Majalah Obat Tradisional (Trad. Med. J.), January-April 2026
Vol. 31(1), p 43-53
ISSN-p : 1410-5918 ISSN-e : 2406-9086

Submitted : 14-11-2024
Revised : 26-07-2025
Accepted : 18-02-2026

Hepatoprotective Effects of Milkfish (*Chanos chanos*) Oil and Silymarin Against Isoniazid–Rifampicin-Induced Liver Injury in Rats

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ABSTRACT

The objectives of the present research were to assess the hepatoprotective activities of milkfish oil (MFO) and silymarin (SL) against rifampicin (RFP) and isoniazid (INH) induced hepatotoxicity. Rats were divided into seven groups: normal control, negative control (INH+RFP), silymarin alone (50 mg/kg BW/day), low-dose MFO (MFO-L), high-dose MFO (MFO-H), low-dose combination (SL+MFO-L), and high-dose combination (SL+MFO-H). Rats receiving RFP and INH showed raised liver enzymes and typical signs of hepatotoxicity. Analyzed parameters comprised proinflammatory mediators (tumor necrosis factor-alpha and interleukin-6), antioxidant markers (catalase, glutathione, malondialdehyde, and superoxide dismutase), cytochrome P450, total protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin and bilirubin. Treatment with a combination of MFO plus SL remarkably decreased hepatic enzyme activities, oxidative stress and inflammation, and suggested a prevention effect against the drug-induce liver injury.

Keywords: Milkfish; Silymarin; Hepatotoxicity; Rifampicin-Isoniazid

INTRODUCTION

Mycobacterium tuberculosis causes tuberculosis (TB), which is one of the most common infections that kills people around the world. In terms of public health, this disease is more serious than HIV/AIDS (Bhatt et al., 2023; Jeong et al., 2023). The number of reported TB cases in Indonesia in 2023 reached 809,000 (Pratiwi et al., 2025). Isoniazid (INH) and rifampicin (RFP) are the two drugs that are utilized most frequently to treat tuberculosis. When these two drugs are used together, they can work well and even better, which lowers the risk of drug resistance. However, this combination also has potentially harmful side effects, including increased hepatotoxicity and a risk of acute liver failure (Akkerman et al., 2023; Djohan et al., 2023; Somasundaram et al., 2014). Previous research has shown that the interaction between the two drugs can change how Cytochrome P450 (CYP450) and its subtypes work, which makes more metabolites that are toxic to the liver (Biswas et al., 2020; Zhuang et al., 2022). Hepatotoxicity produces from the N-acetyltransferase (NAT) enzyme's metabolism of INH, causing toxic metabolites like hydrazine and acetylhydrazine (Li et al., 2021). The combination with RFP strongly induces CYP450 enzymes, accelerating the metabolism of INH and consequently increasing the occurrence of hepatotoxicity (Biswas et al., 2020). This condition requires prevention to reduce hepatotoxicity due to the use of TB drugs.

Silymarin (SL) is an extract from milk thistle seeds that has been widely reported as a hepatoprotective agent (Jabbari et al., 2023; Mukhtar et al., 2021). Traditionally, SL has also been used in treatments for liver and kidney conditions, to increase breast milk production, and to protect the liver from fungal poisoning. Silymarin has been reported to contain flavonoids with antioxidant activity, which can inhibit liver fibrosis caused by CCl_4 induction in rats over 14 days. This study showed that SL provides a hepatoprotective effect by increasing the liver's antioxidant capacity, reducing lipid peroxidation, and protecting hepatocyte membranes from damage (Babu et al., 2023; Okiljević et al., 2024). In addition to SL, various hepatoprotective agents have been widely explored in recent studies. The potential for synergistic or complementary effects among these agents provides a strong rationale for the development of combination therapies, particularly within the context of traditional or herbal medicine. Milkfish oil (MFO) is one of the potential animal sources for

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hepatoprotective agents (Sasongko et al., 2022; Sasongko et al., 2024). Based on previous research, milkfish has been used in treatments due to its significant anti-inflammatory, antioxidant, and DNA protection activities (Liu et al., 2024; Masrukan et al., 2024). Omega-3 fatty acids have been reported to have hepatoprotective functions by reducing oxidative stress in rats induced by fipronil. Other studies have also mentioned that omega-3 boosts the immune system and produces high levels of antioxidants to prevent the formation of free radicals (Masrukan et al., 2024; Refaie et al., 2021). Based on these considerations, this study was conducted to investigate the hepatoprotective effects of the SL+MFO combination in RFP+INH-induced hepatotoxic rats.

MATERIALS AND METHODS

Materials

Milkfish (*Chanos chanos*) was obtained from Bakaran Kulon Village, Juwana Subdistrict, Pati Regency, Central Java, and determined by the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Bentonite, silymarin was acquired from the Sigma Aldrich®, manufactured in Singapore. INH 400 mg was sourced from PT. Mesifarma®, and rifampicin 450 mg was obtained from PT Sanbe Farma®. ELISA assay kit: CYP450-Glo™, catalase, glutathione, malondialdehyde, and superoxide dismutase, TNF-α, IL-6 from Abkine CheKine™. Reagent total protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, and bilirubin from Biosystem®.

Preparation of Experimental Animals

The milkfish was cleaned, with the guts removed, and then cut into small pieces to increase the surface area. The pieces were placed into a pot and boiled with a 1:3 ratio of distilled water at 100ºC for 5 hours. The boiled water was left to stand for 24 hours to obtain an oil deposit. The boiled liquid was then separated between the oil and water phases using a separation funnel and purified by heating to a temperature of 55–60ºC. Subsequently, 1% bentonite (by oil weight) was added as an adsorbent, and the mixture was further heated to 80ºC for 30 minutes. The fish oil was filtered using a vacuum filter (Büchner funnel). Male Wistar rats weighing 160-170 g and aged 2-3 months were provided by CV. Dunia Kaca, Central Java. The rats were maintained in controlled conditions of temperature and humidity. All rats were treated equally, being fed BR1 feed at 20 g/day, with drinking water provided ad *libitum*. All experimental methods were carried out with the agreement of the Dr. Moewardi General Hospital animal test ethics committee (No. 586/IV/HREC/2023).

Animal Testing

The rats were randomly divided into 7 groups (n = 5 per group) and treated for 28 consecutive days (4 weeks) as follows: normal control group, negative control group (INH+RFP group), single-dose silymarin group (SL 50 mg/kgBW/day), low-dose MFO group (MFO-L), high-dose MFO group (MFO-H), low-dose combination group (SL+MFO-L), and high-dose combination group (SL+MFO-H). All rats were treated as follows: (i) normal control group: rats were given standard food and drink, (ii) negative control group: rats were fasted for 1 hour, then given INH 100 mg/kgBW/day + RFP 100 mg/kgBW/day orally, (iii) single-dose silymarin group: rats were given silymarin solution 50 mg/kgBW/day, (iv) MFO-L group: rats were given milkfish oil solution 100 mg/kgBW/day, (v) MFO-H group: rats were given milkfish oil solution 300 mg/kgBW/day, (vi) SL+MFO-L group: rats were given SL solution 50 mg/kgBW/day and milkfish oil 100 mg/kgBW/day, and (vii) SL+MFO-H group: rats were given SL 50 mg/kgBW/day and milkfish oil 300 mg/kgBW/day. One hour after the sample administration, rats were given an INH 100 mg/kgBW/day + RFP 100 mg/kgBW/day solution orally, and then fasted again for 1 hour. At the end of the experiment, blood and liver samples were collected. Serum was separated by centrifuging blood samples at 3,000 rpm for 15 min, and biological activity of liver biomarkers was analyzed. Liver tissues were rinsed with NaCl, dried, weighed and analyzed further.

Liver Biomarker Analysis

The levels of alanine transaminase (ALT), aspartate aminotransferase (AST), total protein, albumin, and total bilirubin (TBIL) were measured in centrifuged blood serum using commercially available equipment and reagents in accordance with the instructions. The protocol supplied by the reagent manufacturer was followed when conducting the sample testing procedure.

Estimation of Antioxidant Enzymes

Liver tissues were homogenized and centrifuged at 2,600 rpm for 10 min. The levels of superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) in the supernatant were measured by Abbkine CheKine™. The sample test method was performed according to the manufacturer's reagent instructions.

Determination of CYP450

The serum CYP450 content was measured from the supernatant using P450-Glo™ kits. The sample testing method was carried out according to the reagent manufacturer protocol.

Determination of inflammation marker

The inflammation cytokines TNF-α and IL-6 were measured from the liver homogenate supernatant by Abbkine CheKine™. The sample examination followed the assay procedure of a reagent manufacturer.

Data Analysis

The quantitative data were expressed as mean values and statistically analyzed using one-way ANOVA, followed by Tukey's post hoc test, with significance levels set at 0.05 and 0.01.

RESULTS

Results of Milkfish Oil Extraction

The yield obtained from milkfish oil extraction was 2.597%, which is comparable to the findings of Aziza et al. (2015), who reported a yield of 2.365%. Aziza explained that the yield from fresh milkfish is lower than that from dried milkfish due to the water content being included in the weight during yield calculations.

Effects of MFO and SL on Liver Index

Figure 1 presents the liver index comparisons between the normal control group and the experimental groups. A significant increase in liver indices was observed in the model group following INH and RFP administration ($P < 0.01$). However, treatment with silymarin and milkfish oil, both individually and in combination, resulted in significantly lower liver indices compared to the model group ($P < 0.01$).

Effects of MFO and SL on CYP450 Levels

As shown in Figure 2, CYP450 levels were significantly elevated in the negative control group following INH and RFP administration compared to the normal control group ($P < 0.01$), suggesting that the treatment induced hepatic metabolic stress. In contrast, administration of silymarin (SL) and milkfish oil (MFO), either as monotherapy or in combination, significantly reduced CYP450 levels relative to the negative control ($P < 0.01$), indicating a modulatory effect on hepatic enzyme expression.

Effects of MFO and SL on Antioxidant Parameters

The antioxidant biomarkers analyzed included glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and malondialdehyde (MDA) (Figure 3). Rats in the negative control group exhibited a significant decrease in GSH and CAT levels following INH + RFP administration ($P < 0.01$), reflecting oxidative stress in liver tissue. A single dose of SL significantly increased GSH levels ($P < 0.05$), whereas both single and combination treatments with MFO produced a more pronounced increase ($P < 0.01$). CAT levels were significantly elevated in all treatment groups compared to the negative control ($P < 0.05$). Similarly, SOD activity was markedly decreased in the negative group ($P < 0.01$), indicating disruption of antioxidant defense mechanisms. Treatment with SL and MFO—either alone or in combination—significantly restored SOD levels, suggesting protective antioxidant effects. Conversely, MDA levels, a marker of lipid peroxidation, were significantly elevated in the negative group ($P < 0.01$). All treatment groups demonstrated a significant reduction in MDA levels compared to the negative control ($P < 0.01$), further supporting the antioxidant efficacy of SL and MFO.

Effects of MFO and SL on Inflammatory Parameters

Inflammatory parameters, including TNF-α and IL-6, significantly increased in the negative group, indicating that RFP + INH also affected these inflammatory markers ($P < 0.05$). SL and MFO treatments, whether single or combined, significantly reduced TNF-α and IL-6 levels. As shown in (Figure 4), SL and MFO were more sensitive to IL-6, leading to a more pronounced decrease.

Effects of MFO and SL on Blood Biochemical Parameters

Based on the results interpreted in (Figure 5), ALT and AST levels in the negative group significantly increased ($P < 0.01$) compared to the control group, demonstrating that INH + RFP induction influenced ALT and AST activity in liver-damaged rats. In contrast, ALT and AST levels significantly decreased in all treatment groups compared to the negative group ($P < 0.01$).

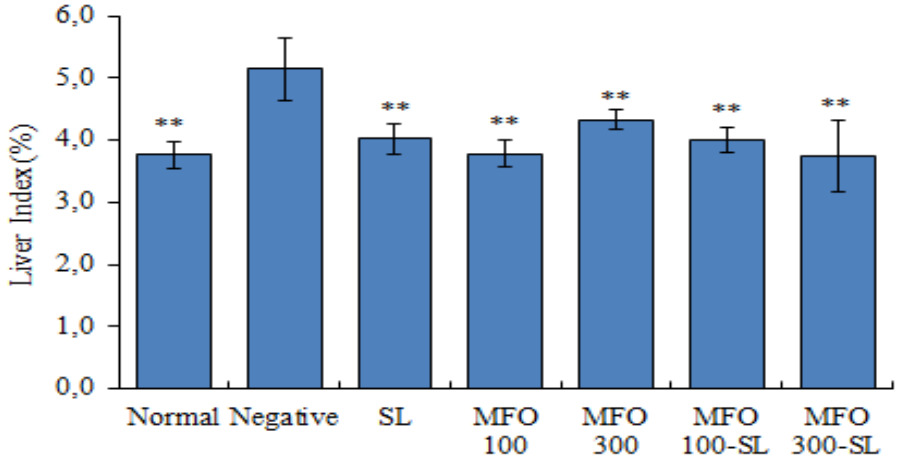

Figure 1. Effect of milkfish oil and silymarin on the liver index percentage in INH and RFP-induced liver injury in rats (n=5). *, Significantly different compared to the negative group (p<0.05). **, Significantly different compared to the negative group (p<0.01).

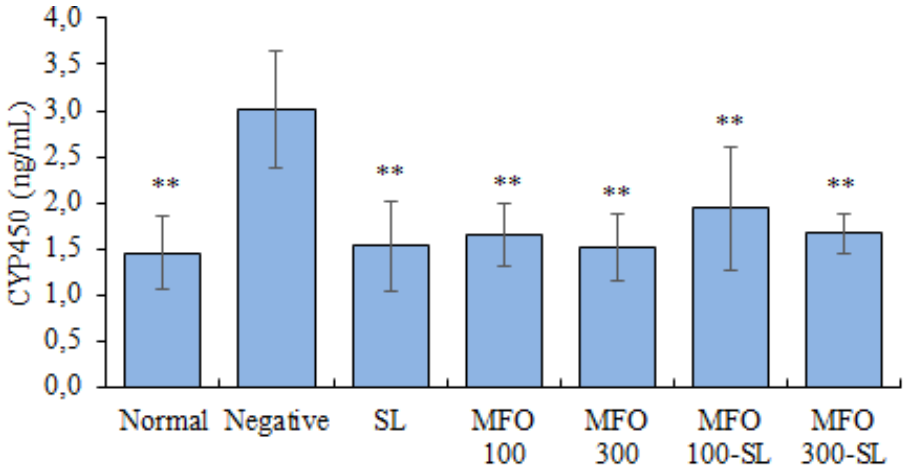

Figure 2. Effect of milkfish oil and silymarin on CYP450 levels in INH and RFP-induced liver injury in rats (n=5). *, Significantly different compared to the negative group (p<0.05). **, Significantly different compared to the negative group (p<0.01).

DISCUSSION

The combination of isoniazid (INH) and rifampicin (RFP) is one of the most commonly prescribed regimens for tuberculosis treatment. However, long-term use is known to cause hepatotoxic side effects, making it a priority to identify agents that can effectively repair liver damage induced by anti-TB drugs (Wang et al., 2022). Both milkfish (high in fatty acids and amino acids) and silymarin (historically, an effective traditional medicine with a spectrum of clinical uses and proven therapeutic effects such as antioxidant, anti-inflammatory and hepatoprotective) (Karimi et al., 2011; Murthy et al., 2016). In the present study, based on the hepatoprotective effect of MFO and SL in RFP and INH-induced rat test system, a strategy for new drug discovery with high effectiveness and low toxicity is proposed to be used in the treatment of drug-induced liver injury.

The liver is INH's main metabolic site, with the metabolization of INH by N-acetyltransferase 2 (NAT2) into hydrazine (Hz) and acetylhydrazine (AcHz), both of which can lead to oxidative stress and hepatocellular injury (Lei et al., 2021). Rifampicin, an established inducer of CYP450, accelerates the metabolic inactivation of INH and therefore elevates the level of these toxic metabolites (Zhuang et al., 2022).

In this research, the negative controls (hepatotoxic model) with RFP and INH were compared with treatment groups with MFO and SL with both single and in combination. There was a marked regulatory effect from treatment groups

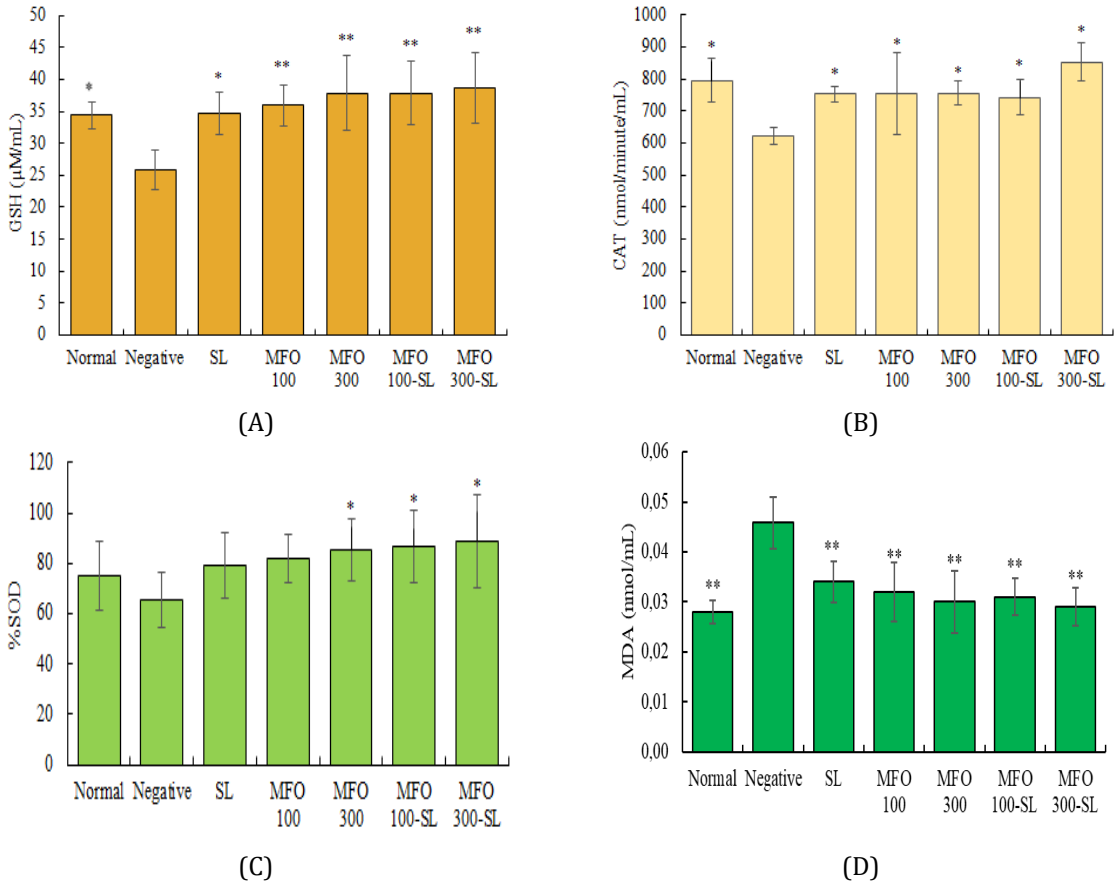

Figure 3. Effect of milkfish oil and silymarin on antioxidant parameters, GSH (A), CAT (B), SOD (C), and MDA (D) levels in INH and RFP-induced liver injury in rats (n=5). *, Significantly different compared to the negative group (p<0.05). **, Significantly different compared to the negative group (p<0.01).

on CYP450 content, and the phenomenon might indicate a metabolic regulation in adaptation to INH- and RFP-induced liver injury. In the negative control group, CYP450 levels increased significantly after INH + RFP treatment, suggesting a disturbance of hepatic enzymes during drug-induced liver injury. However, the CYP450 content in the MFO and SL treated groups were significantly decreased which is in agreement with the possible beneficial hepatoprotective effects by which MFO and SL may help to shift the metabolism of INH and RFP towards an alternative metabolic pathway.

Antioxidant enzymes, including GSH, CAT, SOD, and MDA, are crucial indicators for assessing the extent of tissue damage caused by free radicals (Tekin & Seven, 2022). GSH is the most abundant antioxidant in the body, playing a role in maintaining redox balance and breaking down H_2O_2 into H_2O (Guan, 2023; Wu et al., 2004). SOD initiates the antioxidant process by converting reactive O_2- radicals into the more benign H_2O_2 and O_2 (Zheng et al., 2023), followed by CAT, which converts H_2O_2 into O_2 and H_2O (Ransy et al., 2020). MDA, a byproduct of lipid peroxidation caused by free radical damage, is a biomarker for evaluating the effectiveness of the body's defense against oxidative stress, with high MDA concentrations indicating low antioxidant activity and greater cell damage (Zhang et al., 2019). The groups treated with milkfish oil and silymarin exhibited significantly higher levels of GSH and CAT compared to the negative control group. However, SOD levels did not show a significant increase in the MFO 100 and SL treatment groups. In contrast, the negative control group showed a marked elevation in MDA levels, indicating lipid peroxidation and liver damage, as supported by biochemical enzyme parameters (Figure 5). Conversely, the treatment groups demonstrated hepatoprotective effects, as evidenced by reduced MDA levels and improved antioxidant status. These findings suggest that the test compounds possess detoxifying properties and are effective in

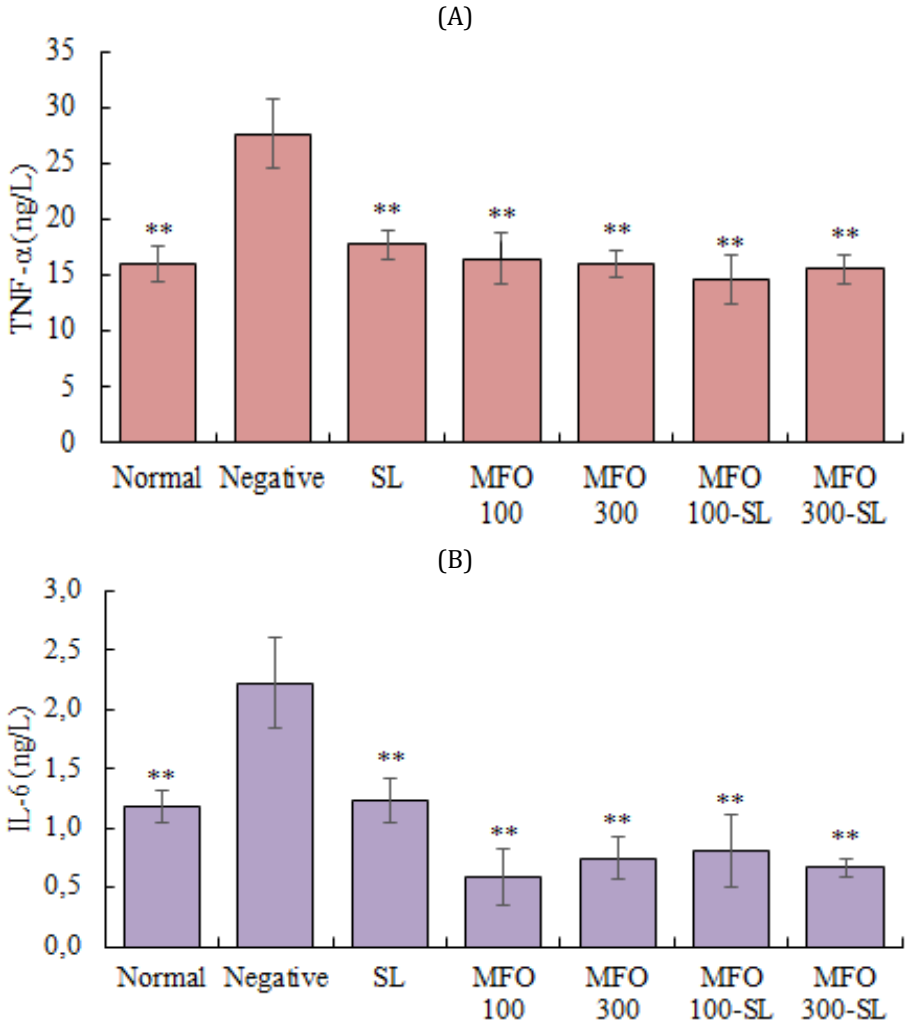

Figure 4. Effect of milkfish oil on inflammatory parameters, TNF-α (A) and IL-6 (B) levels in INH and RFP-induced liver injury in rats (n=5). *, Significantly different compared to the negative group (p<0.05). **, Significantly different compared to the negative group (p<0.01).

preventing liver injury. The observed increase in endogenous antioxidant levels, particularly in the MFO-treated groups, may be attributed to the presence of amino acids that support GSH synthesis and fatty acids that contribute to the maintenance of redox homeostasis. Eicosapentaenoic acid (EPA) enhances CAT and SOD activity, increasing the amount of SOD produced (da Silva et al., 2016; Fazelian et al., 2021; Giustarini et al., 2023). SL, on the other hand, boosts GSH capacity to absorb free radicals, increases GRx levels (responsible for regenerating oxidized GSSG into reduced GSH), and independently scavenges free radicals to maintain redox balance (Borgonovi et al., 2023; Surai, 2015; Taleb et al., 2018).

Inflammation parameters can be observed through TNF-α and IL-6 levels. TNF-α is a key mediator in stimulating liver inflammation and is considered a primary driver of liver tissue damage (Khura et al., 2019). TNF-α and IL-6 levels indicate the severity of tissue inflammation. TNF-α, released by Th1 cells and macrophages, stimulates synovial fibroblasts, promoting excessive tissue growth and attracting inflammatory cells to the area (Jang et al., 2021). IL-6, another important cytokine, stimulates the liver to produce acute-phase proteins like C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen, haptoglobin, and α1-antichymotrypsin, while reducing the production of fibronectin, albumin, and transferrin (Tanaka et al., 2014). The negative control group showed significantly elevated TNF-α and IL-6 levels compared to the normal control group, while the MFO 300 and SL combination groups had the lowest levels of TNF-α and IL-6, indicating that

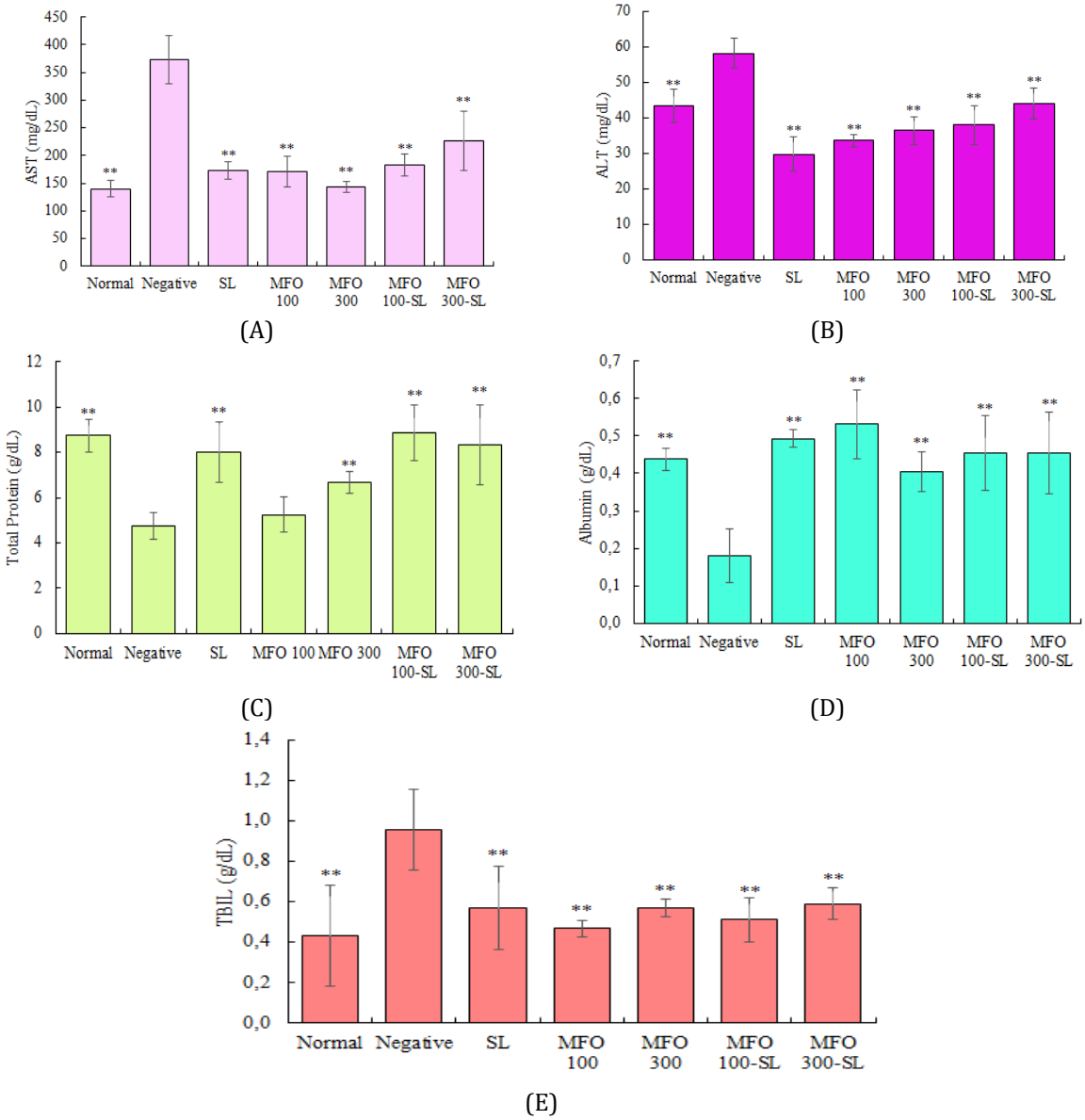

Figure 5. Effect of Silymarin (SL) and Milkfish Oil (MFO) on blood biochemical parameters AST (A), ALT (B), total protein (C), albumin (D), and total bilirubin (E) levels in INH and RFP-induced liver injury in rats (n=5). *, Significantly different compared to the negative group (p<0.05). **, Significantly different compared to the negative group (p<0.01).

MFO and SL have the potential to protect against liver damage.

Other biomarkers, such as total protein, AST, ALT, albumin, and bilirubin, are critical parameters closely associated with liver health. The liver plays a crucial role in protein synthesis, maintaining fluid balance, nutrient transport, and immune function (Trefts et al., 2017). Recent studies show that decreased total protein levels in the blood may indicate liver dysfunction (Okada, 2023). A healthy liver efficiently synthesizes proteins, but during liver injury, its protein synthesis capacity is compromised, leading to lower total protein levels (Simpson et al., 2020). Elevated AST and ALT levels suggest liver cell damage, with AST considered more specific to liver issues as it is predominantly found in the liver (Ndrepepa, 2021). Albumin is the main protein produced by the liver, reflecting its synthetic function, while bilirubin relates to the liver's ability to eliminate waste products, with elevated bilirubin levels indicating liver damage

(Moman et al., 2024; Tufoni et al., 2020). Compared to the negative control group, AST and ALT levels in the MFO and SL test groups, both alone and in combination, significantly decreased, indicating liver protection. Similar trends were observed in total protein levels and albumin levels, which increased significantly compared to the negative control group, while bilirubin levels in the negative control group spiked significantly. In the present study, histopathological analysis could not be performed due to technical constraints, which constitutes a limitation of this research. To provide a more comprehensive evaluation of hepatic injury, future studies should include both histopathological and immunohistochemical assessments. The use of immunohistochemical markers to evaluate inflammatory responses would further enhance the understanding of the underlying mechanisms of liver damage. Nonetheless, numerous studies have demonstrated that biochemical enzyme activities, inflammatory markers, and oxidative stress parameters are reliable indicators for assessing the hepatoprotective potential of test compounds in the absence of histological data.

CONCLUSION

Based on the results of this study, it can be concluded that liver injury induced by rifampicin and isoniazid can be alleviated by milkfish oil and silymarin through the regulation of CYP450 enzymes, and the prevention of oxidative stress and inflammation. The combination of silymarin and milkfish oil was more effective than either treatment alone, indicating a synergistic effect and offering strong potential as a novel therapeutic strategy for the prevention and treatment of drug-induced liver injury, particularly that caused by anti-tuberculosis agents.

ACKNOWLEDGEMENT

The authors are grateful to the Ministry of Education, Culture, Research, and Technology, Indonesia, for funding this study with Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) scheme in 2023 (No. 380.1/UN27.22/PT.01.03/2023).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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