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Research Article
Eucalyptol Ameliorates Rifampicin-Induced Hepatotoxicity via Inhibiting Inflammation, Oxidant Stress and CYP2E1 Gene Expression in a Model of Modest Inflammation in Rats

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Abstract

Background and Objective: Host vulnerability may precipitate certain types of drug toxicity. Also, some concurrent events during drug therapy, such as mild inflammation, may precipitate drug-induced organ toxicity. This study was designed to investigate the ameliorative effect of eucalyptol, a natural component of many foodstuffs, on rifampicin-induced hepatotoxicity in lipopolysaccharide (LPS)-induced modest inflammation in rats. **Materials and Methods:** Sprague Dawley rats were divided into 6 groups (n = 8): Control, LPS (2 mg kg^{-1} IV, non-hepatotoxic), low-dose rifampicin (LRIF, 20 mg kg^{-1} IP, non-hepatotoxic), high-dose rifampicin (HRIF, 50 mg kg^{-1} IP, hepatotoxic), LPS+LRIF (LRIF was given 2 hrs post LPS-administration) and EUC+LPS+LRIF (as LPS+LRIF but eucalyptol 1.0 mg kg^{-1} IP, was given 1 hr before LPS administration). As 6 hrs after the last injection, blood Alanine Transaminase (ALT) and Aspartate Transaminase (AST) were measured. Moreover, hepatic Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), malondialdehyde (MDA), reduced Glutathione (GSH) and Cytochrome P450 2E1 (CYP2E1) mRNA expression were assessed in addition to histopathological analysis. **Results:** The LPS group showed small significant increases of ALT, AST, IL-6, TNF-α, MDA and CYP2E1 mRNA expression, a small significant decrease of GSH and mild hepatic injury. In contrast, the HRIF and LPS+LRIF groups showed large significant changes of all analytes and severe hepatic damage. The LRIF showed normal analyte values with normal hepatic architecture. Eucalyptol in the EUC+LPS+LRIF groups reversed the effects caused by LPS+LRIF resulting in normal analyte values with nearly-normal hepatic architecture. **Conclusion:** In a model of LPS-induced modest inflammation, non-hepatotoxic doses of rifampicin caused hepatotoxicity which was prevented with concurrent administration of eucalyptol. Therefore, eucalyptol supplementation could protect against hepatotoxicity in patients taking rifampicin and suffering simultaneously from systemic low-level inflammation such as in cases of aging, smoking, obesity and chronic diseases. The current data are novel and can help implement a safe, effective and economic protective tool against rifampicin-induced hepatotoxicity.

Key words: Eucalyptol, rifampicin, hepatotoxicity, lipopolysaccharide, inflammation, CYP2E1 gene expression

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INTRODUCTION

Drug idiosyncratic reactions, being rare and related to individual factors, may go unnoticed in the preclinical safety evaluation tests. Therefore, using animal models for drug-induced idiosyncratic hepatic injury increases the prediction and understanding of the human idiosyncratic drug-induced hepatotoxicity[1]. Drug-induced idiosyncratic hepatotoxicity may happen from an accidental decrease in the threshold for drug hepatotoxicity such as in patients with associated modest inflammation. Non-hepatotoxic doses of certain drugs could be hepatotoxic if given to rats having modest inflammation induced by non-hepatotoxic doses of lipopolysaccharide, the main active component of the bacterial endotoxin, such as with ranitidine[2] and with diclofenac[3].

The Cytochrome P450 2E1 (CYP2E1) breaks chemicals and carcinogens and has a key role in both alcohol-induced and immune-mediated hepatic injury[4]. Rifampicin, a widely-used antimicrobial and first-line antitubercular medication, significantly induces the hepatic CYP2E1[5] and has been reported to cause hepatitis when given alone or in combination with other hepatotoxic antituberculosis medications[6]. Risk factors for rifampicin-induced hepatotoxicity include old age, female gender, bad nutritional status, alcoholism, pre-existing liver disease and genetic predisposition[7]. This hepatotoxicity could be due to oxidative stress and lipid peroxidation[8]. It may necessitate cessation of treatment which could be deleterious and even fatal[9], therefore, searching for a medicine which can prevent this hepatotoxicity is essential. Herbal remedies could be useful in this regard, for example, in patients with active tuberculosis, a formulation of *Curcuma longa* and *Tinospora cordifolia* prevented the anti-tubercular medications induced hepatotoxicity and improved the outcome and patient compliance[10]. Moreover, in rats given both rifampicin and isoniazid, kaempferol, a herbal product, inhibited CYP2E1 activity induced by rifampicin and hence inhibited the toxic metabolism of isoniazid[11]. Eucalyptol, a natural terpenoid oxide present in many foodstuffs, given intragastrically for three days, significantly attenuated paracetamol-induced liver damages through inhibition of CYP2E1 activities and hence acceleration of paracetamol non-toxic metabolism[12].

Taken together, this study was designed to investigate the ameliorative effect of eucalyptol on rifampicin-induced hepatotoxicity in a model of modest inflammation in rats.

MATERIALS AND METHODS

Study area: The study was conducted in the research laboratories at King Abdulaziz University, Jeddah, KSA from September, 2022 to October, 2023.

Induction of modest inflammation and experimental groups: Eucalyptol liquid (EUC), rifampicin (RIF) and lipopolysaccharide (LPS, derived from *Escherichia coli* 0128: B12 serotype) were purchased from Sigma Chemical Company (St. Louis, Missouri, USA). Rifampicin and LPS were dissolved in distilled water. The study was approved by the Research Ethics Committee, Faculty of Pharmacy and was carried out in accordance with the guidelines for use of laboratory animals. A total of 48 Sprague Dawley rats (230-250 g) were housed in 22°C room with 12 hrs light-dark cycle and with free access to food and water and were acclimatized for a week before starting the experiments. After 16 hrs fasting, the rats were randomly divided into 6 groups (n = 8) which received the following: (1) Normal control (NC): Vehicles, (2) LPS group: LPS 2 mg kg^{-1} IV (non-hepatotoxic dose) to induce a model of modest inflammation[13], (3) Low-dose Rifampicin (LRIF, 20 mg kg^{-1} IP, non-hepatotoxic dose), (4) High-dose Rifampicin (HRIF, 50 mg kg^{-1} IP, hepatotoxic dose)[14], (5) LPS+LRIF (non-hepatotoxic doses of LPS and Rifampicin): Rifampicin was given 2 hrs post LPS-administration[2] and (6) EUC+LPS+LRIF: Similar to group (5), but eucalyptol (1.0 mg kg^{-1}, IP)[15] was given 1 hr before LPS+LRIF administration[16]. As 6 hrs after the last injection, blood was collected through retro-orbital puncture for measurement of Alanine Transaminase (ALT) and Aspartate Transaminase (AST) and then the rats were sacrificed under anesthesia. Liver was excised and slices of the left hepatic lobe was fixed in 10% neutral-buffered formalin for histopathological analysis, while a part of the right hepatic lobe was kept in liquid nitrogen for determination of inflammatory and oxidant stress markers and CYP2E1 gene expression analysis[3].

Measurement of blood ALT and AST: Blood ALT and AST levels were measured by using ELISA commercially available kits (MyBioSource, Inc., California, USA, Catalog numbers: MBS269614 and MBS264975) following the manufacturer's protocol.

Measurement of Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α), malondialdehyde (MDA) and reduced Glutathione (GSH) in liver homogenate: A 10% hepatic homogenate using phosphate-buffered saline was prepared with the TissueLyser II (Qiagen) at 4°C and following

centrifugation, the supernatant was kept at -80°C[17]. The homogenate protein content was measured by using a colorimetric kit (MyBioSource, Inc., California, USA, Catalog number: MBS355526) and the levels of IL-6, TNF-α, MDA and GSH were evaluated by ELISA kits (Catalog numbers: MBS726707, MBS355371, MBS738685 and MBS265966, respectively) and were expressed/mg protein.

Determination of CYP2E1 gene expression by real-time PCR analysis: Total mRNA was isolated from hepatic tissue with a GeneJET Kit (Thermo Fisher Scientific Inc., Morocco, USA) and was reverse transcribed into cDNA using the RT-PCR kit (Invitrogen). Amplification was performed by the RT-PCR using an Applied Biosystems Cycler (USA) and SYBR Green Kit to assess the mRNA expression levels of CYP2E1 gene. The forward and reverse primers for CYP2E1 were 5'-TGGCTACAAGGCTGTCAAGG-3' and 5'-AGG CTGGCCTTTGGTCTTTT-3'. The β-actin mRNA was used as the normalization control and its forward and reverse primers were 5'-CAGGTCATCACTATCGGCAAT-3' and 5'-TGGCATAGAG GTCTTTACGGA-3'. The relative mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ where ΔΔCt represents "differences in cycle threshold numbers between the target gene and the control gene"[18].

Histopathological analysis: Slices from the rat livers were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections (3-5 μm) were made on glass slides, stained with hematoxylin and eosin and examined for evaluating inflammatory cell infiltration, hepatic cell degeneration and vascular congestion. The injury was reported as mild, moderate or severe[19].

Statistical analysis: Data were expressed as Means±SEM. The SPSS software (version 22, USA) was used. Comparisons between the different groups were made using One-way Analysis of Variance (ANOVA) followed by Tukey's test for multiple comparisons. The difference was considered significant when $p<0.05$.

RESULTS

Effects of combining eucalyptol with rifampicin on plasma ALT and AST in a model of modest inflammation in rats: The LPS group showed small significant increases of ALT and AST while the HRIF and LPS+LRIF groups showed large significant increases compared to the NC group with non-significant variations between each other. The LRIF and EUC+LPS+LRIF groups showed normal values with non-significant differences from the NC group or between each other (Table 1).

Effects of combining eucalyptol with rifampicin on hepatic inflammatory and oxidative markers in a model of modest inflammation in rats: The LPS group showed small significant increases of IL-6, TNF-α, MDA and a small significant decrease of GSH while the HRIF and LPS+LRIF groups showed large significant changes compared to the NC group with non-significant variations between each other. The LRIF and EUC+LPS+LRIF groups showed normal values with non-significant differences from the NC group or between each other (Table 2).

Table 1: Effects of eucalyptol plus rifampicin on plasma ALT and AST in rats having modest inflammation

	NC	LPS	LRIF	HRIF	LPS+LRIF	EUC+LPS+LRIF
ALT (U L^{-1})	38.71±2.29	54.70±2.77**	42.07±2.93*	152.16±3.91***	161.83±3.34***	40.97±1.14*
AST (U L^{-1})	60.13±1.46	75.50±1.52$^{\#}$	54.63±3.40$^{\#\#}$	187.35±5.82$^{\#\#\#}$	175.25±2.77$^{\#\#\#}$	57.89±2.25$^{\#\#}$

Data are expressed as Mean±SEM, NC: Normal control, LPS: Lipopolysaccharide (2 mg kg^{-1} IV, non-hepatotoxic dose), LRIF: Low-dose Rifampicin (20 mg kg^{-1} IP, non-hepatotoxic dose), HRIF: High-dose rifampicin (50 mg kg^{-1} IP, hepatotoxic dose), EUC: Eucalyptol (1.0 mg kg^{-1}, IP), ALT: (*p<0.05) LRIF and EUC+LPS+LRIF vs. LPS (= 0.036 and 0.018), (**p<0.01) LPS vs. NC (= 0.004), (***p<0.001) HRIF and LPS+LRIF vs. NC and vs. LPS, AST: ($^{\#}$p<0.05) LPS vs. NC (= 0.019), ($^{\#\#}$p<0.01) LRIF and EUC+LPS+LRIF vs. LPS (= 0.001 and 0.005), ($^{\#\#\#}$p<0.001) HRIF and LPS+LRIF vs. NC and vs. LPS

Table 2: Effects of eucalyptol plus rifampicin on hepatic IL-6, TNF-α, MDA and GSH in rats having modest inflammation

	NC	LPS	LRIF	HRIF	LPS+LRIF	EUC+LPS+LRIF
IL-6 (pg mg^{-1} protein)	60.63±2.04	96.51±5.23**	63.64±3.20**	182.47±9.23***	178.57±10.38***	55.81±2.72**
TNF-α (pg mg^{-1} protein)	120.74±6.93	179.52±9.02$^{\#\#}$	126.36±7.15$^{\#\#}$	273.56±11.49$^{\#\#\#}$	258.00±14.10$^{\#\#\#}$	130.44±7.15$^{\#}$
MDA (nmol mg^{-1} protein)	6.52±0.37	13.54±0.84$^{\wedge\wedge}$	6.82±0.46$^{\wedge}$	26.38±2.29$^{\wedge\wedge\wedge}$	23.12±2.13$^{\wedge\wedge\wedge}$	7.50±0.22$^{\wedge}$
GSH (ng mg^{-1} protein)	128.68±3.57	102.16±6.56$^{@}$	130.43±8.08$^{@}$	61.40±3.95$^{@@@}$	58.99±5.51$^{@@@}$	135.01±7.39$^{@@}$

Data are expressed as Mean±SEM, NC: Normal control, LPS: Lipopolysaccharide (2 mg kg^{-1} IV, non-hepatotoxic dose), LRIF: Low-dose Rifampicin (20 mg kg^{-1} IP, non-hepatotoxic dose), HRIF: High-dose rifampicin (50 mg kg^{-1} IP, hepatotoxic dose), EUC: Eucalyptol (1.0 mg kg^{-1}, IP), (IL-6): (**p<0.01) LPS vs. NC (= 0.003) and LRIF and EUC+LPS+LRIF vs. LPS (= 0.009 and 0.001), (***p<0.001) HRIF and LPS+LRIF vs. NC and vs. LPS, (TNF-α): ($^{\#}$p<0.05) EUC+LPS+LRIF vs. LPS (= 0.011), ($^{\#\#}$p<0.01) LPS vs. NC and LRIF vs. LPS (= 0.001 and 0.005), ($^{\#\#\#}$p<0.001) HRIF and LPS+LRIF vs. NC and vs. LPS. (MDA): ($^{\wedge}$p<0.05) LRIF and EUC+LPS+LRIF vs. LPS (= 0.012 and 0.032), ($^{\wedge\wedge}$p<0.01) LPS vs. NC (= 0.008), ($^{\wedge\wedge\wedge}$p<0.001) HRIF and LPS+LRIF vs. NC and vs. LPS, (GSH): ($^{@}$p<0.05) LPS vs. NC (= 0.039) and LRIF vs. LPS (= 0.023), ($^{@@}$p<0.01) EUC+LPS+LRIF vs. LPS (= 0.005), ($^{@@@}$p<0.001) HRIF and LPS+LRIF vs. NC and vs. LPS

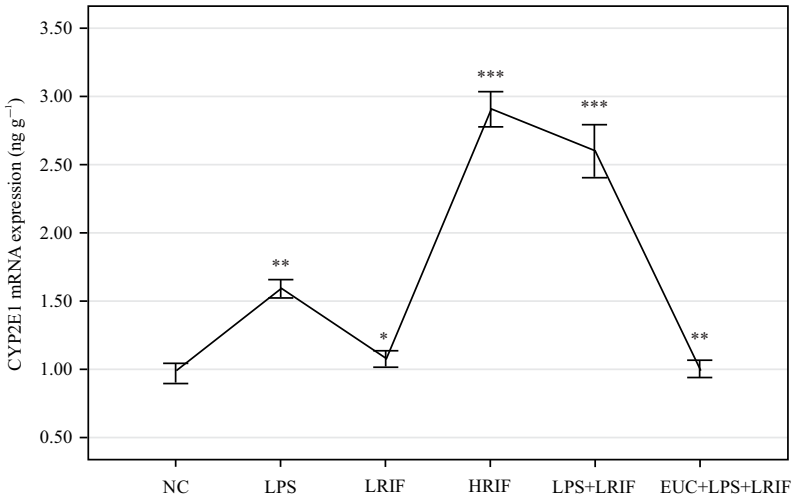

Fig. 1: Effects of eucalyptol plus rifampicin on hepatic CYP2E1 gene expression in rats having modest inflammation

Data are expressed as Mean±SEM, NC: Normal control, LPS: Lipopolysaccharide (2 mg kg^{-1} IV, non-hepatotoxic dose), LRIF: Low-dose Rifampicin (20 mg kg^{-1} IP, non-hepatotoxic dose), HRIF: High-dose rifampicin (100 mg kg^{-1} IP, hepatotoxic dose), EUC: Eucalyptol (1.0 mg kg^{-1}, IP), (*p<0.05) LRIF vs. LPS (= 0.015), (**p<0.01) LPS vs. NC (= 0.002) and EUC+LPS+LRIF vs. LPS (= 0.004), (***p<0.001) HRIF and LPS+LRIF vs. NC and vs. LPS

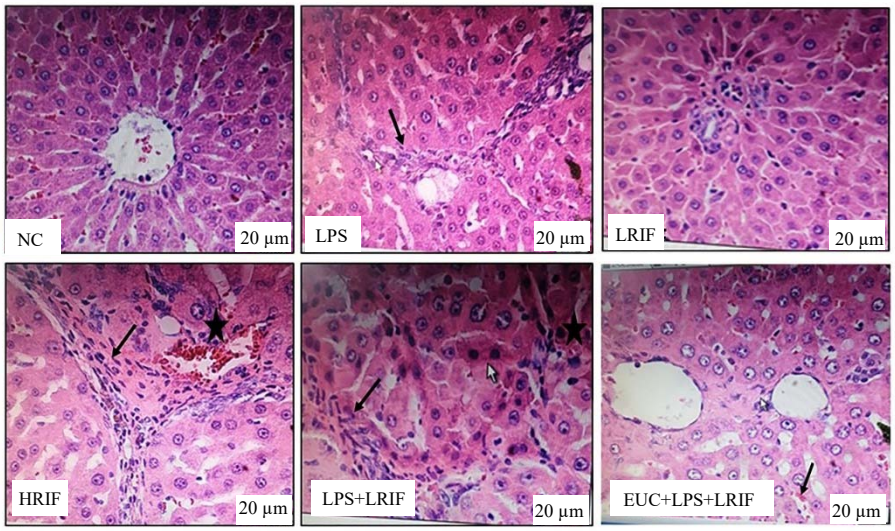

Fig. 2: Effects of eucalyptol plus rifampicin on hepatic structure in rats having modest inflammation (H&E, ×20)

NC: Normal control group showing normal hepatic architecture with normal hepatocytes and central vein, LPS: Lipopolysaccharides group showing mild inflammatory cell infiltration (arrow), and parenchymal edema, LRIF: Low-dose rifampicin group showing normal picture, HRIF: High-dose rifampicin and LPS+LRIF groups showing centrilobular necrosis, marked inflammatory cell infiltration (arrow), severe hemorrhage (star) and parenchymal edema and EUC+LPS+LRIF: Eucalyptol+LPS+LRIF group showing marked protection (improvement) with mild inflammatory cell infiltrate (arrow) and nearly normal picture

Effects of combining eucalyptol with rifampicin on hepatic CYP2E1 gene expression in a model of modest inflammation in rats: The LPS group showed a small significant increase of CYP2E1 gene expression while the HRIF and LPS+LRIF groups showed large significant increases compared to the NC group with non-significant variations between each other. The LRIF and EUC+LPS+LRIF groups showed normal values with non-significant differences from the NC group or between each other (Fig. 1).

Effects of combining eucalyptol with rifampicin on hepatic architecture in a model of modest inflammation in rats: The NC rats showed normal hepatic structure and the LPS rats showed mild damage. The LRIF group showed no damage,

while the HRIF and LPS+LRIF groups showed severe injury in form of marked inflammatory infiltration, hemorrhage and degeneration of hepatocytes. The EUC in the EUC+LPS+LRIF group reversed the changes induced by the LPS+LRIF combination with nearly normal picture (Fig. 2).

DISCUSSION

Modest inflammation could be a risk factor for hepatotoxicity of certain drugs. In rats, LPS-induced modest inflammation rendered a non-hepatotoxic dose of ranitidine (20 mg kg^{-1}) to be hepatotoxic as estimated by increases in ALT, AST and histological changes within 6 hrs after ranitidine administration[2]. The same was reported with diclofenac where non-hepatotoxic doses of diclofenac (up to 40 mg kg^{-1}) exerted hepatotoxicity manifested by elevated ALT and histopathologic injury 6 hrs after diclofenac administration when given to rats having LPS-induced modest inflammation[3]. The mechanisms involved may include binding of LPS to receptors on mammalian inflammatory cells leading to activation of inflammatory cells, synthesis and release of numerous proinflammatory mediators e.g., TNF-α and IL-6[20]. Liver plays an essential role in detoxification of LPS where it induces Kupffer cells to produce inflammatory cytokines which if excessive can cause septic shock, multiple organ failure and acute respiratory distress syndrome[21]. Treatment of rats with isoniazid and rifampicin caused severe oxidative stress, abnormal liver functions and distorted hepatic architecture in form of severe histological vascular changes and lobular necrosis. Heptoplus, an oral polyherbal formulation, protected against this hepatotoxicity[22]. Treatment of Wistar rats with rifampicin (50 mg/kg/day) induced hepatotoxicity as manifested by increased serum ALT, ALT, bilirubin and alkaline phosphatase as well as histological hepatic damage[14]. Oral rifampicin (50 mg/kg/day) for 14 days caused hepatotoxicity in male albino rat as shown by elevations of serum levels of AST, ALT, alkaline phosphatase and bilirubin as well as hepatic MDA content in addition to distorted hepatic structure with dilated central vein[23].

The cytochrome P450 2E1 (CYP2E1) has essential roles in xenobiotic metabolism[24] and its mRNA expression significantly and dose-dependently increased after exposure of rats to low-dose and high-dose X-ray irradiation[18]. It was reported that LPS (5 mg kg^{-1} IP) increased the hepatic mRNA expression of CYP2E1 which started as early as 2-3 hrs post-injection and was sustained for 24 hrs, after which the enzyme activity significantly decreased. Thus, LPS acts at two levels to regulate the hepatic CYP2E1 enzyme, a transcriptional level to induce its mRNA expression and a post-transcriptional level to regulate its protein and activity[25]. Moreover, LPS enhanced isoniazid toxicity through augmenting the inflammatory stress, oxidative stress and CYP2E1 over-expression. Pre-treatment with both dexamethasone and CYP2E1 inhibitor (diallyl sulfide) overcame the inflammatory and oxidative stress and CYP2E1 over-expression respectively causing significant reductions in serum total bilirubin and gamma glutamyl transferase levels and reversal of the liver lesions following isoniazid/LPS treatment and thus strongly protected against isoniazid/LPS-induced hepatic damage[13]. In addition, with low doses of LPS, the liver showed modest leukocyte infiltration, vascular changes, fatty vacuolations and parenchymal edema[3]. In idiosyncratic drug reactions, hepatic lesions are often characterized by inflammatory cell infiltration[1]. It was reported that the hepatotoxic doses of rifampicin caused severe vascular changes, centrilobular necrosis with fatty vacuolations, perivascular infiltration with mononuclear cells and parenchymal edema[23]. Rifampicin actively induces CYP2E1[26] and silymarin, a hepatoprotective agent, restored the rifampicin-induced induction of CYP2E1 expression and activity[5]. Eucalyptol reversed paracetamol-induced hepatocellular injury shown by swollen hepatocytes with marked cytoplasmic vacuolation and condensed nuclei in mice[12].

Based upon the above mentioned data, rifampicin-induced hepatotoxicity could be due to oxidative stress and lipid peroxidation. Co-administering eucalyptol with rifampicin in a model of lipopolysaccharide-induced modest inflammation reversed this hepatotoxicity. Generally, systemic low-level inflammation is common in many conditions such as in cases of aging, smoking, obesity and chronic diseases and it may precipitate rifampicin-induced hepatotoxicity which may necessitate cessation of treatment which could be deleterious and even fatal. Therefore, use of eucalyptol with rifampicin in these cases is recommended to protect against hepatotoxicity. However, the short duration of the current study and using a single concentration of eucalyptol are considered limitations. Conducting a longer duration study for evaluating effects of graded concentrations of eucalyptol is recommended.

CONCLUSION

In a model of LPS-induced modest inflammation, non-hepatotoxic low doses of rifampicin caused hepatotoxicity. Combined administration of eucalyptol, a natural component of many foodstuffs, effectively reversed this hepatotoxicity through inhibiting inflammation, oxidative stress and hepatic CYP2E1 mRNA expression. Therefore, eucalyptol

supplementation could protect against hepatotoxicity in patients taking rifampicin and suffering simultaneously from systemic low-level inflammation such as in cases of aging, smoking, obesity and chronic diseases. To our knowledge, the current data are novel and can help implement a safe, effective and economic protective tool against rifampicin-induced hepatotoxicity.

SIGNIFICANCE STATEMENT

Rifampicin is a widely-used antimicrobial and first-line antitubercular medication. In a model of lipopolysaccharide-induced modest inflammation, non-hepatotoxic low doses of rifampicin caused hepatotoxicity manifested by significant hepatic dysfunction, oxidative stress and inflammation as well as severe histological damage. Combined administration of eucalyptol, a natural component of many foodstuffs, effectively reversed this hepatotoxicity through inhibiting inflammation, oxidative stress and hepatic CYP2E1 mRNA expression. Therefore, eucalyptol supplementation could protect against hepatotoxicity in patients taking rifampicin and suffering simultaneously from systemic low-level inflammation such as in cases of aging, smoking, obesity and chronic diseases. The current data are novel and can help pave the way for developing a safe and effective protective tool against rifampicin-induced hepatotoxicity.

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