Black Seed Oil

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Black Seed Oil (*Nigella sativa L.*) Protects Against Liver Injury induced by Levofloxacin: Chemical, Biomarker, and Histopathological Analysis

Running title: Black seed oil protects against liver dysfunction

Abstract.

Long-term use of levofloxacin can cause alteration of liver function. This study aimed to determine the protective effect of Black Seed Oil (BSO) against liver injury due to levofloxacin administration in rats. Rats were treated with levofloxacin and BSO at different doses (1 ml/kg, 2 ml/kg, or 4 ml/kg) for 28 days. The presence of liver injury was confirmed with serum biomarker levels, liver malondialdehyde (MDA) levels, and histopathological appearance. Levofloxacin administration significantly increased the liver enzyme and MDA levels, and also induced a marked alteration in the liver histological structures. Treatments with 1 ml/kg and 2 ml/kg of BSO decreased the liver enzyme and MDA levels compared to the levofloxacin group ($p<0.05$). The recovery of liver structural damage was also observed. It is concluded that BSO administration could reduce liver dysfunction due to levofloxacin, especially at 1 ml/kg and 2 ml/kg doses.

Keywords: Black seed oil, *Nigella sativa*, levofloxacin, hepatotoxicity

Introduction

Tuberculosis eradication is now challenged by the increased number of multidrug-resistant tuberculosis (MDR-TB) cases worldwide.[1] Levofloxacin, an antibiotic from the fluoroquinolone group, is one of the most common regimens used for MDR-TB cases 2. It has the ability to inhibit *Mycobacterium tuberculosis* DNA supercoiling, thus damaging the DNA replication by interfering with DNA gyrase activity 3. However, fluoroquinolone may induce hepatotoxicity characterized by elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels 4. Hepatotoxicity due to levofloxacin may occur after 5-14 days from therapy initiation 5, 6. Cases of hepatotoxicity have been reported in a number of patients who received levofloxacin in the management of MDR-TB 7. In line with this, retrospective data with 746 subjects showed an increase in the incidence of hepatotoxicity during fluoroquinolone use, especially levofloxacin and moxifloxacin 8.

Therapies to treat hepatotoxicity remain limited due to the lack of approved drugs with hepatoprotective potential. A number of studies have reported the beneficial effects of black seed oil (BSO) from the plant *Nigella sativa* in preventing drug-induced liver injury 9. BSO has been shown to possess several bioactive compounds, including p-cymene, carvacrol, 4-terpineol, t-anethol, thymoquinone, thymohydroquinone, and dithymoquinone 10. Thymoquinone has been demonstrated to be capable of inhibiting oxidative stress by increasing the activity of the antioxidant enzymes, mostly superoxide dismutase and glutathione peroxidase, and also reducing the lipid peroxidase activity 11. Since levofloxacin toxicity mostly occurs due to diminished glutathione supplies 12, strengthening the body's defense system through the use of antioxidants would be essential to overcome oxidative stress-related cellular damage. Therefore, this study aimed to determine the protective effect of black seed oil against levofloxacin-induced toxicity on liver function in rats.

Material and Methods

Chemicals and drugs

Black seed oil (BSO) was obtained from black cumin seed (*Nigella sativa)* purchased from Al-waqia'ah supplier, Indonesia. Levofloxacin tablets (Hexpharm Jaya®) were obtained from a licensed pharmaceutical store in Makassar, Indonesia. Thiobarbituric acid (TBA, Sigma-Aldrich), 1,1,3,3-tetra methoxy propane (TMP, Sigma-Aldrich), and Trichloroacetic Acid (TCA, Merck) were ordered via the official suppliers. Diagnostic kits for gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT), were obtained from Human Diagnostics Worldwide (Germany).

Chemical constituent analysis

Prior to the *in vivo* experiment, a chemical analysis was conducted to identify the chemical compositions of the BSO sample used in this study. The analysis was performed using a Trace 1310 gas chromatography with TSQ 8000 Evo mass spectrometry (Thermo Scientific, USA). The column size was 20 m x 0.18 mm (TG -5MS) and helium gas was used as the carrier. The oven had an initial and final temperature of 50oC and 330oC, respectively, at the rate of 10 to 25ºC/min.

Preparation of Animals

The experimental animals used in this study were 30 male albino rats (*Rattus norvegicus*) at a weight of 200-300 g. The rats were acclimatized for 14 days prior to treatment and were provided with standard pellets and drinking water *ad libitum* throughout the experiment. The animal care protocols were carried out based on the institution's animal standard of care and have been granted an ethical clearance number of 318/UN4.6.4.5.31/PP36/2020.

Preparation of levofloxacin and black seed oil

The dose of levofloxacin used in this study was based on the human dose (15 mg/kg of human body weight per day) that was converted to the animal dose as described in Nair and Jacob (13) study. According to the calculation, the levofloxacin animal dose was 93 mg/kg for rats. With the dose, levofloxacin has been shown to sufficiently induce renal toxicity in rats 14. Levofloxacin powder was suspended in 1% NaCMC immediately before administration. The BSO was diluted with corn oil in three different concentrations, i.e. 10%, 20%, and 40%, and the volume of administration was 1 ml/200 g rat body weight. These concentrations were equivalent to BSO doses of 1 ml/kg, 2 ml/kg, and 4 ml/kg, respectively.

Experimental Protocol

The animals were divided into five groups of six., i.e. the healthy control, the levofloxacin group (received levofloxacin suspension and corn oil as a placebo), and three BSO treatment groups that received BSO in different doses (1 ml/kg, 2 ml/kg, or 4 ml/kg body weight). The BSO treatment was given 2 hours before levofloxacin administration every day for 28 days. Blood samples were taken before (day 0) and after treatment (day 29). Following the final blood sampling, a necropsy was performed to harvest rats' livers for further analysis. The right lobe of the liver was fixed in formaldehyde 10% in Phosphate Buffer Saline (PBS) for histopathological analysis, while the left lobe was immediately immersed in liquid nitrogen for malondialdehyde analysis.

Serum Biomarker Analysis

The blood samples were centrifuged at 3000 rpm for 20 minutes. The levels liver enzymes (ALT, AST, and GGT) were analyzed according to the kits' instructions. The blood plasma (100 µl) was mixed with 1000 µl buffer, and incubated at 37°C for 5 minutes, subsequently, 250 µl of substrate kit was added and reincubated for 1 minute at 37°C. All serum biomarker levels were measured using Humalyzer 3500 (Human, Germany) at 340 nm wavelength.

Liver malondialdehyde (MDA) analysis

The MDA analysis was carried out according to Djabir (15) study. Rat liver weighing 400 mg was ground with a mortar and pestle and homogenized with PBS with a pH of 7.4. The homogenized mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant (0.5 ml) was added with 1 ml of 1% TBA and 1 ml of 10% TCA before being placed in a water bath at 90°C for 20 minutes. The absorbance was measured at a wavelength of 531 nm using a UV-Visible spectrophotometer.

Histopathological examination

After forty-eight-hour storage in formaldehyde, the liver specimens were cut into 0.5-1 cm thickness. The specimens were stored in embedding cassettes and then processed in a tissue processor (Thermo Scientific, USA). The specimens were then prepared in paraffin blocks and sliced into 4-5 μm thickness using a microtome. The slices were then stretched on a floating out with a temperature of 40°C. The specimens were placed on glass slides and dried using an electric hotplate for at least 2 hours before being stained with hematoxylin and eosin (HE) and cover-slipped. The histopathological examination took place under a light microscope (Olympus, Japan) with a camera (Nikon, Japan) attached. The photomicrograph was taken with 100X and 400X magnifications. The intensity of the liver damage was determined by two observers blinded to the treatment groups.

2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activity test

The antioxidative activity of the BSO sample was tested against DPPH radicals. Briefly, the IC_{50} (half maximal inhibitory concentration) value was determined by measuring the absorbance of a series of BSO concentrations using a UV-visible spectrophotometer at a wavelength of 515 nm. The IC_{50} was plotted from a concentration-response curve.

Statistical Analysis

Data are presented in mean ± SD. The normal distribution of data was determined using Shapiro-Wilk analysis, continued with one-way ANOVA analysis, and followed by Tukey's HSD test to determine significant differences between groups. Statistical significance was defined if $p<0.05$.

Results and Discussion

Chemical constituents

The GC-MS analysis of BSO showed 25 peaks on the chromatogram, suggesting 25 volatile chemical compounds were contained in BSO (Table 1). There were six major constituents, i.e. 9,12-octadecadienoic acid(Z,Z)- (44.94%), bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)- (10.23%), thymoquinone (10.14%), o-Cymene (9.05%), n-hexadecanoic acid (8.25%), and 9,12-Octadecadienoic acid(Z,Z)-2,3-dihydroxypropyl ester (6.95%).

Based on Amin and Hossein's study 16, BSO consists of 22-38% fixed oil, such as linoleic acid, oleic acid, tocopherols, retinol, carotenoids, thymoquinone, and about 0.40-1.50% volatile oil. Among the essential oil, the main constituent is mostly thymoquinone (14-28%) 16-18. In fact, as the main constituent with antioxidant and anti-inflammatory activity, thymoquinone has been recommended as the marker for BSO 19. The BSO sample used in this study had a lower thymoquinone concentration (10.14%) compared to those found in the

other studies 16-18. This may emphasize the need to standardize the BSO supplement that is commercially available.

Liver Biomarkers

Fluoroquinolone antibiotics, including levofloxacin, have one of the most serious potential side effects compared to other antibiotics because they can cause permanent damage, and even death, especially if their use is not appropriate 20. The incidences of levofloxacin induced-hepatotoxicity have been reported in many cases, characterized by increased levels of AST, ALT, ALP, and bilirubin, as well as diffuse hepatocellular necrosis and intense cellular degeneration 4.

When rats were given levofloxacin (93 mg/kg) for 28 days, the liver enzyme levels such as AST, ALT, and GGT were significantly elevated ($p<0.05$) (Figure 1). The elevation of AST, ALT, and GGT may suggest the presence of damage in hepatocytes. Simultaneously, the liver MDA levels were also increased approximately three-fold compared to the controls ($p<0.05$). Since MDA is a product of lipid peroxidation, the increase in liver MDA level indicates the increase in lipid peroxidation activity in the liver of rats after levofloxacin administration. The hepatotoxic effect of drugs is often associated with oxidative stress, an imbalance between the antioxidant cellular system, and the production of reactive oxygen species (ROS). ROS can cause severe damage to macromolecules, tissues, and organs predominantly through lipid peroxidation, protein modification, and DNA damage 21.

In this study, when compared to the levofloxacin group, pre-treatment with black seed oil at the lowest dose (1 ml/kg) induced a substantial decrease ($p< 0.05$) in all of the liver biomarker levels (Figure 1). It is worth noting that the reductions were not dose-dependent. When compared to the 1 ml/kg dose, the administration of BSO in a higher dose (2 ml/kg) seemed to reduce the AST, GGT, and MDA levels but failed to prevent the increase in ALT levels. Interestingly, in the groups pre-treated with the highest dose of BSO (4 ml/kg), all of the liver biomarker levels were not reduced, indicating a marked injury still affecting the liver similar to that seen in the levofloxacin group.

Histopathological Analysis

Figure 2 represents the result of histopathological observation using H&E staining under a light microscope. In the healthy controls, without levofloxacin administration, the rat livers showed some normal architecture with no or minimal damage. In contrast, the liver tissues of rats treated with levofloxacin without BSO were characterized by a large area of hydropic degeneration, ballooning hepatocytes, lipid degeneration, vacuolization, congestion and hemorrhage (Figure 2B-C). The histological damage was diffuse and mostly evident in >50% of the observed area at 400x magnification (Figure 2D).

In general, the group pre-treated with BSO 1 ml/kg showed minimal damage with the presence of lipid degeneration and necrotic cells (Figure 2E). These histopathological changes only affected a small number of cells as observed in a microscope at 400x magnification (Figure 2E). Similarly, rats treated with BSO 2 ml/kg also mostly showed a normal appearance of liver tissue structure apart from a low number of lipid degeneration and necrotic cells (Figure 2F). Meanwhile, the group treated with BSO 4 ml/kg appear to have minimal to mild liver damage (Figure 2G). The histopathological changes observed were mostly characterized by the occurrence of lipid degeneration. The occurrence of lipid degeneration is shown by swollen cytoplasm and vacuolization of hepatocytes caused by the accumulation of lipid.

Although most drug-induced hepatotoxicity has been shown to induce an inflammatory reaction in the liver 22, 23, there was a lack of inflammatory cell infiltration observed with levofloxacin administration. Instead, lipid and hydropic degenerations have been the hallmark of histopathological changes seen in our study. A similar result was also observed in mice treated with low to the high dose of levofloxacin, where swollen hepatocytes, necrosis, vacuolization, and pyknosis have been the most evident changes found in the liver 24. With BSO treatment, the presence of histological damage was markedly reduced at all given doses, but more obvious in BSO 1 ml/kg and 2 ml/kg groups.

DPPH scavenging activity

Since the radical scavenging mechanism has been proposed as the main mechanism of the hepatoprotective effect of BSO, a DPPH radical scavenging assay was performed to confirm this. DPPH is a highly stable organic chemical compound that can react with antioxidant compounds by accepting hydrogen atoms 25. Thus, this assay may estimate the antioxidant activity of BSO by the mechanism related to free radical scavenging. It is found that the IC_{50} of the BSO sample used was >250 µg/ml based on the concentration-response curve shown in figure 3.

This IC_{50} value demonstrates that the BSO concentration required to inhibit 50% of DPPH radicals was higher than 250 µg/ml. Another study reported the IC_{50} value of BSO was around 460 mg/mL using a DPPH assay, which was much higher than its individual chemical content such as thymoquinone (211 mg/mL) and carvacrol (29 mg/mL) 17. This result shows that BSO's radical scavenging activity was not as strong as thymoquinone or carvacrol. This may suggest that the protective effect of thymoquinone or carvacrol perhaps is superior to BSO itself. Unfortunately, we did not directly compared the IC_{50} value of the BSO sample with alpha-tocopherol or any other standard antioxidants, which may be necessary to provide a quantitative basis for BSO radical scavenging activity 26. Further study is required to shedding some light on this matter.

Clinical significance

Since the hepatotoxic side effects of levofloxacin might be serious and even life-threatening, it is imperative to find a therapeutic strategy to prevent levofloxacin-induced hepatotoxicity. The use of black seed oil may be found beneficial. Several studies have reported the benefits of black seed oil and some parts of *Nigella sativa* plants as an antioxidant, anti-cancer, anti-inflammatory, and anti-bacterial 27. Based on our study, the black seed oil is beneficial to reduce levofloxacin-induced hepatotoxicity confirmed by reduced liver biomarker levels and improved liver histopathology. This is in accordance with the results of other studies showing hepatoprotective effects of black seed oil against paracetamol 28, carbon tetrachloride 29, and vitaminosis-induced toxicities 30. The protective effects of *Nigella sativa* have been not only demonstrated in the liver, but also in other organs, including the heart 31, gastrointestinal 32, and kidneys 14.

The protective effect of black seed oil may derive from its radical scavenging activity leading to an inhibition of lipid peroxidation activity in the liver, as confirmed in our study through malondialdehyde level measurement. Its antioxidant compound, thymoquinone, has been shown in another study to prevent a decrease in the activity of enzymatic antioxidants, such as glutathione peroxidase, superoxide dismutase, and catalase, as well as non-enzymatic antioxidants such as Vitamins A, E, and C (Alkadri et al., 2019).

Conclusion

In conclusion, black seed oil from the *Nigella sativa* plant has a protective effect against liver toxicity following 28 days of levofloxacin administration. The hepatoprotective effect was confirmed by a decrease in liver enzyme serum levels, recovered liver tissue injury, and a reduction in liver malondialdehyde level. The protection was at the greatest when the black seed oil was given at the doses of 1 ml/kg and 2 ml/kg. This finding may find an application in a clinical setting.

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Authors' contributions
NR: data acquisition, data analysis and manuscript writing: YYD: research concept and design, project leader, data interpretation, manuscript writing and editing; SA and YCP: data acquisition and analysis; AS and RY: data interpretation and manuscript revision.

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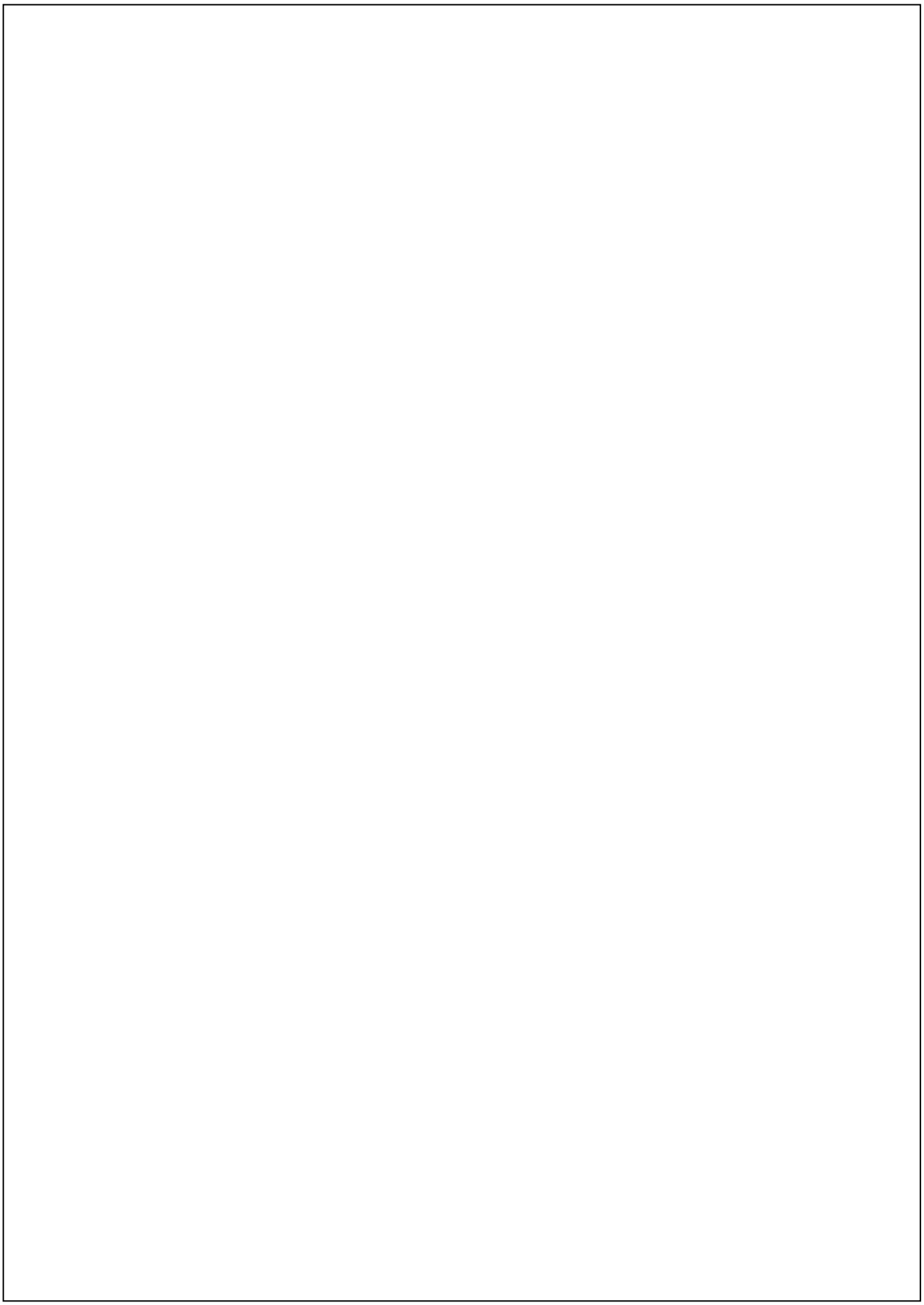

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