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Efeito hepatoprotetor do óleo essencial de capim-limão (*Cymbopogon citratus)* e do seu constituinte majoritário em modelo experimental de lesão hepática induzida por paracetamol

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Tese 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 Doutor em Ciências da Saúde.

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FOLHA DE APROVAÇÃO

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RESUMO

O paracetamol (APAP) é um analgésico e antitérmico muito utilizado mundialmente e é relativamente seguro quando utilizados em doses terapêuticas. No entanto, a sobredose de APAP, pode causar toxicidade hepática. O modelo de toxicidade hepática induzida por APAP é um dos modelos mais populares para testar potenciais agentes hepatoprotetores. Os óleos essenciais são produtos voláteis do metabolismo secundário de plantas aromáticas e possuem propriedades biológicas. *Cymbopogon citratus* é uma planta aromática e medicinal pertencente à família Gramineae, originária da Índia e encontrada no Brasil. Estudos já demostram que o óleo essencial de *C. citratus* ou lemongrass (capim-limão) possui atividade antioxidantes e propriedades inflamatórias. O objetivo deste trabalho foi analisar o efeito hepatoprotetor do óleo essencial de capim-limão (OECL) e do citral (constituinte majoritário) no modelo de lesão hepática aguda induzida por APAP em camundongos. Os marcadores de função hepática alanina aminotransferase (ALT), aspartato aminotransferase (AST), fosfatase alcalina (ALP) e gama-glutamil transferase (γ-GT) foram determinados. Os fígados foram utilizados para determinar a atividade da mieloperoxidase (MPO), produção de óxido nítrico (NO) e análise histológica. A atividade antioxidante (2,2-difenil-l-picrilidrazilo (DPPH)), a viabilidade celular e o efeito do OECL e do citral na quimiotaxia foram avaliados *in vitro*. Nossos resultados demonstraram que OECL e citral possuem atividade antioxidante e reduzem a migração leucocitária. O pré-tratamento com o OECL e o citral (125, 250 e 500 mg/kg), diminuiram a intensidade nas alterações histológicas causadas pela sobredosagem por APAP, corroborando com os achados de ALT, AST, ALP, γ-GT, MPO e a produção de NO. Nossos resultados sugerem que o OECL e o citral, possuem atividade protetora contra a hepatotoxicidade induzida pelo APAP.

Palavras-chave: Óleo essencial, Capim-limão, Citral, Paracetamol, Hepatotoxidade.

Hepatoprotective effect of lemongrass (*Cymbopogon Citratus*) essential oil and its major constituent in experimental model of liver lesion induced by acetaminophen

ABSTRACT

Paracetamol (APAP) is an analgesic and antipyretic widely used worldwide and is relatively safe when used in therapeutic doses. However, APAP overdose can cause liver toxicity. The APAP-induced liver injury model is one of the most popular models for testing potential hepatoprotective agents. Essential oils are volatile products of the secondary metabolism of aromatic plants and have biological properties. *Cymbopogon citratus*, commonly known as lemongrass is an aromatic and medicinal plant belonging to the Gramineae family, originally from India and found in Brazil. Studies have already shown that lemongrass essential oils possess antioxidant activity and inflammatory properties. The aim of this study is to analyze the hepatoprotective effect of lemongrass essential oil (LGO) and its major constituent (citral) in the model of acute liver injury induced by APAP in mice. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GT) markers were determined. Livers were used to determine myeloperoxidase activity (MPO), nitric oxide (NO) production and histological analysis. The antioxidant activity (2,2-diphenyl-1-picrylhydrazyl (DPPH)), cell viability and the effect of LGO and citral on chemotaxis were evaluated in vitro. Our results demonstrate that LGO and citral have antioxidant activity and reduced leukocyte migration. Pretreatment with LGO and citral (125, 250 and 500 mg / kg) decreased the intensity of histological changes caused by APAP overdose, corroborating the findings of ALT, AST, ALP, γ-GT, MPO and NO production. Our results suggest that LGO and citral have protective activity against APAP-induced hepatotoxicity.

***Keywords*:** Essential Oil, Lemongrass, Citral; Acetaminophen, Hepatotoxicity.

LISTA DE ABREVIATURAS

$AINE_S$ – Anti-inflamatórios não-esteroidais

ALP - fosfatase alcalina

ALT – Alanina aminotransferase

APAP – N-acetil-p-aminofenol, paracetamol

AST – Aspartato transaminase

CYP450 – Citocromo P450

DMSO – Dimetilsulfóxido

DNA – Ácido desoxirribonucléico

DPPH – (2,2-difenil-1-picrilhidrazil).

EROs – Espécies reativas de oxigênio

fMLP - formil metionil leucil fenilalanina

GSH – Glutationa reduzida

IL-1β – Interleucina 1-beta

IL-6 – Interleucina 6

IL-10 – Interleucina 10

iNOS - óxido nítrico-sintase induzida

LGO – Lemongrass essential oil

LPS - lipopolissacarídeo

MPO – Mieloperoxidase

MTT – Brometo de [3-(4,5-dimetiltiazol-2-il)-2,5- difeniltetrazólio]

NAPQI – N-acetil-p-benzoquinona imina

NFκB – Fator nuclear kappa B

NO – Óxido nítrico

OECL - Óleo essencial de capim-limão

PBS – Tampão fosfato salino

PMN - polimorfonuclear

RNAm – Ácido ribonucléico mensageiro

SLM - silimarina

TNF – Fator de necrose tumoral

γ GT – gama-glutamil transferase

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CAPÍTULO I

1.1 HEPATOTOXICIDADE INDUZIDA POR PARACETAMOL

O fígado é o principal órgão de biotrasformação de fármacos e está constantemente exposto a uma variedade de xenobióticos. Nos últimos anos, os casos de lesão hepática induzida por drogas aumentou devido ao uso indiscriminado de medicamentos, principalmente aqueles com *status* de venda livre, como o acetaminofeno (paracetamol, N-acetil-p-aminofenol, APAP), conhecidos por causarem lesões e/ou necrose hepática, o que os fazem potencialmente hepatotóxicos (BLACHIER et al., 2013; JAESCHKE, XIE, McGILL, 2014; LORENZONI et al., 2014).

O APAP é uma droga cuja hepatotoxicidade está intimamente relacionada à dose ingerida (RIVEIRA et al., 2017). Em doses terapêuticas, a maior parte do APAP é metabolizado por meio das reações de conjugação e resultam em pequenas quantidades de N-acetil-p-benzoquinonaimina (NAPQI) através do CYP450, que são facilmente detoxificadas pela glutationa (GSH). No entanto, na overdose por APAP as vias de metabolização ficam saturadas, aumentando a formação de NAPQI, que em grandes quantidades irá levar ao esgotamento da GSH, resultando no acumulo de NAPQI no tecido hepático (JAESCHKE, XIE, MCGRILL, 2014).

Sendo assim, a sobredosagem por APAP desencadeia a ligação de NAPQI às proteínas mitocondriais, que também está associada à diminuição dos níveis de Adenosina Trifosfato, à inibição da respiração mitocondrial, à geração de espécies reativas de oxigênio, óxido nitrico e peroxinitrito. Além deste processo de lesão hepática, vários outros eventos sequenciais e em paralelo levam à amplificação e propagação da morte celular, resultando em dano tecidual, com a diminuição da função mitocondrial e a indução do estresse oxidativo por APAP (GHANEM et al., 2016). Associado a este processo de lesão hepática, há uma reação inflamatória intensa, com ativação das células de Kupffer, produção de citocinas, tais como: fator de necrose tumoral, interleucina (IL) -1β, IL- 6; radicais livres, dentre outros, aumentando o dano hepático. Desta forma, ocorre perda de constituintes intracelulares e, consequentemente, uma necrose local grave (MICHEL et al., 2001; KIM et al., 2013).

As alterações estruturais, a degeneração e a necrose tecidual no fígado, podem ser acompanhados com aumento do nível séricos hepáticos, alanina e aspartato aminotransferase (ALT e AST), fosfatase alcalina (ALP), gama-glutamil transferase (γ-GT), e na atividade da mieloperoxidase (MPO) e do óxido nutrico (NO) (FREITAG et al., 2012; YOON et al., 2016). Por isso, é importante a avaliação destes biomarcadores hepáticos, pois servem como indicadores de lesão hepática (SINGH et al. 2011).

1.2 PARACETAMOL

Em 1955, APAP foi introduzido em diversos países, inclusive nos Estados Unidos, com o nome de Tylenol ® pelos laboratórios McNeil, e no Reino Unido com o nome comercial de Panadol produzidos pela Frederick Stearns & Co em 1956. Desde então APAP ganhou popularidade, sendo um medicamento mundialmente utilizado como analgésico e antipirético, de comercialização livre (JÓZWIAK-BEBENISTA, NOWAK, 2014).

O APAP é um fármaco pertencente à classe dos AINEs (analgésicos, antitérmicos e anti-inflamatórios não hormonais), que em doses terapêuticas, é considerado seguro e eficaz, mas a overdose acidental ou intencional pelo APAP pode causar lesão hepática aguda grave em seres humanos e animais (BLACHIER et al., 2013; JAESCHKE, XIE, MCGRILL, 2014).

A dose terapêutica convencional varia de 325 mg a 1g em adultos, não ultrapassando 4 g ao dia. Já em crianças, indica-se administrar uma dose de 10 mg/kg, não utilizando mais que 5 doses em 24 horas (BRUNTON, LAZO, PARKER, 2010). Nos camundongos a dose única ≥ 200 mg/kg em jejum induz a toxicidade hepática entre 6 a 24 horas (JAESCHKE et al., 2013).

De acordo com a Associação Americana para o Estudo das Doenças do Fígado, a incidência de toxicidade hepática relacionada por APAP tem aumentado significativamente nas últimas décadas (GHANEM, 2016). A hepatotoxicidade por APAP contribui para cerca de 70.000 internações por ano nos Estados Unidos e é responsável por 46% de todos os casos de insulficiencia hepática aguda, tornando-se um importante problema de saúde pública (RAMACHANDRAN, JAESCHKE, 2017).

Diante disto, identificar fármaco ou drogas alternativas eficazes para prevenir e/ou tratar a lesão hepática induzida por APAP se faz necesssário (BLACHIER et al., 2013; MADRIGAL-SANDRILA, 2014).

1.3 PRODUTOS NATURAIS

O uso de plantas medicinais na prevenção e tratamento de doenças é utilizado desde a antiguidade devido suas propriedades terapêuticas. Aproximadamente 80% da população mundial utiliza a medicina tradicional para os cuidados de saúde, sendo as drogas originadas a partir de produtos vegetais. Várias investigações científicas de plantas medicinais indicaram que as propriedades responsáveis por seus efeitos benéficos podem ser atribuídos à presença de substâncias biologicamente ativas (MIGUEL, 2010; MACHADO, JUNIOR, 2011; AKBARZADEH et al., 2015).

Dentre as plantas medicinais, existem as aromáticas que por metabolismo secundário, origina grupos de compostos de grande importância econômica devido seu crescente uso nas indústrias farmacêutica, alimentícia e cosmética, tais como os óleos essenciais (OEs) (MACHADO, JUNIOR, 2011; PEREIRA, CARDOSO, 2012).

Os OEs são misturas complexas de compostos bioativos e a grande maioria dos componentes identificados incluem monoterpenos, sesquiterpenos e fenilpropanóides. Geralmente os OEs são extraídos pelas técnicas de hidrodestilação e por arraste a vapor de várias partes da planta (folhas, flores, flores, raízes ou sementes) (BAKKALI et al., 2008).

Diversas atividades biológicas dos OEs são descritas na literatura, como antioxidante, antimicrobiana, antiviral, antimutagênica, anticancerígena e anti-inflamatória, podendo ser potenciamente utilizados para a composição de novos fármacos no tratamento de várias doenças e na conservação de alimentos (BAKKALI et al., 2008; MIGUEL, 2010).

1.4 CAPIM-LIMÃO E CITRAL

Cymbopogon citratus, (DC) Stapf, é uma planta perene pertencente à família Poaceae (Gramineae), internacionalmente conhecida como *lemongrass*, originária da Ásia e amplamente cultivada nos países tropicais e subtropicais. Apresenta longas folhas aromáticas, estreitas, agudas e ásperas com nervura central proeminente. Foi introduzido no Brasil no período colonial e se estendeu por todas as regiões, por se adaptar facilmente ao clima e se desenvolver bem em todos os tipos de solo. No Brasil é popularmente conhecido como capim-limão, capim-santo, capim-cidrão e capim-cidreira, de leve aroma cítrico usado na culinária e na perfumaria (SHAH et al., 2011; VILLAVERDE et al., 2013).

Durante séculos, o chá das folhas do capim-limão é utilizado na medicina popular nos países da América do Sul, da Ásia e da África Ocidental, como digestivos, antigripais, analgésicos, antitérmicos, diuréticos, entre outros (VILLAVERDE et al., 2013; MANVITHA, BIDYA, 2014). Vários estudos têm atribuido atividades biológicas do capim-limão, tais como, atividades anti-inflamatória, anti-séptica, diurética, antimicrobiana e antioxidante (FIGUEIRNHA et al., 2010; MANVITHA, BIDYA, 2014; EKPENYOUNG, AKPAN, NYOH, 2015).

O OE de capim-limão, apresenta como constituinte majoritário o citral (3,7 dimetil, 2,6 octadienal), que é um monoterpenoíde oriundo da mistura de dois isômeros (geranial e neral), empregado na indústria farmacêutica, na indústria de alimentos e na perfumaria (BACHIEGA SFORCIN, 2011; MASWAL, DAR, 2014; BOUZENA et al., 2017).

Diversas atividades biológicas do citral, tem sido demonstradas, tais como: propriedades antibacteriana, antifúngica, antioxidante e anti-inflamatória. Além disso, apresenta potencial de inibição da expressão do óxido nítrico sintase induzível e efeito gastroprotetor contra úlceras induzidas pela indometacina (LEE 2008; BACHIEGA, SFORCIN, 2011; OLORUNNISOLA et al., 2014; NISHIJIMA et al., 2014; BOUZENNA et al., 2017).

1.5 JUSTIFICATIVA

A hepatotoxicidade induzida por fármacos é uma das principais causas de morbidade e mortalidade em vários países (GHANEM, 2016). A orvedose de paracetamol causa lesão hepática grave que pode evoluir para insuficiência hepática e a necrose hepatocelular.

O aumento dos casos de doenças decorrentes de efeitos colaterais associados a drogas sintéticas (APAP), valida a necessidade da busca de substâncias com efeito hepatoprotetor. Portanto, os esforços estão sendo direcionados para descobrir fontes potenciais, a partir de plantas ditas medicinais.

Produtos naturais, como os óleos essenciais das plantas vêm sendo muito estudados devido suas propriedades farmacológicas. Várias atividades biológicas do óleo essencial de capim-limão e do citral foram descritas (MANVITHA, BIDYA, 2014; OLORUNNISOLA et al., 2014; EKPENYOUNG, AKPAN, NYOH, 2015), mas devido a escassez de estudos sobre seus efeitos na lesão hepática, esta investigação é de grande valia para verificar um possível efeito hepatoprotetor do óleo essencial de capim-limão e do citral na hepatotoxicidade pelo paracetamol. O modelo experimental murino de hepatotoxicidade induzido por APAP foi utilizado por ser amplamente utilizado para o *screening* de substâncias naturais com possível potencial hepatoprotetor (JAESCHKE et al., 2011; MADRIGAL-SANTILLÁN et al., 2014; FREITAG et al., 2015).

1.6 OBJETIVOS

GERAL

Avaliar a possível efeito protetor do óleo essencial de capim-limão e seu constituinte majoritário (citral), utilizando o modelo experimental de toxicidade hepática induzida por paracetamol em camundongos.

ESPECÍFICOS

Identificar os principais constituintes majoritários do óleo essencial de capim-limão.

Isolar o citral (constituinte majoritário) do óleo essencial de capim-limão.

Avaliar a atividade antioxidante do óleo essencial de capim-limão e do citral pelo método de DPPH (2,2-difenil-1-picrilhidrazil).

Avaliar a viabilidade celular óleo essencial de capim-limão e do citral pelo teste do MTT (3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina).

Verificar a migração leucocitária em modelo de quimiotaxia *in vitro*.

Analisar o efeito hepatoprotetor do pré-tratamento com o óleo essencial de capim-limão e citral frente as alterações promovidas pela intoxicação induzida pelo paracetamol.

Avaliar o efeito do óleo essencial de capim-limão e citral sobre: as enzimas hepáticas (AST, ALT, ALP e γ-GT), a atividade da mieloperoxidase, a formação de óxido nitrico e as alterações histopatológicas na toxicidade por paracetamol e no pré-tratamento com o óleo essencial de capim-limão e o citral.

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CAPÍTULO II

Artigo 1: "Protective Effect of *Cymbopogon citratus* Essential Oil in Experimental Model of Acetaminophen-Induced Liver Injury"

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Protective effect of *Cymbopogon citratus* essential oil in experimental model of acetaminophen-induced liver injury

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Abstract: To investigate the hepatoprotective effect of *Cymbopogon citratus* or lemongrass essential oil (LGO), it was used an animal model of acute liver injury induced by acetaminophen (APAP). Swiss mice were pretreated with LGO (125, 250 and 500 mg/kg) and SLM (standard drug, 200 mg/kg) during seven days, followed by induction of hepatotoxicity of acetaminophen (single dose, 250 mg/kg). The liver function markers alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase were determined to evaluate the hepatoprotective effects of the LGO. The livers were used to determine myeloperoxidase (MPO) activity, nitric oxide (NO) production and histological analysis. The effect of LGO on leukocyte migration was evaluated in vitro. Antioxidant activity was performed by assessing the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in vitro. LGO pretreatment decreased significantly the levels of ALT, AST and ALP compared with APAP group. MPO activity and NO production were decreased. The histopathological analysis showed an improved of hepatic lesions in mice after LGO pretreatment. LGO inhibited neutrophil migration and exhibited antioxidant activity. Our results suggest that LGO has protective activity against liver toxicity induced by paracetamol.

Keywords: Acetaminophen; *Cymbopogon citratus*; Hepatotoxicity; lemongrass; Acute liver injury; Essential oil.

Introduction

Acetaminophen (paracetamol, N-acetyl-p-aminophenol, APAP) is widely used as analgesic and antipyretic drug. While acetaminophen is a safe and effective drug at therapeutic doses, at large doses can result in severe liver injury, acute liver failure and potentially death (Cigremis *et al*., 2009; Jaeschke *et al*., 2014).

APAP toxicity has been attributed to the accumulation of the toxic reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). At therapeutic doses NAPQI is removed by conjugation with glutathione sulfhydryl (GSH), after overdose of APAP, the sulfonation reaction becomes saturated and the over-production of NAPQI depletes GSH in the liver, causing further accumulation of NAPQI. Unconjugated NAPQI binds to proteins and/or by elevating reactive oxygen species (ROS) resulting in hepatic necrosis (Jaeschke *et al*., 2014).

In recent years the use medicinal plants in the prevention and treatment of diseases have gained importance and popularity because of their safety, efficacy and cost effectiveness. Medicinal plants play a key role in the human health care and it has received lots of attention due to the available scientific evidences. Research with plants used in folk medicine to treat liver diseases and boost liver function has been performed (Grespan *et al*., 2014; Madrigal-Santillán *et al*., 2015). Among plant products, essential oils (EO) have potential pharmacological and biological activities and represent a small fraction of the plant's composition, with a complex composition. In general, the majority components identified in plant essential oils are terpenes, monoterpenes and sesquiterpenes (Miguel, 2010).

The plant *Cymbopogon citratus* (DC) Stapf Poaceae/Gramineae, commonly known as lemongrass, is a source of essential oil used as a component of

ethnopharmaceuticals and largely distributed in tropical and subtropical countries. The essential oil from *C. citratus* mainly contains monoterpene fractions, such as neral (cis-citral, citral B), geranial (trans-citral, citral A) or myrcene. Several studies have shown analgesic, anti-inflammatory, antioxidant, antimalarial and anticonvulsant properties of *C. citratus* essential oil (Silva *et al*., 2010; Ekpenyong *et al*., 2015). However, to our knowledge, information about effects of lemongrass essential oil (LGO) in experimental hepatotoxicity models are scarce, and for this reason, the aim of this study was to investigate the protective effect of LGO on APAP-induced liver injury, for possible use in the treatment of liver disease.

Materials and methods

Chemicals

Transaminases (ALT and AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (γ-GT) were estimated using Analiza Gold enzymatic test kit (Belo Horizonte, MG, Brazil). O-dianisidine dihydrochloride, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide and 2,2-diphenyl-1-picrylhydrazil were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material and essential oils

The leaves of the *Cymbopogon citratus* were commercially purchased in Cercopa-Guarapuava, Paraná, Brazil. The essential oil were extracted by conventional steam distillation using a Clevenger-type apparatus for 3 h. The obtained pale yellow essential

oil was dried over sodium sulfate and stored at 4°C in dark vials until tested. The yield of essential oil of the *C. citratus*, was 0.48 % v/w. LGO was dissolved in Tween (polyoxyethylenesorbitan monooleate - Tween 80® - 0.1% v/v in distilled water, Sigma–Aldrich, USA).

Analysis of the Essential Oil and Compound Identification

The EO was analysed using Gas chromatography – Mass Spectrometry (GC-MS) and Nuclear magnetic resonance spectroscopy.

Gas chromatography was performed with a Thermo Electron Corporation Focus GC model under the following conditions: Agilent DB-5ms capillary column (30 m x 0.25 mm x 0.25 μm), column temperature (60°C for 1 min to 180°C at 3°C/min), injector temperature (220°C), detector temperature (220°C), split ratio (1:10), carrier gas (He), and flow rate (1.0 mL/min). The volume injected (1 μL) was diluted in acetone (1:7). The GC-MS analysis was performed in a Quadrupole mass spectrometer (DSQ II model, Thermo Electron Corporation) that operated at 70 eV.

Nuclear magnetic resonance (NMR) ^{1}H (300.06 MHz) and ^{13}C NMR (75.45 MHz) spectra were recorded in deuterated chloroform ($CDCl_3$) solution in a Mercury-300BB spectrometer, with δ (ppm) and spectra referred to $CDCl_3$ (δ 7.27 for ^{1}H and 77.00 for ^{13}C) as internal standard.

Identification of the individual components was based on comparison of their GC retention index (RI), mass spectra and ^{13}C NMR spectra, with authentic standard purchased from Sigma-Aldrich and compound isolated of the essential oils obtained in our laboratory. The retention index (RI) was calculated relative to a series of the n-alkanes (C_8-C_{40}, Sigma) on DB-5 column, using the Van den Dool and Kratz equation (Van Den

Dool and Kratz, 1963). The thirteen minor components of oil were identified comparing their RI e mass spectra with those reported by Adams (Adams, 2007).

Animals

Male *Swiss* mice (30–40g) were provided by the Central Animal House of the Central Animal House of the State University. The animals were maintained under controlled environmental conditions of temperature (22 ± 2°C) and 12/12 h light/dark cycle. Prior to the experiments, the animals 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 084/2014).

Cell viability analysis

Cell viability was determined by MTT assay according to the method reported by Silva-Filho *et al*. (2014). The MTT (3-[4 5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) assay is based on the mitochondrial enzyme reduction of tetrazolium dye to detect and determine cell viability. Leukocytes were obtained from the peritoneal cavity of mice 4 h after zymosan injection (1 mg/cavity, i.p.). Briefly, the cells were plated at a density of 5×10^5 cells/well in a volume of 100 μL RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin + 100 μg/mL streptomycin in 96-well plates. The cells were incubated with varying concentrations of LGO (3, 10, 30, and 90 μg/mL) at 37°C in 5% CO_2 for 90 min followed by the addition of 10 μL MTT (5 mg/mL) stock solution to each well. After 2 h of incubation at 37°C, 150 μL of the supernatant was removed, and 100 μL dimethyl sulfoxide was added to each well. The cells were incubated at 25°C for a further 10 min, and absorbance was measured using a

Biochrom Asys Expert plus microplate reader at a wavelength of 540 nm. The values of the blank wells were subtracted from each well of treated and control cells. Viability was determined using the equation:

Viability (%) = (At – Ab) / (Ac – Ab) × 100

where At, Ab and Ac are the absorbance of treated cells, blank and control, respectively.

Radical scavenging activity-DPPH assay

Free radical scavenging capacity (RSC) was evaluated by measuring the 2,2-diphenyl-1-picrylhydrazil (DPPH)-scavenging activity of LGO. The DPPH assay was performed as previously described by Espin *et al*. (2000), with minor modifications. The samples 125 - 8750 mg/mL were mixed with 1 mL of 25 mM of DPPH• solution, 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 the 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.

Treatment of Animals

The experimental animals were divided into six 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. Group II, the mice received LGO vehicle (saline containing 0.1% Tween 80). Groups III–V, the mice were pretreated with LGO at doses of 125, 250, and 500 mg/kg, respectively. The group VI, the mice were pretreated with the hepatoprotective standard drug silymarin (SLM) (200 mg/kg). After this time, the animals fasted for 8 h and then received oral acetaminophen on the seventh day at a dose of 250 mg/kg in Groups II–VI. Group I orally received saline that contained 0.1% Tween 80 (APAP vehicle). After 12 h, the mice was anesthetized with halothane, and blood was collected for the biochemical determination (Grespan *et al*., 2014).

Determination of Seric ALT, AST, ALP and γGT Levels

Blood samples were collected and centrifuged at 3000 × g for 15 min at 4 °C. Biochemical parameters in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GT) levels were estimated using commercially available diagnostic kits.

Chemotaxis Assay

To evaluate the effects of LGO on chemotaxis, was used the method described by Silva-Filho *et al*. (2014). Neutrophils were obtained 4 h after zymosan injection (1 mg/cavity, i.p.) of *Swiss* mice by peritoneal washing with 3 mL of phosphate-buffered saline (PBS). The cell number was adjusted to 1×10^6 cells/mL in RPMI 1640 medium that contained 0.1 % bovine serum albumin (BSA). A chemotaxis assay was performed using a 48-well microchemotaxis plate (Neuro Probe), in which the chambers were separated by a

polyvinylpyrrolidone-free polycarbonate membrane (5 μm pore size). The chemoattractant, N-formyl methionyl leucyl phenylalanine (fMLP; 10^{-6} M), and vehicle (RPMI 1640) were placed in the lower chamber. A neutrophil suspension (1×10^6 cells/mL) pretreated with LGO (1, 3, 10, 30, 60 and 90 μg/mL) for 30min was then placed in the upper chamber. The cells were allowed to migrate into the membrane for 1 h at 37°C in 5% CO_2. Following incubation, the membrane was washed in PBS, fixed in methanol, and stained with Instant Prov. The membrane area of each well was scored using light microscopy to count the cells present in five random fields. The results are expressed as the mean number of neutrophils per field and are representative of triplicate measurements from three separate experiments.

Myeloperoxidase (MPO) assay

The homogenate supernatant of the liver sections, were used to determine MPO enzyme activity (Bradley *et al*., 1982), which were placed in potassium phosphate buffer that contained hexadecyltrimethylammonium bromide in a Potter homogenizer. The homogenate was stirred in a vortex and centrifuged. Ten microliters of the supernatant was added to each well in triplicate in a 96-well microplate. Two hundred microliters of the buffer solution that contained 16.7mg *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 addition of sodium acetate. Enzyme activity was determined by absorbance measured at 460 nm using a Spectra Max Plus microplate spectrophotometer.

Determination of Nitric Oxide (NO) Production

The NO production was determined by the Griess method in the supernatant of liver tissue sections, which determines the nitrite production (Saleh *et al*., 1999). Fifty microliters of the supernatant was added to each well in triplicate in a 96- well microplate. Sequentially, solution (50 µL) was added to Griess (1 g sulfanilamide in 2.5 mL phosphoric acid and 0.1 g dihydrochloride of N-(1-naphtyl) ethylenediamine *Milli-Q* water) at room temperature. The reading was taken using an ELISA plate reader at a wavelength of 550 nm. NO production was calculated from a standard curve of sodium nitrite. The results were expressed as µM.

Histopathological Analysis

The livers were collected and fixed in 10% formaldehyde solution. Subsequently, they were dehydrated with increasing concentrations of alcohol (80–100%, v/v) and paraffin embedded and sectioned in semiserial at a 5 μm thickness on a Leica rotary microtome (Leica Microsystems, Gladesville, New SouthWales, Australia). The sections were stained with hematoxylin and eosin (H&E) and examined for visualization of changes using light microscopy (Olympus BX-41, Tokyo, Japan). The histological sections from each group were examined using a grading scale of 0–3, according to the proportion of liver injury: 0 (absent injury); 1 (mild, liver destruction in one to two areas); 2 (moderate, liver destruction in three or more areas) and 3 (marked, severe liver injury). Cellular infiltration: 0 (infiltrated cells equivalent to normal), 1 (poorly infiltrated cells), 2 (moderately infiltrated cells), and 3 (densely infiltrated cells). The semi quantitative scale was adapted from Lobenhofer *et al*. (2006) and Yamada *et al*. (2013).

Statistical analysis

The data are expressed as mean ± SEM for each experimental group. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The software used was GraphPad Prism version 5.01, GrahPad Software, Inc. Differences were considered significant at $p < 0.05$.

Results

Analysis of lemongrass essential oil

The chemical composition of LGO investigated by GC-MS revealed high areas for the compounds 1. Myrcene (30.75%), Neral (24.98%) and Geranial (27.3%), summarized in Fig 1. A complete list of the components and their relative percentages are presented in Table 1. To confirm the structure of the main compounds, LGO was studied by ^{13}C NMR (Fig. 2). The chemical shift of each carbon in the experimental spectrum was compared with the shifts of the spectra of pure compounds.

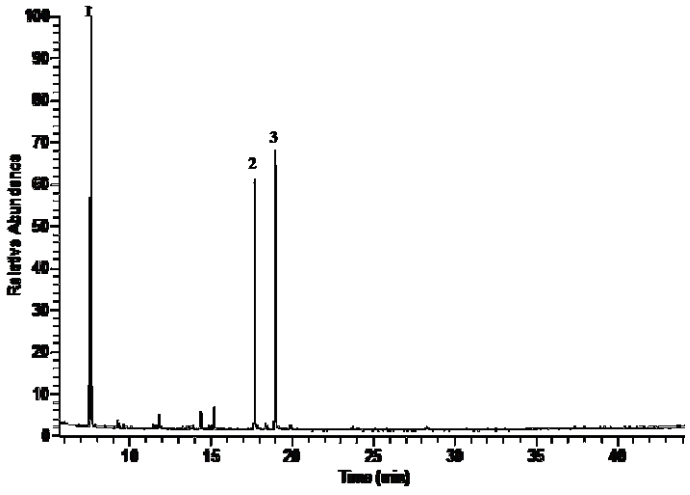

Figure 1. Chromatogram of the C*ymbopogon citratus* essential oil. The numbers on the peaks are attributed to majority compounds: 1 myrcene, 2 neral and 3 geranial.

Table 1 Chemical Composition of Essential Oil of C*ymbopogon citratus*

RI[a]	Compounds	% RA[b]	Identification methods[c]
992	6-methyl-5-Hepten-2-one	2,71	MS, RI
995	Myrcene	30,75	MS, RI
1041	(Z)-β-ocimene	0,82	(MS, RI)*
1051	(E)-β-ocimene	0,41	(MS, RI)*
1098	6,7-epoxymyrcene	0,43	(MS, RI)*
1102	6-camphenone	0,36	(MS, RI)*
1108	Linalool	1,39	MS, RI
1143	(E)-epoxymyrcene	0,25	(MS, RI)*
1150	exo-isocitral	0,34	(MS, RI)*
1154	(E)-5,6-epoxy-β-ionone	0,30	(MS, RI)*
1158	Citronellal	0,48	MS, RI
1170	(Z)-isocitral	1,69	(MS, RI)*
1181	rosefuran epoxide	0,46	(MS, RI)*
1188	(E)-isocitral	2,34	(MS, RI)*
1248	Neral	24,98	MS, RI
1264	Geraniol	0,76	MS, RI
1278	Geraniale	27,32	MS, RI
1300	2-undecanone	0,37	(MS, RI)*
1391	geranylacetate	0,38	MS, RI
1398	β-elemene	0,24	(MS, RI)*
1441	trans-bergamotene	0,20	(MS, RI)*
1504	tridecanone	0,41	(MS, RI)*
---	Others	2,13	-----

[a]RI= Retention Index, obtained with reference to *n*-alkane series C_8H_{18} – $C_{20}H_{42}$ on DB-5 column, using the Van den Dool and Kratz equation (1963). [b]Relative area (peak area relative to the total peak area) [c]Identification based on retention index (RI) and mass spectra (MS) of authentic compounds. [*]Identification based in the literature (Adams, 2007).

Cell viability

In cell viability assay LGO at concentrations 3, 10, 30, and 90 µg/mL showed cell viability of 85.7, 97.1, 85.7, and 80.1%, respectively. Thus, our data indicate that LGO has low cytotoxicity at any tested concentrations.

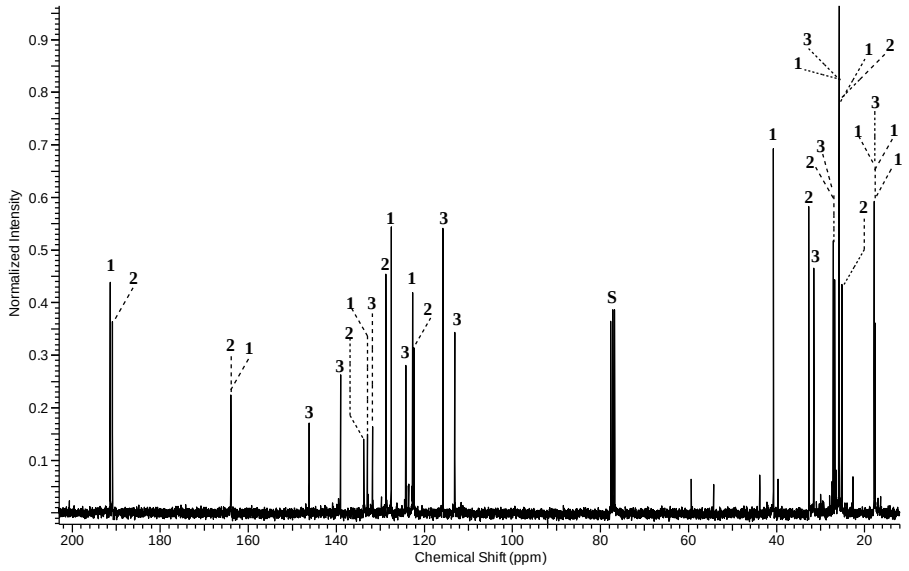

Figure 2. ^{13}C NMR Spectra of the *Cymbopogon citratus* essential oil in Solvent (S) deuterated chloroform ($CDCl_3$). The numbers on the peaks are attributed to majority compounds: 1 geranial, 2 neral, and 3 myrcene.

DPPH scavenging activity

The antioxidant capacity of LGO was measured spectrophotometrically by the DPPH test. The LGO at doses of 125 - 8750 µg/mL showed antioxidant activity *in vitro* (IC50 = 8650 ± 0.07 µg/mL). The IC50 value of ascorbic acid (positive control) was 13.72 ± 0.24 µg/mL in the DPPH assay (Fig. 3). These results show that, in the DPPH assay, free radical inhibition increased with the elevation of the concentration of the LGO.

Effects of lemongrass essential oil and APAP on Serum Hepatic Biomarkers

The mice treated with APAP (250 mg/kg) exhibited a serum elevation of ALT, AST, ALP and γGT serum increased significantly ($p < 0.05$), showing the characteristic acute liver injury caused by APAP, as revealed by H&E staining and sections of livers. Pretreatment

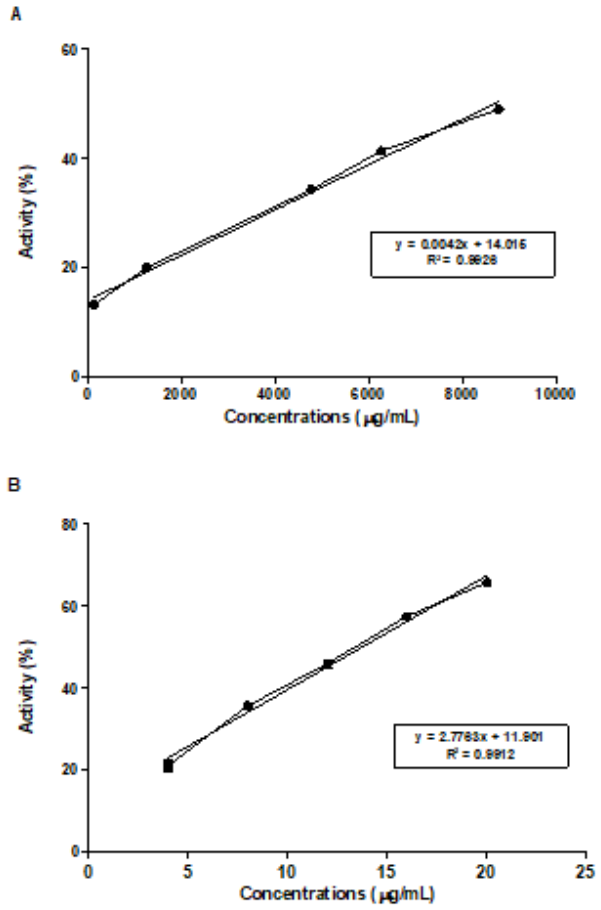

Figure 3. Antioxidant activity of the essential oil from lemongrass. The figure shows the percentage of neutralization of DPPH by (A) the essential oil of lemongrass and (B) ascorbic acid in the DPPH assay (μg/mL).

with 125, 250 and 500 mg/kg LGO showed a significant reduction in levels of serum markers ALT (95.35, 95.47, 97.70%, respectively); AST (91.22, 88.92, 94%, respectively); ALP (65.79, 67.72, 79.46%, respectively) and γGT at doses of 125 or 500 mg/kg (42.63 or 42% respectively) compared with APAP group (Fig. 4A-D). The SLM group also had levels of ALT, AST and ALP (95.95, 89, 64.23%, respectively) decreased compared with APAP animals.

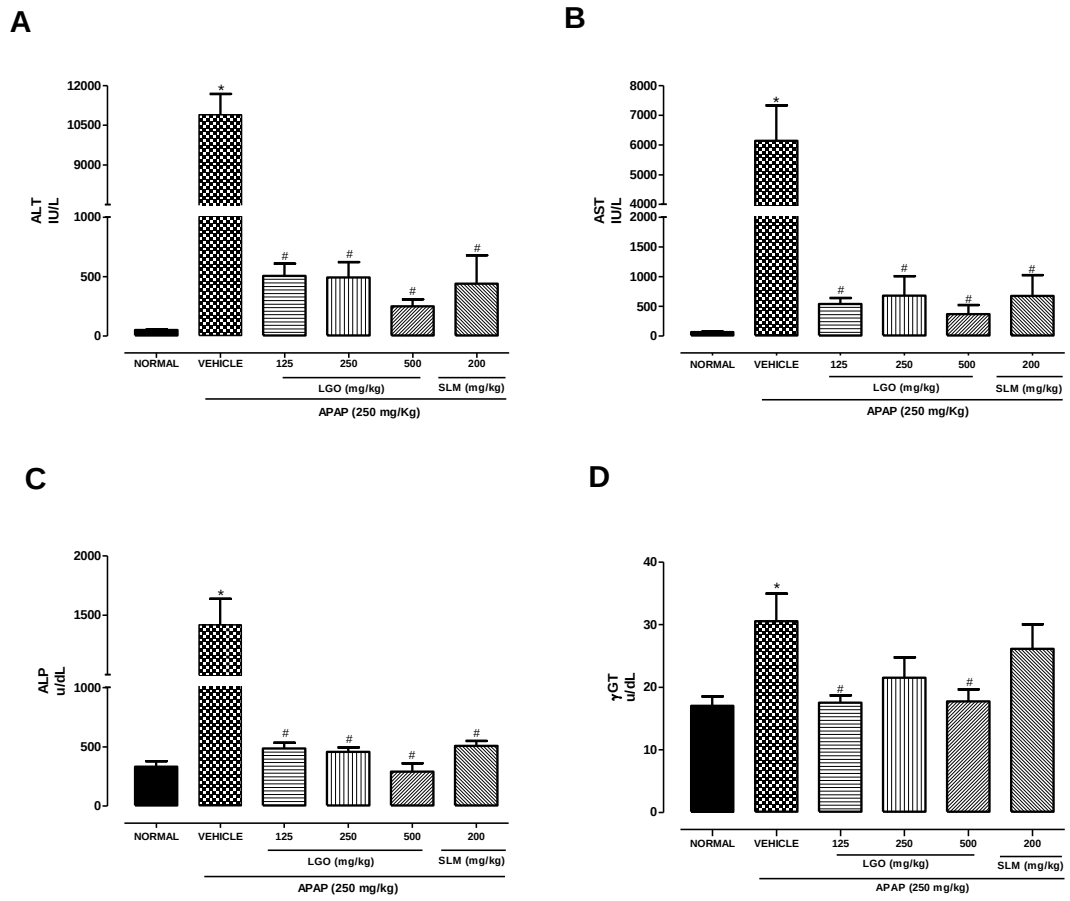

Figure 4. Effects of lemongrass essential oil (LGO), Silymarin (SLM) against acetaminophen-induced liver toxicity in biomarkers of hepatic damage. Serum ALT (A); AST (B); ALP (C) and γGT (D) enzyme levels. Values are mean ± SEM (n=5). * $p < 0.05$ acetaminophen (APAP) treated group vs. animals in control groups. # $p < 0.05$ lemongrass essential oil or Silymarin treated group vs. acetaminophen group.

Effect of lemongrass essential oil on chemotaxis

To investigate the effect of LGO on leukocyte migration, *in vitro* chemotaxis assay was performed. fMLP induced a significant leukocyte migration when compared with the control group (RPMI 1640). LGO in all concentrations tested (1, 3, 10, 30, 60 and 90 μg/mL) caused a significant reduction ($p < 0.05$) of leukocyte migration toward fMLP (36.75 ± 1.13, 35.80 ± 1.59, 33.65 ± 2.25, 33.45 ± 1.39, 22.50 ± 1.47 and 19.60 ± 0.79 respectively). The results obtained are shown in Fig. 5.

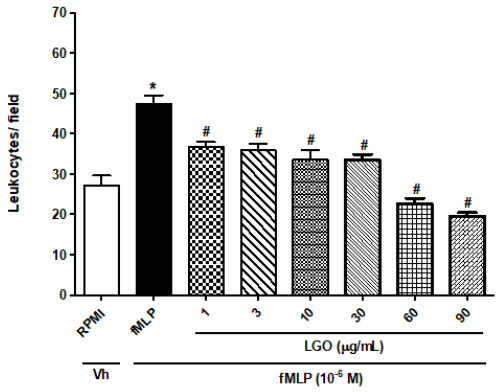

Figure 5. Effect of lemongrass essential oil on *in vitro* leukocyte chemotaxis. Leukocytes were obtained from zymosan-induced peritonitis (200 µg/cavity) and stimulated with fMLP (10^{-6}) after 30 min of treatment with LGO at doses of 1, 3, 10, 30, 60 and 90 µg/mL. Values are mean ± SEM (n = 5) and are representative of three independent experiments. * $p < 0.05$ vs. vehicle, # $p < 0.05$ vs. group of leukocytes stimulated with fMLP.

Myeloperoxidase activity and nitric oxide production

In the MPO assay, pretreatment with LGO at doses 125, 250, 500 mg/kg and SLM decreased the activity of MPO (75.51, 68.60, 70.20, 69.32% respectively) (Fig. 6A). NO production was decreased in the groups treated with LGO and SLM, compared to APAP group (55.09, 33.46, 47.46, 52.36% respectively) (Fig. 6B).

Histopathological Examination

Histological sections of livers of normal mice showed a lobular architecture and hepatocytes structure normal with mild inflammatory infiltrate (Fig. 7A). A severe injury was visible in the APAP-treated group, characterized by necrotic areas with centrilobular vein congestion, presence of inflammatory cell infiltration and vacuolization of hepatocytes (Fig. 7B). In the SLM group, it was observed portal area preserved hepatocytes, some vacuolated cells and mild injury were found in the centrilobular region

(Fig. 7C). Animals treated with 125 mg/mL of LGO showed mild injuries, whereas hepatic parenchyma without necrosis with centrilobular area preserved. Furthermore, a mild vacuolization and the presence of binucleate hepatocyte were observed (Fig. 7D). The groups that received treatment with 250 mg/mL of LGO showed the centrilobular region preserved, presence of binucleate hepatocytes, mild vacuolization of hepatocytes in liver parenchyma and mild lesion area (Fig. 7E). After treatment with 500 mg/kg of LGO, the liver architecture was preserved with apparently normal hepatocytes, presence of binucleate hepatocytes, less vacuolation and mild injury (Fig. 7F). Thus, our results indicate that pretreatment with LGO, improved the liver morphology showing less inflammatory cell infiltration, and preserved hepatocytes with less of an area of necrosis compared with the severe centrilobular necrosis observed in acetaminophen-treated mice. The histological grading scale is presented in the Table 2.

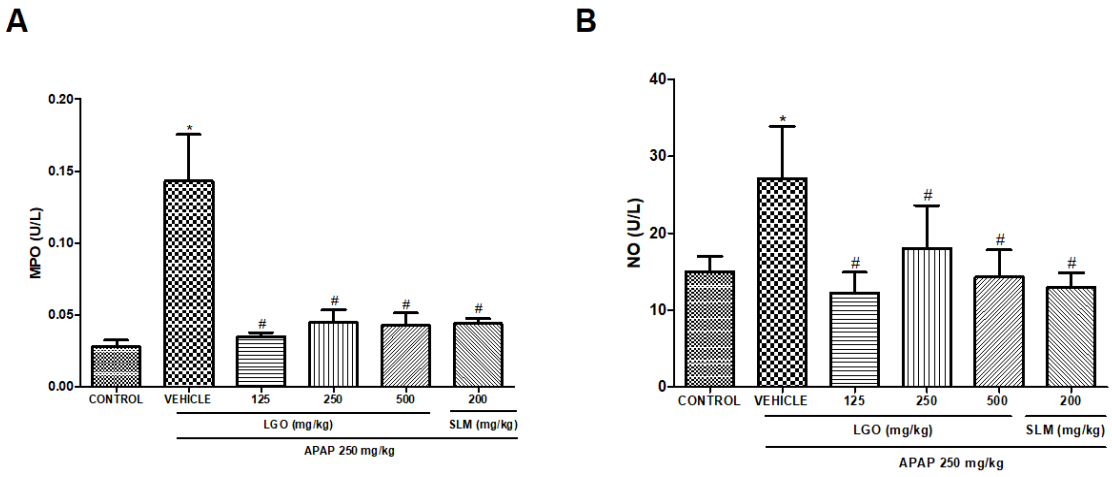

Figure 6. Effect of lemongrass essential oil (LGO) and Silymarin (SLM) in MPO activity (A) and NO production (B) in mice serum after APAP intoxication. Values are mean ± SEM (n=5). * $p < 0.05$ acetaminophen (APAP) treated group vs. animals control groups; # $p < 0.05$ lemongrass essential oil or Silymarin treated group vs. acetaminophen.

Table 2. Histological Grading Scale

Category	Control (n=5)	APAP (n=5)	SLM (n=5)	LGO (mg/kg) 125	250	500 (n=5)	Score
Cell infiltrate							
Normal	5		1	1	1	1	0
Poorly infiltrated cells			4	4	4	4	1
Moderate infiltrated cells							2
Densely infiltrated cells		5					3
Liver lesions							
Absent	5		1	1		1	0
Mild injury			4	4	5	4	1
Moderate injury							2
Severe injury		5					3

Note: Modified from the scale described by Lobenhofer *et al*. (2006) and Yamada *et al*. (2013). Histological analysis of liver sections were evaluated.

Discussion

Natural products are believed to have liver protective functions and researchers are concerned to investigate the medicinal plants with more safety profile and effectiveness is needed to protect or cure the liver disease (Koh *et al*., 2012; Madrigal-Santillán *et al*., 2014).

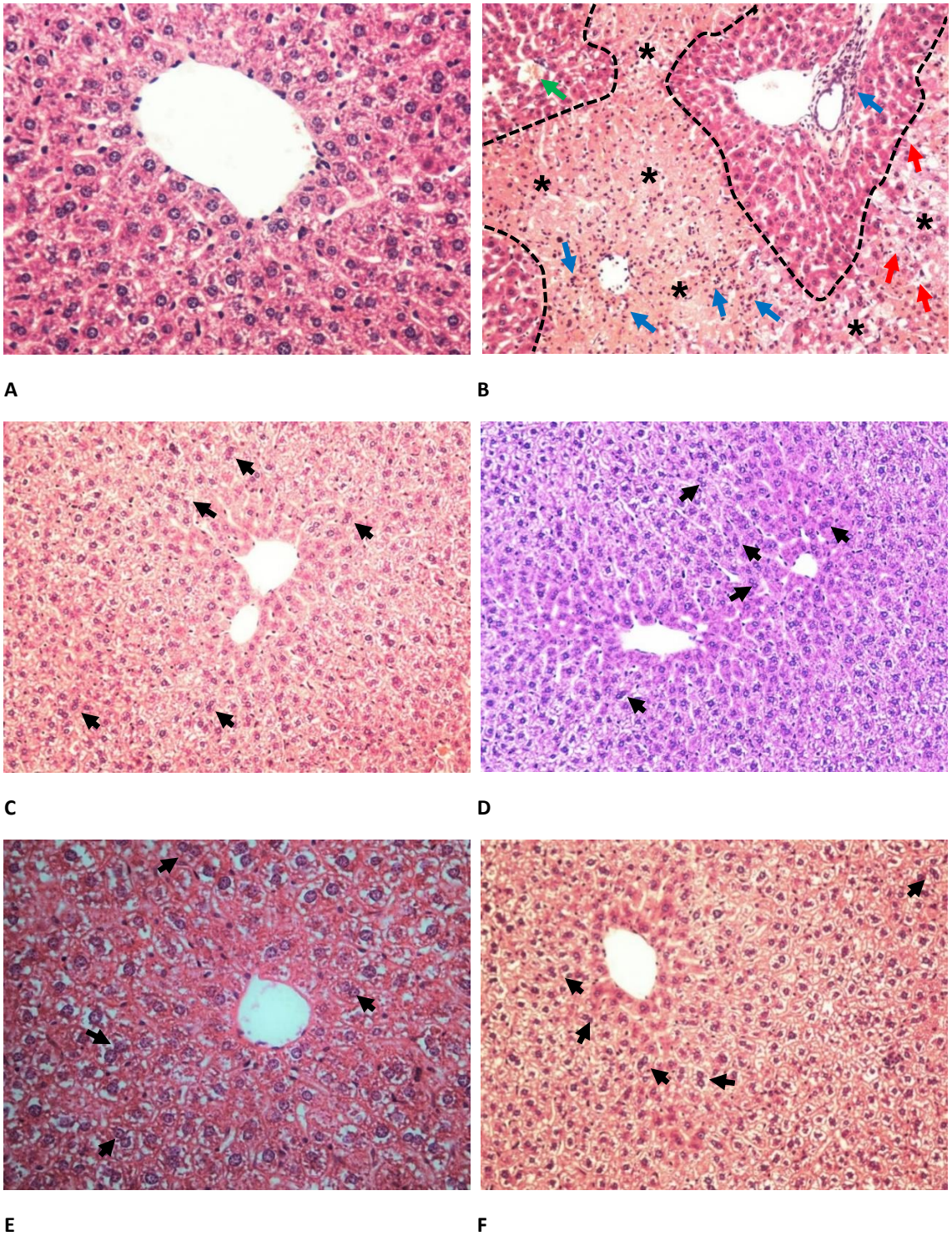

Figure 7. Histological sections. (A) control mice liver showed normal morphology, absent lesion area; (B) acetaminophen group (mice liver that received orally APAP on last day of treatment, 250mg/kg): presence of severe necrosis (*) in the defined area, polymorphonuclear inflammatory infiltrates (arrows blue), congestion (arrows green), vacuolated hepatocytes (arrows red); (C) group pretreated with silymarin (200mg/kg) that received APAP and ((D)–(F)) group pretreated with essential oil of lemongrass (LGO), 125, 250, and 500 mg/kg, respectively that received APAP. In ((C)–(F)) presence of binucleate hepatocytes (arrows black) and mild lesion area. Liver sections were stained with H&E. Original magnification 40x in (A) and (E); original magnification 20x in (B), (C), (D) and (F).

C. citratus (DC) Stapf. (lemongrass) essential oil has anti-inflammatory, analgesic and antipyretic properties. Previous studies showed that citral is the main component of LGO and constitutes the mixture of stereoisomers geranial and neral. Citral has been reported to be useful in the management of inflammatory processes and the quality of LGO is generally determined by its citral content (Boukhatem *et al*., 2014; Ekpenyong *et al*., 2015). In our work, analysis of LGO by GC-MS, revealed the majority component was citral, indicating high quality this oil.

LGO was used in our study to analyze its effect on the liver damage induced by APAP. The capacity of LGO to scavenge the stable radical DPPH formed in the solution by donation of a hydrogen atom or an electron were determined in the DPPH assay. In our test, LGO revealed an antioxidant property against DPPH and this result is consistent with other studies (Adesegun *et al*., 2013; Ekpenyong *et al*., 2015). If essential oils are able to scavenge some free radicals, they can also act as anti-inflammatory agents, because one of the inflammatory responses is the oxidative burst that occurs in many cells (monocytes, neutrophils, eosinophils, and macrophages) (Miguel, 2010).

Several works have considered APAP as hepatotoxic agent in experimental animal models and has been widely used for screening purposes in liver protection activity (Grespan *et al*., 2014; Jaeschke *et al*., 2014). Hepatotoxicity induced by acetaminophen are initiated by the formation of the presumed reactive metabolite N-acetylp-benzoquinone imine (NAPQI), which is generated mainly by the CYP P450 enzymes Cyp2e1 and 1a2. Thus the availability of GSH is limited and NAPQI levels are increased. Consequently, sulfhydryl groups of hepatic proteins may react with the reactive metabolite, causing direct mitochondrial damage and the induction of an inflammatory response, resulting in liver damage (Jaeschke *et al*., 2014). An, overdose with APAP promotes destruction of liver cells that in turn result on the elevation in serum level of

enzymes aminotransferases (ALT, AST, ALP or bilirubin). When the hepatopathy is installed, these enzymes leak into the blood stream in conformity with the extent of liver injury (Green *et al*., 2010; Freitag *et al*., 2015). The serum hepatic biomarkers analysis are important for identification of liver lesion. In this study, acute liver injury induced by APAP was established as demonstrated through high levels of serum liver enzymes, ALT, AST, ALP and γGT. In addition, liver damage induced by APAP, were confirmed histopathologically, as observed in some previous studies (Koh *et al*., 2012; Grespan *et al*., 2014).

The *in vitro* MTT assay was performed to evaluate cell viability with the treatment of LGO and even at a higher concentration, LGO showed no toxicity, because did not affect the viability of neutrophils. In the present work, we demonstrated that the pretreament with LGO can prevent acetaminophen-induced hepatic damage in mice, because was able to decrease the biochemical parameters (ALT, AST and ALP) in all doses tested. In serum γGT activity was found at a dose of 125 and 500 mg/kg of the LGO. Koh *et al*. (2012) and Rahim *et al*. (2014), also obtained similar results using *C. citratus* extract against liver damage induced by carbon tetrachloride and hydrogen peroxide in rats, respectively.

The previous biochemical parameters analysed in this study, were supported by the livers histopathological analysis obtained from the LGO-pretreated group that showed an improvement in the liver injury when compared in the acetaminophen-treated mice, such as preserved hepatocytes, less inflammatory cell infiltration, decrease the necrotic area and parenchyma similar morphology to the control group. Some vacuolated hepatocytes were found in the treatment groups, suggesting a non-lethal injury, because the presence of inflammatory cells were scarcely observed. Furthermore, binucleate hepatocytes were also found in our animal groups treated with LGO, indicating

regeneration of liver cells, suggesting a hepatoprotective effect of the oil. Plant extracts protect the liver from acetaminophen overdose (Madrigal-Santillán *et al*., 2014), indicating that the hepatoprotective effect can be considered, probable as an expression of the functional improvement of hepatocytes that results from accelerated cellular regeneration. In general, hepatoprotective plant activity can be explained by containing a variety of chemical compounds (monoterpenes, flavonoids, phenols, etc), as observed for SLM that has been employed as a protective treatment liver disease by its antioxidant properties deriving from the phenolic nature of flavonolignans (Grespan *et al*., 2014; Madrigal-Santillán *et al*., 2014; Freitag *et al*., 2015).

Increased levels of MPO activity and NO production hepatocellular inflammatory disorders were demonstrated in APAP group, indicating an acute inflammatory process characterized by polymorphonuclear leukocyte recruitment (Freitag *et al*., 2015). The migration and accumulation of neutrophils at the inflammation site are crucial for host defense and these processes are mediated by a signaling cascade that involves adhesion molecules, several cytokines, and production of nitric oxide (Secco *et al*., 2003). Cytokines are critical mediators of APAP hepatotoxicity and studies report that the enhanced release of tumor necrosis factor (TNF) and interleukin-1 beta (IL-1β) may be responsible for further hepatic damage (Blazka *et al*., 1995). Furthermore, TNF-induced hepatocyte injury provides a vital signal for PMN transmigration from sinusoids into the parenchymal tissues, which leads to cellular necrosis (Bradham *et al*., 1998). In the *in vitro* chemotaxis assay, it was used fMLP a chemotactic agent which promotes the release of cytokines. Our results show in all LGO doses tested, a significant reduction in leukocyte migration was observed, suggesting an antiinflammatory activity of this essential oil.

The MPO is an indirect marker of neutrophil infiltration into tissue and in our study, we evaluated MPO enzyme activity. The group of animals treated with LGO has a MPO activity levels significantly lower than to that of APAP group, suggesting decreased of neutrophil infiltration. These results were also correlated with a discrete lymphocytic infiltrate observed in LGO-pretreated group obtained by histopathological examination of the liver. These results were similar to those obtained by Silva *et al*. (2010), that demonstrated the *C. citratus* and *C. winterianus* oils inhibit the MPO release from PMA-stimulated human neutrophils. NO exerts roles important as mediator of acetaminophen toxicity in the liver. NO is a well-established marker of inflammation, and inhibition of its production, might be a useful therapeutic strategy in inflammatory diseases (Cigremis *et al*., 2009; Figueirinha *et al*., 2010; Freitag *et al*., 2015). Our data demonstrate that LGO pretreatment produced a marked decrease in hepatic NO contents. Our data suggest an antiinflammatory activity and anti-free radicals properties of this oil.

Previous studies using extracts of *C. citratus* and citral, have shown inhibitory effects on cytokines production by inhibiting of the transcription factor NF-kB (Bachiega and Sforcin, 2011) and the IL-1 and IL-6 production by macrophages inhibited after incubation *in vitro* with lemongrass essential oil (Sforcin *et al*., 2009). Figueirinha *et al*. (2010), demonstrated that *C. citratus* leaf infusion significantly inhibited inflammatory NO production and inducible NO synthase expression by LPS-stimulated mouse skin dendritic cells, suggesting its anti-inflammatory activity. Our data and previous studies support that LGO has beneficial effects as hepatoprotector, probably associated with the inhibition of free radical-scavenging properties (NO) or their performance on proinflammatory cytokines.

Conclusion

We conclude that the *Cymbopogon citratus* essential oil showed an important protective effect against acute hepatotoxicity induced by APAP. Further studies are necessary to elucidate the mechanism of action of lemongrass essential oil and what are specific constituents responsible for this hepatoprotection.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Artigo 2: “Hepatoprotective Effect of Citral on Acetaminophen-Induced Liver Toxicity in Mice”

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Hepatoprotective effect of citral on acetaminophen-induced liver toxicity in mice

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Abstract

High doses of acetaminophen (APAP), leads to acute liver damage. In this study, we evaluated the effects of citral in a murine model of hepatotoxicity induced by APAP. The liver function markers alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and gamma-glutamyl transferase (γGT) were determined to evaluate the hepatoprotective effects of citral. The livers were used to determine myeloperoxidase (MPO) activity, nitric oxide (NO) production and histological analysis. The effect of citral on leukocyte migration and antioxidant activity was evaluated *in vitro*. Citral pretreatment decreased significantly the levels of ALT, AST, ALP and γGT; MPO activity and NO production. The histopathological analysis showed an improved of hepatic lesions in mice after citral pretreatment. Citral inhibited neutrophil migration and exhibited antioxidant activity. Our results suggest that citral protects the liver against liver toxicity induced by APAP.

Keywords: *Citral, Acetaminophen, Hepatoprotection*

1. Introduction

Drug-induced liver injury is a significant clinical problem worldwide [1]. Most drug-induced liver injury and acute liver failure occurs due to either accidental or intentional overdose of acetaminophen (N-acetyl-p-aminophenol, paracetamol, APAP) [2]. APAP is an antipyretic and analgesic drug used widely in clinics. When used at therapeutic doses, APAP is metabolized by glucuronidation or sulfation by the cytochrome p450 system into the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI). Under normal circumstances, NAPQI is rapidly converted to nontoxic metabolites by glutathione (GSH). Meanwhile, at large doses of APAP, NAPQI accumulate, can react directly with hepatic proteins, resulting liver injury [3, 4]. Because of its dose-dependent toxicity, APAP-induced hepatic damage can be studied in animal models and most mechanisms are translatable to humans [2].

APAP-induced hepatotoxicity has been a significant issue for several years and different strategies have been studied, including the use of natural compounds with hepatoprotective effects [5, 6].

Natural products are attracting the interest of many researchers to investigate its potential as drugs for the treatment of various diseases. Furthermore, there are many bioactive substances that are synthesized from constituents of essential oils (mixture of volatile and natural substances) that has some pharmacological activities [7]. In this context, monoterpene citral, an isomeric mixture of neral and geraniale, a component of lemongrass oil, has been reported to have many biological activities such as anti-bacterial and anti-inflammatory [8–10]. However, the protective effect of citral on APAP-toxicity remains unclear. Thus, the aim of the present study was to evaluate the effect of citral in hepatotoxicity induced by APAP.

2. Materials and Methods

2.1. Plant material and constituent of essential oils. The constituent citral was isolated from lemongrass essential oil as fractions of hydrodistillated oil, and was identified by GC-MS and NMR as previous described [11].

2.2. Animals. Male *Swiss* mice (30–40g) were provided by the Central Animal House of the Central Animal House of the State University. The animals were maintained under controlled environmental conditions of temperature (22 ± 2°C) and 12/12 h light/dark cycle. Prior to the experiments, the animals 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 084/2014).

2.3. Cell viability analysis. The MTT (3-[4 5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide) assay is based on the mitochondrial enzyme reduction of tetrazolium dye to detect and determine cell viability. Leukocytes were obtained from the peritoneal cavity of mice 4 h after zymosan injection (1 mg/cavity, i.p.). Briefly, the cells were plated at a density of 5×10^5 cells/well in a volume of 100 μL RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin + 100 μg/mL streptomycin in 96-well plates. The cells were incubated with varying concentrations of citral (3, 10, 30, and 90 μg/mL) or vehicle (0.1% Tween 80 solution, used as control), at 37°C in 5% CO_2 for 90 min followed by the addition of 10 μL MTT (5 mg/mL) stock solution to each well. After 2 h of incubation at 37°C, 150 μL of the supernatant was removed, and 100 μL dimethyl sulfoxide was added to each well. The cells were incubated at 25°C for a further 10 min, and absorbance was measured using a Biochrom

Asys Expert plus microplate reader at a wavelength of 540 nm. The values obtained of the blank (RPMI 1640) wells were subtracted from each well of treated and control cells. Viability was determined using the equation:

$$\text{Viability (\%)} = (At - Ab) / (Ac - Ab) \times 100$$

where At, Ab and Ac are the absorbance of treated cells, blank and control, respectively.

2.4. DPPH radical-scavenging. Free radical scavenging capacity (RSC) was evaluated by measuring the 2,2-diphenyl-1-picrylhydrazil (DPPH)-scavenging activity of citral. The DPPH assay was performed as previously described [12] with minor modifications. The samples 125 - 5000 µg/mL were mixed with 1 mL of 25 mM of DPPH• solution, 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 the RSC was calculated using the following equation: $RSC\ (\%) = 100 \times (A_{blank} - A_{sample} / A_{blank})$. The IC_{50} value, representing the concentration of the citral that caused 50% RSC inhibition, was determined by linear regression analysis from the obtained RSC values [11, 13].

2.5. Treatments. The experimental animals were divided into six 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 (normal). Group II, the mice received citral vehicle (0.1% Tween 80 solution). Groups III–V, the mice were pretreated with

citral at doses of 125, 250, and 500 mg/kg, respectively. The group VI, the mice were pretreated with the hepatoprotective standard drug silymarin (SLM) (200 mg/kg). After this time, the animals fasted for 8 h and then received oral APAP on the seventh day at a dose of 250 mg/kg in groups II–VI. Group I orally received saline that contained 0.1% Tween 80 solution (APAP vehicle). The stock solution was used as the first concentration of 50 mg/mL and after was diluted in 0.1% Tween 80 solution to prepared the solutions of 25 and 12.5 mg/mL. After 12 h of APAP administration, serum samples and liver tissue were collected and biochemistry and histological analysis [11, 13].

2.6. Markers of liver function. Blood samples were collected and centrifuged at 3000 × g for 15 min at 4 °C. Biochemical parameters in serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (γ-GT) levels were estimated using the Analyze Gold enzymatic test kit.

2.7. Chemotaxis Assay. To evaluate the effects of citral on chemotaxis neutrophils were obtained 4 h after zymosan injection (1 mg/cavity, i.p.) of *Swiss* mice by peritoneal washing with 3 mL of phosphate-buffered saline. The cell number was adjusted to 1×10^6 cells/mL in RPMI 1640 medium that contained 0.1 % bovine serum albumin . A chemotaxis assay was performed using a 48-well microchemotaxis plate (Neuro Probe), in which the chambers were separated by a polyvinylpyrrolidone-free polycarbonate membrane (5μm pore size). The chemoattractant, N-formyl methionyl leucyl phenylalanine (fMLP; 10^{-6} M), and negative control (vehicle: RPMI 1640) were placed in the lower chamber. A neutrophil suspension (1×10^6 cells/mL) pretreated with citral (1, 3, 10, 30, 60 and 90 μg/mL) for 30 min was then placed in the upper chamber. The

cells were allowed to migrate into the membrane for 1 h at 37° C in 5% CO_2. Following incubation, the membrane was washed in PBS, fixed in methanol, and stained with Instant Prov. The membrane area of each well was scored using light microscopy to count the cells present in five random fields. The results are expressed as the mean number of neutrophils per field and are representative of triplicate measurements from three separate experiments.

2.8. Determination of myeloperoxidase activity (MPO) on liver tissue. The homogenate supernatant of the liver sections, were used to determine MPO enzyme activity [14] which were placed in potassium phosphate buffer that contained hexadecyltrimethylammonium bromide in a Potter homogenizer. The homogenate was stirred in a vortex and centrifuged. Ten microliters of the supernatant was added to each well in triplicate in a 96-well microplate. Two hundred microliters of the buffer solution that contained 16.7mg *O*-dianisidine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA), 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 addition of sodium acetate. Enzyme activity was determined by absorbance measured at 450 nm using microplate spectrophotometer (Asys Expert Plus) [11, 13].

2.9. Determination of Nitric Oxide (NO) Production. The NO production was determined by the *Griess* method in the supernatant of liver tissue sections, which determines the nitrite production [15]. Fifty microliters of the supernatant was added to each well in triplicate in a 96- well microplate. Sequentially, solution (50 µL) was added to Griess (1 g sulfanilamide in 2.5 mL phosphoric acid and 0.1 g dihydrochloride of N-(1-naphtyl)

ethylenediamine *Milli-Q* water at room temperature. The reading was taken using an ELISA plate reader at a wavelength of 550 nm. NO production was calculated from a standard curve of sodium nitrite [11, 16]. The results were expressed as µM.

2.10. Histopathological Analysis. The livers were collected and fixed in 10% formaldehyde solution. Subsequently, they were dehydrated with increasing concentrations of alcohol (80–100%, v/v) and paraffin embedded and sectioned in semiserial at a 5 μm thickness on a Leica rotary microtome (Leica Microsystems, Gladesville, New SouthWales, Australia). The sections were stained with hematoxylin and eosin (H&E) and examined for visualization of changes using light microscopy (Olympus BX-41, Tokyo, Japan). The graded lesions were subjectively classified as absent, mild, moderate, or severe according to lesion area [13] and the cellular infiltration as infiltrated cells equivalent to normal, poorly infiltrated cells, moderately infiltrated cells, and densely infiltrated cells [17].

2.11. *Statistical analysis*. The data are expressed as mean ± SEM for each experimental group. The results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. The software used was GraphPad Prism version 5.01, GrahPad Software, Inc. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Cell viability analysis (MTT assay). Citral was the major component of the lemongrass essential oil (data not shown) and did not induce cell death in viability

assay. Citral at different concentrations of 3, 10, 30, and 90 µ/mL presented cell viability of 95.53, 86.52, 81.10, and 96.87%, respectively, indicating that citral did not induce cell death in any tested concentrations.

3.2. *Radical Scavenging Assay.* The DPPH radical-scavenging activity of citral was evaluated spectrophotometrically at doses of 125 - 5000 µg/mL showed antioxidant activity *in vitro* (IC50 = 2341 ± 0.07 µg/mL). Ascorbic acid (positive control) scavenged DPPH radicals completely, and its IC50 value was 9 µg/mL (Figure 1).

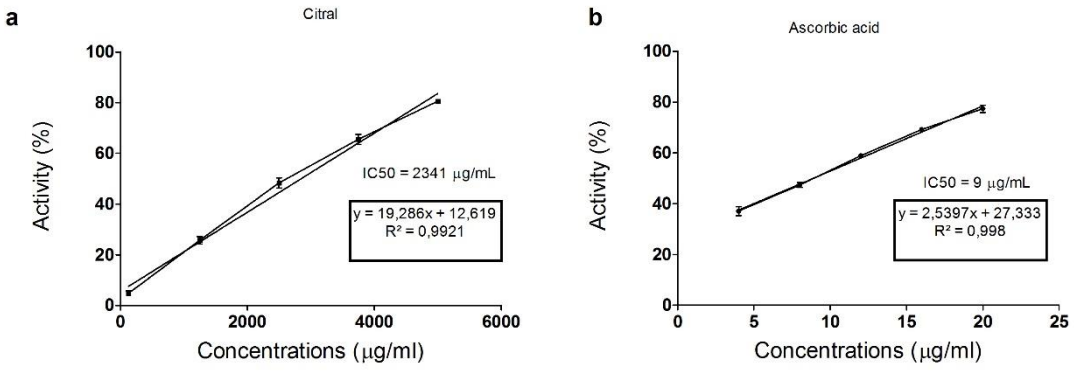

FIGURE 1: Antioxidant activity of citral. The figure shows the percentage of neutralization of DPPH by (a) citral and (b) ascorbic acid in the DPPH assay (*µ*g/mL).

3.3. Effect of Citral on serum transaminases and phosphatases activity from APAP-induced hepatotoxicity. In order to assess the effect of the pretreatment with citral a serum analysis of ALT, AST, ALP and γGT were performed (Figure 2). The mice administered with APAP caused significant ($p < 0.001$) liver damage and necrosis of cells as evidenced by the elevated serum hepatic enzymes ALT, AST, ALP and γGT compared with normal group. Conversely, effects of pretreatment with different doses of citral (125, 250 and 500

mg/kg), exhibited a significant ($p < 0.05$) decrease in serum activities of ALT (91.79%, 93.07% and 95.61%, respectively), AST (93.40%, 91.89% and 96.52%, respectively), ALP (39.29%, 37.07% and 59.80%, respectively) and γGT (39.09%, 39.69% and 44.82%, respectively), when compared to the APAP group. Similar results were found in pretreatment with SLM on the activity of ALT (95.90%), AST (95.03%), ALP (70.52%) and γGT (41.68%).

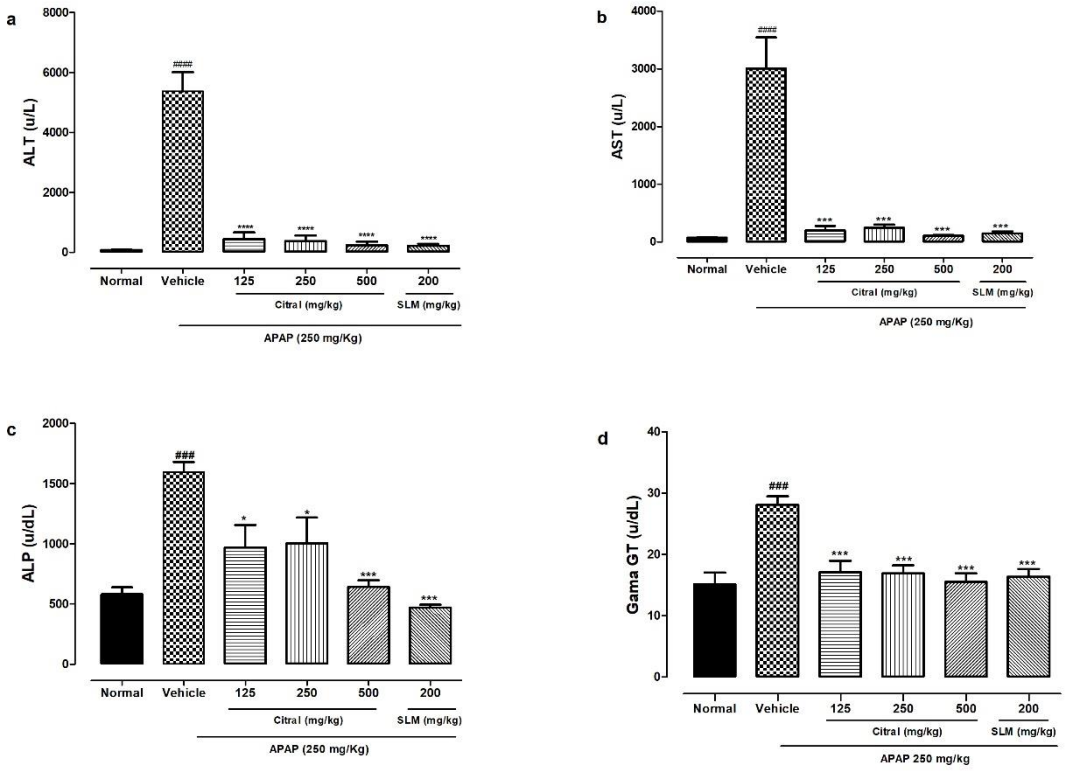

FIGURE 2: Effects of citral, SLM against APAP-induced liver toxicity in biomarkers of hepatic damage. Serum ALT (a); AST (b); ALP (c) and γGT (d) enzyme levels. Values are mean ± SEM. ### $p < 0.001$ vs. normal group. * $p < 0.05$ vs. APAP group, *** $p < 0.001$ vs APAP group.

3.4. *Citral reduces leukocytes chemotaxis* in vitro. To investigate the effect of citral on leukocyte migration, *in vitro* chemotaxis assay was performed (Figure 3). fMLP induced a significant leukocyte migration ($p < 0.001$) when compared with the control group

(RPMI 1640). Citral significantly reduced neutrophil migration toward fMLP (10^{-6} M) at doses of 3, 10, 30, 60 and 90 μg/mL (34.06 ± 3.44, 13.70 ± 2.77, 14.15 ± 2.00, 12.55 ± 1.93 and 6.85 ± 0.89, respectively).

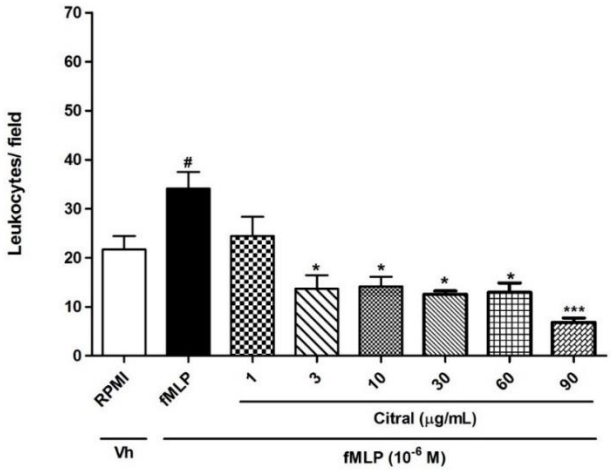

FIGURE 3: Effect of citral on *in vitro* leukocyte chemotaxis. Leukocytes were obtained from zymosan-induced peritonitis (1 mg/cavity) and stimulated with fMLP (10^{-6}M) 30 min after citral treatments at doses of 1, 3, 10, 30, 60 and 90 μg/mL. Values are mean ± SEM. and are representative of three independent experiments. $^{\#}$ $p < 0.05$ vs RPMI, * $p < 0.05$, *** $p < 0.001$ vs group of leukocytes stimulated with fMLP.

3.5. Citral decreased of MPO activity and NO production. The APAP overdose increased significantly MPO activity compared with normal group (Figure 4(a)). Moreover, the MPO activity was significantly reduced at all doses (125, 250 and 500 mg/kg) in citral-pretreated mice (87.41%, 86.87% and 87.45%, respectively), compared with the group that received only APAP ($p < 0.001$). Conversely, NO concentration in liver tissue was decreased in the citral groups (79.17%, 79.80% and 83.16%, respectively), when compared to the APAP group. The SLM group decreased considerably the MPO activity (93.09%) and NO concentration (81.78%) in the liver compared to APAP group. The results are shown in Figure 4(b).

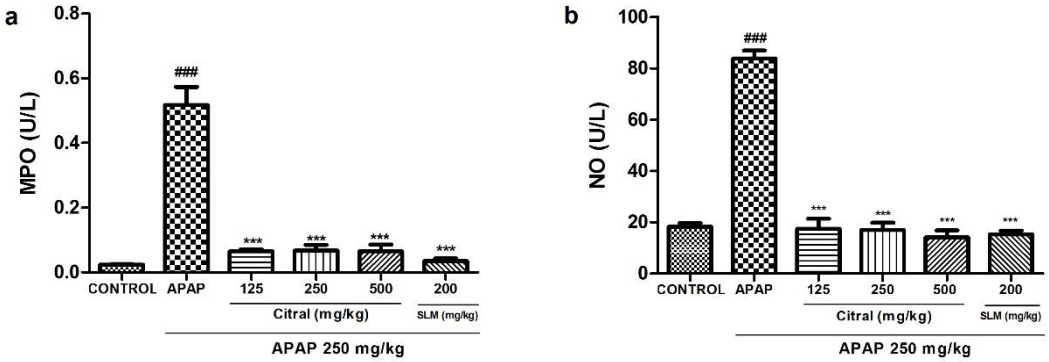

Figure 4: Effect of citral and SLM in: (a) myeloperoxidase activity and (b) nitric oxide production. Values are mean ± SEM (n=5). ### $p < 0.001$ vs animals control groups. *** $p < 0.001$ citral or SLM pretreated groups vs APAP group.

3.6. Liver Histopathology. Histological sections of livers of normal mice showed a lobular architecture and hepatocytes structure normal (Figure 5(a)). On the hand, severe necrotic areas were visible in the APAP-treated group, characterized by large areas of necrosis with centrilobular vein congestion, presence of a dense e/or moderate polymorphonuclear infiltrate and vacuolization of hepatocytes (Figure 5(b)). In the SLM group, it was observed poorly infiltrated cells, a portal area preserved hepatocytes, some vacuolated cells and mild injury were found in the centrilobular region (Figure 5(c)). The severity of hepatic injury has improved with citral pretreatment (Figure 5(d-e)). Animal treated with citral showed mild injuries, whereas hepatic parenchyma without necrosis with centrilobular area preserved and poorly infiltrated cells. Furthermore, a mild vacuolization and the presence of binucleate hepatocyte were observed with citral-pretreatment. Additionally, pretreatment with 500 mg/kg of citral, showed the hepatic parenchyma similar morphology to the control group. The liver architecture was preserved with apparently normal hepatocytes, presence of binucleate hepatocytes and the cellular infiltration as infiltrated cells equivalent to normal (Figure 5(f)).

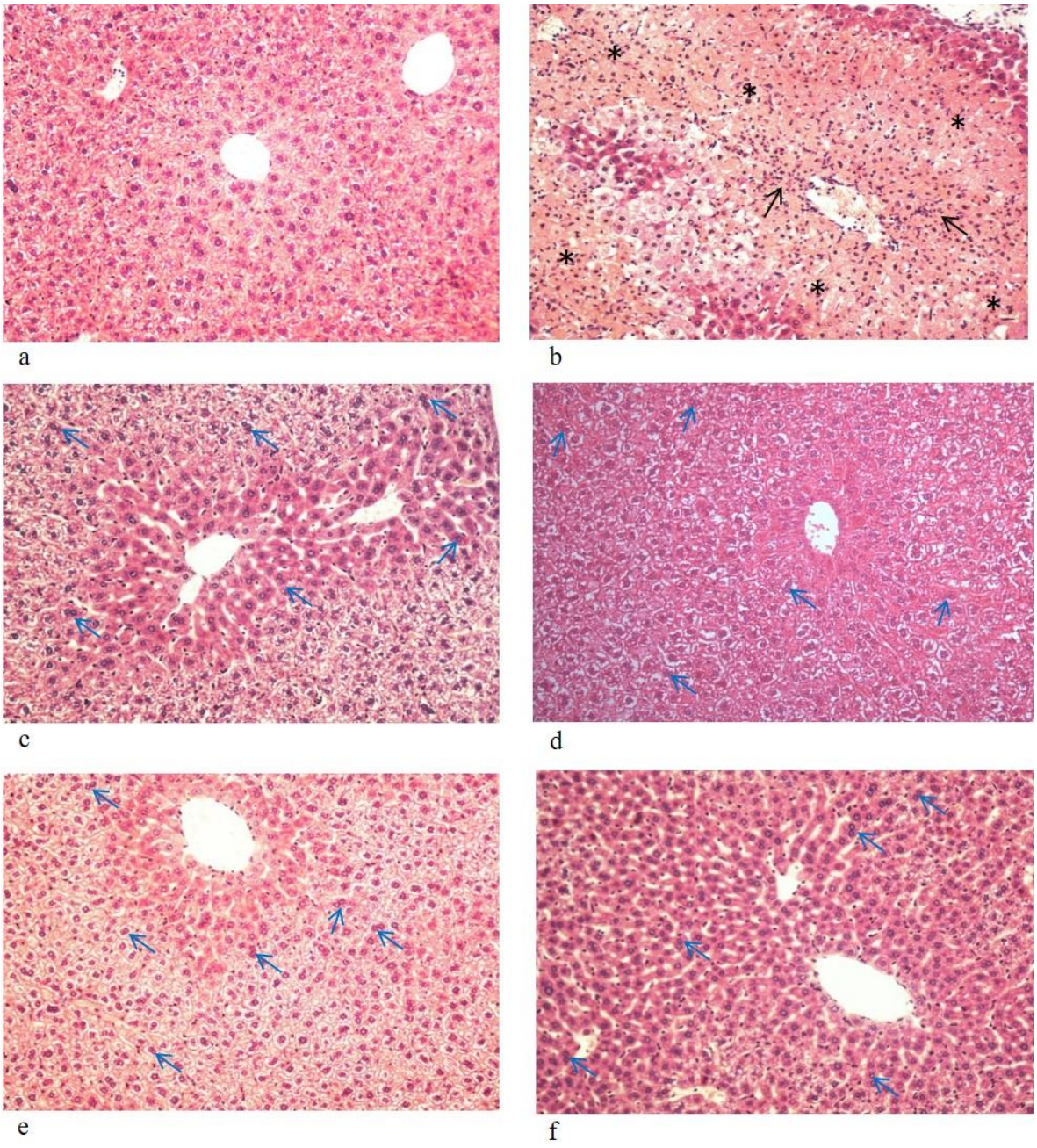

FIGURE 5: Effect of pretreatment with citral in the liver tissue morphology. (a) control mice liver showed normal morphology, absent lesion area; (b) APAP group (mice liver that received orally APAP on last day of treatment, 250 mg/kg): presence of severe necrosis (*), inflammatory infiltrate (arrows); (c) group pretreated with SLM (200 mg/kg) + APAP; (d) 125 mg/kg citral + APAP; (e) 250 mg/kg citral+APAP, (f) 500 mg/kg citral + APAP. In ((c)–(f)) presence of binucleate hepatocytes (arrows blue), and mild lesion area. Original magnification 20x. The sections stained with hematoxylin and eosin.

4. Discussion

Cymbopogon citratus, commonly known as lemongrass, is widely distributed worldwide and is commonly used in traditional Indian, Chinese, and Brazilian medicines [18]. The essential oils from *C. citratus* contain various monoterpenes, being the citral (mixture of neral and geranial), the constituent most pharmacologically and physiologically important [6, 19]. In previous study [11], we demonstrated that lemongrass essential oil improves the hepatic injury caused by APAP, and in this present study, the effect of citral, the major component of this oil was evaluated.

Natural products have important biological properties in disease prevention as in hepatoprotective capacity. This activity of natural products can be explained by its antioxidant properties deriving from monoterpenes, flavonoids, phenols, etc [20]. The DPPH assay has been widely used to determine the free radical scavenging capacity of various samples because of DPPH stability, simplicity, and fast assay [21]. In this assay, citral showed antioxidant properties due to their DPPH scavenging activity, as also observed in previous studies [22–24]. In general, the hepatoprotective activity of plants can be considered as an expression of the functional improvement of hepatocytes that results from accelerated cellular regeneration [13, 20, 25]. Therefore, SLM that has been employed as a protective treatment liver disease by its antioxidant properties deriving from the phenolic nature of flavonolignans [13, 16, 26]. The MTT assay *in vitro* performed was used to evaluate the cell viability after citral treatment. The results showed that citral, even at a higher concentration, did not affect cell viability. Our data were also observed by Bachiega [9], Bouzenna et al. [23] and Shen et al. [8].

In high doses of APAP, the oxidation pathway is initiated by the formation of the reactive metabolite NAPQI, which is generated mainly by the cytochrome P450 enzymes Cyp2e1 in mice and humans [3]. Excessive NAPQI formation after APAP overdose

depletes cellular glutathione, adducts proteins, including mitochondrial proteins, and induces mitochondrial oxidant stress and dysfunction, this results in nuclear DNA fragmentation and necrotic cell death [27].

APAP overdose results in destruction of liver cells that in turn result on the elevation in serum level of enzymes aminotransferases [28–30]. The measuring serum levels of specific liver enzymes such as ALT, AST, ALP and γGT are most commonly used markers in hepatotoxic studies. Therefore, serum hepatic biomarkers analysis are important for identification of liver lesion [16, 28, 30, 31]. In our work, APAP administration (250 mg/kg) caused acute liver injury in mice, characterized by an increase in serum activity of transaminase and phosphatases (AST, ALT, ALP and γGT). In addition, the toxic effects of APAP were also observed histologically, showing severe necrotic areas with centrilobular vein congestion, presence of inflammatory cell infiltration and vacuolization of hepatocytes. In contrast, our results showed that pretreatment with citral, was able to reduce levels of AST, ALT, ALP and γGT at all doses employed, improving liver damage when compared with the APAP-treated mice. The live histopathological analysis in groups pretreated with citral, showed hepatocytes preserved, infiltrated cells equivalent to normal, mild lesion areas and the morphology of the hepatic parenchyma similar to the control group, suggesting a protective effect of citral. Differently of our results, Li et al. [24], did not show a significant difference in the levels of AST and ALT among rats pretreated with citral compared with APAP group. However, in their work, the rats were fed a pelleted laboratory diet with a single dose of citral and the APAP was given intraperitoneally. This difference in the experimental design between the work of Li et al. [24], and our study, may have contributed to the difference in AST and ALT results.

In APAP hepatotoxicity, the oxidant stress results in nuclear DNA fragmentation followed by an inflammatory response, that includes the release of pro-inflammatory cytokines and the activation of immune cells [2, 27]. Neutrophils and macrophages can also potentially aggravate the injury, as well established in hepatic ischemia reperfusion injury and obstructive cholestasis [32, 33]. In the present study, neutrophil migration was measured indirectly by MPO activity and citral-pretreatment decrease the MPO activity compared to the group APAP, suggesting decreased of neutrophil infiltration. These findings were consistent with the reports by Shen et al. [34], in which citral-treatment decreased significantly MPO activity induced by LPS. Moreover, our study of the *in vitro* chemotaxis assay, it was used fMLP as a chemotactic agent which promoted neutrophil migration and citral reduced the leukocyte migration, suggesting an anti-inflammatory activity of this monoterpene. These results were also correlated with a discrete lymphocytic infiltrate observed in citral-pretreated group obtained by histopathological observation of the liver.

NO exerts roles important as mediator of toxicity of APAP on the liver. NO is a well-established marker of inflammation, and inhibition of its production, can be a useful therapeutic strategy in inflammatory diseases [10, 35, 36]. Additionally, others inflammatory mediators, such as TNF-α, IL-1β and IL-6 are associated with the severity of hepatic injury [8, 37, 38]. Several studies demonstrated that citral inhibits the release of important inflammatory mediators, such as TNF-a, IL-1β, IL-6 and NO [8, 9, 38–40], probably by inhibition of iNOS expression, NO production and various LPS-induced pathways, including p38 mitogen-activated protein kinase (MAPK), c-jun NH2-terminal kinase 1/2 and the transcription factor NF-kB, as demonstrated by Lee and collaborators [7, 37]. In our work, the pretreatment with citral, decrease the NO production, suggesting an anti-inflammatory activity of this compound.

Therefore, in this study, we demonstrated that citral has antioxidant activity. The pretreatment with citral prevented hepatic alterations caused by APAP acute toxicity, preventing the increase of hepatic injury markers (ALT, AST, ALP and γGT), MPO activity and NO concentration in hepatic tissues. Some studies have shown the hepatoprotective effect of lemongrass on hepatotoxicity in experimental model in rats or mice [11, 41, 42] and according to our results, we suggest that this improvement in liver damage by pretreatment with lemongrass essential oil, may be associated with citral content, since citral is the major constituent of *C. citratus* essential oil.

5. Conclusion

In conclusion, pretreatment with citral showed hepatoprotective effects on hepatic lesions caused by APAP overdose. This effect may be associated with the reduction of oxidative stress, or have an influence on inflammatory events. Therefore, further studies are necessary to investigate the mechanism by which citral is metabolized to protect the liver.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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CAPÍTULO III

3.1 CONCLUSÃO

Este estudo avaliou o efeito do óleo essencial de capim-limão e citral na hepatotoxicidade induzida por APAP em camundongos. Os resultados indicaram que óleo essencial de capim-limão e o citral possuem efeitos protetores sobre os danos hepáticos induzidos pelo APAP, tanto funcionais quanto estruturais. Além disso foram efetivos em restaurar as alterações da resposta inflamatórias ocorridas durante a intoxicação. Estes efeitos hepatoprotetores podem ser devido as propriedades antioxidantes que o óleo essencial de capim-limão e o citral apresentam, podendo estarem associados também as suas atividades anti-inflamatórias.

3.2 PERSPECTIVAS FUTURAS

Estudos que elucidem alternativas eficazes na prevenção e tratamento das doenças hepáticas ou intoxicações são importantes na saúde pública. Os resultados sugerem um efeito hepatoprotetor do óleo essencial de capim-limão e do citral na toxicidade induzida por paracetamol. Porém mais estudos são necessários para determinar quais mecanismos de ação deste óleo e de seus constituintes para concretizar e compreender seus efeitos hepatoprotetores a novas drogas.

Neste sentido, devido as atividades já descritas para o óleo essencial de capim-limão e o citral mostrando efeito sobre a lesão hepática, nova pesquisa deve ser realizada para validar o óleo essencial de capim-limão e o citral na clínica.