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Hepatoprotective effect of red ginger rhizome extract in deep frying oil-fed male wistar rats

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ABSTRACT

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BACKGROUND

Non-alcoholic steatohepatitis (NASH) is a chronic liver disease associated with increased free fatty acids, trans fatty acids and free radical compounds due to the consumption of deep frying oil (DFO). Ginger has hypolipidemic and antioxidant properties, and acts as an insulin sensitizer. The present study was conducted to evaluate the hepatoprotective effect of an ethanol extract of *Zingiber officinale* Roxb var rubrum (EEZO) in DFO-fed male Wistar rats.

METHODS

A study of experimental design was conducted involving 24 non-alcoholic steatohepatitis (NASH) male Wistar rats that were randomly divided into 4 groups. Group K1 (control) was given DFO-3 (3 times heated DFO) at a dose of 10 µl/g BW/day. The treatment groups (P1, P2, P3) were given DFO-3 and red ginger rhizome extract at doses of 100, 200, and 400 mg/kg BW orally for 30 days. The histopathology examination assessed fatty degeneration, and degree of fibrosis. The data were analyzed with the Kruskal-Wallis and Mann-whitney test.

RESULTS

The Kruskal-Wallis test showed that fatty degeneration (steatosis, lobular inflammation, hepatocyte ballooning and NASH) were significantly different among the four groups ($p < 0.05$). Mann-Whitney test proved that fatty degeneration was not significantly different between control and treatment groups ($p > 0.05$).

CONCLUSION

Deep frying oil increased fatty degeneration and NASH, and none of red ginger rhizome extract doses was able to inhibit fatty degeneration significantly. For the future research it is intended to prolong the induction time and the administration of red ginger rhizome extract.

Keywords: NASH, red ginger rhizome extract, fatty degeneration, fibrosis, rats

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a chronic condition that is histopathologically characterized by more than 5 to 10% of macrovesicular liver steatosis and is not due to alcohol consumption. Non-alcoholic fatty liver disease is categorized into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH). The NASH is a chronic condition that is histopathologically characterized by hepatic steatosis and inflammation with hepatocyte injury (ballooning) with or without fibrosis.^(1,2) The prevalence of NAFLD worldwide is estimated to be between 6.3% and 33%.⁽²⁾ In the United States the incidence of NAFLD in 2012 in the United States was up to 27-34%, in Europe it reached 20-30% and in Asian countries such as Sri Lanka, Malaysia, and Indonesia it reached 15-20%, while 2-3% of the general population is estimated to have NASH.⁽³⁾

Non-alcoholic steatohepatitis is considered to be one of the causes of chronic liver disease. The hazards of fatty liver are hepatic cirrhosis and liver cancer, due to liver inflammation from steatosis.⁽⁴⁾ One of the causes are free fatty acids, free radical compounds and trans fatty acids, which can be produced by deep frying oil (DFO).⁽⁵⁻⁷⁾ Trans fatty acids are able to increase lipid peroxidation, and the lipid peroxides formed will damage the rough endoplasmic reticulum so that the synthesis of lipoprotein-forming proteins is impaired. Low lipoprotein levels cause some of the fat in the liver to not bind to lipoproteins to be transported to other parts of need. The accumulation of fat in the liver causes fatty degeneration of the liver cells. Degeneration is a change in cell morphology due to injury. Cell injury causes changes in intracellular metabolism that subsequently alter the cell structure.⁽⁸⁾

Lipid peroxidation is characterized by increased malondialdehyde (MDA) and decreased antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), that cause oxidative stress.⁽⁹⁾

The resulting oxidative stress or reactive oxygen species (ROS) destroys mitochondrial deoxyribonucleic acid (DNA) and increases the expression of proinflammatory cytokines [tumor necrosis factor alpha (TNF- α), transforming growth factor beta (TGF- β), interleukin-8 (IL-8)], so increasing the formation of Mallory bodies and collagen synthesis in the stellate cells. The ROS will also cause apoptosis directly through the nuclear factor- κ B (NF- κ B) activation, thereby causing injury to the liver, inflammation and development of fibrosis.^(10,11)

Zingiber officinale Roxb var *rubrum* (ZO) reportedly contains flavonoids, polyphenols and tannins, that are known to have antioxidant activity.⁽¹²⁾ And is able to inhibit lipid peroxidation, to increase antioxidant enzymes, to regulate the low density lipoprotein (LDL) receptors and 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) that affect the absorption of cholesterol in the liver.⁽¹³⁾ The ZO component zingerone is particularly effective as a free radical scavenger of superoxide, hydroxyl, nitric oxide (NO), peroxy and peroxy nitrite radicals *in vitro*. Gingerol is also an effective peroxy radical scavenger and inhibits NO formation. Therefore, 6-gingerol (the most abundant gingerol) and zingerone effectively inhibit lipid peroxidation due to the generation of ROS.⁽¹⁴⁾

Phytochemical studies showed that ZO is rich in a large number of substances, including gingerols and shogaols, the dehydrated form of gingerols. These compounds display diverse biological activities such as antioxidant, anti-inflammatory, and anticarcinogenic properties.⁽¹⁵⁾ A study on 30 male Wistar rats given DFO at a dose of 0.42 ml per day for 14 days found increased steatosis, leading to the development of fatty liver.⁽¹⁶⁾ Another study, in which 48 rats were fed a high cholesterol diet and received an aqueous infusion of ginger orally at doses of 100, 200 and 400 mg/kg BW in comparison with atorvastatin, found that the doses of 200 and 400 mg/kg BW lowered cholesterol more effectively than did atorvastatin.⁽¹⁷⁾ Yet another study showed that oils from *Z. officinale* (200 mg/kg) exhibited

hepatoprotective activity in acute ethanol-induced fatty liver in male Wistar rats.⁽¹⁸⁾

A study on male Wistar rats that were fed used frying oil showed that there were a large number of dark-red patches, necrosis, and bleeding in the liver, while the renal cells were also obviously damaged.⁽¹⁹⁾ However, no information could be obtained from previous studies on the effects of an ethanol extract of red ginger rhizome on NASH. The objective of the present study was to evaluate the hepatoprotective effect of an ethanol extract of red ginger rhizome on the histopathology of NASH in DFO-fed male Wistar rats.

METHODS

Research design

This study was of a randomized post-test only control group design, in which the control and treatment groups were each treated differently. The study was conducted in the animal breeding house in the Pharmacy Laboratory, while the histopathology examination was conducted in the Laboratory of Histology, Medical Faculty, Universitas Sumatera Utara from September 2016 to January 2017.

Plant material

Red ginger rhizomes were collected from Raya Simalungun and have been identified in the Herbarium Medanese Laboratory of Universitas Sumatera Utara under number 871/MEDA/2016

Preparation of red ginger rhizome extract

Red ginger rhizomes were washed and then dried in a drying oven at a temperature of 60°C. After drying, the rhizomes were then crushed in an electric blender. The simplicia homogenate powder was macerated in 96% ethanol. After filtration, the extract was evaporated using a rotary evaporator at a temperature range of 40-60°C until it had thickened. The solutions made from the extract were prepared freshly each time and were administered orally once daily for 30 days to the treatment groups.

Standardization of simplicia and extract was based on the Indonesian Pharmacopeia 2014,⁽²⁰⁾ to calculate the water content, water soluble extract, ethanol soluble extract, total ash content and acid soluble ash content.⁽²⁰⁾ Identification of the phytochemicals in red ginger rhizome ethanol extract was based on Yang et al.⁽²¹⁾ to identify flavonoids, alkaloids, tannins, saponins, steroids and terpenoids as well as glycosides.

Preparation of deep frying oil

Deep frying oil was made by frying 5 kg catfish 3 times in 2.5 liter cooking palm oil at 200°C (measured with a cooking thermometer) for 15 minutes. After each frying the oil was left to cool for \pm 5 hours at room temperature. The 3-times heated DFO preparation will subsequently be referred to as DFO-3.

Experimental animals

The experimental animals were 24 healthy male Wistar rats aged 2-3 months and weighing between 200 and 250 g. The rats were divided by simple randomization into 4 groups of 6 animals per group, by using the formula of Federer: $(t-1)(n-1) \geq 15$.⁽²²⁾ The animals were acclimatized at room temperature (25-30°C) for one week under a 12 hour light-and-dark cycle. Food was given orally *ad libitum* in the form of the CP 551 standard animal feed concentrate from PT. Charoen Pokphand, Tanjung Morawa, Medan.

Experimental procedure

The interventions were based on the treatment group. Group K1 (control) was given standard diet and DFO-3 + distilled water + 1 ml of 1% sodium carboxymethyl cellulose (Na-CMC), and treatment groups P1, P2, P3 were given orally standard diet and DFO-3 + distilled water + 1 ml of 1% Na-CMC and red ginger rhizome extract at stratified dosages of 100, 200 and 400 mg/kg BW, administered daily for 30 days. At the end of the treatment on the 37th day (of acclimatization and treatment), the rats were anesthetized by using ketamine xylazine at doses of 75-100 mg/kg + 5-10 mg/kg intraperitoneally

for 10-30 minutes. Then surgical laparotomy was performed to remove the liver, which was prepared for histological analysis.

Histological analysis

The slide preparations for histological analysis were stained with hematoxylin and eosin (H&E) for the assessment of lipid degeneration and with Masson's trichrome stain for the assessment of fibrosis, then examined microscopically. The preparations successively underwent fixation, washing, dehydration, clearing, embedding, sectioning, affixing, staining, mounting and labeling.⁽²³⁾

The stained slides were analyzed under the light microscope at 400x magnification. Blind labeling was performed randomly on 24 slides. Fat droplet-containing liver cells were counted in four areas around the central veins of randomly selected liver lobules and then the mean number of fat droplets was calculated from the total number of fat droplets in all areas. The degree of fibrosis was determined according to Brunt.⁽²⁴⁾

Statistical analysis

The data were not normally distributed. The Kruskal-Wallis test was followed by the Mann-Whitney test to analyze the data. Significance was set at $p < 0.05$.

Ethical clearance

The study was conducted according to the ethical clearance from the Animal Research Ethics Committee of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara (FMIPA USU), Medan, under number 589/KEPH-FMIPA/2016.

RESULTS

The standardization of the ZO ethanol extract (EEZO) yielded the following results: water content of simplicia (7.50%), extract (4.90%), water soluble content (21.70%), ethanol soluble concentration (10.43%), total ash content (3.42%) and acid soluble ash (1.32%). The phytochemical screening of simplicia and EEZO traced the presence of alkaloids, saponins, tannins, flavonoids, steroids, triterpenes and glycosides.

Fatty degeneration was found in 24 male Wistar rat liver tissue samples with H & E staining, as shown in Figures 1A and 1B. The histologic sections in some groups showed steatosis, lobular inflammation and hepatocyte ballooning after 30 days of DFO administration. Figure 1C shows decreased lobular inflammation with neutrophils in normal sinusoids after administration of red ginger extract at the dose of 200 mg/kg BW.

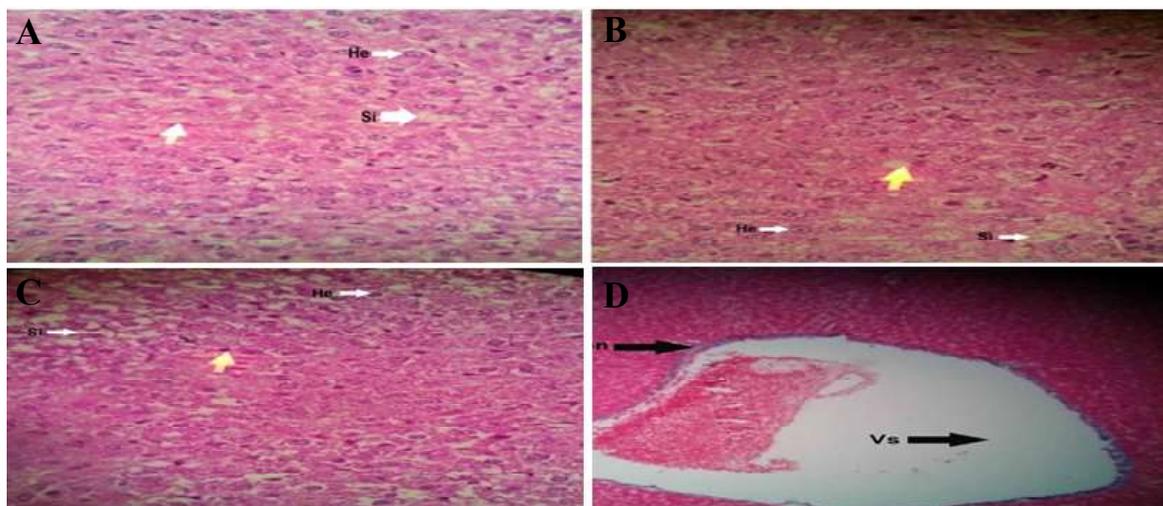


Figure 1. Histopathology of lipid degeneration; Si=sinusoid, He=hepatocyte, Vs=central vein, A. Steatosis B. ballooning of hepatocyte C. lobular inflammation (H&E stain), D. Arrows show collagen fibers clearly visible surrounding the central vein (F0) (Masson's trichrome stain)

Determination of the degree of fibrosis was performed on 24 male Wistar rat liver tissue samples with Masson's trichrome staining, as shown in Figure 1D. The image shows that the Masson's trichrome staining was successful, with a perfect color of collagen fibers clearly visible between the central veins. Therefore it can be concluded that in this study the collagen fibers were normal (F0=no fibrosis).

The result of Kruskal-Wallis test showed that the number of hepatocytes with steatosis ($p=0.001$), lobular inflammation ($p=0.001$), hepatocyte ballooning ($p=0.000$) and NASH ($p=0.001$) were significantly different between the four groups (Table 1).

The result of Mann-Whitney test showed the comparison between KI (control) and treatment groups (P1, P2, P3) with regard to the effect of DFO-3 on the number of hepatocytes with steatosis, lobular inflammation, hepatocyte ballooning and NASH grade, there was no significant difference between the four groups ($p>0.05$). There was only a significant difference in the amount of steatosis between the control group and the treatment group P1 at the dose of 100 mg/kgBW. It can be seen from the result that the amount of steatosis was greater in the treatment group than in the control group (data not shown).

DISCUSSION

This study showed that the administration of deep frying oil can increase the amount of steatosis, lobular inflammation, hepatocyte ballooning and NASH grade. Although the

administration of red ginger rhizome ethanol extract did not significantly decrease the histopathologic abnormalities, at a dose of 200 mg/kg BW the result was better than that in the other groups. In another study on the administration of aqueous extract of ginger to Sprague Dawley rats at a dose of 400 mg/kg BW, it was found that EEZO can be used as an anti-inflammatory agent.⁽²⁵⁾ In another histopathological study on acute and chronic models of inflammation in Wistar rats, it was found that administration of an aqueous ginger extract decreased granulation tissue, inflammatory cells and fibroblasts as compared to controls.⁽²⁶⁾

This is supported by another study, which found that red ginger rhizome can decrease fatty degeneration which is influenced by anti oxidant compounds such as flavonoids and tannins as free radical scavengers that can decrease the formation of proinflammatory cytokines and increase the formation of lipoprotein as a fat carrier in the liver.⁽²⁷⁾

In previous studies the doses of 100, 200, and 400 mg/kg BW could decrease fatty degeneration of the liver using an assessment of the enzymatic system, but in the present study only the dose of 200 mg/kg BW was able to reduce fatty degeneration of the liver. The present study was directly assessed on the gold standard of liver histology, especially hepatocyte degeneration (amount of steatosis, lobular inflammation, hepatocyte ballooning), NASH and fibrosis.

Decreased fatty degeneration is due to the fact that the red ginger rhizome has an antioxidant content of flavonoids. Flavonoids are compounds

Table 1. Effect of ethanol extract of red ginger rhizome on liver histopathology in DFO-fed male Wistar rats

Histopathology	K1 (n=6)	P1 (n=6)	P2 (n=6)	P3 (n=6)	p
Steatosis	0.00	1.00*	0.00	1.00	0.001
Lobular inflammation	1.00	1.00	0.00	1.00	0.001
Hepatocyte ballooning	0.00	0.00	0.00	0.00	0.000
NASH	0.50	1.00	0.00	1.00	0.001

Data presented as median; *Significant at $p<0.05$ by Mann-Whitney test; NASH : non-alcoholic steatohepatitis; K1: normal diet +DFO-3 (10 μ l/g/day) as control; P1: normal diet + DFO-3 + EEZO (100 mg/kg once daily); P2: normal diet + DFO-3 + EEZO (200 mg/kg once daily); P3: normal diet + DFO-3 + EEZO (400 mg/kg once daily)

belonging to the phenolic group. The main phenolics in red ginger are gingerol and shogaol, that are more effective than quercetin.⁽²⁸⁾

A phenolic has one aromatic ring A, one aromatic ring B, and an oxygen-containing middle ring and contains one or more hydroxyl groups.⁽²⁹⁾ The hydroxyl group in phenolics is a group that has antioxidant activity and plays an important role as free radical scavenger, due to its ability to donate hydrogen atoms, thereby stabilizing free radical compounds.^(30,31)

High temperature frying at 200°C converts trans fatty acids through thermal polymerization and oxidation reactions after a second repeat frying for 30 minutes.⁽³²⁾ Trans fatty acids (TFA) inhibit the activity of enzymes for lipid metabolism, such as fatty acid desaturase elongase and lecithin cholesterol acyl transferase (LCAT); the latter plays a role in the metabolism of high-density lipoprotein cholesterol (HDL), i.e. the transport of cholesterol from the tissues to the liver, to be reconverted into very low density lipoprotein.⁽³³⁾ In our study the formation of fatty acids in the liver was due to excessive accumulation of fat after administration of DFO-3 in volumes of 10 µl/g BW for 30 days. Heating oil at the high temperatures for frying will decrease the quality of the oil and can induce NAFLD.⁽³⁴⁾

Deep frying oil has a high trans fatty acid (TFA) and mono-unsaturated fatty acid (MUFA) content.⁽³⁵⁾ It decreases the efficiency of antioxidant enzyme systems by increasing pro-inflammatory signals, thus increasing oxidative stress. It can cause failure of the liver in synthesizing apolipoprotein B-100 (apo B-100) to transport fat from the liver and prevent triglyceride and LDL accumulation.⁽³⁶⁾

When viewed from the effect of the EEZO dose, it was found that a dose of 200 mg/kg BW/day was effective in reducing steatosis, lobular inflammation and degree of NASH in comparison with the control group (K1). The effectiveness of EEZO at the dose of 200 mg/kg BW/day, proves the concept of drug or vitamin consumption, because each drug or

vitamin has different dosage rules so as not to harm or cause new diseases. Previous results also stated that EEZO at the dose of 200 mg/kg BW significantly decreased pro-cytokines such as (IL-1β, IL-2, IL-6 and TNF-α).⁽³⁷⁾ The results of our study in using multilevel dosages in the administration of EEZO with DFO-3 showed no significant difference in the decrease in steatosis, lobular inflammation, hepatocyte ballooning, NASH and fibrosis (F0). This may be induced and it requires a longer duration of therapy to affect a more optimal improvement of the liver tissue. Therefore, high doses of EEZO increase the intake of gingerol in the body. The gingerol compound is spicy, so high doses cause irritation which has the potential to increase liver injury, thus exacerbating hepatocyte steatosis. This study did not measure the duration of EEZO administration, so further studies need to consider the duration of treatment to evaluate the hepatoprotective effect of EEZO.

CONCLUSIONS

Ethanol extract of *Z. officinale* effectively reduces the amount of steatosis, lobular inflammation and NASH. However, there was no significant difference in the treatment at the doses of 100, 200 and 400 mg/kg BW. This study concludes to favor ginger but further research is required before its efficacy can be claimed.

CONFLICT OF INTEREST

The authors declare that there was no conflict of interest between the authors and the study subjects.

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CONTRIBUTORS

SN contributed to the design of the study, drafting of the manuscript and was responsible for the final content. SN, EF, UH, and DY contributed to data collection, analysis and interpretation. TW and LDL contributed to revising the manuscript critically for important intellectual content. All authors read and approved the final version. ✚

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