e ALT.

A atividade antioxidante dos óleos REO e GEO são sugeridas na análise na eliminação de radicais livres nos ensaios de DPPH e Peroxidação Lipídica.

PERSPECTIVAS FUTURAS

Embora estes resultados sugerem efeitos antioxidantes dos óleos essências REO e GEO, outras análises terão que ser realizadas para efetiva comparação dos dados apresentados, assim como estudos químicos mais detalhados para elucidar quem são os componentes responsáveis por promover esse efeito hepatoprotetor sugerido. Sendo assim, com novas análises e dados mais precisos poderemos ter a sugestão de um possível candidato a novo fármaco para doenças hepáticas causadas por excesso de formação de radicais livres.

ANEXO

Instructions For Authors

African Journal of Traditional, Complementary and Alternative medicines (AJTCAM)

Regular articles

These should describe new and carefully researched findings that have arisen from experimental procedures. The articles should be given in sufficient detail for others to verify the work. Authors should submit data that have arisen from animal and human studies in an ethically proper way by following guidelines as set by the World Health Organization. Papers that violate these principles will not be accepted.

The African Journal of Traditional, Complementary and Alternative medicines (AJTCAM) provides rapid publication of papers on ethnomedicines and veterinary ethnomedicines. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published approximately two to three months after acceptance.

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Kamanzi AK, Koné M, Terreaux C, Traore D, Hostettmann K, Dosso M. Evaluation of the antimicrobial potential of medicinal plants from the Ivory Coast. Phytother. Res., 2002; 16:497–502.

Vinegar, R.; Truax, J.F.; Selph, J.L.; Voelker, F.A. Pathway of onset, development and decay of carrageenan pleurisy in the rat. Fed. Proc. 1982, 41:2588-2595.

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AJTCAM ISSN: 0189-6016