Acta Pharm. Sci. Vol 61:(2), 2023
DOI: 10.23893/1307-2080.APS6113

Syzygium aromaticum essential oil ameliorates levofloxacin-induced hepatic injury in rats: antioxidant, biomarker and histopathological analysis

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ABSTRACT

Prolonged levofloxacin treatment can trigger hepatotoxicity. This study aimed to examine *Syzygium aromaticum* L. essential oil effect on levofloxacin-induced hepatic injury in rats**.** The chemical constituents and radical scavenging activity were also examined. Rats (n=30) were grouped to receive per oral administration of either placebo, levofloxacin (93 mg/kg), *S. aromaticum* L. oil (10, 25, or 50 mg/kg), or curcuma (6 mg/kg) with levofloxacin for 28 days. Serum liver biomarkers, liver malondialdehyde (MDA) levels, and histopathological changes were analyzed. *S. aromaticum* L. oil contained eugenol (63.63%) and the IC_{50} was 35.1 µg/mL. Levofloxacin induced significant increases in serum biomarker and tissue MDA levels ($p < 0.05$), with significant damage in hepatocytes. The oil treatment (10 mg/kg) reduced the levels of alanine aminotransferase, total bilirubin, lactate dehydrogenase, tissue MDA ($p < 0.05$), and the hepatic injury. In conclusion, *S. aromaticum* L. oil alleviated levofloxacin-induced liver injury, possibly related to its eugenol content and radical scavenging activity.

Keywords: Clove essential oil, hepatotoxicity, levofloxacin, *Syzygium aromaticum* L.

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(Received 28 Jan 2023, Accepted 10 Mar 2023)

INTRODUCTION

Levofloxacin is a broad-spectrum antibiotic that clinicians widely prescribe to treat various infections. Moreover, it is commonly used in the second-line anti-tuberculosis (AT) regimen in patients who are intolerant to first-line AT agents, have latent tuberculosis infection (LTBI), or multidrug-resistant tuberculosis (MDR TB), for up to 24 months [1, 2]. Levofloxacin is considered well-tolerated; however, there is evidence that levofloxacin treatment can trigger hepatotoxicity [3]. Although unusual, fatality can result [4]. The prolonged use of levofloxacin in TB patients has raised concerns about the increased risk of levofloxacin-induced hepatotoxicity [5]. Studies on the use of levofloxacin regimen have shown hepatotoxicity in 9.7% to 14.3% of MDR TB patients [6, 7].

Even though the exact mechanism of levofloxacin-induced liver injury remains elusive, many believe it relates to the increased release of intracellular reactive oxygen species (ROS) that leads to mitochondrial damage [8]. The alteration of mitochondrial electron transport chain activity may also result in ROS accumulation [9]. Since oxidative stress is involved in the pathogenesis of levofloxacin-induced liver injury, using antioxidants may relieve the damage.

Antioxidants obtained from natural sources might serve as alternatives to standard antioxidants. These antioxidant compounds are abundant in essential oils from spices or herbs [10]. *Syzygium aromaticum* L. (clove), a member of the Myrtaceae family native to eastern Indonesia [11], is one of the most valuable kitchen spices and has been used in folk medicines for centuries. Its essential oil has demonstrated very potent antioxidant activity comparable to, and even superior to, butylated hydroxytoluene (BHT), a synthetic antioxidant [12, 13]. The main active compound in *S. aromaticum* L. oil, eugenol, has demonstrated a protective effect in induced hepatotoxicity in many animal models [14, 15]. The purpose of this study is to examine the potential use of *S. aromaticum* L. essential oil as an antioxidant to reduce levofloxacin-induced hepatic injury in rats.

METHODOLOGY

Drugs and chemicals

Diethyl ether, formaldehyde 10%, sodium carboxymethyl cellulose (Na CMC), methanol, hematoxylin, and eosin were purchased from a registered chemical store in Makassar, Indonesia. Levofloxacin tablets (Hexpharm Jaya®) and Curcuma tablets (Soho®) were purchased from a licensed pharmaceutical store in Makassar, Indonesia. 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 1,1,3,3-tetramethoxypropane (TMP) were purchased from Sigma Aldrich. Diagnostic kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT),

total bilirubin, lactate dehydrogenase (LDH) were purchased from Human Diagnostics Worldwide (Magdeburg, Germany).

The essential oil was purchased from Happy Green®, Indonesia, batch No. 20171526-08-23). This oil was obtained from flower buds of *Syzygium aromaticum* L. plants from Indonesia using steam distillation. The oil is a dark, clear liquid and *S. aromaticum* L. bud odor, with a certificate of analysis from the manufacturer showing a specific gravity (20°C) of 1.0639, a refractive index (20°C) of 1.5217, and a density (20°C) of 1.0620.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis was performed to obtain the profiles of chemical constituents in the *S. aromaticum* L. essential oil by using the Trace 1310 GC coupled with TSQ 8000 Evo MS (Thermo Scientific, USA). The column used was TG-5MS with a size of 20 m x 0.18 mm. The oven had an initial temperature of 50°C that increased to 150°C at a rate of 25°C/min, then to 230°C at a rate of 15°C/min, and finally to 330°C at the rate of 10°C/min. The holding time was 5 min. The sample volume was 1 µL, and helium was used as the carrier gas.

Free radical scavenging assay

The free radical scavenging activity was examined using a 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method as described in a previous study[16]. DPPH reagent (5 mg) was dissolved in 100 mL of methanol to obtain a DPPH concentration of 50 µg/mL. The *S. aromaticum* L. oil solution was prepared in methanol with a series of concentrations: 10, 20, 30, 40, and 50 µg/mL. Each concentration of *S. aromaticum* L. oil was pipetted (1 mL) and mixed with DPPH solution to a total volume of 5 mL in the measuring flask. The prepared mixtures were incubated at room temperature for 30 minutes (mins), and the absorbances were measured with a UV spectrophotometer (Shimadzu, Japan) at a wavelength of 517 nm. The same treatment was carried out on the control solution (4 mL of DPPH solution and 1 mL of methanol). All sample measurements were carried out in triplicate. The percentage of inhibition was calculated using the equation:

$$\% \text{ Inhibition} = \frac{A_1 - A_2}{A_2} \times 100\%$$

A_1: the absorbance of the control
A_2: the absorbance of samples

The value of IC_{50} (inhibitory concentration at 50%) was obtained using a linear regression equation between sample concentration (x) and % inhibition (y).

Animal preparation

Albino Wistar rats (200–250 g) were procured from a rodent breeding house in Yogyakarta, Indonesia. Prior to the experiment, the rats were acclimatized for 14 days to adapt to the laboratory environmental conditions (25°C with a 12-hour light/dark cycle). Rats were provided with regular food pellets and water *ad libitum*.

Evaluation of hepatoprotective effects

Thirty male Wistar rats were assigned to one of the six groups. The control group only received per oral (p.o) administration of a placebo (1% Na CMC suspension), while the LFX group received only p.o administration of levofloxacin. The *S. aromaticum* L. (clove oil, CO) groups (CO 10 + LFX, CO 25 + LFX, and CO 50 + LFX) received p.o administration of *S. aromaticum* L. oil at a dose of 10, 25, or 50 mg/kg, respectively, followed by levofloxacin administration with a 2-hour interval. Another group received curcuma (Curcuma + LFX) as a comparison to *S. aromaticum* L. oil. Curcuma was applied in this study as the comparison treatment since it has been used in a clinical setting to treat patients with hepatotoxicity[17].

Levofloxacin and curcuma tablets were prepared in 1% Na CMC suspension, while the *S. aromaticum* L. oil was diluted in corn oil to facilitate oral administration. Levofloxacin and curcuma doses were chosen based on human daily doses (93 mg/kg for levofloxacin and 6 mg/kg for curcuma) as explained in Nair and Jacob[18]. The dose of *S. aromaticum* L. oil was chosen based on the pilot study, where three daily doses were compared (10 mg/kg, 50 mg/kg and 100 mg/kg). From the pilot study, it was found the 10 mg/kg dose had the most efficacy in preventing hepatotoxicity (unpublished data). Therefore, in this present study, 10 mg/kg, 25 mg/kg and 50 mg/kg were used. The treatments were administered for 28 days. The rats were sacrificed 24 hours after the last treatment using diethyl ether and cervical dislocation. A laparotomy was performed, and the liver was harvested.

Analysis of serum biomarkers

Before and after 28 days of treatments, the blood samples (3 mL) were collected from the lateral saphenous veins of the rat using vacuum tubes containing an anticoagulant. This method allows rapid blood sampling and the site of puncture can be repetitively used to withdraw blood if needed[19]. All animals were anesthetized with diethyl ether prior to blood withdrawal. Blood samples were centrifuged at 3000 rpm for 15 mins to separate serum and blood cells. The serum was analyzed spectrophotometrically (Humalyzer 3500) using reagent kits for AST, ALT, total bilirubin, and LDH (Human Diagnostics Worldwide, Germany). All protocols were performed according to the manufacturer's instructions.

Analysis of liver malondialdehyde

Malondialdehyde (MDA) was analyzed using a thiobarbituric acid reactive substance (TBARS) method as explained in a previous study[20]. 1,1,3,3-tetramethoxypropane (TMP) was used to obtain the standard curve. The liver tissues were weighed (400 mg), homogenized with 2 mL of phosphate-buffered saline (PBS), and then centrifuged at 3000 rpm for 10 mins. The supernatant (0.5 mL) was mixed with 1 mL of 1% thiobarbituric acid and 1 mL of 10% trichloroacetic acid. The mixture was heated at 90°C in a water bath for 10 mins before centrifugation to remove precipitation. The absorbance of the supernatant was measured using a spectrophotometer (Agilent, USA) at 531 nm. The MDA concentration of the samples was obtained by plotting the absorbances against the standard curve, multiplied by the dilution factor. The final concentration of liver MDA was expressed as ng/mL.

Examination of liver histopathology

Once harvested, the liver tissues were rinsed in normal saline and immersed in 10% formaldehyde for 48 hours. The liver tissues were then processed as described in previous studies[21, 22], and stained with hematoxylin-eosin. The histopathological analysis was conducted by a forensic pathologist blinded to the treatments using a light microscope coupled to a camera (Olympus).

Statistical analysis

The data (presented as mean ± SEM) were analyzed for normal distribution using the one-sample Kolmogorov-Smirnov test. If the data were normally distributed ($p > 0.05$), a one-way ANOVA analysis was performed, followed by Tukey's post hoc test to determine any significant differences among groups. A significant difference was indicated by a p-value < 0.05.

RESULTS AND DISCUSSION

We used *S. aromaticum* L. oil as a protective agent to alleviate levofloxacin hepatotoxicity. Previously, hepatotoxicity-induced levofloxacin has been demonstrated in rats [20], which shown intense degeneration of hepatocytes accompanied by elevation of serum ALT, AST and GGT levels.

Chemical composition and free radical scavenging activity of *S. aromaticum* L. oil

The acceptable daily human intake of *S. aromaticum* L. or clove oil, used in food additives and medicines for centuries, has been established by the World Health Organization (WHO) at 2.5 mg/kg of body weight [13].

The quality of *S. aromaticum* L. oil is determined by the content of its phenolic compounds, especially eugenol as the main constituent. Based on the GC-MS analysis (Table 1), the *S. aromaticum* L. oil had 23 constituents with 4 major compounds: eugenol (63.63%), 3-allyl-6-methoxyphenyl acetate (20.53%), caryophyllene oxide (4.75%) and phenol,2-methoxy-3-(2-propenyl)-acetate or eugenol acetate (2.44%).

Table 1. The chemical compounds of the *S. aromaticum* L. oil based on GC-MS analysis

No	Retention Time	Molecular Formula	Compound Name**	%Area
1	4.56	$C_8H_{14}O$	5-Hepten-2-one, 6-methyl-	0.10
2	4.93	$C_{10}H_{16}$	D-Limonene	0.11
3	5.92	$C_9H_{10}O_2$	Benzoic acid, ethyl ester	0.10
4	6.51	$C_9H_{10}O$	Phenol,4-(2-propenyl)-	1.41
5	6.12	$C_8H_8O_3$	Methyl salicylate	1.74
6	7.08	$C_7H_{12}O_5$	Glycerol 1,2-diacetate	0.80
7	7.62	$C_{10}H_{12}O_2$	Eugenol	63.63
8	8.54	$C_{12}H_{14}O_3$	3-Allyl-6-methoxyphenyl acetate	20.53
9	9.06	$C_{15}H_{24}O$	Caryophyllene oxide	4.75
10	9.99	$C_{10}H_{12}O_3$	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	0.10
11	10.39	$C_{15}H_{24}O$	Caryophyllene oxide	0.12
12	10.67	$C_{15}H_{24}O$	Isoaromadendrene epoxide	0.28
13	11.10	$C_{15}H_{26}O_2$	4,4,8-Trimethyltricyclo[6.3.1.0(1,5)]dodecane-2,9-diol	0.39
14	11.24	$C_{11}H_{18}O_6$	1,3-Dioxolane-2,2-diacetic acid, diethyl ester	0.27
15	12.63	$C_{21}H_{36}O_4$	9,12,15-Octadecatrienole acid, 2,3-dihydroxypropyl ester, (Z,Z,Z)-	0.11
16	15.55	$C_{11}H_{16}O$	3-Methyl-2-pent-2-enyl-cyelopent-2-enone	1.16
17	15.41	$C_{19}H_{24}$	Benzene, 1,2,4,5-tetramethyl-3-(3-phenylpropyl)-	0.16
18	15.88	$C_{10}H_{12}O_2$	Phenol,2-methoxy-6-(1-propenyl)-	0.10
19	16.17	$C_{15}H_{20}O_3$	Butanoic acid,2-methyl-2-methoxy-4-(2-propenyl) phenyl ester	0.19
20	17.20	$C_{12}H_{14}O_3$	Phenol,2-methoxy-4-(2-propenyl)-, acetate	0.91
21	17.74	$C_{12}H_{14}O_3$	Phenol,2-methoxy-3-(2-propenyl)-acetate	2.44
22	18.91	$C_{27}H_{54}O_4Si_2$	1-Monolinoleoyglycerol trimethylsilyl ether	0.05
23	19.20	$C_{27}H_{54}O_4Si_2$	1-Monolinoleoyglycerol trimethylsilyl ether	0.09
		Total		**100**

Radical scavenging activity was determined to evaluate the ability of *S. aromaticum* L. oil to halt free radical reactions in biological systems. In the DPPH assay, *S. aromaticum* L. oil reduced the radical DPPH to diphenyl-picrylhydrazine, as demonstrated by the yellow color in the solution. At a concentration of 10 to 50 µg/mL, *S. aromaticum* L. oil demonstrated a powerful reducing capacity with an IC_{50} of 35.1 µg/mL (R^2 = 0.9963) (Figure 1). This value suggests a potent radical scavenging capacity, which can protect liver cells against the damaging effects of free radicals and oxidative reactions.

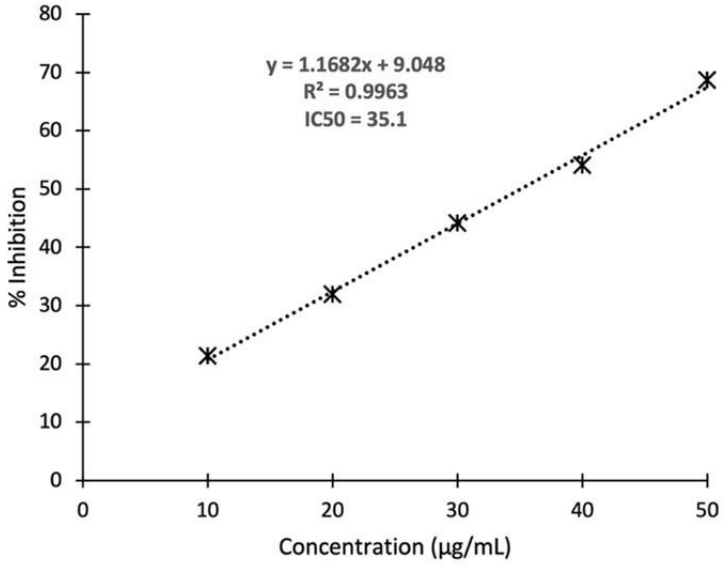

Figure 1. The dose-response curve of free radical scavenging activity of the *S. aromaticum* L. oil using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay

The antioxidant activity of *S. aromaticum* L. essential oil is mainly derived from its eugenol content, but other polyphenols may also contribute [23]. Eugenol is a very potent antioxidant and can reduce ROS production and significantly improve oxidative stress [24]. It is believed that the antioxidant activity of eugenol strongly contributes to its pharmacological effects, such as anti-inflammatory, analgesic, anticancer, and anti-dyslipidemia [25, 26]. Apart from eugenol, three other major components were found in *S. aromaticum* L. oil: 3-allyl-6-methoxyphenyl acetate, caryophyllene oxide, and eugenol acetate. These compounds are also known to have pharmacological activities. A study by Kamadatu and Santoso shows that 3-allyl-6-methoxyphenyl acetate possesses a potent cytotoxicity against human breast cancer cell line [27]. Caryophyllene oxide has also been found to elicit anticancer and analgesic activities [28]. Whereas, in sepa-

rate studies, eugenol acetate and caryophyllene have been reported to possess nematocidal and antifungal properties [29, 30].

Hepatoprotective effect of *S. aromaticum* L. oil

The results of the liver function test and liver MDA analysis are shown in Figure 2. Significant increases in the levels of ALT (77.9 ± 8.4 U/L), AST (245.6 ± 21.4 U/L), total bilirubin (1.32 ± 0.15 mg/dl), and LDH (841.8 ± 97.4 mg/dl) were observed in the group treated with levofloxacin (LFX) for 28 days compared to the controls ($p < 0.05$). The *S. aromaticum* L. oil pre-treatment, especially at the dose of 10 mg/kg, significantly reduced the elevation of (53.2 ± 2.2 U/L), total bilirubin (0.35 ± 0.11 mg/dl), and LDH (440.7 ± 69.2 mg/dl) levels ($p < 0.05$), although the AST level (214.2 ± 10.0 U/L) was not significantly lowered. However, the reduction of ALT, bilirubin, and LDH were not observed with *S. aromaticum* L. oil treatment at the higher doses (25 mg/kg and 50 mg/kg). Nevertheless, the liver MDA levels in all *S. aromaticum* L. oil groups improved compared to the LFX group ($p < 0.05$). As a comparison, curcuma pre-treatment was also found to significantly reduce the total bilirubin, LDH, and liver MDA levels in LFX-treated rats ($p < 0.05$).

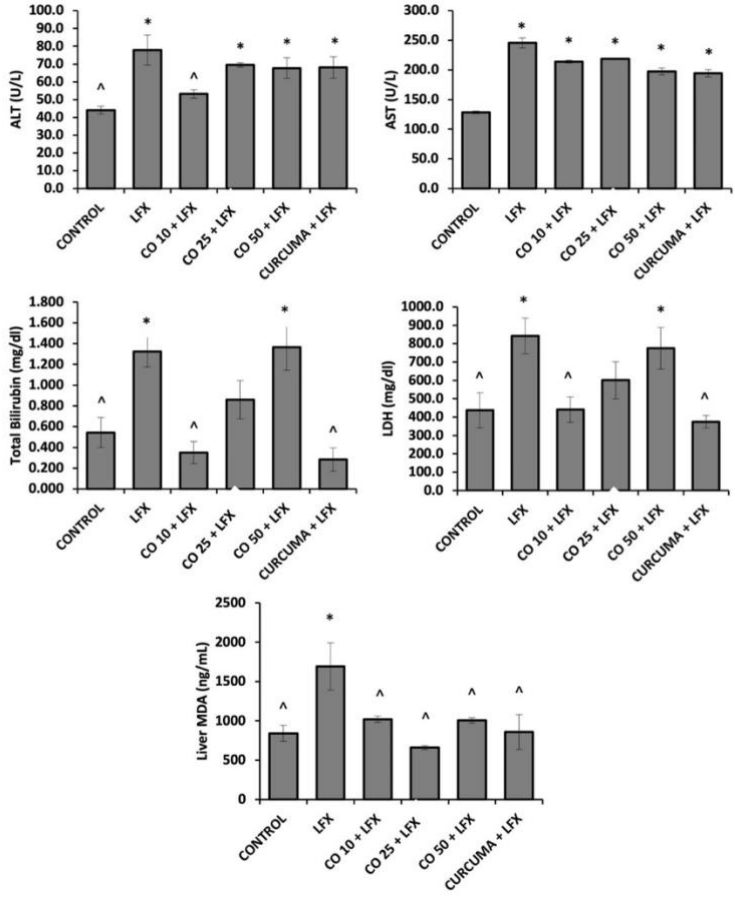

Figure 2. The levels of liver injury biomarkers following 28 days of different treatment administration in rats. A. Alanine aminotransferase (ALT), B. Aspartate aminotransferase (AST), C. Total bilirubin, D. Lactate dehydrogenase (LDH), and E. Liver malondialdehyde. (MDA). LFX: levofloxacin; CO: clove oil. *p<0.05 compared to the control group; ^p<0.05 compared to the LFX group

Photomicrographs of rat liver histology are shown in Figure 3. The liver histology of the control tissues demonstrates a normal architecture of hepatocyte lobules with portal triads at the vertices. The sinusoidal space was clear and sometimes contained Kupffer cells and blood cells. The hepatocytes had eosinophilic cytoplasm and clear nuclei containing nucleoli (Figure 3a). In contrast, the LFX-treated rats had significant histopathological changes in their liver tissues. Congestion of hepatic veins and hyperplasia of the bile ducts were evident (Figure 3b), along with infiltration of inflammatory cells (Figure 3c). Some areas of hepatocyte lobules experienced both hydropic and lipid degen-

eration (Figure 3d). Curcuma treatment led to improved liver histology (Figure 3e) similar to that of tissues treated with *S. aromaticum* L. oil at 10 mg/kg dose (Figure 3f). Pre-treatment with *S. aromaticum* L. oil at higher doses also led to improved hepatocyte structures, although some liver sections still presented hepatic degeneration and an increased number of Kupffer and inflammatory cells in the sinusoid (Figure 3g, h).

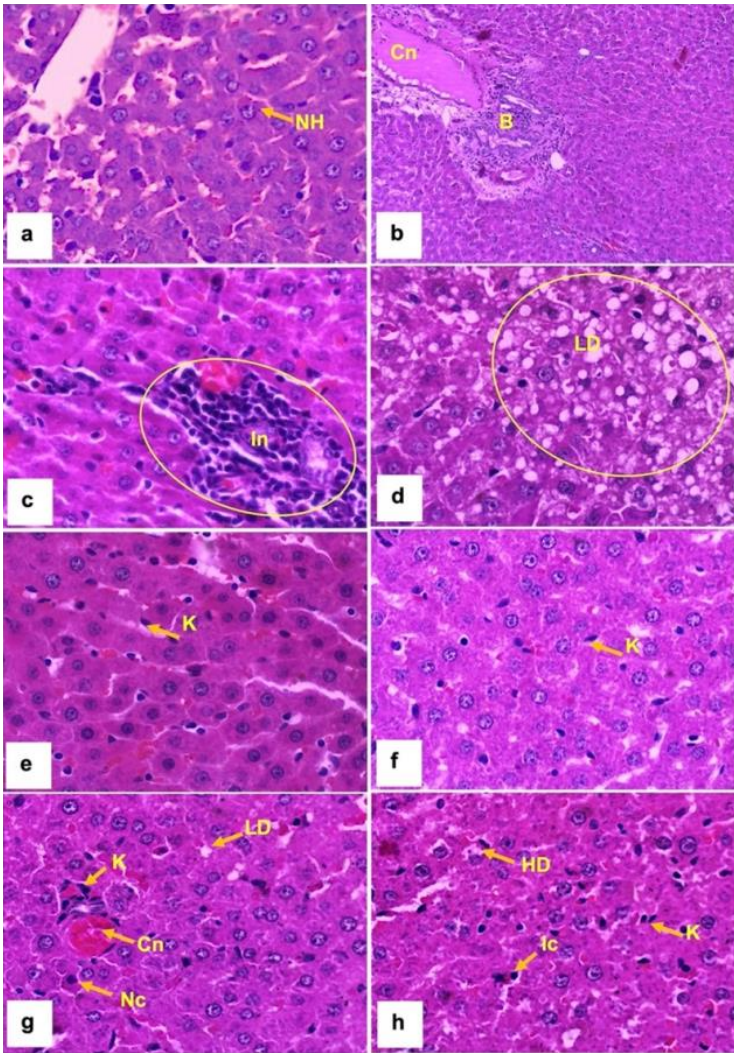

Figure 3. Representative photomicrograph of rat liver tissue in the control (a), LFX (b,c,d), Curcuma + LFX (e), CO 10 + LFX (f), CO 25 + LFX (g), CO 50 + LFX (h) groups. LFX: levofloxacin; CO: Clove oil; NH: normal hepatocyte; Cn: congestion; B: proliferation of biliary ducts; In: Inflammation; LD: lipid degeneration; HD: hydropic degeneration; K: Kupffer cell; Nc: necrotic cells

Hepatotoxicity was evident in levofloxacin-treated animals after 28 days of administration, as demonstrated by marked elevation of liver enzyme, total bilirubin, and oxidative product (MDA) levels. The histological examination supported the serum biomarker and liver oxidant levels. The signs of liver tissue injury were prominent, characterized by marked hepatocyte degeneration and infiltration of inflammatory cells in many areas of the hepatocyte lobules. The main finding of this study is that the liver damage induced by levofloxacin was alleviated by a pre-treatment with *S. aromaticum* L. oil, especially at a preventative dose of 10 mg/kg. Administration of *S. aromaticum* L. oil treatment not only allowed for recovery of serum biomarker levels and MDA but also improved liver histological features. Notably, the present study found that the low dose of *S. aromaticum* L. oil (10 mg/kg) provided better protection than the higher doses. The 10 mg/kg *S. aromaticum* L. oil returned ALT, bilirubin, LDH, and liver MDA levels to near normal, comparable to those of the curcuma treatment. Curcuma is a hepatoprotective agent that has been clinically used to treat hepatitis or drug-induced liver injury [17], hence, it was used in this study as a comparison.

The hepatoprotective effect of *S. aromaticum* L. oil had been evaluated previously against carbon tetrachloride-induced hepatotoxicity in rats [31], where the essential oil was administered 3 times a week (for 4 weeks) using higher doses (100 mg/kg and 200 mg/kg). However, carbon tetrachloride-induced hepatotoxicity may have different pathogenesis from that of levofloxacin. Since every drug has its own particular mechanism, it is important to mimic the mechanism of drug-induced liver injury, levofloxacin in this case, to examine if *S. aromaticum* L. oil can still elicit liver protection. In our study, daily administration of 10 mg/kg dose for 28 days (4 weeks) was sufficient to provide liver protection against levofloxacin-induced toxicity.

It is important to acknowledge that although it is considered safe for ingestion, some evidence has pointed out possible inducement of liver dysfunction by *S. aromaticum* L. oil in high doses. Shalaby et al.[32] showed that the sub-chronic administration of *S. aromaticum* L. oil at > 300 mg/kg dose per day might induce the elevation of ALT and AST levels and congestion and inflammation in the liver tissue. Meanwhile, a subacute toxicity study suggested that the no-observed-adverse-effect level (NOAEL) of eugenol was 50 mg/kg in rats, whereas higher doses might trigger unwanted effects, including liver injury [33].

The present study showed that 10 mg/kg *S. aromaticum* L. oil was the only dose effective at ameliorating levofloxacin-induced hepatotoxicity. Higher doses were not necessarily superior to the lower dose. Consistent with this

finding, an earlier study showed that 10 mg/kg of *S. aromaticum* L. oil treatment was more effective at reducing renal damage than greater doses (25 mg/kg and 50 mg/kg) [34]. Unfortunately, the toxic effects of *S. aromaticum* L. essential oil in rats, using daily administration for a longer period, were not evaluated. Further investigation of the effects of *S. aromaticum* L. oil treatment is needed to conclusively resolve the safe use parameters of *S. aromaticum* L. oil as a hepatoprotective agent.

STATEMENTS OF ETHICS

All animal protocols were performed according to the guide for the care and use of laboratory animals, and the study was granted an institutional ethical clearance (# UH20050197) in the Faculty of Medicine, Hasanuddin University, Indonesia.

CONFLICT OF INTEREST

Authors declare to have no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept: YYD; Design: YYD, AF, SL, AS; Data Collection or Processing: AF, FZ, NF; Analysis or Interpretation: YYD, AF, SL, AS; Literature Search: YYD, AF, FZ, NF; Writing: YYD, AF, FZ, NF; Revision and Proofreading: YYD, AF, SL, AS, FZ, NF

FUNDING SOURCES

This study obtained financial support from the Ministry of Research, Technology, and Higher Education of the Republic of Indonesia.

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