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ORIGINAL ARTICLE

Phytochemical screening and antidyslipidemic effect of lime fruit (*Citrus aurantifolia*) ethanol extract on high-density lipoprotein levels of high-fat diet-induced rats

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ABSTRACT

Dyslipidemia is a significant health problem in Indonesia and a major cause of coronary heart disease (CHD). According to the 2018 Riset Kesehatan Dasar Nasional (RISKESDAS), 24.3% of Indonesians aged ≥15 years had low high-density lipoprotein (HDL) levels, while 13.8% had high HDL levels. Treatment with traditional medicine is a popular approach in Indonesia. Lime (Citrus aurantifolia) contains flavonoids that can inhibit the activity of the hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase) enzyme. This study aimed to identify the chemical compounds found in lime peel and fruit, as well as to determine the effective dose of ethanol extract from lime fruit in increasing HDL levels. This research is a true experimental study conducted in the laboratory using an in vivo approach. The research used a randomized post-test only control group design involving both control and treatment groups. Based on the results of phytochemical screening, the lime fruit ethanol extract contains flavonoids, alkaloids, tannins, polyphenols, saponins, and quinones. Using the Saphiro-Wilk normality test, the data obtained are normally distributed with a p-value of 0.809 ($p > 0.05$). Statistical analysis was continued with the one-way ANOVA parametric test, and a p-value of 0.146 ($p > 0.05$) was obtained. An increase in the average HDL levels was also observed in the treatment group given a dose of 0.875 g/KgBW of lime fruit ethanol extract, but there was no significant difference in the average HDL levels between the groups.

Keyword: *ethanol extract, flavonoids, high density lipoprotein, lime (Citrus aurantifolia), phytochemical*

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INTRODUCTION

Dyslipidemia is a significant health problem in Indonesia and a major cause of coronary heart disease (CHD).[1] This condition can be triggered by dietary habits, lifestyle, tobacco exposure, or genetic factors. Dyslipidemia refers to an imbalance of lipids such as total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), and high-density lipoprotein cholesterol (HDL-C).[2,3] High-density Lipoprotein (HDL) is a heterogeneous component of particles that differ in density, size, electrophoretic mobility, and apolipoprotein content.[4] HDL functions by transporting low-density lipoprotein (LDL) cholesterol from the endothelium of blood vessels to prevent cholesterol accumulation in the endothelium, then carrying it to the liver for disposal via the digestive system.[5] Normal HDL-C levels in the blood range from 40 mg/dL to 60 mg/dL, with high levels being ≥60 mg/dL and low levels being <40 mg/dL.[3] According to the 2018 *Riset Kesehatan Dasar Nasional* (RISKESDAS), 24.3% of Indonesians aged ≥15 years had low HDL levels, and 13.8% had high HDL levels.[6] Compared to the 2013 RISKESDAS results, the percentage of Indonesians aged ≥15 years with low HDL levels increased by 1.4%.[7] A 1 mg/dL decrease in HDL-C levels increases the risk of coronary artery disease by 2-3%.[8]

Treatment for dyslipidemia includes both non-pharmacological and pharmacological approaches. Non-pharmacological therapy involves lifestyle changes, increased physical activity, exercise, and smoking cessation.[9] Simvastatin is a pharmacological intervention that can be administered. This drug reduces the synthesis of apolipoprotein B100 (Apo B100) and decreases cholesterol synthesis in the liver through competitive inhibition of the enzyme hydroxymethylglutaryl-coenzyme A reductase (HMG-CoA reductase), which plays a role in cholesterol biosynthesis.[10] However, long-term use of simvastatin can lead to drug intolerance, which may cause side effects such as myopathy.[11] Treatment with traditional medicine is a popular approach in Indonesia. Indonesian medicinal plants that have been proven to lower and prevent cholesterol formation include soybeans, green tea leaves, peanuts, garlic, wheat bran, omega-3 fatty acids, blueberries, and lime.[12] Lime (*Citrus aurantifolia*) belongs to the Rutaceae family and the Citrus genus.[13] It contains flavonoids, saponins, and essential oils.[14,15] Flavonoids have a significant effect on lowering total cholesterol (TC) levels, LDL cholesterol (LDL-C) levels, and increasing HDL cholesterol (HDL-C) levels. Flavonoids inhibit the activity of the HMG-CoA reductase enzyme, which reduces cholesterol synthesis in the liver, leading to a decrease in Apo B100 synthesis and cholesterol synthesis.[16]

Based on the research conducted by Mende, Simbala, and Mauda in 2021, the treatment group receiving ethanol extract of lime peel at a dose of 7.2 mg and the treatment group with lime juice at a dose of 0.3 ml were proven effective in reducing the total cholesterol levels in male Wistar rats. This study suggests examining other parameters such as LDL, HDL, and triglyceride (TG) levels.[17] Research conducted by Cyndi, Andriane, and Nur in 2016 demonstrated that the ethanol extract of lime leaves in a hypercholesterolemic mouse model at a concentration of 3.5 g/kg body weight (BW) was effective as an anti-dyslipidemia agent. This is attributed to the flavonoids contained in the ethanol extract of lime leaves, which can inhibit HMG-CoA reductase activity, reduce cholesterol absorption by stimulating the expression and transcription of the LDL receptor gene, and reduce apolipoprotein B secretion by hepatocytes.[12] Based on the research conducted by Kemit, Widarta, and Nocianitri in 2016, it was shown that the type of solvents and maceration time affect the flavonoid content of avocado leaf extracts. The highest flavonoid content was

obtained using 90% ethanol as a solvent and a 3—hour maceration time.[18] Based on these results, this research uses 90% ethanol to extract lime. Research on the use of whole lime fruit to increase HDL levels is still limited. Therefore, this study aimed to identify the chemical compounds found in lime peel and fruit, as well as to determine the effective dose of ethanol extract from lime fruit in increasing HDL levels.

METHODS AND SUBJECT

This *in vivo* true experimental laboratory study used a randomized post-test-only control group design involving both control and treatment groups. Ethical approval was obtained from the Research Ethics Committee of the Faculty of Medicine, Universitas Jenderal Achmad Yani, (approval number 014/UH2.09/2024).

Research Subject

The research subjects were male Wistar rats (*Rattus norvegicus*) aged 2-3 months, weighing 150-250 grams, and obtained from the Cytohistotechnology Laboratory of the Faculty of Health Sciences and Technology. Inclusion criteria were healthy and active rats with no anatomical abnormalities. Rats experiencing weight loss greater than 10% after acclimatization or death during the research were excluded.

The rats were divided into six groups: a negative control group (KN), a positive control group (KP) induced by high-fat diet (HFD), and four treatment groups. The treatment group consisted of rats induced by High Fat Diet (HFD) and treated with simvastatin at 0,9 mg/kgBW (P1), or lime extract at 0,875 g/kgBW (P2), 1,75 g/kgBW (P3), or 3,5 g/kgBW (P4).

Place and Time of Research

Extraction was performed in the Pharmacology and the Biochemistry Laboratories, while HDL level analysis was conducted in the Clinical Pathology Laboratory, Faculty of Medicine, Unjani. Animal treatment and blood sampling procedures took place in the Cytohistotechnology Laboratory, Faculty of Health and Technology, Unjani. The study was conducted from July to November 2024.

Research Material

The materials used in this study included lime fruit (*Citrus aurantifolia*) obtained from the Manoko Experimental Garden; 90% ethanol; sodium carboxymethyl (Na CMC); 10 mg simvastatin; Dragendorf's reagent; Mayer's reagent; dilute ammonium hydroxide (NH4OH); chloroform; powdered material; amyl alcohol; iron (III) chloride reagent (FeCl3); potassium hydroxide (KOH); 1% gelatin solution; 10% vanillin-sulfuric acid reagent ; acetone; quail egg yolk; 100 mg propylthiouracil (PTU), standard pellet feed; and blood samples from the experimental animals.[13]

The equipment used includes both glassware and non-glassware, such as analytical balances, ovens, rotary evaporators, mortars, test tubes, volumetric flasks, beakers, graduated cylinders, stirring rods, spatulas, filter paper, measuring pipettes, micropipettes, stopwatches, test tube racks, dropper pipettes, spectrophotometers, 5 ml syringe pumps, animal cages, and syringes.[13]

Making of Simplicia

The lime fruit was cleaned under running water to remove dirt or foreign substances, then drained. The lime fruit was then thinly sliced (without peeling the skin) and the seeds were removed. The slices were dried in an oven at 50ºC until they became dry simplicia.[13]

Processing of extracts

The dried simplicia was ground into a powder using a blender. A total of 300 grams of powder was added to 3L of 90%

ethanol and macerated at room temperature for four days with occasional stirring. The mixture was then filtered thorugh filter paper, and the filtrate was collected and evaporated using a rotary evaporator at 50ºC to obtain lime extract.[13,17,18]

Phytocemical Screening

Flavonoid Test

One gram of lime fruit ethanol extract was heated in a water bath with water before filtration. One milliliter of 2N hydrochloric acid and magnesium powder were added to five milliliters of filtrate in a test tube. After heating in a water bath, the mixture was filtered. Five milliliters of amyl alcohol were added to the orange filtrate in a test tube. The mixture was shaken well and allowed to separate. A reddish-brown color in the amyl alcohol layer indicated the presence of flavonoids in the extract.[13]

Alkaloid Test

One gram of lime fruit ethanol extract was basified with 5mL of dilute ammonia and homogenized in a mortar. Then, 20mL of chloroform was added with continuous grinding, and the mixture was then filtered. 5 mL of 2 N HCl was added to the filtrate in a test tube. The mixture was shaken vigorously until two layers formed. The formed layers were separated and divided into 3 portions.

The first portions served as a blank control. The second portion was treated with 2 – 3 drops of Mayer's reagent; a white or yellowish precipitate indicated a positive result. The third portion was treated with 2 – 3 drops of Dragendorff's reagent; an orange-brown or red-brown precipitate indicated a positive reults.[13]

Tannin and Polyphenol Test

One gram of lime fruit ethanol extract was heated with water in a water bath, and filtered while hot. The filtrate was divided into two equal portions. To the first portion, 2-3 drops of FeCl3 solution were added; a green-black color indicatedthe presence of natural polyphenols. The second portion was tested by adding 5 drops of 1% gelatin solution; a white precipitate forms indicated of tannins in the lime extract.[13]

Saponin Test

The lime fruit ethanol extract was stored in a test tube placed over a water bath and mixed with hot water for a while, then filtered. After cooling, the filtrate was shaken vigorously for 30 seconds. The formation of a foam at least 1 cm high that persisted for several minutes indicated that the extract contained saponin compounds.[13]

Quinone Test

One gram of lime fruit ethanol extract was heated with water over a water bath, then filtered. The resulting filtrate was added with 2-3 drops of KOH solution; a deep red color indicated the presence of quinone.[13]

Making of Simvastatin Suspension

0.5 grams of sodium carboxymethyl cellulose (Na CMC) was weighed and transferred into a 100 mL volumetric flask. The volume was adjusted to 100 mL with warm distilled water and mixed until homogeneous. This solution was used as a suspending agent for the simvastatin suspension.[19]

The simvastatin dose for a 200-gram rats , using a conversion factor of 0.018 from a 70 kg human dose , was calculated as follows: 10 mg x 0.018 = 0.18 mg / 200 gr = 0.9 mg/kgBW of rats.[20]

Making of High Fat Diet

The high-fat diet used is a 7:3 mixture of quail egg yolk and used cooking oil, administered at a dose of 2 mL per rat each morning. Drinking water was supplemented with a 0.01% propylthiouracil (PTU) solution and provided *ad libitum*.

Experimental Animal Treatment

After a 7-day acclimatization period with a standard feed (20 g per rat per day) and ad libitum access to drinking water to prevent stress or the emergence of exclusion criteria, rats were assigned to their respective treatment groups. Treatments were administered for 14 days, as shown in Table 1.

Cholesterol Measurement

Blood samples were collected on day 22. # mL of blood was collected from each rat via cardiac puncture a 12-hour fast. The blood samples were placed in a tube and separated between blood and serum through a centrifugation process. HDL levels were measured using the precipitation + enzymatic colorimetry (CHOD / PAP) method.

Serum samples and HDL precipitate reagents were added, homogenized, and centrifuged to separate the sediment and supernatant. Cholesterol reagent was added to standard tube, and the supernatant was added into the sample tube. After mixing until homogeneous, the tubes were incubated at 37ºC for 10 minutes. The absorbance of the standard and sample against the reagent blank was the read using a spectrophotometer at 500 nm.[21]

Table 1. Treatment of Experimental Animal

No	Group	Standard feed	High fat diet	Water	Simvastatin	Lime Ethanol Extract	Treatment time
1	KN	+	-	Without PTU	-	-	14 days
2	KP	+	+	With PTU	-	-	14 days
3	P1	+	+	With PTU	0,9mg/kgBW	-	14 days
4	P2	+	+	With PTU	-	0,875g/kgBW	14 days
5	P3	+	+	With PTU	-	1,75g/kgBW	14 days
6	P4	+	+	With PTU	-	3,5g/kgBW	14 days

Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test. If the data were normally distributed and the variances were homogenous ($p > 0.05$), differences among the six groups were analyzed using a one-way ANOVA. If significant differences were found ($p < 0.05$), a Post Hoc test was performed.

RESULTS AND DISCUSSION

Results

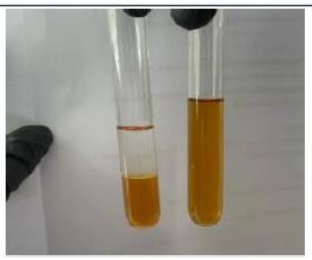
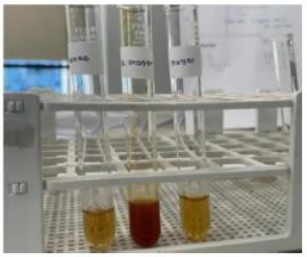
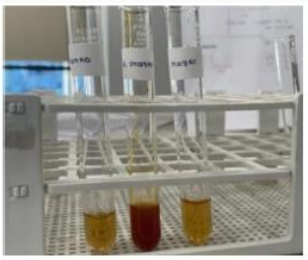
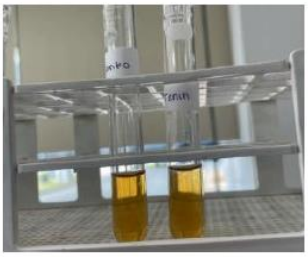
This section presents the results of phytochemical tests and data analysis regarding HDL levels in the treated rats.

The phytochemical test (Table 2) revealed the presence of flavonoids, alkaloids, tannins, polyphenols, saponins, and quinones in the lime fruit (*Citrus aurantiifolia*).

During the treatment period, several rats died, meeting the study's dropout criteria. Specifically, two rats from the simvastatin group, three rats from the 1.75g/kgBW lime extract group, and three rats from the 3.5g/kgBW lime extract group died.

Consequently, HDL levels were measured only in negative control, positive control and 0.875g/kgBW lime extract groups. These death may be caused by the possibility that the dose of the extract given fell within a toxic range.[22]

Table 2. Result of Phytochemical Screening

Phytochemical Test	Result	Images	Description
Flavonoid	+		A ring of reddish-brown color formed
Alkaloid (Mayer)	+		A white precipitate formed
Alkaloid (Dragendorf)	+		A brick-red precipitate formed
Tannin	+		A white precipitate formed

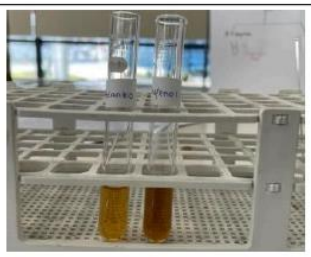
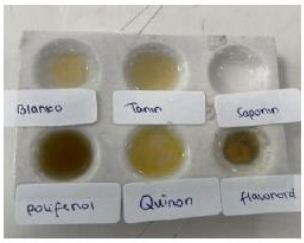
Phytochemical Test	Result	Images	Description
Polyphenol	+		A green-black color formed
Saponin	+		A foam of at least 1 cm was formed and persisted for several minutes
Quinone	+		A deep red color formed

Table 3. HDL Cholesterol Level in Negative, Positive and Lime extract 0.875g/kgBW groups

Groups	HDL cholesterol level (mg/dL)	p-value
	Mean ± SD	
Negative control	27.5 ± 2.52	0.146
Positive control	20.25 ± 3.40	
Lime extract 0.875g/kgBW	25.25 ± 7.14	

The Saphiro Wilk normality test indicated that the data were normally distributed (p-value = 0.809, p > 0.05). A one-way ANOVA was performed to determine the effect of lime fruit ethanol extract on HDL levels in rats induced by a high-fat diet. The ANOVA test yielded a p-value of 0.146 (p >0.05), indicating that the administration of lime fruit ethanol extract had a less significant effect on increasing HDL levels in the high-fat diet-induced rats. Therefore, no post hoc test was conducted.

Discussion

The test results conducted on the ethanol extract of lime fruit (*Citrus aurantifolia*) were positive for flavonoids.

The red color produced indicates the presence of flavonoids, resulting from their reduction by concentrated hydrochloric acid and magnesium.[23] Flavonoids are knows to contribute to lower cholesterol levels by inhibiting HMG-CoA reductase enzyme, thereby suppressing cholesterol synthesis.[16,24] In addition, flavonoids may also exhibit antibacterial activity through the inhibition of bacterial DNA synthesis.[25]

In this alkaloid testing analysis, the ethanol extract of lime (*Citrus aurantifolia*) tested positive for alkaloids. The principle of this analysis method is the precipitation reaction that occurs due to ligand substitution. The nitrogen atom with a free electron pair in alkaloids can replace the iodo ion in these reagents, forming a coordinate covalent bond with the metal ion. Mayer's reagent contains potassium iodide and mercuric chloride [potassium tetraiodomercurate (II)], while Dragendorff's reagent contains bismuth nitrate and potassium iodide in glacial acetic acid solution [potassium tetraiodobismuthate (III)].[23] According to research conducted by Al Namani et al. in 2018, alkaloids have been shown to possess antioxidant activity, and according to research conducted by Hamidah and Adrianto in 2017, alkaloids have the ability to be toxic to mosquito larvae.[25]

Tannins are highly complex organic compound, consisting of phenolic compounds that are difficult to separate and crystallize. They are known for their ability to precipitate proteins from solution, forming complexes with them. In this study, the formation of a white precipitate indicated the presence of tannins in the lime fruit ethanol extract.[23,26] Tannins possess diverse biological activities, including antioxidants, anti-cholesterol, anti-diarrheal, and antibacterial effects. Their antibacterial action is partly attributed to the inhibition of protein synthesis for bacterial peptidoglycan.[25]

Polyphenols were positively found in the tests performed on the lime fruit ethanol extract sample. When FeCl3 was added, it reacted with one of the existing hydroxyl groups, resulting in a dark greenish color.[23] Polyphenols hold great promise as a profitable raw material for the manufacturing of medicines, cosmetics, and functional foods. They have been shown to have a number of health advantages, including anti-aging, anti-mutagenic, anti-carcinogenic, and anti-allergenic properties.[25,27]

The results of the saponin test in this study were positive, as evidenced by the formation of stable foam with a height of 1-10 cm that persisted for several minutes.[23] Foam is obtained because of the ability of saponins as natural foaming agents, which is inseparable from the hydrophilic and hydrophobic groups they possess.[28] Saponin has benefits as an antioxidant, anti-cholesterol and antibacterial by degrading the lipid cell membrane of bacteria.[25]

The test results showed the formation of a deep red color in the extract with added KOH, compared to the extract without added KOH. The formation of this color indicates that the lime extract contains quinone. Quinone is one of the derivatives of phenol that exhibits biological activities such as antifungal, antimalarial, antibacterial, anticancer, and antioxidant.[29]

A high-fat diat (HFD) lowers HDL levels by increasing lipid intake and absorption, resulting in an elevated levels of lipids, including cholesterol and triglycerides, in both low-density lipoproteins and peripheral cells. Consequently, reverse cholesterol transport activity increases initially but then becomes unbalanced and begins to decrease, ultimately leading to reduced HDL levels. The observed decrease in HDL levels following HFD administration underscores the significant role of high-fat foods consumption in the reduction of HDL levels, as can be seen from the mean value of the HDL cholesterol level in positive group (Table 3).

While Cyndi, Andriane, and Nur (2016) reported an effective dose of 3.5 g/KgBW for increasing HDL levels, this study found a lower effective dose of 0.875 g/kg/BW. This discrepancy is likely due to the different parts of the lime used and the resulting variations of flavonoids content. The research conducted by Cindy, Andriane, and Nur in 2016 used lime leaves, whereas in our research, we used lime fruit and peel.[12] According to the study conducted by Chinelo, Okeke, and Bibian in 2013, the flavonoid content in lime leaves was 0.06 ± 0.07%, which was lower compared to the lime peel at 0.51 ± 0.02%.[30]

The administration of 0.875 g/kgBW lime fruit ethanol extract increased HDL levels in high-fat diet-induced rats, although the increase was not statistically significant compared to the positive control. The increase may be attributed to the chemical compounds present in lime, particularly flavonoids, tannins, and saponins.

Flavonoids inhibit hepatic HMG-CoA reductase and increase the hepatic acyl-CoA: cholesterol transferase (ACAT) activity, thus limiting cholesterol synthesis in the liver and cholesterol absorption from the diet. Additionally, flavonoids also increase the production of Apo-A1, the main component of HDL, leading to increased HDL levels.[25,31,32]

Tannins act as inhibitors of the enzyme HMG-CoA reductase, which plays a role in cholesterol synthesis, and the enzyme ACAT, which is responsible for cholesterol esterification. By inhibiting the enzyme HMG-CoA reductase, cholesterol synthesis in the liver is reduced, leading to decreased synthesis of apo B-100 and increased LDL receptors on the liver surface. Consequently, LDL will be drawn into the liver, reducing LDL and VLDL levels. Thus, HDL levels will increase.[25,31]

Saponins also exhibit hypolipidemic effect through several mechanisms. They inhibit HMG-CoA reductase activity and promote excretion of bile acids by enhancing the conversion of cholesterol into bile acids. Saponins can also interfere with cholesterol and bile acid absorption by interrupting micelle formation, thereby preventing cholesterol uptake. Additionally, saponins also enhance the turnover or shedding of intestinal cells (membranolytic action), leading to increased cholesterol loss from the cell membrane into the shed cells.[25,31]

CONCLUSION

This study identified flavonoids, alkaloids, tannins, polyphenols, saponins, and quinones in lime fruit. An increase in the average HDL levels was observed in the group treated with 0.875 g/kgBW of lime fruit ethanol extract, but there was no significant difference in the average HDL levels between the groups.

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DECLARATION OF INTERESTS

The author hereby declares that there is no conflict of interest in the scientific articles that we write.

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