

Lavender (*Lavandula officinalis*) essential oil prevents acetaminophen-induced hepatotoxicity by decreasing oxidative stress and inflammatory response

Óleo essencial de lavanda (*Lavandula officinalis*) previne a hepatotoxicidade induzida pelo paracetamol ao diminuir o estresse oxidativo e a resposta inflamatória

El aceite esencial de lavanda (*Lavandula officinalis*) previene la hepatotoxicidad inducida por el paracetamol al disminuir el estrés oxidativo y la respuesta inflamatoria

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Abstract

Acetaminophen (N-acetyl-p-aminophenol, APAP) is the most popular drug recommended and consumed for relieving mild and moderate pain and fever. Although effective in therapeutic doses, APAP overdose induces hepatotoxicity, causing acute liver failure. In this study, the hepatoprotective effects and the underlying mechanisms of *Lavandula officinalis* essential oil (LEO) were investigated in APAP-induced hepatotoxicity. To evaluate the hepatoprotective effect, Balb/c mice were pretreated with LEO at doses of 200 and 400 mg/kg, once daily for seven days. On the seventh day, mice were treated with APAP (250 mg/kg) to induce hepatotoxicity. LEO significantly decreased serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transferase (γ -GT) compared to the APAP group. Besides, a decrease in myeloperoxidase (MPO) activity, nitric oxide (NO), and pro-inflammatory cytokines levels were observed in liver samples of the LEO treated mice. Moreover,

pretreatment with LEO showed significant antioxidant activity by decreasing the levels of malondialdehyde (MDA), carbonylated proteins, reactive oxygen species (ROS), and glutathione (GSH), in addition to increasing levels of the hepatic superoxide dismutase (SOD), catalase (CAT), and oxidized glutathione (GSSG). Our results showed that LEO improved liver functions altered by APAP by inhibiting oxidative stress and inflammatory induced by APAP and other oxidative stress-mediated toxicities.

Keywords: Acute hepatic injury; Lavender essential oil; Oxidative stress; Antioxidant; Hepatotoxicity.

Resumo

O acetaminofeno (N-acetil-p-aminofenol, APAP) é o medicamento popular mais recomendado e consumido para o alívio da dor e febre leves e moderadas. Embora eficaz em doses terapêuticas, a overdose de APAP induz hepatotoxicidade, causando insuficiência hepática aguda. Neste estudo, os efeitos hepatoprotetores e os mecanismos subjacentes do óleo essencial de *Lavandula officinalis* (LEO) foram investigados na hepatotoxicidade induzida por APAP. Para avaliar o efeito hepatoprotetor, camundongos Balb/c foram pré-tratados com LEO nas doses de 200 e 400 mg/kg, uma vez ao dia, durante sete dias. No sétimo dia, os camundongos foram tratados com APAP (250 mg/kg) para induzir a hepatotoxicidade. LEO diminuiu significativamente os níveis séricos de alanina aminotransferase (ALT), aspartato aminotransferase (AST), fosfatase alcalina (ALP) e gama-glutamil transferase (γ -GT) em comparação com o grupo APAP. Além disso, uma diminuição na atividade da mieloperoxidase (MPO), óxido nítrico (NO) e níveis de citocinas pró-inflamatórias foram observados em amostras de fígado dos camundongos tratados com LEO. Além disso, o pré-tratamento com LEO apresentou atividade antioxidante significativa pela diminuição dos níveis de malondialdeído (MDA), proteínas carboniladas, espécies reativas de oxigênio (ROS) e glutathione (GSH), além de aumentar os níveis da superóxido dismutase hepática (SOD), catalase (CAT) e glutathione oxidada (GSSG). Nossos resultados mostraram que o LEO melhorou as funções hepáticas alteradas pelo APAP ao inibir o estresse oxidativo e a inflamação induzida pelo APAP.

Palavras-chave: Lesão hepática aguda; Óleo essencial de lavanda; Estresse oxidativo; Antioxidante; Hepatotoxicidade.

Resumen

El acetaminofén (N-acetil-p-aminofenol, APAP) es el fármaco más popular recomendado y consumido para aliviar el dolor y la fiebre leves y moderados. Aunque es eficaz en dosis terapéuticas, la sobredosis de APAP induce hepatotoxicidad, provocando insuficiencia hepática aguda. En este estudio, se investigaron los efectos hepatoprotectores y los mecanismos subyacentes del aceite esencial de *Lavandula officinalis* (LEO) en la hepatotoxicidad inducida por APAP. Para evaluar el efecto hepatoprotector, se pretrató a ratones Balb/c con LEO en dosis de 200 y 400 mg/kg, una vez al día durante siete días. El séptimo día, los ratones se trataron con APAP (250 mg/kg) para inducir hepatotoxicidad. LEO redujo significativamente los niveles séricos de alanina aminotransferasa (ALT), aspartato aminotransferasa (AST), fosfatasa alcalina (ALP) y gamma-glutamil transferasa (γ -GT) en comparación con el grupo APAP. Además, se observó una disminución en los niveles de actividad de mieloperoxidasa (MPO), óxido nítrico (NO) y citocinas proinflamatorias en muestras de hígado de los ratones tratados con LEO. Además, el pretratamiento con LEO mostró una actividad antioxidante significativa al disminuir los niveles de malondialdehído (MDA), proteínas carboniladas, especies reactivas de oxígeno (ROS) y glutatión (GSH), además de aumentar los niveles de superóxido dismutasa hepática (SOD), catalasa (CAT) y glutatión oxidado (GSSG). Nuestros resultados mostraron que LEO mejoró las funciones hepáticas alteradas por APAP al inhibir el estrés oxidativo e inflamatorio inducido por APAP y otras toxicidades mediadas por estrés oxidativo.

Palabras clave: Lesión hepática aguda; Aceite esencial de lavanda; Estrés oxidativo; Antioxidante; Hepatotoxicidad.

1. Introduction

Acetaminophen or paracetamol (N-acetyl-p-aminophenol, APAP) is the most popular drug recommended and consumed for relieving mild and moderate pain and fever due to its antipyretic and analgesic properties (Subramanya et al., 2018). Although its efficacy has been confirmed in therapeutic doses, APAP overdose induces hepatotoxicity causing acute liver failure (ALF). More than 30,000 are admitted to the hospital each year, being APAP toxicity responsible for most cases of ALF (Athersuch et al., 2018; Blieden et al., 2014; Ghanem et al., 2016). Besides, liver transplantation is the only effective treatment for APAP toxicity (Tezcan et al., 2018).

At therapeutic doses, APAP is metabolized to nontoxic metabolites in the liver and easily excreted by the kidney via glucuronidation and sulfation. Only a small fraction is metabolized by oxidation via cytochrome P450s (CYPs) to a highly toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). After an overdose, the glucuronidation and sulfation routes become saturated and produce excess NAPQI, leading to the rapid depletion of the hepatic glutathione (GSH) pool. Subsequently, if

glutathione is not replenished, NAPQI covalently binds to proteins, modify their structure and function, initiate lipid peroxidation mediated by reactive oxygen species (ROS), inducing oxidative stress and liver tissue damage (Du et al., 2016; Eugenio-Pérez et al., 2016; Lee, 2017). The hunt for alternative, safe, and therapeutically effective compounds against APAP-induced liver toxicity may be an important strategy to attenuate liver damage.

Currently, medicinal plants are an alternative strategy applied in the treatment of various diseases to avoid the side effects of drugs, due to their low toxicity and relevant antioxidant potential (Xie et al., 2015). Several studies reported the hepatoprotective effect of natural products against APAP-induced hepatotoxicity (da Rocha et al., 2017; Freitag et al., 2015; Pinho et al., 2014; Uchida et al., 2017; Uchida et al., 2017). In this context, the constituents of lavender essential oil (LEO) are secondary metabolites extracted from *Lavandula officinalis* with many pharmacological effects such as anti-inflammatory (Cardia et al., 2018), antidepressant and anxiolytic (Woelk & Schläfke, 2010), cardioprotective (Ziaee et al., 2015), analgesic (Silva et al., 2015), wound healing (Mori et al., 2016), antimicrobial and antioxidant (Niksic et al., 2017). Therefore, the protective effects of LEO on acetaminophen-induced hepatic damage in mice were investigated. Use the paragraph as a template

2. Methodology

2.1 Chemicals

APAP, Silymarin (SLM), Zymosan, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide), O-dianisidine dihydrochloride, Carboxymethyl cellulose (CMC), Dinitrophenylhydrazine (DNPH), 2',7'-dichlorofluorescein diacetate (DCFH-DA) were provided by Sigma-Aldrich (St. Louis, MO, USA). The enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), the hepatobiliary injury biomarkers alkaline phosphatase (ALP), and gamma-glutamyl transferase (γ -GT) were purchased from Gold Analisa Diagnostica (Belo Horizonte, MG, Brazil). Kits for the measurement of interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and tumor necrosis factor α (TNF- α) were purchased from Life Technologies Corp (Carlsbad, CA, USA). Routinely used chemicals and standards were provided by Sigma-Aldrich (St. Louis, MO, USA).

2.2 Plant Material

The aerial parts (leaves and stem) without inflorescence of the *Lavandula officinalis* were commercially purchased in June of 2019 from Cercopa Guarapuava, PR, Brazil. A voucher specimen was identified and deposited by Herbarium of the State University of Maringá (HUEM000004622), Maringá, PR, Brazil.

2.3 Essential oil extraction

The lavender essential oil was obtained by hydrodistillation of its leaves and stem (1.0 kg) for 3 hours using a Clevenger-type apparatus. The essential oil was extracted, dried over sodium sulfate, and stored at 4°C in dark vials until analysis. Essential oil yield (w/w %) was calculated on a dry weight basis of the plant (Sumalan et al., 2020).

2.4 Essential oil analysis

The lavender essential oil was analyzed using Gas chromatography–Mass Spectrometry (GC-MS) and Nuclear magnetic resonance spectroscopy following the conditions described in our previous study (Nancy Sayuri Uchida et al., 2017).

2.5 MTT assay

The cell viability of lavender essential oil was determined according to the MTT assay (Silva-Filho et al., 2015). The mice received a zymosan solution (1 mg/cavity, i.p.) for collection of leukocytes from the peritoneal cavity. Then, cells were incubated with LEO in concentrations of 3, 10, 30, or 90 μ g/mL for 90 min. After the incubation, a volume of 10 μ L of MTT

(5 mg/mL) was added. Two hours later, 150 μ L of supernatant was removed, and 100 μ L of dimethyl sulfoxide was added to a 96-well plate. The absorbance of the solution was read in the ELISA reader (ASYS Expert Plus; Biochrom Ltd., Cambridge, UK) at a wavelength of 540 nm, and the percentage of viability was determined as previously reported (Cardia et al., 2018).

2.6 Animals

The experiments were performed on male Balb/c mice (6 weeks old), weighing 25 ± 2 g. They were obtained at the Central Animal House of the State University of Maringá, PR, Brazil, and maintained in our animal housed under standard laboratory conditions with temperature (24 ± 1 °C) and humidity (60–65%) for a 12 h light-dark cycle. Standard pellet food and water ad libitum were provided. The animals use protocol was appreciated and approved by the Committee on Animal Care of State University of Maringá (CEUA/UEM N° 2534270418).

2.7 Experimental Design of APAP-Induced Hepatotoxicity

Mice were acclimated for 1 week before initiation of any experiment. The animals were randomly assigned to the following five groups (n = 8/group), as follows: Group I: The normal control group received CMC, the vehicle used in dissolving APAP and LEO. Group II: The APAP group received a single administration of acetaminophen (250 mg/kg) on day 7. Groups III and IV, pre-treatment groups, mice were treated with lavender essential oil at doses of 200 and 400 mg/Kg, respectively. Group V: The mices were pretreated with SLM (200 mg/kg), which is universally used as a standard drug for its hepatoprotective properties (Papackova et al., 2018). The treatments with CMC, SLM, or LEO was carried once a day orally for seven days. All selected doses for LEO or APAP are within the dose range used in previous studies (Rocha et al., 2017; Grespan et al., 2014; Uchida et al., 2017; Uchida et al., 2017).

On day 7, groups II-V received 250 mg/kg APAP to induce acute liver injury. Twelve hours after APAP administration, the animals were anesthetized with a single dose of thiopental (50 mg/kg i.p.). Blood was collected from the cava vein in heparin-containing tubes for hepatic enzyme assays. Livers samples were delicately extracted, weighed, and stored at -80 °C for further analysis.

2.8 Determination of serum liver biomarkers

Approximately 1 mL of blood was collected from the vena cava of the animals. The serum was immediately separated using a centrifuge (5,000G for 15 min at 4°C) for biochemical assessment. The serum activities of ALT, AST, ALP and, γ -GT were used as liver toxicity markers and measured by using the corresponding commercial kits (Gold Analisa® Diagnostic, Brazil) according to the manufacturer's instructions.

2.9 Myeloperoxidase Assay

Myeloperoxidase Activity (MPO) was evaluated by the colorimetric method as described by Bradley et al. (Bradley et al., 1982). In brief, tissue samples were homogenized in 1 mL of sodium phosphate buffer in a Potter homogenizer. Next, the homogenate was centrifuged at 5000G for 15 min at 4 °C. Lastly, a 10 μ L aliquot of the supernatant was transferred in triplicate to each well of a microplate, which previously received 200 μ L of solution that contained O-dianisidine dihydrochloride (16.7 mg), distilled water (90 mL), potassium phosphate buffer (10 mL), and 1% hydrogen peroxide (50 μ L). Fifteen minutes later, sodium acetate was added to stop the enzymatic reaction. The absorbance was determined using a microplate spectrophotometer (ASYS Expert Plus; Biochrom Ltd., Cambridge, UK) with a wavelength of 460 nm.

2.10 Nitric Oxide Production

The nitrite concentration was determined as an indicator of nitric oxide (NO) and quantified by the Griess method (Cardia et al., 2018). Fifty microliters of the liver tissue samples were plated in triplicate into a 96-well plate were added to 100 μ L of Griess reagent (0.1% naphthyl ethylenediamine and 1% sulfanilamide in 5% H₃PO₄), and incubated for 10 min. Next, the absorbance was measured at 550 nm using an ELISA plate reader (ASYS Expert Plus; Biochrom Ltd., Cambridge, UK).

2.11 Cytokine Assays in liver tissue

The cytokines IL-1 β , IL-6, and TNF- α levels were evaluated in the supernatant of liver tissue sections of the different groups by immunoassay method using a MAGPIX Luminex[®] Analyzer (Luminex Corporation, USA), based on the manufacturer's instructions.

2.12 Liver Oxidative Stress Parameters

In this study, the oxidative damage parameters were determined in liver homogenates. The livers samples were homogenized in potassium phosphate buffer using a Potter homogenizer. One part obtained was separated, and the other was centrifuged at 10,000 rpm for 15 minutes and the supernatant was removed for subsequent evaluation.

Protein carbonyl groups were evaluated according to the method described by Levine et al (Levine et al., 1990). The carbonyl content was measured by the absorbance of the samples at 370 nm and the values were presented as nmol.per.mg protein. Lipid peroxidation was determined using the thiobarbituric acid-reactive substances (TBARS) assay. The amount of lipoperoxides was measured at 532 nm using a standard curve of malondialdehyde (MDA) (Buege & Aust, 1978).

The levels of reactive oxygen species (ROS) were determined spectrofluorimetrically in liver homogenate using 2',7'-dichlorofluorescein diacetate (DCFH-DA) assay. The assay quantifies the oxidation of DCFH-DA in the presence of ROS. Briefly, the formation of DCF was determined using a spectrofluorimeter RF-5301 (Shimadzu Corporation, Kyoto, Japan) at an excitation wavelength of 504 nm and an emission wavelength of 529 nm (Siqueira et al., 2005).

GSH levels and oxidized glutathione (GSSG) were determined in liver homogenate spectrofluorimetrically through the o-phthalaldehyde (OPT) assay. In brief, OPT reacts with the samples, generating fluorescence. Thus, the contents of GSH and GSSG were determined from comparisons with a standard curve measured spectrofluorimetrically (Hissin & Hilf, 1976).

The activities of antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were determined by spectrophotometric. The CAT activities were determined in supernatants of the liver samples by the method described by Aebi (Aebi, 1984). The rate of reduction with a superoxide anion is inhibited by SOD. Therefore, the inhibition activity of SOD can be determined by a colorimetric method measured at 450 nm, as described by Marklund (Marklund & Marklund, 1974).

2.13 Statistical analysis

Experimental data were reported as the mean \pm standard error of the mean (S.E.M). Significance was assessed by one-way analysis of variance (ANOVA), followed by Tukey's test using GraphPad Prism 5.0 (GraphPad Software[®], San Diego, CA, USA) to assess differences between means. P values of < 0.05 were taken to be statistically significant.

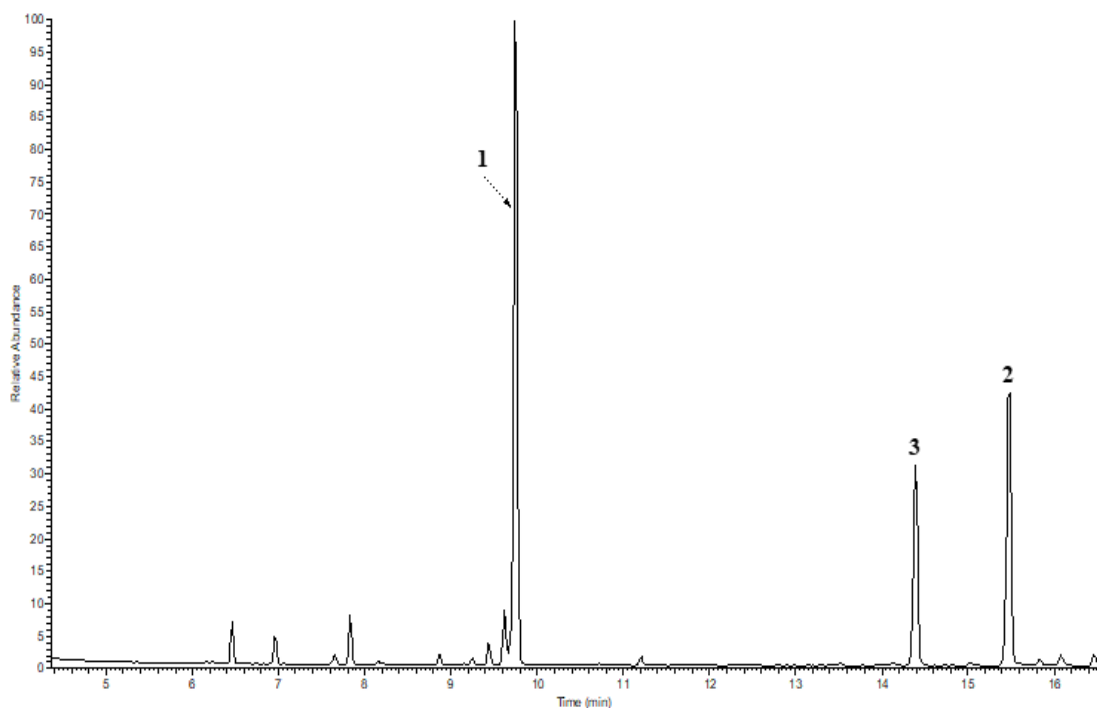
3. Results

3.1 Analysis of lavender essential oil

Hydrodistillation of leaves and stem of the *Lavandula officinalis* provided a pale-yellow essential oil with a yield of 0.45% (w/w). GC-MS analysis of the lavender essential oil (Table 1 and Figure 1) showed the presence of various compounds. The major chemical constituents are 1,8-cineole (41.22%), borneol (21.69%) and, camphor (14.83%). The components of the oil

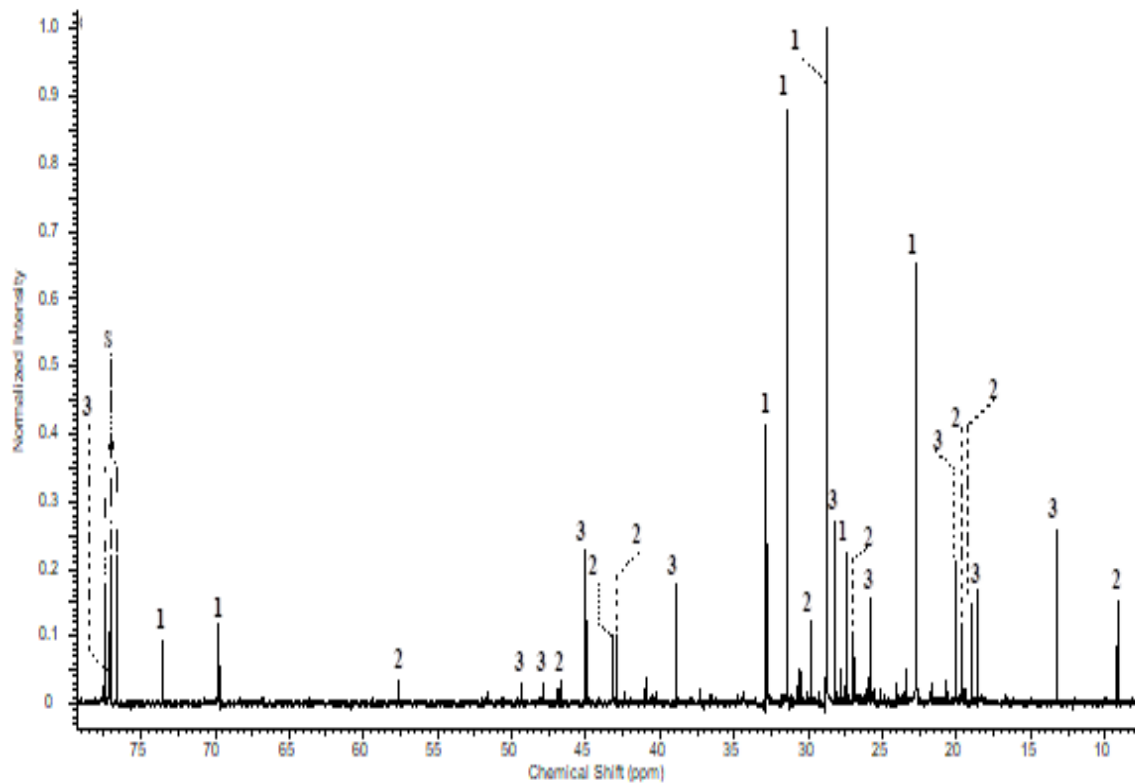
were identified by comparing their RI and mass spectra with the literature (Adams, 2007). To identify the main compounds of LEO, the chemical shifts of each carbon in the ^{13}C NMR spectrum (Figure 2) were compared with carbons signals in the spectrum of pure compounds.

Figure 1. GC-MS analysis of the *Lavandula officinalis* essential oil. The numbers on the peaks are attributed to main phytochemicals of LEO: (1) 1,8-cineole, (2) borneol, and (3) camphor.



Source: Authors.

Figure 2. ^{13}C NMR Spectrum of the *Lavandula officinalis* essential oil. The numbers on the peaks are attributed to carbon signals of the majority of compounds: (1) cineole, (2) borneol, and (3) camphor.



Source: Authors.

Table 1. Chemical Composition of Essential Oil of *Lavandula officinalis*.

| RI ^a | Compounds | % RA ^b | Identification methods ^c |
|-----------------|--------------------------------------|-------------------|-------------------------------------|
| 931 | α -pinene | 2.05 | MS, RI |
| 947 | camphene | 1.72 | MS, RI |
| 970 | sabinene | 0.68 | MS, RI |
| 976 | β -pinene | 2.56 | MS, RI |
| 987 | α -phellandrene | 0.30 | MS, RI |
| 1018 | <i>p</i> -cymene | 0.48 | MS, RI |
| 1023 | <i>o</i> -cymene | 1.22 | (MS, RI)* |
| 1027 | limonene | 3.21 | MS, RI |
| 1030 | 1,8-cineole | 41.22 | MS, RI |
| 1068 | <i>cis</i> -sabinene hydrate | 0.60 | (MS, RI)* |
| 1145 | camphor | 14.83 | MS, RI |
| 1160 | pinocarvone | 0.27 | (MS, RI)* |
| 1170 | borneol | 21.69 | MS, RI |
| 1179 | terpinen-4-ol | 0.56 | MS, RI |
| 1184 | cryptone | 0.97 | (MS, RI)* |
| 1193 | dihydrocarveol | 1.00 | (MS, RI)* |
| 1226 | <i>cis</i> -sabinene hydrate acetate | 0.69 | (MS, RI)* |
| 1240 | cumin aldehyde | 0.70 | (MS, RI)* |
| 1416 | caryophyllene | 0.49 | MS, RI |
| 1509 | γ -cadinene | 0.72 | MS, RI |
| 1577 | caryophyllene oxide | 0.30 | MS, RI |
| ----- | other minor compounds | 3.74 | - |

^aRI= Retention indices relative to *n*-alkane series C₈H₁₈ – C₂₀H₄₂ on the DB-5 column (Van Den Dool & Kratz, 1963).

^bRelative area. ^cRetention index (RI) and mass spectra (MS). *Identification based on the literature (Sparkman, 2005).
Source: Authors.

3.2 MTT assay

In this study, the cell viability of LEO was evaluated at concentrations of 3, 10, 30, and 90 μ g/ml. The results presented cell viability of 85, 84, 78, and 76%, respectively, indicating that LEO has low cytotoxicity *in vitro* with cell viability greater than 75%.

3.3 Serum biochemical parameters

The values of liver marker enzymes in groups of animals treated with APAP, SLM, and LEO are shown in Table 2. The levels of ALT, AST, ALP, and γ -GT in the serum were increased significantly with APAP administration, indicating liver injury. On the other hand, changes in hepatic enzymes increased by APAP were restored by treatment with LEO at doses of 200 and 400 mg/kg in AST activity (85.2% and 93.6%, respectively), ALT (88.8% and 91.5%, respectively), ALP (46.8% and 47%, respectively), and γ -GT (58.6% and 67.2%, respectively), when compared to the APAP group. Besides, 200 mg/kg of silymarin reduced toxicity, and the serum parameter were restored to normal values.

Table 2. The effects of the pretreatment with LEO on the serum activities in acetaminophen-induced hepatotoxicity

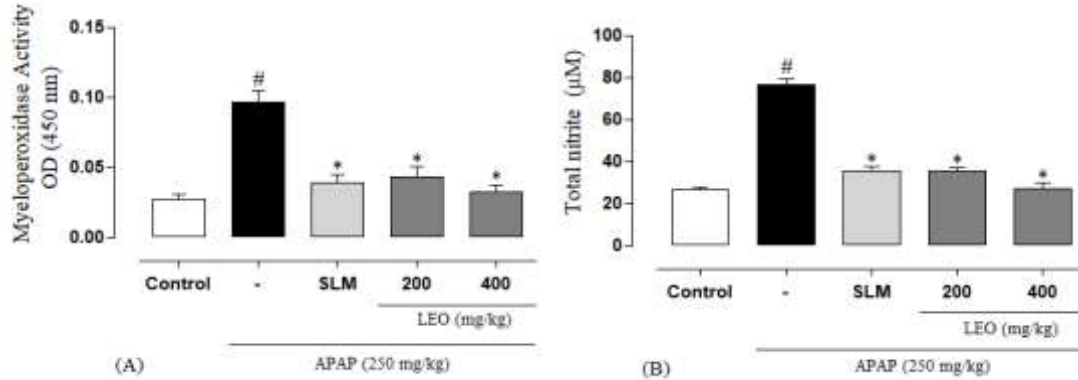
| | AST (U/L) | ALT (U/L) | ALP (U/L) | γ -GT (U/L) |
|---------------------------|------------------------------------|------------------------------------|--------------------------------|--------------------------------|
| Control | 47.67 \pm 6.11 | 27.00 \pm 4.77 | 27.12 \pm 2.40 | 7.75 \pm 1.11 |
| APAP (250 mg/kg) | 1683.00 \pm 159.80 ^{##} | 3434.00 \pm 308.80 ^{##} | 51.83 \pm 4.33 [#] | 22.17 \pm 2.99 ^{##} |
| LEO (200 mg/kg) + APAP | 249.00 \pm 41.48 ^{**} | 383.30 \pm 113.40 ^{**} | 27.14 \pm 3.26 ^{**} | 9.10 \pm 0.91 ^{**} |
| LEO (400 mg/kg) + APAP | 107.90 \pm 24.98 ^{**} | 290.00 \pm 68.34 ^{**} | 27.05 \pm 1.68 ^{**} | 7.25 \pm 0.84 ^{**} |
| SLM (200 mg/kg) + APAP | 461.80 \pm 75.98 ^{**} | 508.20 \pm 54.27 ^{**} | 34.88 \pm 4.01 [*] | 7.08 \pm 0.75 ^{**} |

Results are expressed as mean \pm S.E.M, n = 8/group; # p < 0.01, ## p < 0.001 statistically significant compared to control animals. * p < 0.01, ** p < 0.001 statistically significant to APAP treated animals. LEO: Lavender essential oil, APAP: N-acetyl-p-aminophenol, SLM: Silymarin, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, γ -GT: Gamma-glutamyl transferase, U/L: Units per litre. Source: Authors.

3.4 MPO activity and NO production

The APAP overdose increased hepatic NO content and MPO activity to about 185% and 416%, respectively, when compared with the control animals. On the other hand, pretreatment with LEO at doses of 200 and 400 mg/kg significantly decreased hepatic NO content to about 53% and 64%, and MPO activity to about 74% and 79%, respectively, as compared to the APAP group, which may partially reduce the inflammatory injury in the liver (Figure 3). Moreover, the SLM group as well showed a significant reduction in the MPO activity (82%) and NO concentration (53%).

Figure 3. The effect of lavender essential oil (LEO) and silymarin (SLM) on myeloperoxidase activity (A) and nitric oxide production (B) in liver samples. Values are mean \pm SEM n = 8/group; #p < 0.001 versus animals control groups. *p < 0.001 versus APAP group.

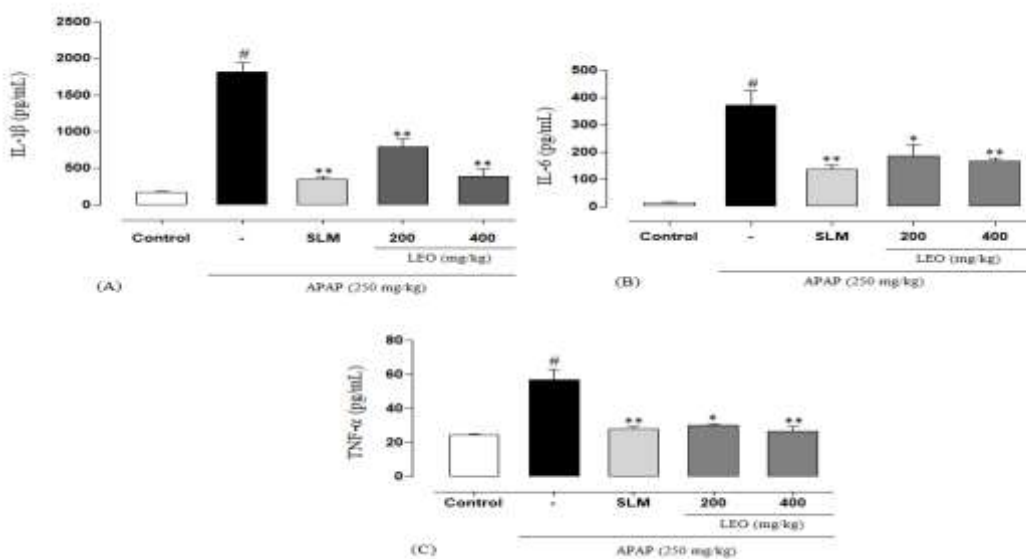


Source: Authors.

3.5 LEO Inhibits APAP-Induced overproduction of proinflammatory cytokine

To investigate the effect of LEO on the APAP-induced inflammatory response, we determined the levels of IL-1 β , IL-6, and TNF- α in the liver of animals. Proinflammatory cytokines are considered relevant in the control of hepatic injury-associated inflammation. The IL-1 β , IL-6, and TNF- α levels were increased in APAP-treated mice, while LEO and SLM (reference drug) treatment significantly decreased the cytokine production. As shown in Figure 4.

Figure 4. Effects of LEO on modulation of proinflammatory cytokines (A) interleukin-1 β , (B) interleukin-6, and (C) tumor necrosis factor- α in APAP-intoxicated mice. The results are expressed as mean \pm S.E.M n = 8/group; # p < 0.001 statistically significant compared to control animals. * p < 0.01 and ** p < 0.001 statistically significant to APAP treated animals.



Source: Authors.

3.6 Oxidative Stress Assays and Antioxidant Enzyme

To determine the effects of LEO on oxidative stress induced by APAP, levels of different oxidative markers and antioxidant enzyme activities were evaluated. Lipid peroxidation (TBARS) caused by APAP is commonly associated with Reactive oxygen species (ROS) generation. Our results demonstrated that administration of APAP resulted in increased levels of TBARS, ROS, and protein carbonyl, when compared with the control group, indicating oxidative stress in the liver. Remarkably, the pretreatment with SLM or LEO at doses of 200 and 400 mg/Kg significantly diminished the levels of TBARS in 29.8%, 24.3% and 20.7%, respectively. These treatments also reduced carbonyl protein content and the generation of ROS to a level comparable to control (Table 3).

APAP overdose reduced the liver homogenate GSH levels when compared to the normal control group, while LEO at doses of 200 and 400 mg/Kg and SLM treatment significantly reversed this reduction. The content of GSSG in the liver of the APAP group was 187% higher than that in the controls. However, the treatment with LEO or SLM reduced GSSG to levels close to the control values, demonstrating that LEO could be modulating the glutathione system (Table 3).

To further confirm the antioxidant effects of LEO, the activities of Hepatic SOD and CAT were measured. CAT and SOD activities were decreased significantly in the APAP group compared to the control group, as shown in Table 3. However, the pretreatment with LEO 200 and 400 mg/kg caused a significantly enhanced SOD (57% and 52%, respectively) and CAT (128% and 130%, respectively) compared with those of the APAP group. Similar results were obtained in the SLM group..

Table 3. The effects of pretreatment with LEO on liver oxidative stress parameters.

| | Control | APAP | SLM | LEO 200mg/kg | LEO 400mg/kg |
|---|--------------|----------------------------|----------------|----------------|----------------|
| TBARS, nmol·mg ⁻¹ | 87.16 ± 3.79 | 189.10 ± 4.65 [#] | 132.60 ± 3.89* | 142.90 ± 5.09* | 149.80 ± 5.91* |
| ROS, nmol·mg ⁻¹ | 0.64 ± 0.04 | 0.97 ± 0.04 [#] | 0.69 ± 0.09* | 0.74 ± 0.04* | 0.63 ± 0.03* |
| Protein carbonyl, nmol·mL ⁻¹ | 1.17 ± 0.02 | 4.61 ± 0.04 [#] | 3.10 ± 0.27* | 3.06 ± 0.23* | 1.53 ± 0.06* |
| GSH, nmol·mg ⁻¹ | 37.25 ± 1.01 | 16.84 ± 0.95 [#] | 36.63 ± 3.13* | 38.98 ± 2.28* | 39.89 ± 3.37* |
| GSSG, nmol·mg ⁻¹ | 1.86 ± 0.15 | 5.34 ± 0.56 [#] | 3.72 ± 0.33* | 3.2 ± 0.25* | 2.43 ± 0.23* |
| CAT, nmol·min·mg ⁻¹ | 1.25 ± 0.03 | 0.39 ± 0.02 [#] | 0.81 ± 0.02* | 0.89 ± 0.03* | 0.90 ± 0.04* |
| SOD, U·mg ⁻¹ | 2.74 ± 0.07 | 1.49 ± 0.03 [#] | 2.28 ± 0.06* | 2.35 ± 0.04* | 2.27 ± 0.06* |

The results are expressed as mean ± S.E.M, n = 8/group; # p < 0.001 statistically significant compared to control animals. * p < 0.01 statistically significant to APAP treated animals. LEO: Lavender essential oil, APAP: N-acetyl-p-aminophenol, SLM: Silymarin, TBARS: Thiobarbituric acid-reactive substances, ROS: reactive oxygen species, GSH: Reduced glutathione, GSSG: oxidized glutathione, CAT: Catalase, SOD: Superoxide dismutase, U·mg⁻¹: Units per mg protein. Source: Authors.

4. Discussion

Aromatic plants are often used in traditional medicine and plant secondary metabolites are widely used as antioxidants and anti-inflammatory for the prevention and treatment of various diseases (Ames-Sibin et al., 2018; da Rocha et al., 2017; Edris, 2007; Freitag et al., 2015; Silva-Comar et al., 2014; Silva-Filho et al., 2015). Lavender essential oil isolated from *Lavandula officinalis* is characteristic of showing strong fragrance, indicating the presence of monoterpenes as demonstrated by GC-MS analysis. Several of these phytochemicals have important pharmacological effects (da Rocha et al., 2017; Freitag et al., 2015; Pinho et al., 2014; Uchida, et al., 2017; Uchida et al., 2017; Xie et al., 2015). For example, the main constituents such as 1,8-cineol and camphor have been reported with anti-inflammatory, antioxidant, antinociceptive, and hepatoprotective activities

(Ciftci et al., 2011; Jiang et al., 2019; Santos et al., 2001; Silva-Filho et al., 2015). Besides, the *in vitro* MTT assay has shown that LEO does not present cytotoxicity, similar to a previous study (Cardia et al., 2018). The APAP overdose results in oxidative damage, hepatocellular necrosis, depletion of hepatic glutathione, inflammatory infiltrates, hepatocyte vacuolization, and mitochondrial dysfunction promoting the elevation in serum level of enzymes transferases such as ALT and AST, as well as, the simultaneous increase in hepatobiliary injury biomarkers (ALP and γ -GT) (Lee, 2017; Robles-Diaz et al., 2015; Shan et al., 2018).

In the present work, we demonstrated that pretreatment with LEO considerably reduced the elevation of the serum AST, ALT, ALP, and γ -GT at all doses employed. In agreement, another study showed that lavender essential oil treatment protects against malathion-induced liver injury reducing the increase in AST, ALT, ALP, and γ -GT activities (Selmi et al., 2015). The capacity of LEO to attenuate the increase of these enzymes indicates protection against APAP toxicity, which may be associated with its antioxidant components.

In APAP-induced liver injury, ROS produces fragmentation in nuclear DNA followed by an exacerbated inflammatory response, promoting an increase in the release of proinflammatory cytokines. Since neutrophils and macrophages may aggravate hepatotoxicity (Du et al., 2016; Woolbright & Jaeschke, 2017), these cells migration was determined by MPO activity, an indirect marker of oxidative stress and tissue inflammation (Bradley et al., 1982). The activity of MPO increased significantly after APAP-induced liver injury in mice. However, pretreatment with LEO reduced this effect. Therefore, we suggest that LEO decreases leukocyte infiltration by reducing the inflammatory response, as described in our previous work, where LEO treatment reduced MPO activity in inflammatory models (Cardia et al., 2018).

The APAP toxicity modulated by inflammatory cells and pro-inflammatory cytokines has been studied for many years. Previous works have demonstrated the process of activation of Kupffer cells and increased levels of cytokines induced by APAP (Hinson et al., 2010; Liang et al., 2018). Our results suggest that LEO possesses the ability to prevent hepatotoxicity by down-regulate the levels of pro-inflammatory cytokines, attenuating the inflammatory response induced by APAP.

NO is a reactive oxidant produced from L-arginine by NO synthase, which can be increased by the high doses of APAP. NO may react with ROS and produce peroxynitrite, which induces a cytotoxic effect on neutrophils, DNA biosynthetic inhibition, cell proliferation suppression, apoptosis induction, and aggravate lipid peroxidation (Dutta et al., 2019; Saito et al., 2010; Zhang et al., 2018). In the present study, LEO decreased NO production. It was also demonstrated in PC12 cells where LEO treatment decreased the production of NO, TNF, IL-1 β , and IL-6 in β amyloid peptide-induced inflammation (Khan et al., 2014) and the NO increase induced by croton oil and carrageenan in mice (Cardia et al., 2018). These data suggest that LEO can prevent and reduce liver damage due to an anti-inflammatory effect.

Several studies have demonstrated that oxidative stress can generate deleterious processes in acute liver injury (Casas-Grajales, 2015; Chiu et al., 2018; Kumari & Kakkar, 2012). When there is an overdose of APAP, an excess of NAPQI induces GSH depletion and interrupts the conversion of the balance between glutathione (Cao et al., 2013; Chen et al., 2013; Khayyat et al., 2016). GSH is considered the main enzyme against free radicals and may react with NAPQ to diminish toxic responses. Moreover, GSH can be oxidized to GSSG, regulating redox homeostasis, reducing oxidative stress. In this work, LEO considerably decreased the APAP-induced oxidative stress in the liver by increasing the levels of GSH and reducing the levels of GSSG, ROS, and carbonyl protein, due to the antioxidant activity of the LEO constituents.

Our results indicated that APAP caused a significant increase in MDA levels in the liver of mice. MDA is used as an indicator of oxidative damage to cells and tissues through the oxidative degradation of polyunsaturated fatty acids (Dutta et al., 2019). The effect of LEO decreasing the MDA levels was also observed in previous reports, where treatment with LEO reduced lipid peroxidation, suppressed proinflammatory responses, improved antioxidant systems, reduced the level of MDA in the myocardium (Ziaee et al., 2015). Extrapolating to our findings, the hepatoprotective effect of LEO can be attributed to its ability

to reduce lipid peroxidation.

To confirm the hepatoprotective effect of LEO via down-regulation of oxidative stress, we evaluate some parameters associated with oxidative stress. Free radical scavenging enzymes like SOD and CAT are the main defense enzymes counter to oxidative stress. SOD activity in the liver can be used to evaluate the intensity of liver damage. SOD converts the greatly reactive superoxide radicals to hydrogen peroxide, which consecutively is metabolized by CAT to molecular oxygen and water, preserving the cell from toxicity (Arauz et al., 2016; Younus, 2018). The present study showed that SOD and CAT activities increased in mice treated with LEO. The increase in the activity of SOD and CAT reduced the effects of NAPQI and ROS promoting attenuation in the hepatic injury.

It has been demonstrated that LEO exerts protective effects in malathion-induced liver injury owing, in part, by its antioxidant properties, protecting against the depletion of CAT and SOD activities (Selmi et al., 2015). One study reported that LEO promoted neuroprotection on cerebral ischemia by inhibition of protein oxidation and lipid peroxidation, augmentation in endogenous antioxidant defense, reduction in mitochondria-generated ROS, and also regulating the levels of the SOD and CAT (Wang et al., 2012). In this way, we can suggest that the hepatoprotective effects of LEO against acute APAP-induced liver injury are probably involved with these antioxidant enzymes.

Lavandula officinalis contains high levels of monoterpene and phenols compounds having antioxidant properties. Previous studies have shown that 1,8-cineole decreases proinflammatory cytokines such as TNF- α and IL-1 β , and arachidonic acid metabolism, besides reducing NF- κ B and toll-like receptor 4 expression, and MPO activity (Juergens et al., 1998; Santos & Rao, 2000; Zhao et al., 2014). Other studies have confirmed its antioxidant activity, demonstrating that 1,8-cineole can restore the function and integrity of liver cells, thus decreasing the leakage of serum aminotransferases into the blood, in addition to increasing the amount of antioxidant enzymes such as CAT (Abdallah et al., 2019; Ciftci et al., 2011; Santos, 2004; Santos et al., 2001). Likewise, the use of camphor decreased edema formation and MPO activity in the acute inflammation model (Silva-Filho et al., 2015). Additionally, it was demonstrated that the Rosemary essential oil and Sage essential oil (containing 1,8-cineole and camphor) reduced the elevation of AST, ALT, ALP, and γ -GT activities in serum (El-Hosseiny et al., 2016; Rašković et al., 2014). Therefore, we suggest that the hepatoprotective activity of LEO can be due to an individual or synergistic effect of its major constituents.

5. Conclusion

In this work, we provide evidence that LEO significantly improved liver injuries induced by APAP. Its hepatoprotective effect may involve modulating of detoxification pathways in the liver, which eliminates the toxic metabolite NAPQI. Also, LEO acts by inhibiting oxidative stress and the inflammatory response, reducing the production of NO and the proinflammatory cytokines (TNF, IL-1 β , and IL-6), mainly due to its free radical scavenging antioxidant activity. Our study proposes that LEO might have a protective effect against APAP-induced acute liver injury and other oxidative stress-mediated toxicities in mice.

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