Potential of Olive Oil Extract (*Olea europaea*) For Affecting Lipid Profile, Lipid Oxidative and Fatty Liver on Hiperlipemic Rats (*Rattus norvegicus*)

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Abstract

Olive oil (*Olea europaea*) contains 55 to 83% oleic acid which is a single chain unsaturated fatty acid or mono-unsaturated fatty acid (MUFA), and 2% phenolic components in the form of hydroxytyrosol and tyrosol. This study aims to determine the potential of olive oil extract (*Olea europaea*) in reducing cholesterol and malondialdehyde levels, along with inhibiting fatty liver development in hiperlipemic rats. Sixteen rats were divided into four groups, the first group was healthy control group, the second group was positive control group, received a high-fat diet containing 100 mg of cholesterol powder, 25 mg of cholic acid powder and 1 mL of quail egg yolk for 28 days. Third and fourth treatment groups were received for 28 days, plus 0.828 g (1 mL) and 1.656 g (2 mL) of olive oil extract daily for 14 days, respectively. The measurement of total cholesterol used cholesterol test strips based on oxidation enzyme reaction cholesterol esterase and cholesterol oxidase that produce hydrogen peroxidation, then analyzed with biosensor refractophotometry. Measurement of malondialdehyde used thiobarbituric acid (TBA) test. Histological observation of fatty liver was assessed using a NAS (Non-alcoholic fatty liver disease score). The results showed that 1.656 g (2 mL) of olive oil extract per day decreased total cholesterol level up to 44.41 %, malondialdehyde level up to 61.75%, and NAS score up to 50%, compared with positive control. It was concluded that olive oil extract was decreasing total cholesterol level, as an anti-oxidant and prevent fatty liver development.

Keywords: fatty liver, hyperlipidemia, malondialdehyde, *Olea europaea*.

INTRODUCTION

Hypercholesterolemia causes an enzymatic oxidation reaction occurs, between oxygen radicals and lipoprotein, which produces lipid radicals or LDL oxidation. Lipid hydroperoxide (ROOH) is the first stable product of a lipid peroxidation reaction [1]. Malondialdehyde (MDA) is the result of a reaction from the decomposition of lipid peroxidation. The accumulation of triglycerides in the liver, or steatosis, increases the susceptibility of hepatocytes, which are mediated by the second stage, such as inflammatory cytokines and adipokines, mitochondrial dysfunction and oxidative stress, which cause steatohepatitis and or fibrosis [2].

Olea europaea contains oleic acid and a minor phenolic component, hydroxytyrosol, which can reduce LDL cholesterol levels, by increasing triglyceride clearance. Oleic acid can also reduce LDL oxidation and the production of reactive oxygen species, which can reduce levels of malondialdehyde. Olive oil can reduce inflammation and lipid formation and inhibit the occurrence of liver steatosis [3]. This study was conducted to analyze the potential of olive oil containing oleic acid in improving lipid profiles, reduce oxidative stress, and reduce fatty liver in hiperlipidemic rats.

MATERIAL AND METHOD

Animal Preparation

The male Wistar strain (*Rattus norvegicus*), around two to three months olds, weighing between 180-200 g obtained from UPHP (Experimental Animal Development Unit), Gadjah Mada University, Yogyakarta and having received the Ethical Clearance Certificate University of Brawijaya with Number 919-KEP-UB. Rats were acclimatized for two weeks, placed in 16 cages and received standard food. Rats were divided into 4 groups, each group consisting of 4 rats. Healthy control group (K1) with a standard diet, positive control group (K2) with a high fat diet for 28 days, two treatment groups (P1) and (P2), with a high-fat diet for 28 days plus olive oil extract of 0.858 g (1 mL) and 1.656 g (2 mL) every day, for 14 days.

The composition of a High-Fat Diet

A high-fat diet consists of 100 mg of pure high-grade cholesterol powder Bioworld brand, 25 mg of cholic acid powder from the Tokyo Chemical Japan Industry brand and 1 mL of egg

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yolk, coupled with water to a solution of 3 mL. Based on the composition of the hyper cholesterol induction diet which consisting of 1% cholesterol powder, 0.25% bile salt or cholic acid and 4% animal fat, for 21 days [4].

Determination of Dosage of Olive Oil Extract

Bertoli extra virgin olive oil, produced by Carapelli Florence, Italy, the dosage of olive oil to reduce the risk of cardiovascular disease, is 2 tablespoons or 23 g per day [5]. Dosage was converted between species, from human doses, with a human body weight of 70 kg, equivalent to 200 g of rat body weight, obtained 0.018, based on Laurence formula. The dose of 2 tablespoons or 23 g is converted to rats weighing 200 g, 23 times multiplied by 0.018 resulting in 0.414 g or 0.5 mL per day. Giving olive oil, in the treatment groups 3 and 4, with a dose of 0.828 g (1 mL) and 1.656 g (2 mL) every day, for 14 days.

Analysis of Oleic Acid in Olive Oil, Using Liquid Chromatography-Mass Spectrometry, Electro Spray Ionization (LC-MS-ESI)

Total of 3 mL olive oil inserted into the liquid chromatography solvent reservoir, will separate the oleic acid compound with other compounds, based on differences in composition and molecular weight. The oleic acid compound is converted into an electrically charged molecular ion, to calculate its molecular mass, into a chromatogram.

Examination of Total Cholesterol Levels

Total cholesterol levels were measured using the easy touch, cholesterol test strips based on the presence of the cholesterol esterase enzyme oxidation reaction and cholesterol oxidase which produced hydrogen peroxidation, then analyzed by biosensor reflectophotometry.

Examination of Malondialdehyde (MDA) Levels

Measurement of MDA levels was carried out with the Bioassay System Quantichrom TBARS Assay Kit (DTBA-100), based on the formation of a reaction between malondialdehyde and thiobarbituric acid, which produces pink fluorescence at an intensity of 560 nm. Serum for the examination of malondialdehyde, obtained from blood taken through the heart using 10 mL syringe as much as 10 mL, then put into vacutainer. Total of 5 mL blood is stored at room temperature for two to ten hours, then centrifuged at 2000 rpm for 20 minutes, then stored at -40^{o}C.

Histological Preparations and Liver Histology Examination

The liver organ is taken and cut into a size of 1 x 1 cm and then fixed in neutral buffer formalin (NBF) of 10% for 24 hours. Tissue cassettes are included in the tissue processor for the stages of dehydration, clearing, embedding. After blocking with paraffin, cut with a microtome in a thickness of 5-6 microns. Furthermore, the preparations were stained with staining of hematoxylin-eosin (HE).

Calculation of Non-Alcoholic Fatty Liver Disease Score (NAS) Score

The calculation was conducted based on the histology of liver tissue in the form of steatosis, hepatocyte ballooning, and inflammation. Observations with microscopes were seen in twenty view fields with 200 times magnification. The NAS score is between zero and eight, based on the amount of steatosis, ballooning, and inflammation.

Data Analysis

This study was tested using ANOVA with a confidence level of 95%, every treatment was replicated four times, followed by the test Tukey's real difference with a confidence level of 95%.

RESULTS AND DISCUSSION

The Presence of Oleic Acid in the Olive Oil Extract

Analysis of extra virgin olive oil using MS-ESI LC, there is an ion mass per charge ($m.z^{-1}$) with a range of 280.50 - 281.50 $m.z^{-1}$ which corresponds to the mass of ions per electronic charge of oleic acid (Fig. 1A). Based on data from Mass Bank Records MT000029 that used LC-MS-ESI, the ion mass per electronic charge ($m.z^{-1}$) of oleic acid is 281.3 $m.z^{-1}$.

Besides that, there is an ion mass per charge ($m.z^{-1}$), with a range of 254.50 - 255.50 $m.z^{-1}$, and 278.50 - 279.50 $m.z^{-1}$, which corresponds to the mass of ions per charge of palmitic acid and linoleic acid (Fig. 1B and 1C). Based on Mass Bank Record data MT000114, ion mass per electronic charge ($m.z^{-1}$) of palmitic acid and linoleic acid using MS-ESI LC-255.3 $m.z^{-1}$and 279.3 $m.z^{-1}$. In addition to these three types of fatty acids, there is an ion mass per electronic charge ($m.z^{-1}$) in the range 298.50 - 299.50 $m.z^{-1}$ which corresponds to the ion mass of tyrosol glucoside, which is a phenolic component of olive oil (Fig. 1D).

So that it can be concluded, from the examination of Bertolli extra virgin olive oil, using

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LC-MS - ESI, there are oleic acid, palmitic acid and linoleic acid which are the three most fatty acids in olive oil.

Oleic acid is a monounsaturated fatty acid (MUFA), while palmitic acid is a type of saturated fatty acid, and linoleic acid is a double chain unsaturated fatty acid (PUFA). In addition to fatty acids, the phenolic component is tyrosol glucoside, which is a derivative of tyrosol, where tyrosol is a major substance from the phenolic component.

Every 100 g of olive oil contains fatty acids, namely 73.7 g of oleic acid which includes monounsaturated fatty acids (MUFA), 13.5 g of palmitic acid which is a saturated fatty acid, 7.9 g of linoleic acid and alpha-linolenic acid, which is polyunsaturated fatty acid (PUFA) [6].

Olive Oil Extract for 14 Days Decrease Total Cholesterol Levels

The administration of 0.828 g of olive oil extract (1 mL) and 1.656 g (2 mL) daily for 14 days in P1 and P2, significantly reduced ($p < 0.05$) cholesterol levels, compared with the positive control group. Total cholesterol levels in the group of P1 and P2 were 99 $mg.dL^{-1}$ and 94.5 $mg.dL^{-1}$, decreased 41.76% and 44.41% compared to the positive control group (Table 1).

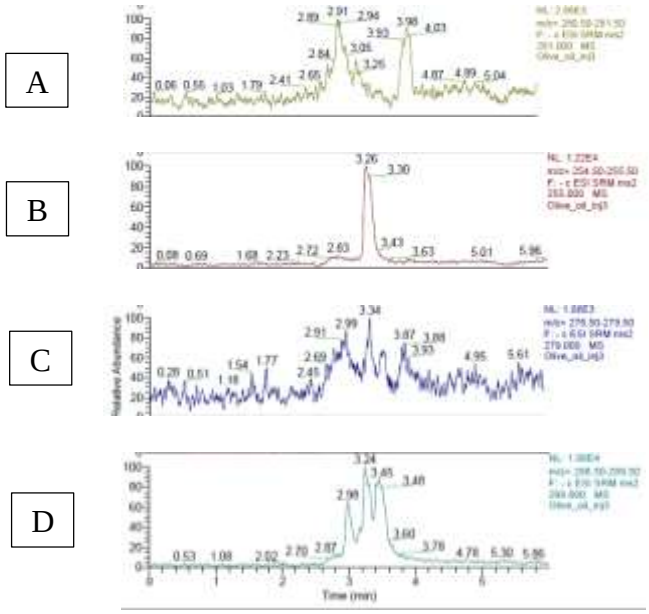

Figure 1. Ion Mass per Charge of Fatty Acids and Phenolic Components in the Extract Olive Oil
A. Ion mass per charge of oleic acid
B. Ion mass per charge of palmitic acid
C. Ion mass per charge of linoleic acid
D. Ion mass per charge of tyrosol glucosi

Table 1. Total Cholesterol Levels of Mice After a 28-day High-fat Diet and Oil Olives Extract for 14 days

Group	Total Cholesterol ($mg.dL^{-1}$)		
	High fat diet for 14 days	High fat diet for 28 days + olive oil extract for 14 days	Decrease of total cholesterol Compared with positive control (%)
K1	51.25 ± 0.95^{a}	49.25 ± 0.95^{a}	-
K2	176.54 ± 3.41^{b}	170.00 ± 10.8^{c}	-
P1	177.00 ± 2.16^{b}	99.00 ± 0.81^{b}	41.76
P2	173.00 ± 6.21^{b}	94.50 ± 4.50^{b}	44.41

Notes: K1 = Healthy control, K2 = Positive control, high fat diet for 28 days, P1 = a high fat diet for 28 days + olive oil extract 0.828 g (1 mL) per day for 14 days, and P2 = high fat diet for 28 days + olive oil extract 1.656 g (2 mL) per day for 14 days

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The decrease of cholesterol levels in the P1 and P2 group caused by the content of oleic acid and tyrosol in olive oil extract detected by LC-MS-ESI. Olive oil extract containing oleic acid and phenolic components can increase HDL capacity to accelerate the removal of cholesterol from macrophages by increasing the expression of adenosine triphosphate (ATP)-binding membrane cassette system (ABCA1 and ABCG1) [7]. Oleic acid can reduce HDL oxidation in humans, so it can increase the disposal of cholesterol from macrophages because HDL oxidation can damage HDL function and reduce the ability of HDL to eliminate cholesterol from macrophages [8]. Olive oil phenolic compounds can increase the size and stability of HDL, which can reduce triglycerides, and reduce HDL oxidation by increasing polyphenol metabolism in a lipoprotein [9].

Olive Oil Extract Decrease MDA Levels

The administration of 0.828 g of olive oil extract (1 mL) per day and 1.656 g (2 mL) daily for 14 days in P1 and P2 group, significantly reduced ($p < 0.05$) MDA levels, compared to the positive control group.

The average MDA levels in the P1 and P2 group were 8.07 $\mu mol.L^{-1}$ and 7.41 $\mu mol.L^{-1}$, decreased 58.29% and 61.75%, compared to the positive control group (Fig. 3, Table 2). Giving a high fat diet plus olive oil extract 0.828 g (1 mL) and 1.656 g (2 mL) every day for 14 days in P1 and P2 group, significantly reduced MDA levels, because olive oil contains oleic acid and phenolic components which can inhibit LDL lipoprotein oxidation process, which results in a decrease in MDA value.

Olive oil can reduce lipid peroxidation, in fresh meat put into gastric simulation media with a pH of 3, thereby reducing levels of malondialdehyde, from 121.7 ± 3.1 $\mu mol.L^{-1}$ to 48.2 ± 1.3 $\mu mol.L^{-1}$. Oleic acid in olive oil can reduce the release of hydrogen atoms so that oxygen does not occur, which inhibits the occurrence of lipid oxidation [10].

Olive Oil Extract Decrease Non-Alcoholic Fatty Liver Disease Activity Score (NAS)

The NAS score or the Non-Alcoholic Fatty Liver Disease Activity Score is a histological assessment based on the presence of steatosis, lobular inflammation, and ballooning of hepatocytes, which describes the presence of active injury in the liver. The NAS score is from 0 to 8, which is the sum of the number of steatosis, inflammation of the liver lobe and ballooning of liver cells that occur (Table 3) [11].

The administration of olive oil extracts for 14 days at a dose of 0.828 g (1 mL) and 1.656 g (2 mL) per day, in the P1 and P2 group, decreased the Non-Alcoholic Fatty Liver Disease Activity Score (NAS), when compared to the positive group. The P1, P2 group with an average NAS score of 2.5 and 1.5, reduced NAS score of 16% and 50% lower than the positive control group (Table 4). Giving olive oil extract as much as 1.656 g (2 mL) every day for 14 days in P2 group can reduce 50% NAS scores, compared to the positive control group, because olive oil extract can reduce the accumulation of triglycerides in the liver, accelerating recovery of liver steatosis, slow fibrosis growth and prevent oxidative stress in the liver. Tyrosol which is a polyphenol, act as an anti-inflammatory in the liver [12]. Olive oil can increase the formation of anti-oxidation enzymes; improve liver tissue, by repairing hepatocyte cell membranes [13].

Table 2. Levels of Malondialdehyde after a 28-day High-fat Diet and Olive Oil Extract for 14 days

Group	MDA level average ($\mu mol.L^{-1}$)	Increase of MDA level compared with healthy control (%)	Decrease of MDA level compared with positive control (%)
K1	6,25 ± 0,73[a]	-	-
K2	19,35 ± 2,78[b]	209	-
P1	8,07 ± 1,63[a]	29,12	58,29
P2	7,41 ± 0,42[a]	18,56	61,75

Notes: K1 = Healthy control, K2 = Positive control, high fat diet for 14 days, P1 = a high fat diet for 28 days + olives oil extract 0.828 g (1 mL) per day for 14 days, and P2 = high fat diet for 28 days + olive oil extract 1.656 g (2 mL) per day for 14 days

Table 3. Stadium of Non-Alcohol Fatty Liver Disease (NAFLD), based on NAFLD activity score (NAS)

NAS	Steatosis	Ballooning	Lobular Inflammation
0	<5% (0)	None (0)	None (0)
3	5-33% (1)	Rare or few (1)	1-2 focci per 20 x field (1)
6	34-66% (2)	Many (2)	2-4 focci per 20 x field (2)
8	> 66% (3)	Many (2)	> 4 focci per 20 x field (3)

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Table 4. NAS Score After a High-fat Diet for 28 and Olive Oil Extracts for 14 days

Group	NAS score average	Increase NAS score compare with healthy control (%)	Decrease NAS score compare with positive control (%)
K1	$1 \pm 0{,}81^{a}$	-	-
K2	3 ± 0^{c}	200	-
P1	$2{,}5 \pm 0{,}57^{b,c}$	150	16
P2	$1{,}5 \pm 0{,}57^{a,b}$	33,33	50

Notes: K1 = Healthy control, K2 = Positive control, high fat diet for 14 days, P1 = a high fat diet for 28 days + olives oil extract 0.828 g (1 mL) per day for 14 days, and P2 = high fat diet for 28 days + olive oil extract 1.656 g (2 mL) per day for 14 days. NAS scores ranged 0-8, i.e. the sum of many steatosis (0-3), ballooning hepatocytes (0-2), and hepatocyte inflammation (0-3) [11].

In this study, the healthy control group (normal hepatocytes) showed in green arrows (Fig. 5A). In the positive control group, many inflammations of the lobules hepatocytes containing infiltrates (orange arrow) (Fig. 5B). In the P1 and P2 group, inflammatory hepatocytes (orange arrows), was found less than the positive control group and a few of ballooning hepatocytes (yellow arrows) (Fig. 5C) and (Fig. 5D).

CONCLUSION

The results showed that olive oil extract 1.656 g (2 mL) per day decreased total cholesterol level up to 44.41%, MDA level up to 61.75%, and NAS score up to 50%, compared to the positive control. It was concluded that oleic acid and tyrosol in the olive oil extract can decrease total cholesterol level, thereby reducing lipid oxidation and inhibiting fatty liver development.

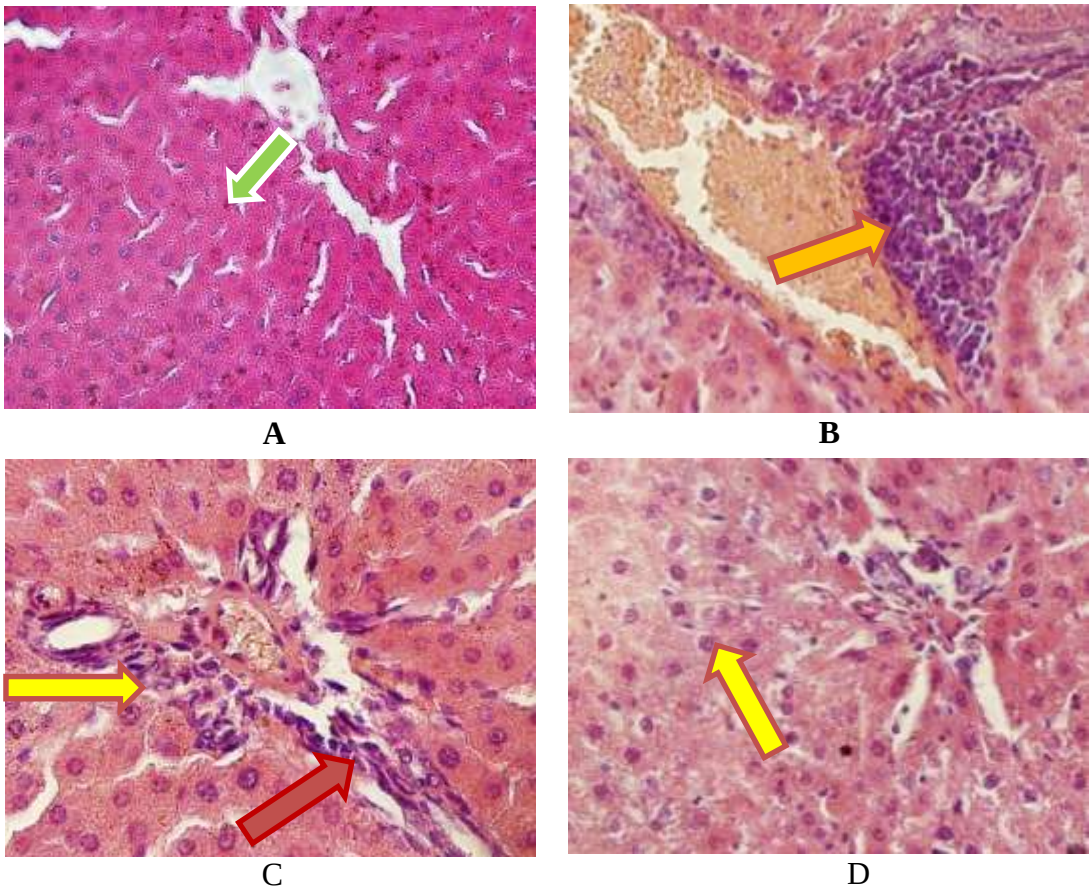

Figure 5. Hepatocyte Cells Histology.

Description:

(A) Hepatocyte cells of the healthy control group, shows there are normal hepatocytes (green arrow),

(B) Hepatocyte cell of the positive control group, with 28-day high-fat diet, shows there are many inflammation hepatocytes and infiltrates containing polymorphonuclear cells (orange arrows).

(C) Hepatocyte cells of the P1 group, with a high-fat diet for 28 days and olive oil extract 0.828 g per day (1 mL per day) shows there are hepatocytes inflammation (orange arrows) and ballooning hepatocytes (yellow arrow).

(D) Hepatocyte cells of the P2 group , with a high-fat diet for 28 days and olive oil extract 1.656 g per day (2 mL per day) shows there are few ballooning hepatocytes (yellow arrow).

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