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Protective effect of ethanolic *Feronia elephantum* Correa fruit extract on high-fat diet induced steatohepatitis in rats

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ABSTRACT

BACKGROUND
A high-fat diet can lead to hyperlipidemia which will end up as liver damage (steatohepatitis). Ethanolic *Feronia elephantum* Correa fruit extract (EFEC) has an antioxidant activity which is expected to overcome hyperlipidemia in the liver. The objective of this study was to determine the effects of EFEC on liver function and morphological changes in rats.

METHODS
This was an experimental study with a post-test only group design. A total of 20 male Wistar rats aged 2-4 months were randomized into 5 groups, A= negative control, B= positive control (high fat diet + 10 mg/kgBW simvastatin), C = high fat diet + 500 mg/kgBW EFEC fruit extract, D = high fat diet + 600 mg/kgBW EFEC, and E = high fat diet + 700 mg/kgBW EFEC). High-fat diet was given for 4 weeks (quail egg yolks, 10ml/200gBW). EFEC was administered for 4 weeks after induction of hypercholesterolemia. Examination of liver serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) was performed on rat blood serum and histopathological examination was carried out using steatohepatitis grading. One way ANOVA test and Post-Hoc Tamhane's test were used to analyze the data.

RESULTS
Administration of EFEC at 700 mg/kgBW improved the liver enzymes (p=0.00 for SGPT and SGOT) and steatohepatitis grading in high-fat diet induced rats (mild condition, E = 75% vs A = 100% mild)

CONCLUSION
Ethanolic *Feronia elephantum* Correa fruit extract at 700 mg/kgBW was able to improve steatohepatitis in high-fat diet induced rats.

Keywords: *Feronia elephantum* Correa, liver enzyme, steatohepatitis, high-fat diet, rats

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INTRODUCTION

A high-fat diet can induce hyperlipidemia and disrupt organ functions. In the liver, hyperlipidemia is frequently associated with non-alcoholic fatty liver disease (NAFLD),[1–4] which is histologically characterized by features of steatosis (lipid accumulation in hepatocytes), inflammation, and cell degeneration.[5] This disorder is characterized by elevated liver enzyme levels, including higher serum glutamic oxaloacetic transaminase/SGOT (also known as alanine aminotransferase/ALT), serum glutamic pyruvic transaminase/SGPT (also known as aspartate aminotransferase/AST), and alkaline phosphatase (ALP), as well as increased triglyceride and cholesterol levels.[6,7] Higher levels of hepatic enzymes and progressive morphological changes in the liver can lead to irreversible liver disease, such as fibrosis and hepatic cirrhosis.[5,8]

Statins are drugs used for hypercholesterolemia that reduce steatohepatitis, but not liver fibrosis.[9] The use of statin therapy for hypercholesterolemia is also inseparable from its side effects. Statins can cause muscle-related problems, such as myopathy (including cardiomyopathy) and rhabdomyolysis, as well as damage to the kidneys and liver. Hepatic disorders caused by statins can be indicated by elevated levels of transaminase enzymes (SGOT and SGPT).[10] The side effects of statins can increase the risk of progression of such comorbidities as hepatic disorders in patients who also experience hyperlipidemia.

Exogenous antioxidant supplements emerge as an alternative therapy to substitute statins as standard drugs for hyperlipidemia, particularly in hyperlipidemia with coexisting liver disease. Consumption of a diet containing antioxidants can reduce the risk of NAFLD.[11] Many studies have examined the presence of antioxidants in plants, one of which is related to their effect on the liver.[12]

Feronia elephantum Correa (also known as kawista; wood apple; *Limonia acidissima* Linn; *Feronia lemonia* Swingle) and subsequently in this article referred to as FEC, are a potential herbal medication for conditions that are high in reactive oxygen species (ROS). An in-vitro study found that methanolic *Feronia elephantum* Correa fruit extract (MFEC) can also reduce the proliferation of breast cancer cells.[13] FEC fruit at doses of 500 mg/kgBW, 600 mg/kgBW, and 700 mg/kgBW dissolved in 1 ml distilled water (for each administration) can prevent increased MDA levels in the blood serum of rats exposed to cigarette smoke.[14] With regard to hyperlipidemia, FEC is able to improve cholesterol profiles in animal models of hyperlipidemia, such as Triton WR-1339 induced hyperlipidemia in rats.[15]

In addition, FEC is also able to improve carbohydrate metabolism in the liver (by improving the activity of the hepatic enzyme glucose-6-phosphatase and increasing liver glycogen). The antihyperlipidemic effects of FEC can be explained with different mechanisms. Saponins and phytosterols can inhibit pancreatic lipase and reduce intestinal absorption of lipids. Fibers of the extract can lower LDL level. In addition, flavonoids and ascorbate increase HDL and decrease LDL.[16]

The studies of Pandit et al.[15] and Vasant et al.[16] have demonstrated the antihyperlipidemic effect of methanolic FEC fruit extract and FEC fruit powder, respectively. In contrast, the present study did not aim to examine the effect of FEC on lipid profiles in hyperlipidemic conditions, but rather its effect on the liver. The objective of this study was to determine the effects of EFEC fruit extract on liver function and morphological changes in rats.

METHODS

Research design

This research involved an experimental design with a posttest-only control group approach. The experiment lasted for four months (January-April 2020) and was conducted in several laboratories. The preparation of EFEC

fruit extract, animal care, group treatment, and termination of experimental animals were carried out in the Pharmacology Laboratory, Faculty of Medicine, Universitas Mataram. The observation and image retrieval (tissue micrographs) were done in the Laboratory of Histology and Anatomical Pathology, Faculty of Medicine, Universitas Islam Indonesia.

Collection of plant material and extraction

Ripe kawista (FEC) fruits were obtained from Bima Regency, West Nusa Tenggara (NTB), Indonesia. To prepare the extract, the fruit pulp was first dried under the sun and crushed to obtain a coarse powder, which was then sieved to yield the finest particles. The extraction process used maceration in 70% ethanol (1:3) at room temperature and was repeated for 3 days. The viscous extract obtained after evaporation was stored in a closed container at room temperature and given to the experimental animals as per predetermined doses.

Animals and treatment

The rats were obtained from the Pharmacology Laboratory, Universitas Mataram. The inclusion criteria were male Wistar rats aged 2-4 months, weighing 150-200 grams, and apparently healthy without any physical disabilities. A total of 20 rats (4 per group) were used according to the Festing research equation.[17] Based on this formula, the minimum total number of subjects required in this study was 15-25 (3-5 in each group). Therefore, this study used four animals per group. The research subjects were selected through a random sampling technique. The rats were randomized into five research groups in this study, namely A = healthy controls (regular feed), B = positive controls (high-fat diet + 10 mg/kg BW simvastatin), C = high-fat diet + 500 mg/kgBW EFEC fruit extract, D = high-fat diet + 600 mg/kgBW EFEC fruit extract, and E = high-fat diet + 700 mg/kgBW EFEC fruit extract. EFEC fruit extract was administered for 4 weeks as soon as the rats were confirmed to have hypercholesterolemia. The EFEC fruit extract was dissolved in 0.5% carboxymethyl cellulose (CMC). The high-fat diet and therapy (simvastatin or EFEC fruit extract) were given via a nasogastric tube, and the rats received feed and water ad libitum during the study. The three different intervention groups C to E were each treated with a different dose of EFEC fruit extract (500 mg /kgBW= C, 600 mg/kgBW= D and 700 mg/kgBW= E). All treatments were performed by the researchers.

High fat diet induced hyperlipidemia

A high-fat diet was provided by feeding 10 ml/200 g BW quail egg yolk for 4 weeks. The intervention involving EFEC fruit extract was provided if the rats experienced a hypercholesterolemic condition which was confirmed if the total cholesterol level was >200 mg/dl (using the Easy-Touch® rapid test on peripheral/tail blood). If the rats showed no hypercholesterolemia after four weeks of induction through feeding on quail egg yolk, they were excluded from this study.

Steatohepatitis grading

The livers obtained after termination (after 4 weeks of hypercholesterolemia induction and 4 weeks of FEC therapy) were stored in a 10% formalin phosphate-buffered saline fixative for 24 hours. The liver tissues were then placed in 70% ethanol for further processing. The liver samples were transversely sliced, blocked in paraffin, and stained with hematoxylin eosin (HE). All of the liver tissue processing was performed in the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Mataram (Unram).

The histological changes were observed with the Brunt system (grading of steatohepatitis). According to this system, changes in the liver are categorized into mild, moderate, and severe.[18] The parameters observed in these three categories include the degree of steatosis, ballooning cells, and

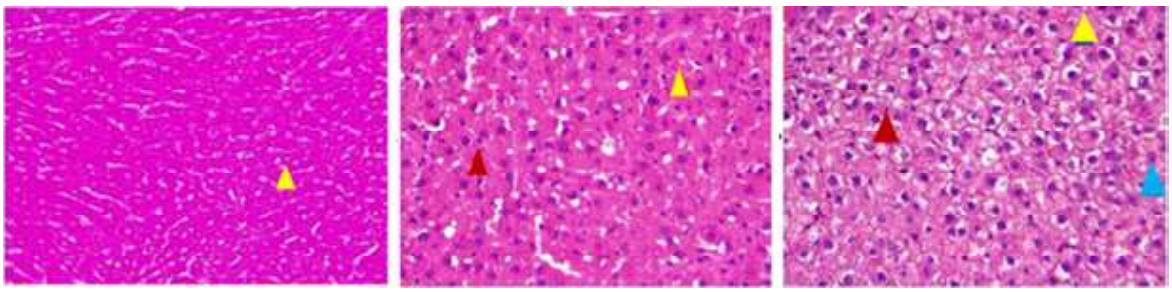

Figure 1. Histopathological changes in liver structure. A: mild grade, B: moderate grade, C: severe grade. Yellow arrows: cell steatosis, red arrows: lymphocytes, blue arrow: ballooning cell (400x magnification, HE staining).

inflammation. Liver damage is deemed mild if steatosis (to a maximum of 2/3 of the liver) and ballooning cells appear to be minimal, and inflammation seems diffuse. The moderate degree is marked by visible steatosis and ballooning cells (in zone 3, i.e. the area of hepatic lobules near the central vein), and inflammation appears as a result of ballooning cells. Severe liver damage is signified by a high degree of steatosis (>2/3), and ballooning cells are clearly visible in zone 3.

The histological observation was performed with single blinding by an experienced, independent anatomical pathologist from the Laboratory of Histology and Anatomical Pathology, Faculty of Medicine, Universitas Islam Indonesia, using a light microscope. Imaging was conducted using a light microscope with a camera (OptiLab) attached and connected to a computer. Observations were made in all fields of view on each sample. A low magnification (100x) was used when observing the location of the damage, while high magnification (400x) was employed to observe morphological changes in detail.

Laboratory analysis

The liver protection of high-fat diet induced rats was observed from the liver enzyme (SGOT and SGPT) levels, which were examined from the rat blood serum obtained during termination. The blood samples were collected via retro-orbital blood sampling. Both examinations were carried out in the Pharmacology Laboratory, Faculty of Medicine, Universitas Mataram using spectrophotometric analysis.

Statistical analysis

The histological changes in the liver were stated as a percentage (the number of positive samples compared to the total samples). The levels of liver enzymes were analyzed using the Shapiro-Wilk normality test, One Way Analysis of Variance/ANOVA (significance test), and Tamhane's T2 test (post-hoc test). The data were declared as significantly different if $p < 0.05$.

Ethical clearance

The ethical research permit was obtained from the Ethics Committee, Faculty of Medicine, Universitas Mataram prior to the study (Number 354/UN18.F7/etik/2019).

RESULTS

All of the rats survived until the end of the study and met the inclusion and exclusion criteria. From the examination of liver tissue, no significant histopathological changes were found in the liver parenchyma of the normal healthy (negative control) group (A). However, changes in the liver were observed in the positive control group (B = 50% severe) and the groups C – E receiving EFEC fruit extract. Therapy using 700mg/kgBW EFEC fruit extract showed the ability to reduce the steatohepatitis induced by a high-fat diet (E = 75% mild, Table 1).

Table 1. Grading system for steatohepatitis in the study groups

Group	n	Grade	n	%
A	4	Mild	4	100
		Moderate	0	0
		Severe	0	0
B	4	Mild	2	50
		Moderate	0	0
		Severe	2	50
C	4	Mild	0	0
		Moderate	2	50
		Severe	2	50
D	4	Mild	0	0
		Moderate	1	25
		Severe	3	75
E	4	Mild	3	75
		Severe	0	0
		Moderate	1	25

Note: A = healthy control (normal feed, no therapy), B = positive control (high-fat diet + 10 mg/kgBW simvastatin), C = high-fat diet + 500 mg/kgBW EFEC fruit extract, D = high-fat diet + 600 mg/kgBW EFEC fruit extract, E = high-fat diet + 700 mg/kgBW EFEC fruit extract). #Percentage obtained from the ratio of n to N

There were significant differences in the levels of SGOT and SGPT from the five study groups ($p<0.001$). The administration of EFEC fruit extract at 500 mg/kgBW (C), 600 mg/kgBW (D), and 700mg/kgBW (E) resulted in reduced SGOT and SGPT levels compared to the group receiving standard therapy (Table 2). In the post-hoc Tamhane's test, significant differences in SGPT levels were seen in all of the treatment groups with EFEC fruit extract therapy as opposed to the positive control group ($p<0.001$, Table 3). Similar results were found in the SGOT levels with the p values for SGOT levels in groups C, D, and E reaching 0.008, 0.000, and 0.002, respectively (Table 3).

There was no significant difference in the SGPT levels between the group receiving 700 mg/kgBW EFEC fruit extract and the normal healthy group A ($p=0.535$). Similarly, the SGOT levels of the groups given 500 mg/kgBW and 600 mg/kgBW EFEC fruit extract did not seem significantly different from group A. Meanwhile, the SGOT levels of rats in the group with 700 mg/kgBW EFEC fruit extract were lower than those in group A ($p = 0.013$) (Table 3).

DISCUSSION

This study showed that the administration of EFEC fruit extract can improve the functional and morphological features of a high-fat diet induced steatohepatitis in rats. Compared with the group receiving simvastatin therapy, the EFEC fruit extract therapy at doses of 500 mg/kgBW and 600 mg/kgBW was able to improve only the features of liver function without improving the hepatic morphological changes. The therapeutic effect of EFEC fruit extract can be found at 700 mg/kgBW (SGOT and SGPT levels and steatohepatitis grades were similar to those of the normal healthy group).

The protective effect of FEC extract can be observed in a number of induced tissue-damage models. In animal models of skin wounds (incision, excision, and dead space), the administration of methanolic FEC fruit extract (MFEC) at doses of 200mg/kgBW and 400mg/kgBW can accelerate wound healing. The potential dose in that study is 400 mg/kgBW.[19]

Table 2. Levels of SGOT and SGPT by treatment groups

Variable	Group	n	Mean ± SD	p-value
SGPT	A	4	57.25 ± 3.04	0.00*
	B	4	122.05 ± 9.52	
	C	4	92.90 ± 4.96	
	D	4	85.0 ± 3.71	
	E	4	48.44 ± 6.19	
SGOT	A	4	159.85 ± 14.8	0.00*
	B	4	221.3 ± 13.2	
	C	4	158.1 ± 5.85	
	D	4	120.3 ± 10.6	
	E	4	77.62 ± 2.1	

Note: * $p<0.05$. A = healthy control (normal feed, no therapy), B = positive control (high-fat diet + 10 mg/kgBW simvastatin), C = high-fat diet + 500 mg/kgBW EFEC fruit extract, D = high-fat diet + 600 mg/kgBW EFEC fruit extract, E = high-fat diet + 700 mg/kgBW EFEC fruit extract)

Table 3. Post-hoc test of liver enzyme levels in rat blood serum

Group		p-value				
		A	B	C	D	E
SGPT	A		0.000*	0.000*	0.000*	0.535
	B			0.000*	0.000*	0.000*
	C				0.798	0.000*
	D					0.000*
SGOT	A		0.008*	1.00	0.060	0.013*
	B			0.008*	0.000*	0.002*
	C				0.019*	0.000*
	D					0.032*

Note: Post hoc test using Tamhane's T2 test; A = healthy control (normal feed, no therapy), B = positive control (high-fat diet + 10 mg/kgBW simvastatin), C = high-fat diet + 500 mg/kgBW EFEC fruit extract, D = high-fat diet + 600 mg/kgBW EFEC fruit extract, E = high-fat diet + 700 mg/kgBW EFEC fruit extract)

Although the prepared extract in that study is different from this present research (ethanol), the therapeutic effect of FEC extract on tissue damage is similar to that of the present study. At a dose of 2000mg/kgBW, no side effects of FEC treatment were found.[15,19] Therefore, the dose of 700 mg/kgBW in this study remains potential as it is still far below the toxic dose.

F. elephantum Correa extract also has a protective effect in numerous tissue-damage models. MFEC fruit extract has a neuroprotective effect on stroke animal models.[20] Research by Sharma et al.[21] showed that premedication using aqueous *F. elephantum* Correa (AQFEC) leaf extract at doses of 400 mg/kgBW and 800 mg/kgBW can reduce the percentage of deaths and prevent the increase in weight and volume of rat liver induced by alloxan (induction of type-1 DM) and thioacetamide (a hepatotoxic substance). With regard to the functional parameters (liver enzymes), premedication involving AQFEC leaf extract can also prevent elevated hepatic enzymes (SGOT and SGPT).[21] Similarly, the administration of EFEC fruit extract at doses of 500mg/kgBW, 600mg/kgBW, and 700mg/kgBW in the present study can improve the features of hepatic function (SGOT and SGPT) compared to the group receiving a standard drug.

Administering FEC extract can also improve the morphological features of the liver. Aqueous *F. elephantum* Correa leaf extract has the potential to reduce the degree of hepatocellular necrosis in alloxan-thioacetamide-induced rats (mild injury). However, premedication using AQFEC leaf extract is unable to make a significant difference to the degree of inflammation, congestion (lobular as well as vascular), and hepatocyte regeneration.[21] The increasing protective effect of FEC corresponds to the higher dose administered. In line with the studies by Sharma et al.[21], the present study also showed that administration of EFEC fruit extract at a dose of 700 mg/kgBW results in hepatic features that are close to the normal liver architecture (75% mild). The presence of moderate damage of up to 25% in the high-fat diet-induced group treated with EFEC fruit extract at a dose of 700 mg/kgBW indicates that the ability of the liver to regenerate at such dose remains imperfect. Meanwhile, the liver regeneration ability has yet to be shown at lower doses (500 mg/kgBW and 600 mg/kgBW).

The varied results of liver enzyme and steatohepatitis grading improvement in the present study indicates that the effect of EFEC fruit extract is dose-dependent. EFEC fruit extract therapy at doses of 500mg/kgBW and 600mg/kgBW is only able to improve the liver enzyme levels alone without significant improvement in the steatohepatitis grading. Liver enzymes and steatohepatitis grading are concurrently observed at a dose of 700mg/kgBW. In line with the therapeutic and protective dose of FEC fruit extract, the higher the dose

used, the more significant the effects that can be observed. In line with that, a more considerable improvement in steatohepatitis condition in this study can be found at a higher dose (700 mg/kgBW EFEC fruit extract).

In addition, FEC fruit extract also has a therapeutic effect on metabolic disorders. Administration of MFEC fruit extract can reduce the serum hyperlipidemia parameters (in hyperlipidemia animal models).[15] With regard to such findings, the therapeutic effect on liver enzymes and steatohepatitis grading in this study is made possible by the ability of FEC fruit extract to reduce cholesterol levels.

High ROS levels will induce oxidative stress conditions which will damage the cell membrane and the DNA.[22] The liver is the main target of high ROS levels which induce changes in cell metabolism. The increased metabolism of ethanol by hepatocytes in oxidative stress conditions will result in steatosis. In addition, activated Kupffer cells will also induce inflammation, apoptosis (through TNF-α induction) and fibrosis (occurrence of collagen production). Inflammation will further exacerbate oxidative stress conditions.[23]

Reducing the ROS levels is one of the potential strategies to prevent worsening of steatosis conditions in oxidative stress. Steatosis is characterized by an increase in Cytochrome P450 Family 2 Subfamily E Member 1 (CYP2E1).[24] Studies show that flavonoids can suppress inflammation (reduce TNF-α), and reduce liver enzyme and MDA levels and also reduce CYP2E1. This shows that flavonoids have an antioxidant activity and are also hepatoprotective in oxidative stress conditions.[25] The FEC used in this study also contain flavonoids, alkaloids and phytosterols.[15] In line with Xiang et al.[25] it seems that the flavonoids in FEC play an important role in preventing the worsening of steatosis in rats with oxidative stress induced by high fat diet.

The protective effects of EFEC fruit extract in this study are strongly associated with the antioxidant content. MFEC fruit extract has proved to be able to increase the level of antioxidants (SOD and catalase/CAT) in the granulation tissue that forms during incisions, excisions, or dead space wounds in animal models. [19] Reduced nitrite and lipid peroxidation levels as well as elevated catalase levels can also be observed in stroke animal models.[20] A study by Darsini et al [26] shows that long-term FEC supplementation (30 and 60 days in fish) results in increased levels of antioxidants (SOD, glutathione S-transferase/GST, and glutathione peroxidase/GPX) in the blood serum as well as in liver tissue and muscle. The highest SOD level is found in the liver. With FEC administration, GPX shows activities in the liver similar to those in the blood serum. In contrast, GST shows the lowest activity in the liver as opposed to the blood serum and muscle. Meanwhile, the MDA free radical levels in hepatic tissue decrease significantly after 60 days of FEC supplementation. These studies suggest that the activation of antioxidant pathways is associated with the type of damage models and the kind of organs affected. In line with Darsini et al.,[26] the protective effect of EFEC fruit extract in the present study is likely to occur through increased antioxidant activities of SOD and GPX in liver tissue.

However, the present study has some limitations in that the researchers did not involve a FEC negative control group (high-fat diet-induced, unhealthy group). In addition, the researchers did not further investigate the antioxidant content in EFEC fruit extract and the response of the observed tissue to ROS. The researchers did not reexamine as well the total cholesterol levels in the rats after the administration of EFEC fruit extract, thereby leaving unexplained the correlations among the changes in cholesterol levels, antioxidant levels, and ROS as well as changes in liver morphology and function. Despite such limitations, this study has proved that the hepatic functional and morphological changes in the group induced by a high-fat diet and given EFEC fruit extract at a therapeutic dose of 700mg/kgBW are nearly the

same as in normal liver. Further research is recommended to determine the ranges of therapeutic and toxic doses as well as the mechanism of action of EFEC fruit extract in hyperlipidemia.

CONCLUSION

EFEC fruit extract at 700 mg/kgBW was able to improve steatohepatitis in high-fat diet induced rats.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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CONTRIBUTORS

DNA interpreted the data and prepared the manuscript for publication, MWD and KYS collected the data, conducted the study and prepared the manuscript. All of the authors have read and approved this manuscript.

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