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Polyunsaturated Fatty Acids from Eel Extract Attenuate Gastritis and Hepatotoxic by Indomethacin-Induced in Rats

(Asid Lemak Politaktepu daripada Ekstrak Belut Melemahkan Gastritis dan Hepatotoksik oleh Aruhan Indometasin pada Tikus)

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) potentially induce hepatotoxicity and ulcerative lesions in the stomach. Several studies have been conducted on the benefits of natural resources to overcome the problem of drug side effects. However, the use of fishery resources, such as the eel (*Anguilla bicolor*), for medicine has not been widely studied. Eels are rich in omega-3 polyunsaturated fatty acids with antioxidant and anti-inflammatory effects. Therefore, this study aims to determine the gastroprotective and hepatoprotective effects of polyunsaturated fatty acids extracted from eel (FaE). The experimental models were 42 male rats divided into seven groups. The experiment was performed for 14 days by orally administering FaE samples. On the 15th day, the rats were orally induced with indomethacin (48 mg/kg). After 8 h of induction, they were euthanized using ketamine and xylazine. Furthermore, blood samples were collected from the animals to measure the levels of aspartate aminotransferase and alanine aminotransferase. Malondialdehyde (MDA) and glutathione (GSH) levels were measured, while the histology of the liver and stomach were examined. The results showed that the administration of FaE from a dose of 1000 mg/kg significantly attenuated the liver and stomach damage caused by indomethacin. The influence of oxidative stress inhibition on damage is characterized by inhibition of the increase in MDA and a decrease in GSH. This research shows that FaE has the potential to inhibit liver and gastric damage due to the use of NSAIDs. However, further investigation needs to be conducted on other specific parameters of the inflammatory mechanism.

Keywords: Drug injury; fish; omega-3; oxidative stress

ABSTRAK

Dadah anti-radang bukan steroid berpotensi menyebabkan kehepatotoksikan dan lesi ulseratif di dalam perut. Beberapa kajian telah dijalankan tentang kebaikan sumber alam bagi mengatasi masalah kesan sampingan dadah. Walau bagaimanapun, penggunaan sumber berasaskan ikan, seperti belut (*Anguilla bicolor*), untuk tujuan rawatan belum dikaji dengan meluas. Belut kaya dengan asid lemak tak tepu omega-3, yang mempunyai kesan antioksidan dan anti-radang. Oleh itu, kajian ini bertujuan untuk menentukan kesan gastropelindung dan gastropelindung asid lemak tak tepu yang diekstrak daripada belut (FaE). Model uji kaji terdiri daripada 42 ekor tikus Jantan yang dibahagikan kepada tujuh kumpulan. Uji kaji ini dilakukan selama 14 hari dengan memberikan sampel FaE secara oral. Pada hari ke-15, tikus telah diinduksi dengan indometasin (48 mg/kg) secara oral. Selepas 8 jam induksi, mereka dimatikan menggunakan ketamin dan xilazina. Kemudian, sampel darah dikumpulkan daripada haiwan tersebut untuk mengukur

tahap *aspartate aminotransferase* dan *alanine aminotransferase*. Tahap malondialdehid (MDA) dan glutation (GSH) diukur, manakala histologi hati dan perut diperiksa. Hasil kajian menunjukkan bahawa pemberian FaE daripada dos 1000 mg/kg dengan ketara melemahkan kerosakan hati dan perut yang disebabkan oleh indometasin. Pengaruh perencatan tekanan oksidatif terhadap kerosakan dicirikan oleh perencatan peningkatan MDA dan penurunan GSH. Penyelidikan ini menunjukkan bahawa FaE mempunyai potensi untuk menghalang kerosakan hati dan gastrik akibat penggunaan NSAID. Walau bagaimanapun, kajian lanjut perlu dijalankan terhadap parameter khusus yang lain tentang mekanisme keradangan.

Kata kunci: Ikan; kecederaan dadah; omega-3; tekanan oksidatif

Introduction

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) used for the treatment of rheumatoid arthritis, osteoarthritis, and other diseases (Liu et al. 2022; Yang et al. 2021). Since almost all cases of the condition require painkillers, the widespread use of NSAIDs causes people to worry about the side effects (Jahangiri et al. 2022). Indomethacin is reported to have side effects of erosion, ulcerative lesions, and petechial bleeding in the abdomen (Mohamed et al. 2022; Salinas-Nolasco et al. 2022). Furthermore, the development of gastric mucosal lesions induced by this drug is mainly mediated by the formation of oxygen free radicals and the depletion of endogenous prostaglandins through inhibition of the cyclooxygenase enzyme (Stachowicz 2020). The drug can cause an increase in the activity of ALT, AST, ALP, Bilirubin, stress oxidative damage as a marker of liver function decline (Hilal Ahmad et al. 2018; Lu et al. 2015), as well as liver histology damage (Bagheri et al. 2018).

The current prevention strategy for NSAID-related gastric mucosal disorders, such as using a proton pump inhibitor (PPI), is not yet fully effective (Seid & Mamo 2022). Despite being the primary choice in reducing gastric acid secretion, it has been reported to cause side effects due to long-term chronic use (Sri Rethinavel et al. 2022). The current treatment for the adverse effects of indomethacin on liver function includes silymarin and curcumin (Aboelhadid et al. 2019). This option does not fully provide the most effective solution, requiring other alternative materials. Furthermore, the fishery is a renewable resource that can be developed into medicine (Sasongko et al. 2022). The fatty acids from fish oil, such as omega-3, including its precursors, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to have gastric injury effects. (Pineda-Peña et al. 2018; Tihista & Echavarría 2018). However, no research has been conducted on the effect of fish extracts, especially eel (Anguilla bicolor), on the ability to protect against stomach and liver damage due to the influence of NSAIDs.

Eel is a fish species with great potential but not optimally exploited by the world community (Sasongko et al. 2017). It contains omega-3 fatty acids, including EPA and DHA (Ahn et al. 2015; Arai et al. 2012; Kusharto et al. 2014; Nafsiyah et al. 2018). DHA is a compound from the group of omega-3 polyunsaturated fatty acids that is abundant in fish oil. It exerts gastroprotective and hepatoprotective effects in indomethacin-induced rats (He et al. 2017). This compound could help prevent lipid peroxidation and protect erythrocytes from oxidative stress in brain, liver, kidney, and heart tissue by increasing levels of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) as well as by lowering levels of malondialdehyde (MDA) (Pineda-Peña et al. 2018). Meanwhile, EPA can be converted into cytokines such as prostaglandins, thromboxane, and leukotrienes, giving it the potential to exert gastroprotective and hepatoprotective effects (Patterson et al. 2012).

There has been no previous research on the effect of eel extract on the inhibition of gastric and liver damage due to the use of NSAIDs. Therefore, this research aims to analyze the activity of polyunsaturated fatty acids from eel extract (FaE) as a gastroprotection and hepatoprotection in indomethacin-induced rats.

Materials and Methods

Materials

Eel (*Anguilla bicolor*) is obtained from eel farm in the Surakarta area, Jawa Tengah, Indonesia. Wistar rat strain was collected from the Faculty of Medicine, Universitas Sebelas Maret, Surakarta. Furthermore, Biosystem Germany supplied indomethacin (Dialon®), silymarin (Liver-L-Aactin®), aminotransferase reagent (AST), alanine aminotransferase (ALT), bilirubin, Sodium Buffer Phosphate 10% Hematoxylin, and Eosin.

EXTRACTION AND ANALYSIS OF TOTAL FATTY ACID

The extraction method refers to previous research by Sasongko et al. (2017) with some modifications. 1000 gram of eel samples were washed, gutted, and cut into small pieces. They were extracted by wet rendering using sterile water at 70-80 °C for 5 h. The solvent was attempted in a water bath, and starch (10% w/w of wet eel) was added to speed up the drying process and increase the stability of the extract. A sufficient amount of starch was added to the extract by heating it in a water bath at 50 °C until dried. Subsequently, the product was re-dried in an oven at 50 °C for an hour. The dry extract was calculated as the yield of eel extract (FaE). The fatty acid content of the eel extract was then determined through hydrolysis and methylation processes using Gas Chromatography (Hidayah et al. 2022).

ANIMALS

The experimental animals were Wistar white rats aged 2-3 months with a weight of 150-180 grams. The rats were conditioned in 12 h of light/dark lighting for a week and received adequate food and water. The health research ethics committee of Dr. Moewardi Hospital, Surakarta, Jawa Tengah has approved the testing methods on experimental animals (No: 966/VII/HREC/2022).

In vivo EXPERIMENT

In this study, 42 male Wistar rats were divided into seven groups consisted of normal group (control), negative control (indomethacin), hepatoprotective drug control (silymarin 100 mg/kg), gastroprotective drug control (omeprazole 2.7 mg/kg), and three groups of FaE samples of 1000, 2000, and 4000 mg/kg dosages. The experiment was conducted for 14 days, and FaE treatments were administered orally suspended in 0.5% carboxymethyl cellulose sodium (Na-CMC) using a sonde instrument. On the 15th day, the rats were fasted and orally induced with indomethacin (48 mg/kg), except for the normal group (Bagheri et al. 2018). After 8 h of induction, they were euthanized using ketamine and xylazine. Furthermore, the blood samples of the experimental animals were collected to measure aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels. Liver and stomach organs were obtained for histological observation, as well as the measurement of malondialdehyde (MDA) and glutathione (GSH) levels, as shown in Figure 1.

ASSESSMENT OF ULCERATIVE LESIONS

The degrees of ulceration in the indomethacin-treated animals were quantified using the procedure outlined by Ahmed et al. (2020). The rat stomach was cut at the *curvature major*. Briefly, the stomachs of the rats were cleaned and pinned on a corkboard, while the area was calculated using a caliper to measure the gastric mucosa. Additionally, a *raster image* application was used to determine the number and area of ulcers produced in the stomach.

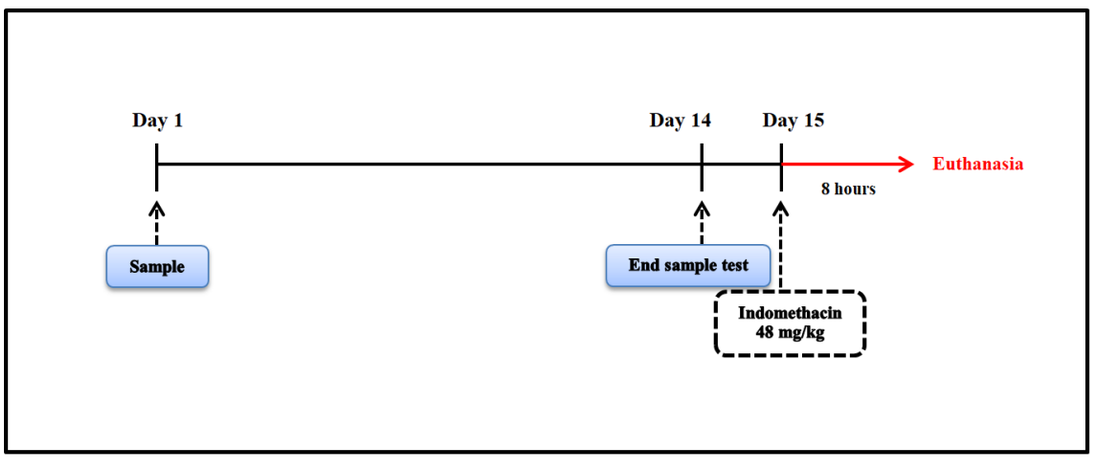


FIGURE 1. Gastroprotective and hepatoprotective examination procedures in rats

The mean ulcer score for each animal was expressed as ulcer index (UI). The following expression was used to determine the percentage of inhibition against ulceration:

$$UI = \frac{\text{Total ulcer area (mm}^2\text{)}}{\text{Total gastric area (mm}^2\text{)}}$$

$$\text{Ulcer inhibition (\%)} = \frac{\text{(UI in Indomethacin} - \text{UI in treated rats)}}{\text{UI in Indomethacin}} \times 100$$

LIVER FUNCTION TEST

Liver damage parameter tests were conducted through *pretest* and *posttest*. Blood samples were centrifuged at 4000 rpm for 10 min at 4 °C to obtain serum. The biochemical markers of liver damage measured using the testing protocol from Commercial kits (Biochemistry Analyzer by Biosystem Germany) were ALT and AST.

DETERMINATION OF OXIDATIVE STRESS PARAMETERS

A 10% b/v homogenate was obtained by adding 500 mg of liver and stomach organs to 5 mL of 0.15 M tris-HCl solution (pH 7.4) and homogenizing the mixture. The plasma malondialdehyde (MDA) was measured using the thiobarbituric acid reactive substances (TBARS) method. Furthermore, 0.2 mL hepatic homogenate, supplemented with 0.2 mL sodium decyl sulfate (SDS) 8.1%; 1.5 mL of 20% acetic acid, 1.5 mL of 0.8% TBA, and aqua dest. The mixture was heated at 95 °C for 60 min. Subsequently, 4 mL of 10% TCA was added and centrifuged at 3000 rpm for 10 min. The absorption read from a UV-Vis spectrophotometer was λ=532 nm (Sasongko, Efendi & Sugiyarto 2018).

The GSH levels were measured according to the method developed by Ellman (1959). The liver homogenate (0.75 mL) was added with 0.75 mL of 10% TCA and centrifuged at 200 rpm for 10 min. Subsequently, 1.8 mL of Elman's reagent was added (5, 5'-dithio bis-2-nitrobenzoic acid). The absorbance was measured using a UV-Vis spectrophotometer λ=412 mm.

HISTOLOGICAL RESEARCH

Histological testing of the stomach and liver was carried out to determine their general morphology. Organ fixation was performed using a 10% NBF solution. Furthermore, liver and stomach tissues preparation were colored with Hematoxylin-Eosin (HE). Histological examination was qualitatively performed to determine the general morphology of epithelial and hepatocyte cells in the stomach and liver by counting the number of normal cells and those undergoing necrosis in the form of nuclear shrinkage (pyknosis), ruptured nuclei (karyolysis) and loss of nuclei (karyolysis).

STATISTICAL ANALYSIS

Data were presented for each group with a mean ± standard error (SEM). The statistic analysis was performed using one-way analysis of variance (ANOVA) and alpha was set at 5%. To distinguish among experimental animal groups, a post hoc LSD test was applied using IBM SPSS statistic version 25.0.

RESULTS

TOTAL FATTY ACID ANALYSIS

The total FaE of the eel samples was 18.88%. This study showed that eel extract contained 45.30% saturated fatty acids (SFA), 33.14% monounsaturated fatty acid (MUFA), and 21.56% polyunsaturated fatty acid (PUFA). In addition, 22 types of fatty acids consisting of nine SFA, seven MUFA, and six PUFA were discovered in this study (Table 1).

GASTROPROTECTIVE ANALYSIS

The effects of eel extract on the ulcer index and % inhibition in the experimental animals are shown in Figures 2 and 3. Oral administration of 48 mg/kg BW indomethacin significantly increased in the degree of ulceration (ulcer index) in rats ($p<0.05$). Furthermore, the highest ulcer index as a result of indomethacin was discovered in the negative control group.

A significant improvement of inhibition level against ulceration was observed in the extracts-treated animals. The 4000 mg/kg extracts provided better protection than the 1000 mg/kg BW and 2000 mg/kg regimens and compared well with the standard drug (omeprazole). Pretreatment with the extracts resulted in a considerable decrease in ulcer index with a significant increase in % inhibition compared with ulcerated control rats.

TABLE 1. Composition of saturated and unsaturated fatty acids eel extract

Fatty acid parameters	Average (%)
Saturated Fatty Acid (SFA)	
M Palmitate	23.00
M Octadecanoate	6.96
M Butyrate	2.93
M Docosanoate	2.74
M Tetradecanoate	2.58
M Heptadecanoate	2.34
M Arachidonate	2.17
M Pentadecanoate	1.61
M Laurate	0.97
Total SFA	45.30
Monounsaturated Fatty Acid (MUFA)	
Cis-9-Oleic Methyl ester	14.95
Methyl cis-11-eicosenoate	5.42
M Palmitoleate	3.87
M Erucate	3.57
M Nervonate	3.30
Myristoleic acid methyl ester	1.06
Cis 10-Heptadecenoic acid Methyl Ester	0.97
Total MUFA	33.14
Polyunsaturated Fatty Acid (PUFA)	
M Linoleate	7.76
Cis-11,14-Eicosadienoic acid methyl ester	5.51
Cis-4,7,10,13,16,19-Docosahexaenoate	4.50
Cis-13-16-Docosadienoic acid methyl ester	1.66
M Cis-5,8,11,14-Eicosatetraenoic	1.16
M Cis-5,8,11,14,17-Eicosapentaenoate	0.98
Total PUFA	21.56
Total fatty acid	100.00

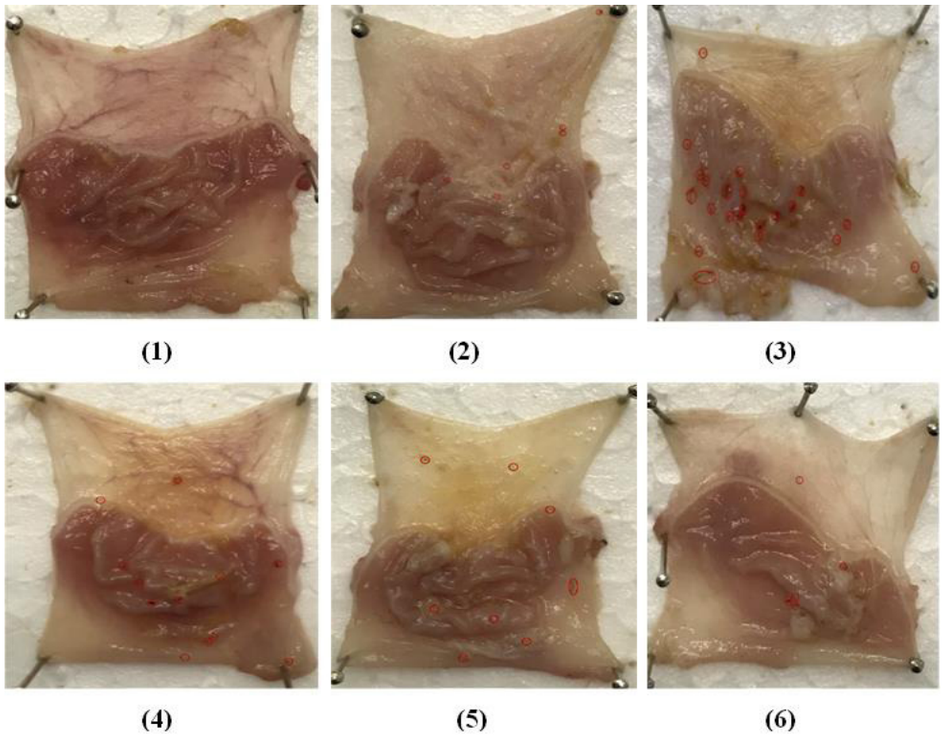


FIGURE 2. Photos of rat's stomachs subjected to indomethacin-induced ulcerogenesis in pyloric ligation model. (1) Normal (control), (2) Omeprazole group, (3) Indomethacin group, (4) FaE 1000 mg/kg, (5) FaE 2000 mg/kg, (6) FaE 4000 mg/kg

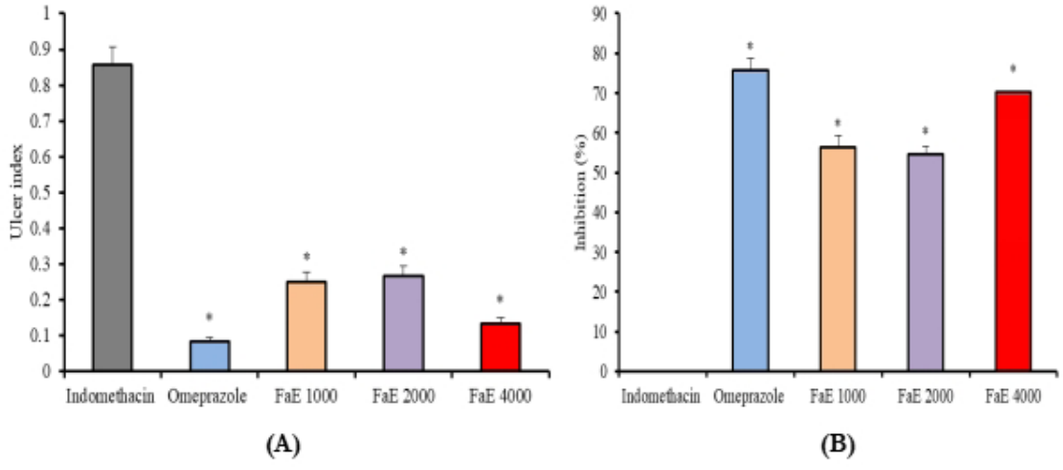


FIGURE 3. Bar graphs showing the effect of indomethacin, Omeprazole, FaE 1000 g/kg, FaE 2000 mg/kg, and FaE 4000 mg/kg. FaE= fatty acids eel extract, (A) The ulcer, and (B) inhibition index. Data (n = 6) are presented as Mean SEM, with a (*) $p<0.05$ difference when compared to the indomethacin group

LIVER FUNCTION ANALYSIS

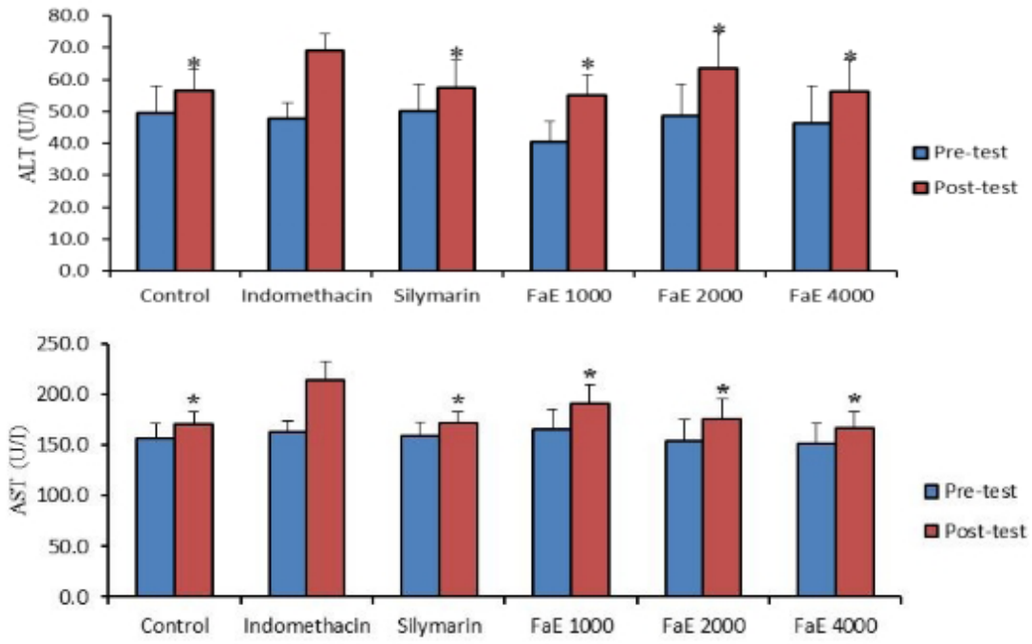
Elevated ALT and AST levels occurred in all groups after indomethacin induction (Figure 4). The control group had the lowest blood biochemical profile. ALT and AST levels were highest in the indomethacin-induced animal group without medication. An increase in ALT and AST enzymes in the blood serum showed that there was damage to the liver. Higher doses in the rats given FaE resulted in lower levels of ALT and AST than in the group without the drug ($p<0.05$).

OXIDATIVE STRESS ANALYSIS

The administration of indomethacin significantly increased MDA and decreased GSH levels ($p<0.05$) (Figure 5). This indicated that this drug can increase oxidative stress in the liver and stomach organs. The administration of FaE animal groups at all dose levels showed significantly different results compared to the negative group (indomethacin) ($p<0.05$). Omeprazole can prevent oxidative stress due to the damage caused by indomethacin to the stomach organ. In addition, the same effect was shown in the group given silymarin which is able inhibit liver damage. Moreover, the FaE 4000 mg/kg dose produced the highest inhibition of oxidative stress compared to the 1000 mg/kg and 2000 mg/kg.

LIVER AND GASTER HISTOLOGICAL ANALYSIS

Histological observations are presented in Figures 6 and 7. Normal cell histology was indicated by a round cell shape with a clear nucleus, uniform cell shape, and no edema. The liver and stomach organs in the negative control group had the highest number of necrotic cells. This indicates more significant damage from indomethacin induction. Furthermore, the administration of FaE to rats showed lower total cell damage in liver and stomach histology compared to negative controls.



Note: (a) Significantly different from negative control $p<0.05$ (b) significantly different from positive control $p<0.05$

FIGURE 4. Effects of oral administration of FaE on liver enzyme status in rats induced by indomethacin. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FaE, fatty acids eel extract. Data are presented as mean ± SEM (n = 6), with a (*) $p<0.05$ difference compared to the indomethacin group

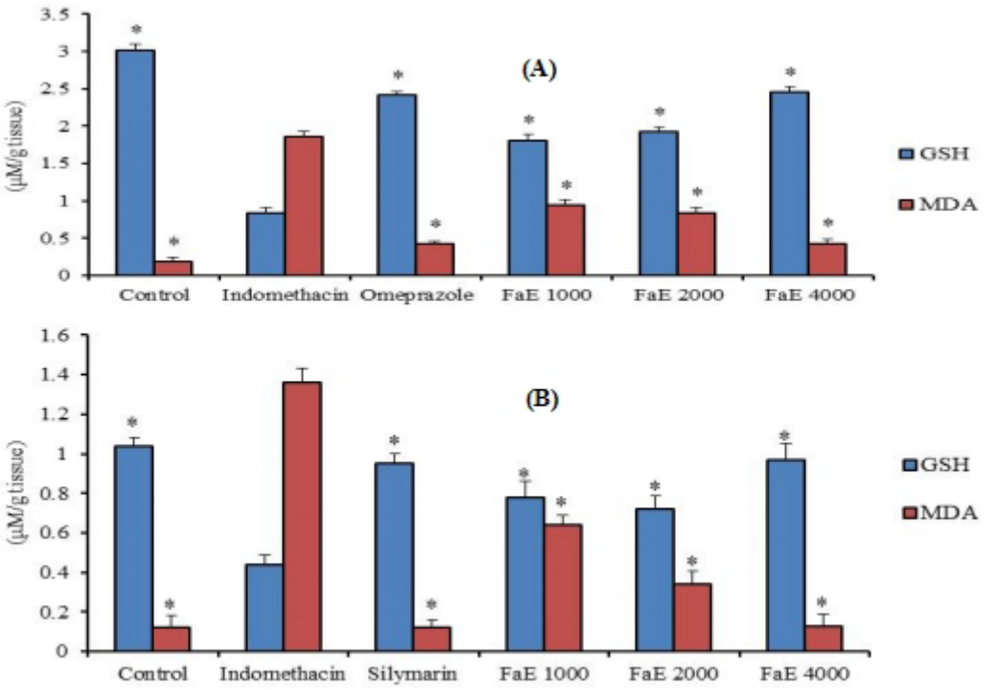


FIGURE 5. Effects of oral administration of FaE on gastric and hepatic oxidative stress status with indomethacin-induced in rats. (A) stomach and (B) liver; FaE, fatty acids eel extract; GSH, glutathione; MDA, malondialdehyde. Data are presented as mean ± SEM (n = 6) with a (*) $p<0.05$ difference when compared to the indomethacin group

DISCUSSIONS

The activity of FaE as a gastroprotection and hepatoprotection in indomethacin-induced rats has been evaluated in this present study. The FaE contained fatty acids, especially EPA and DHA which are previously reported to have positive effects on health, especially as hepatoprotection (Dias et al. 2022; El-Gendy et al. 2021; Sasongko, Zulpadly & Farida 2023) and gastroprotection (Pineda-Peña et al. 2018, 2012). The complex compounds in the nutrition or traditional medicine provides a synergistic effect. However, not all of fatty acid content of processed fish (FaE) have an effect on health. According to Carta et al. (2017), the palmitic acid compound, part of SFA, which accounts for 20-30% of the total fatty acids, does not have a beneficial effect on health. On the other hand, marker compounds such as omega-3 (EPA and DHA) are often associated with drug action. The hepatotoxicity induced by this drug is evidenced by elevated ALT and AST activity (Lucas 2016) and changes in liver histology (Kakisaka et al. 2018). The increase in specific liver enzymes is related to the metabolism of Indomethacin in the liver. This study proved that administration of FaE started at 1000 mg/kg can reduce ALT and AST levels. Linear results were also shown in the histopathological profile of experimental rats administrated by FaE where hepatic organ necrosis decreased when compared to indomethacin-induced rats. The metabolism of indomethacin through N-deacylation reaction produces a reactive metabolite iminoquinone. These radicals cause an increase in reactive oxygen species (ROS) and oxidative stress, which initiates lipid peroxidation, thereby triggering liver damage (Beiranvand 2021). In this study finding that found lower MDA and higher GSH levels in animal models administrated by FaE. This proves that FaE is able to inhibit oxidative stress in the liver due to indomethacin administration.

Administration of FaE started at 1000 mg/kg BW can inhibit ulcer index based on analysis of the gastric organs. The gastric damage induced by toxic doses of indomethacin is associated with an inflammatory

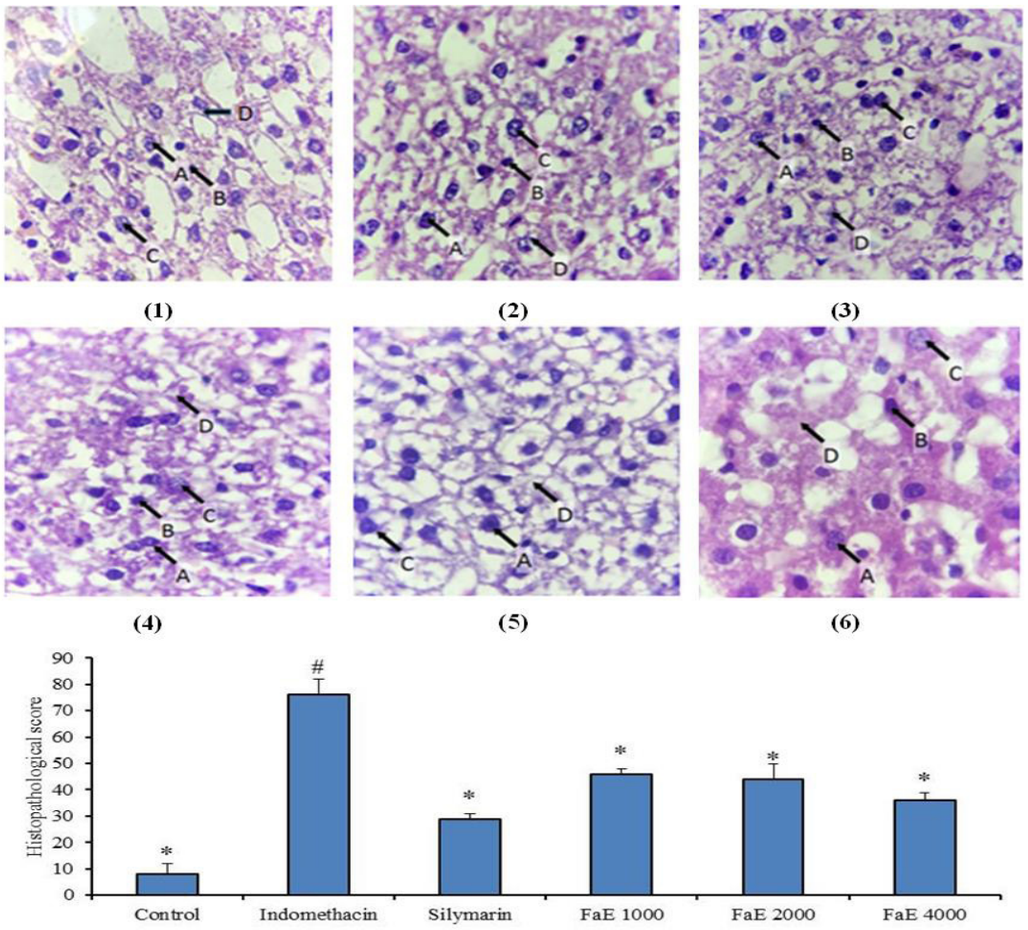


FIGURE 6. Histology of liver with HE staining. (1) normal (control), (2) indomethacin group, (3) omeprazole group, (4) FaE 1000 mg/kg, (5) FaE 2000 mg/kg, (6) FaE 4000 mg/kg. (A) normal cell (B) picnotic cell (C) karyoreksis cell (D) karyolisis cell. Data are presented as mean ± SEM (n = 6) with a (*) $p<0.05$ difference when compared to the indomethacin group

reaction that produces ROS and lipid peroxidation, leading to severe ulceration (Grimstad et al. 2012). Consumption of this drug can also increase the levels of *Tumor Necrosis Factor Alpha* (TNF-α) because of the inhibition of COX-2. TNF-α induces *Intercellular Adhesion Molecule 1* (ICAM-1), which increases the attachment of neutrophils to vascular endothelial cells (Ahmed et al. 2020). Indomethacin causes severe gastric inflammation, as well as activation of the NF-κB pathway and up-regulation of downstream signals, including TNF-α, IL-6, and MPO (Kim et al. 2017). Omega-3 has gastroprotective functions with an anti-inflammatory mechanism by modulating leukocyte recruitment or activity, which is observed in the decrease in *Myeloperoxidase* (MPO) levels, *Intercellular Adhesion Molecule 1* (ICAM-1), *Leukotriene B4* (LTB4), and TNF-α to minimize the inflammatory response (Calder 2013). DHA inhibits the activation of *Nuclear Factor Kappa-B* (NF-κB), as oxidative stress has a primary role in the activation (Ali & Rifaai 2019). The nuclear translocation of NF-κB has been shown to stimulate the expression of proinflammatory cytokines (He et al. 2017). Furthermore, DHA acts as a chain-breaking antioxidant in membranes that can prevent cell damage by lipid peroxidation and inhibit the formation of free radicals (Leng, Winter & Aukema 2018).

Omega-3 fatty acids consist of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic

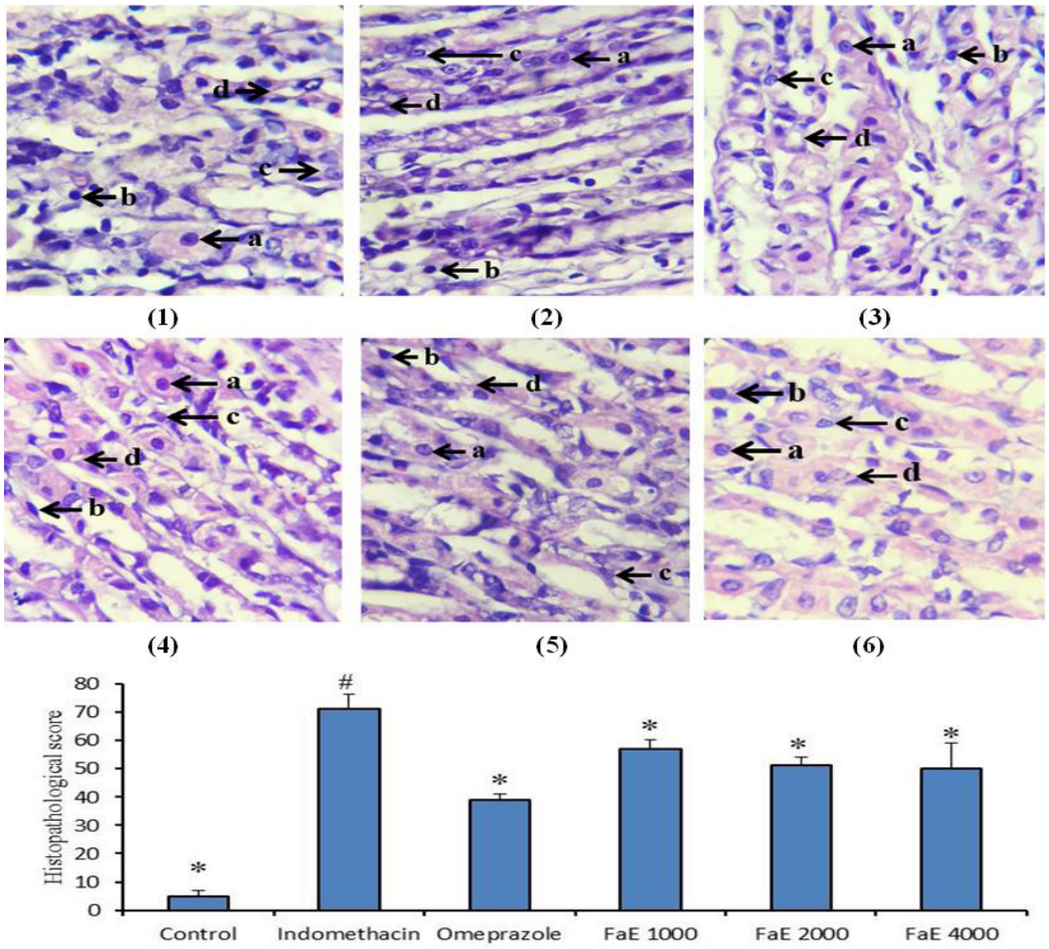


FIGURE 7. Histology of stomach with HE staining. (1) normal (control), (2) indomethacin group, (3) omeprazole group, (4) FaE 1000 mg/kg, (5) FaE 2000 mg/kg, (6) FaE 4000 mg/kg. (A) normal cell (B) picnotic cell (C) karyoreksis cell (D) karyolisis cell. Data are presented as mean ± SEM (n = 6) with a (*) $p<0.05$ difference when compared to the indomethacin group

acid (DPA), and docosahexaenoic acid (DHA) (Ahmed et al. 2020). This study suggested that the administration of FaE containing omega-3s in the formation of MDA demonstrates its capacity to prevent oxidative damage to lipids and proteins required for cellular homeostasis, even in the presence of indomethacin. In addition, the data shows that the administration of FaE can maintain normal redox potential by maintaining basal GSH levels and enhancing endogenous antioxidant defenses against free radicals and cytotoxicity. Several studies have shown that the cellular antioxidant response to the production of omega-3-related reactive oxygen species can occur in the mitochondria in vitro research (Garrel et al. 2012; Weydert & Cullen 2010). The data obtained demonstrated antioxidant capacity after FaE administration in indomethacin-induced liver damage. Furthermore, the reduction in oxidative stress associated with FaE administration was consistent with an increase in liver function with a decrease in the amount of ALT and AST enzymes, as well as in the amount of liver and stomach necrosis.

Conclusions

Eel extract has gastroprotective and hepatoprotective activity in rats induced by indomethacin, starting from the smallest dose of eel extract, 1000 mg/kg. The

oxidative stress activity of FaE affected the inhibition of gastric and liver damage by decreasing MDA and maintaining endogenous antioxidant (GSH) levels. The higher the dose of eel extracts, the greater the protective effect. In addition, administration of FaE in rats has the potential to attenuate the damage that NSAIDs cause to the liver and stomach. However, further study should be taken into account on other specific parts of the inflammatory mechanism.

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