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Hepatoprotective Effect of Pretreatment with Rosemary and Ginger Essential Oil in Experimental Model of Acetaminophen-induced Injury

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RJP and RPA designed the study. Author RAS performed the statistical analysis. Authors SESF wrote the protocol. Author FMSSC wrote the first draft of the manuscript. Authors EMN and CABA managed the analyses of the study. Authors RJP and RPA managed the literature searches. Author RKNC revised the manuscript. All authors read and approved the final manuscript

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ABSTRACT

Aims: 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.
Methodology: 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), alanine aminotransferase (ALT) in mice. Their livers were then used 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.
Results: REO and GEO reduced the levels of the serum marker enzymes AST, ALT, and MPO activity. The essentials oils also exhibited antioxidant activity, reflected by its DPPH radical-scavenging effects and in the lipid peroxidation assay.
Conclusion: These results suggest 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; acetaminophen.

1. INTRODUCTION

Many aromatic plants are considered important sources for the extraction of essential oils and have many applications in ethno-medicine [1]. 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 [2,3].

In folk medicine ginger, *Zingiber officinale* Roscoe (*Zingiberaceae*), has been used for pain, inflammation, arthritis, urinary infections and gastrointestinal disorders [4,5]. In our laboratory, we have demonstrated the anti-inflammatory, antinociceptive and immunomodulatory effects of ginger essential oil in animal models [6,7].

Rosemary (*Rosmarinus officinalis* L., *Lamiaceae*) is a spice widely used in folk medicine, cosmetics, phytopharmacy, and the flavoring of food products. Studies have been focused on various biological activities of the secondary metabolites of this spice such as phenolic compounds, which are powerful antioxidants, hepatoprotective, antimicrobial, antinociceptive and anti-inflammatory agents [4,5,6,7,8,9].

Liver diseases have become one of the major causes of morbidity and mortality all over the world. Acetaminophen (APAP) at large doses causes serious liver injury that may develop into liver failure [7]. Hepatocellular degeneration and necrosis are also associated with elevated enzyme markers, such as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) that indicate hepatotoxicity [2]. Liver injury induced by acetaminophen in mice is a commonly used experimental model for screening substances with potential hepatoprotective activity [10].

To our knowledge, scarce information is available about the effects of ginger (GEO) and rosemary essential oils (REO) in experimental hepatotoxicity models. Therefore, the present

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

2. MATERIALS AND 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 4ºC.

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 and II, received saline that contained 0.1% Tween 80 (APAP, GEO or REO vehicle). 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 only 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 4ºC. Serum ALT and AST levels were then measured using the Analyze Gold® enzymatic test kit.

2.5 Determination of MPO Activity

MPO activity was measured in liver tissues as previously described by Bradley et al. [11]. The livers were used to determine enzymatic 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 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 by Espin et al. [5], 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 x (A_{blank} - A_{sample} / A_{blank}). The IC50 value, representing the concentration of the essential oil that caused 50% RSC inhibition, was determined by linear regression analysis from the obtained RSC values.

2.7 Lipid Peroxidation Assay

A lipid peroxidation assay was performed as previously reported by Dasgupta & De (2004), with a minor modification. Egg yolk homogenates were prepared as lipid-rich media. Briefly, 0.1 ml of REO and GEO (0.5; 5.0, 50.0 and 500 x 10^{-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 37.5ºC. 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 95ºC 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} / $A_{control}$) x 100. $A_{control}$ was considered the absorbance of the control (i.e., methanol, instead of the sample). The IC50 value, representing the concentration of the essential oil that caused 50% inhibition of lipid peroxidation in the Fe2+/ascorbate system, was determined by linear regression analysis from the obtained inhibition (%) values [4].

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

3.1 Determination of Serum ALT and AST

We evaluated the effects of GEO and REO on serum enzyme markers. As shown in (Fig. 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.

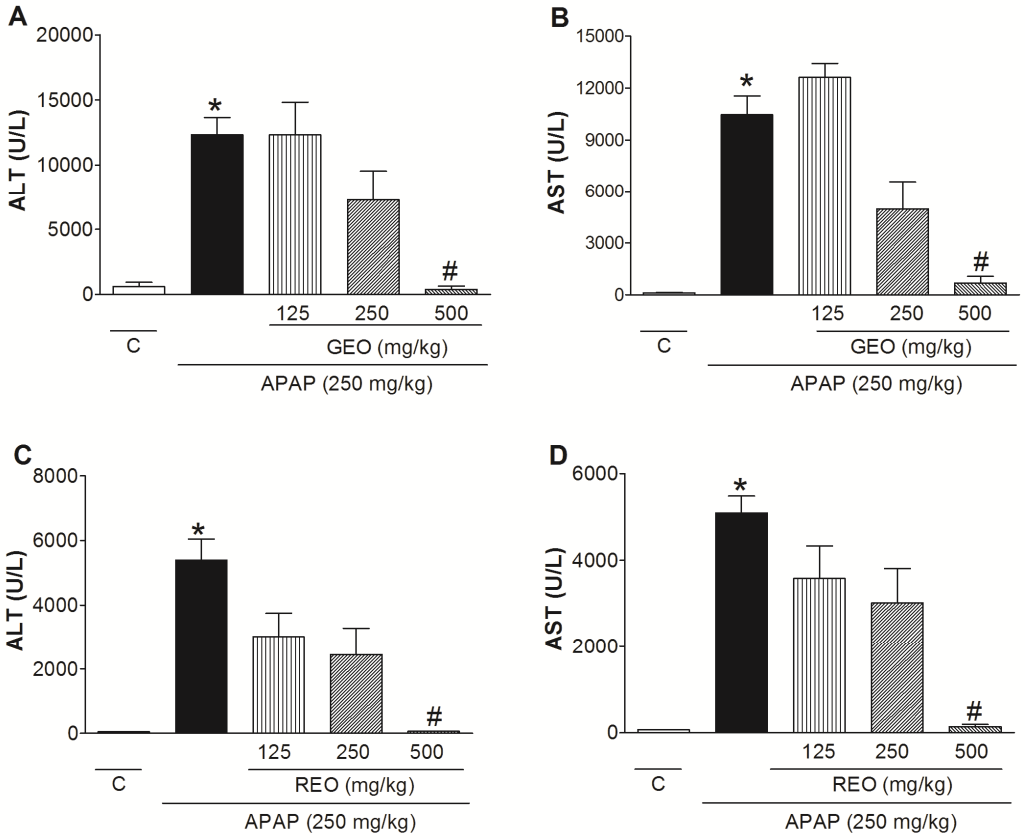

Fig. 1. Ginger essencial oil (GEO) and rosemary essencial oil (REO) reduced the serum ALT and AST on acetaminophen-induced hepatotoxicity in mice

*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*

3.2 Determination of MPO Activity

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

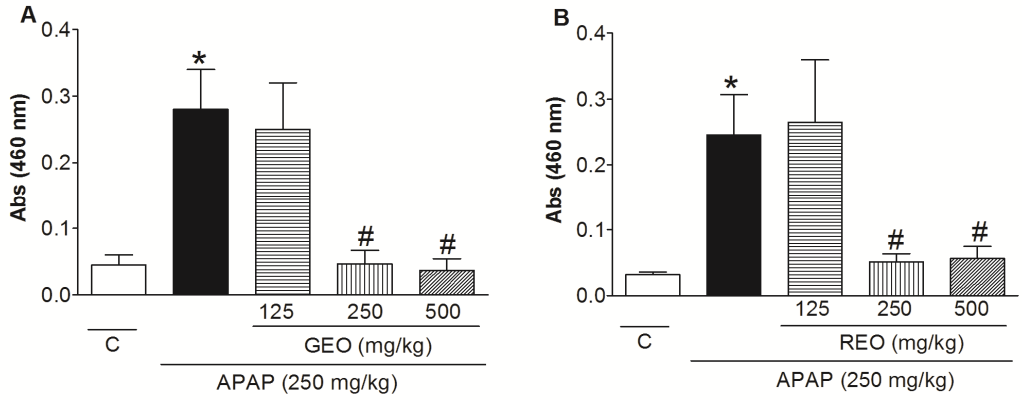

Fig. 2. Effect of the ginger essential oil (GEO) and rosemary essential oil (REO) on MPO enzyme activity in liver of mice

*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*

3.3 DPPH Assay

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).

Table 1. Assessment of DPPH free radical scavenging activity of rosemary essential Oil (REO) and ginger essential Oil (GEO)

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

3.4 Lipid Peroxidation Assay

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).

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

Concentration (mg/ml)x10^{-3}	% Inhibition of radical scavenging	
	REO	GEO
0.5	1	3
5	2	3
50	10	3
500	15	4

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 [8,9,12]. 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 [13,14,15].

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 [16]. 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 [17]. 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 [16,17]. On the other hand, it has been describe by El-Demerdash et al [18] and Pratap & Indira [19], extracts of REO and GEO, respectively, decrease the activity of enzymes, such as SOD (Superoxide Dismutase), GST (Glutathione-S-Transferase), GSH (Glutathione Synthase), CAT (Catalase), GPx (Glutathione peroxidase), reinforcing its important antioxidant activity and maintenance of liver function by the damage caused by oxidative stress.

Various essential oils have been shown to have antioxidant activity [20,21,22]. 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 partially 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 [23]. The mobilization of polymorphonuclear leukocytes to the site of acute inflammation is one of the key process in the host response to tissue injury. MPO is a marker of polymorphonuclear leukocytes, and MPO activity is directly related to the amount of leukocyte infiltration, which is indicative of an inflammatory reaction [24]. Previous results shown by our research group confirmed the anti-inflamatory activity of GEO and REO [6,25,26]. 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 [27,28]. Furthermore, previous studies have also shown that some essential oils have properties of scavenging free radicals and antioxidants hepatoprotective activity [20]. 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.

CONSENT

Not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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