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Antihyperlipidemic activity of kaffir lime leaf extract (*Citrus hystrix* DC) on hypercholesterolemic model mice

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ABSTRACT

Kaffir lime (*Citrus hystrix* DC) is rich in various bioactive substances like flavonoids, tannins, saponins, hesperidin, and several monoterpenes, which are believed to have antihyperlipidemic activity. Hyperlipidemia is a metabolic issue that can lead to high blood pressure and weight gain. This condition is the result of an imbalance in cholesterol levels, including total cholesterol, elevated LDL, and reduced HDL. This study aimed to investigate the antihyperlipidemic activity of kaffir lime leaf extract (*C. hystrix* DC) on hypercholesterolemic model mice. It was pre- and post-control group design study. Tweenty-five male mice (*Mus musculus*) weighing 16-21g at 12 wk of age were used in this study. Five mice as normal control mice group (Group 1) were only administered 1% CMC-Na solution and 20 mice were administered an atherogenic diet to induce hyperlipidemic model. The hyperlipidemic mice were then randomly divided into four groups i.e. positive control mice group (Group 2) were administered simvastatin suspendet in 1% CMC-Na at dose of 0.03 mg/g BW, treatment mice group (Group 3-5) were administered *C. hystrix* DC leaf extract suspendet in 1% CMC-Na at doses of 0.21; 0.28; and 0.35 mg/g BW, respectively. The interventions were administered one daily for 14 d. Blood sampling from the retroorbital plexus of the mice was conducted before and 14 d after the intervention for lipid profile analysis. The serum total cholesterol, LDL, HDL, and triglyceride levels were measured by the CHOD-PAP (cholesterol oxidase-phenyl amino phyrazolone) enzymatic method. A significant decrease in total cholesterol, LDL, triglycerides levels, and a significant increase in the HDL level were observed in the treatment group at doses of 0.28; 0.35 mg/g BW compared to the normal control group ($p<0.05$). The maximal antihyperlipidemic activity was observed at dose of 0.28 mg/g BW. In conclusion, the *C. hystrix* DC leaf extract has antihyperlipidemic model mice.

ABSTRAK

Jeruk purut (*Citrus hystrix* DC) kaya akan berbagai zat bioaktif seperti flavonoid, tanin, saponin, hesperidin, dan beberapa monoterpen, yang diyakini memiliki aktivitas antihiperlipidemia. Hiperlipidemia adalah masalah metabolik yang dapat menyebabkan tekanan darah tinggi dan peningkatan berat badan. Kondisi ini merupakan akibat dari ketidakseimbangan kadar kolesterol, termasuk kolesterol total, peningkatan LDL, dan penurunan HDL. Penelitian ini bertujuan untuk mengkaji aktivitas antihiperlipidemia dari ekstrak daun jeruk purut (*C. hystrix* DC) pada model tikus hiperkolesterolemia. Penelitian ini menggunakan desain kelompok kontrol pra dan pascaintervensi. Sebanyak 25 ekor tikus jantan (*Mus musculus*) dengan berat 16–21 g pada usia 12 minggu digunakan dalam penelitian ini. Lima ekor tikus sebagai kelompok kontrol normal (Kelompok 1) hanya diberikan larutan CMC-Na 1%, dan 20 ekor tikus lainnya diberikan diet aterogenik untuk menginduksi model hiperlipidemia. Tikus hiperlipidemik kemudian dibagi secara acak menjadi empat kelompok, yaitu Kelompok kontrol positif (Kelompok 2) diberikan simvastatin yang disuspensikan dalam CMC-Na 1% dengan dosis 0,03 mg/g BB, Kelompok perlakuan (Kelompok 3–5) diberikan ekstrak daun *C. hystrix* DC yang disuspensikan dalam CMC-Na 1% dengan dosis masing-masing 0,21; 0,28; dan 0,35 mg/g BB. Intervensi diberikan satu kali sehari selama 14 hari. Pengambilan sampel darah dilakukan dari pleksus retroorbital tikus sebelum dan 14 hari setelah intervensi untuk analisis profil lipid. Kadar kolesterol total, LDL, HDL, dan trigliserida dalam serum diukur dengan metode enzimatik CHOD-PAP (*cholesterol oxidase-phenyl amino phyrazolone*). Penurunan signifikan pada kadar kolesterol total, LDL, dan trigliserida, serta peningkatan signifikan pada kadar HDL diamati pada kelompok perlakuan dosis 0,28 dan 0,35 mg/g BB dibandingkan dengan kelompok kontrol normal ($p<0,05$). Aktivitas antihiperlipidemia maksimal diamati pada dosis 0,28 mg/g BB. Kesimpulannya, ekstrak daun *C. hystrix* DC memiliki aktivitas antihiperlipidemia pada tikus model hiperlipidemia.

Keywords:
hyperlipidemia;
Citrus hystrix DC;
leaf extract;
total cholesterol;
triglyceride

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INTRODUCTION

High blood cholesterol levels in the blood, often referred to as hyperlipidemia, pose a risk for heart-related issues, brain blood vessel problems, and diseases affecting the blood vessels in the limbs.[1] Hyperlipidemia is a condition related to body metabolism that can lead to the emergence of additional metabolic issues like high blood pressure and excessive weight. Hyperlipidemia occurs when there is an imbalance in cholesterol levels in the blood, including overall cholesterol levels, elevated low density lipoprotein (LDL), and diminished high density lipoprotein (HDL).[2]

The World Health Organization reported that approximately 36% of the population experiences dyslipidemia. The prevalence is notably higher in women (38.2%) compared to men (33.1%).[6] Commonly, total cholesterol levels increase on both genders after the age of 20 yr. However, men between the ages of 30 and 40 yr, generally have higher total cholesterol levels. In contrast, women over the age of 50 yr tend to exhibit increased total cholesterol levels.[3]

The lipid profile is closely linked to the proportion of visceral fat present in the body. Visceral fat is a key element connecting various features of metabolic syndrome, including glucose intolerance, hypertension, dyslipidemia, and insulin resistance. Studies reported that the amount of visceral fat positively correlates with levels of total cholesterol, triglycerides, and LDL cholesterol. Conversely, HDL cholesterol levels show a negative correlation with the amount of visceral fat in the body.[4]

The primary objective in treating dyslipidemia is to lower both LDL and triglyceride levels. Implementing lifestyle changes can significantly enhance lipid profiles. Actions to consider include decreasing intake of saturated fats, increasing fiber consumption, cutting back on carbohydrates and alcohol, boosting daily physical activity, reducing excess weight, and quitting smoking.[5] Additionally, the first-line drugs for managing dyslipidemia are statins, designed to lower cholesterol to meet LDL targets.[5] However, statin use can lead to various unexpected side effects, such as a rise in liver enzymes in 0.5-2% of those using high doses of these medications. Statins are also linked with the potential onset of new diabetes and an increased risk of memory issues in affected individuals.[6] Nonetheless, this particular study only focused on the reduction of cholesterol levels and changes in the body weight of test mice, meaning it could not definitively confirm whether there would be liver enzyme increases, new instances of diabetes, or heightened memory disorder risks.

Citrus hystrix DC, commonly known as kaffir lime, is a citrus species recognized as a traditional healing plant in many emerging nations, particularly in Indonesia.[7] This plant is abundant in antioxidants, making it popular for alleviating oxidative stress and chronic inflammation linked to long-lasting health issues such as obesity, diabetes, and heart disease.[8] *Citrus hystrix* DC contains numerous bioactive elements, which include flavonoids, tannins, saponins, glycosides, coumarin, bergamottin, phenolic acids, limonoids, glycerolipids, tocopherols, hesperidin, cystenols, and a variety of monoterpenes. Studies reported that hesperidin may enhance the count of LDL cholesterol receptors while also diminishing HMG CoA reductase activity, which is involved in cholesterol production within the liver.[9] Similarly, cystosterol is recognized for its ability to lower LDL cholesterol levels and limit cholesterol uptake in the gut.[8] Despite the widespread availability of *C. hystrix* DC in Indonesia, its use has been mainly restricted to cooking enhancement and aromatherapy. The intent of this study

is to find additional value in the leaf of *C. hystrix* DC.[10] Furthermore, statins are commonly used as the primary treatment for dyslipidemia, but they can be costly and may cause adverse effects.[11] This study aimed to evaluate the antihyperlipidemic activity of the ethanol extract from kaffir lime leaf (*C. hystrix* DC) on hypercholesterolemic model mice.

MATERIAL AND METHODS

Extract preparation

Leaf of *C. hystrix* DC were collected from local farmers in Batu, Malang, East Java and authenticated by a botanist at the Herbal Laboratory of Materia Medica Batu, Malang. The collected leaves were washed and dried in oven at a temperature of 55-60 ^{o}C. Once dried, the leaves were ground into a fine powder using a blender and sieve. The powder was then macerated 3 times using 70% ethanol for 72 h. In each maceration, the mixture was stirred using an orbital shaker to facilitate the extraction process. After 72 h of maceration, the mixture was filtered using Whatman filter paper. The macerate was separated and collected. The collected macerates were then evaporated using water bath at a temperature of 50 ^{o}C to obtain a dried extract. The dried extract was then kept in refrigerator untul used.

Preliminary phytochemical screening

Preliminary phytochemical analysis of the *C. hystrix* DC leaf extract was carried out to identify different phytoconstituents using standar methods such as flavonoid, tannin, steroid, and triterpenoid. The flavonoid was identified using Shinoda's test. The extract was dissolved with 95% ethanol, and a small piece of magnesium foil metal was added followed by 3-5 drops of the concentrated HCl. A positive result is indicated by the formation of the intense orange color. The tannin was identified using ferric chloride test. The extract was dissolved with 95% ethanol, and 1% $FeCl_3$ solution was added. A positive result is indicated by the formation of a black-green color. The steroid and terpenoid were identified using Liebermann-Burchad's test. The extract was dissolved with acetic anhydride. The concentrated H_2SO_4 was then added. A positive result is indicated by the formation of a greenish blue for steroid, and a blackish brown for terpenoid.

Design and experimental procedure

It was pre- and post-control group design study. Twenty-five male mice (*Mus musculus*) weighing 16-21g at 12 wk of age were used in this study. The mouse were adapted in individual cages for one wk prior to the experiment. During this time, the mice had unlimited access to standard food and water. Five mice as normal control mice group (Group 1) were only administered 1% CMC-Na solution and 20 mice were induced by the oral administration of atherogenic diet consisting a mixture of cooked egg yolk 5g, goat fat 100g, and corn rice 1000g daily for 14 d as hyperlipidemic model. The hyperlipidemic mice were then randomly divided into four groups i.e. positive control mice group (Group 2) were administered oral simvastatin suspendet in 1% CMC-Na at dose of 0.03 mg/g BW, treatment mice group (Group 3-5) were administered oral *C. hystrix* DC leaf extract suspendet in 1% CMC-Na at dose of 0.21; 0.28; and 0.35 mg/g BW, respectively. The 1% CMC-Na, simvastation, and *C. hysteric* DC leaf extract were administered one daily for 14 d. Blood sampling (1 mL) from the retroorbital plexus of the mice was conducted before and 14 d after the intervention.

Biochemical analysis

The blood samples were then allowed to clot at room temperature for 30 min and centrifuged at 3000 rpm for 20 min. The serum was separated and stored at -20°C until biochemical analysis. The serum total cholesterol, LDL, HDL, and triglyceride levels were measured by the CHOD-PAP (cholesterol oxidase-phenyl amino phyrazolone) enzymatic method using a spektrofometer at a wavelength of 546 nm.

Data analysis

Data normality was checked using the Shapiro-Wilks Test, and homogeneity was examined with the Levene Test. For data that showed a normal distribution and homogeneity, analysis proceeded with One Way Anova followed by the Duncan test to evaluate the differences in HDL level increases and reductions in total cholesterol, LDL, and triglycerides across the various treatment groups.

RESULTS

Extraction and phytochemical screening

From 500g of *C. hystrix* leaf powder macerated with 70% ethanol yielded a dried extract weight of 88.604g (17.72%). Phytochemical screening of the extract showed the existence of phytoconstituents such as flavonoids, tannin, and steroids (TABLE 1).

Antihiperlipidemic activity of the *C. hystrix* DC leaf extract

The increase of the total cholesterol, LDL, triglycerides levels, and the decrease of in the HDL level were higher in hyperlipidemia-induced group compared to the normal group. Furthermore, a significantly decrease in the total cholesterol, LDL, triglycerides levels, and a significantly increase in the HDL level were observed after intervention compared to before intervention in all groups ($p<0.05$). The defference of lipid profil (Δ) before and after intervention in all groups are presented in TABLE 2 and FIGURE 1.

A significant decrease in total cholesterol, LDL, triglycerides levels, and a significant increase in the HDL level were observed in the treatment group at dose 0.28; 0.35 mg/g BW, and the positive control groups compared to the normal control group ($p<0.05$). The maximal antihyperlipidemic activity was observed after administration of the *C. hystrix* DC leaf extract at dose of 0.28 mg/g BW. There was no significant difference in lipid profile after administration of the *C. hystrix* DC leaf extract at dose of 0.21 mg/g BW compared to the after administration of the simvastatin at dose 0.03 mg/g BW as positive control ($p>0.05$). It was indicated that both groups have similar antihyperlipidemic activity.

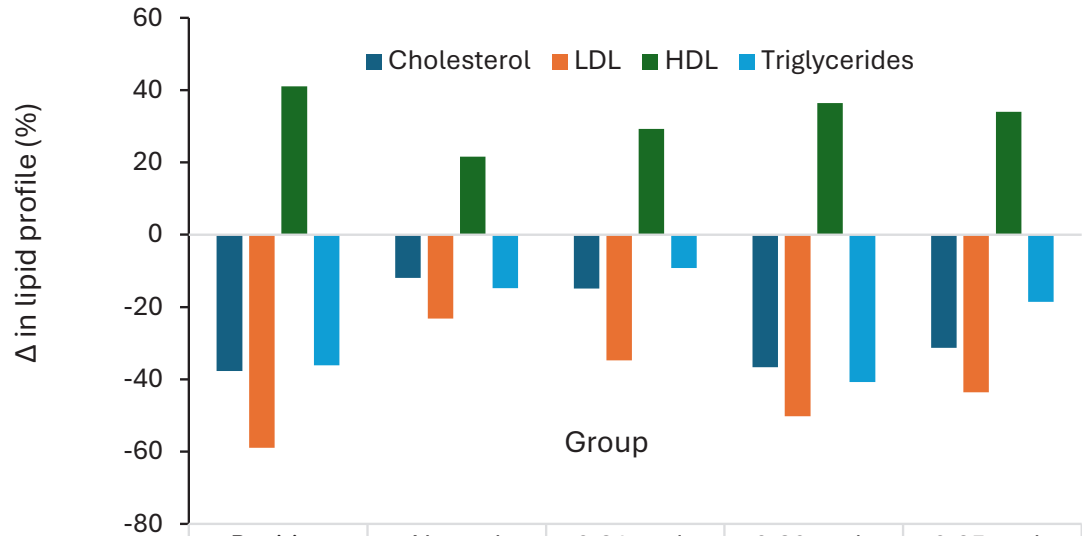
TABLE 1. Result of the phytochemicall screening of *C. hystrix* DC extract

Phytoconstituents	Test	Result	Note
Flavonoid	Shinoda's test	Orange	+
Tannin	Ferric chloride test	Greenish black	+
Steroid	Liebermann-Burchad's test	Greenish blue	+
Triterpenoid	Liebermann-Burchad's test	Blackish brown	+

Note: (+) results indicate the presence of tested compounds

TABLE 2. The defference of lipid profil (± SD mg/dL) before and after administration of the *C. hystrix* DC leaf extract one daily for 14 d compared to positive control and normal control

Variabel	Positive control	Normal control	0.21 mg/g BW	0.28 mg/g BW	0.35 mg/g BW
Total cholesterol	-56.00±22.91	-14.00±14.88	-21.80±10.82	-62.80±25.82	-53.20±28.06
LDL	-58.40±33.70	-17.80±13.81	-35.40±26.54	-65.40±30.09	-50.40±18.23
HDL	20.20±3.83	8.20±5.16	12.20±4.20	17.40±2.07	14.20±3.76
Triglycerides	-27.20±7.85	-13.20±8.76	-17.40±8.79	-29.00±3.46	-25.60±15.60



	Positive control	Normal control	0.21 mg/g BW	0.28 mg/g BW	0.35 mg/g BW
Cholesterol	-37,68	-11,92	-14,93	-36,63	-31,33
LDL	-58,98	-23,17	-34,77	-50,23	-43,59
HDL	41,05	21,57	29,32	36,4	33,97
Triglycerides	-36,17	-14,74	-9,16	-40,77	-18,59

FIGURE 1. The defference of lipid profil (Δ in %) before and after administration of the *C. hystrix* DC leaf extract one daily for 14 d compared to positive control and normal control

A significant decrease in the body weight of mice in day 14 compared to day 1 in the threatment group at dose of 0.21; 0.28; and 0.35 mg/g BW as well as the positive control group was observed ($p<0.05$). Whereas in the normal group, there was no significant difference in the body weight in day 14 compared to day 1 (TABLE 3).

TABLE 3. The body weight of mice (g) of all groups on day to day.

Day	Positive control	Normal control	0.21 mg/g BW	0.28 mg/g BW	0.35 mg/g BW
1	31.4	33.4	27.4	32.6	28.0
7	29.0	32.4	28.4	32.0	26.2
14	26.0	31.4	24.0	22.8	25.4

DISCUSSION

This study aimed to evaluate the activity of *C. hystrix* DC leaf extract in lowering total cholesterol, LDL, and triglyceride levels, while also boosting HDL levels in mice (*Mus musculus*) given a high-fat diet. The results showed that the administration of *C. hystrix* DC leaf extract can reduce the total cholesterol, LDL, and triglycerides levels, and increase the HDL levels (TABLE 2 and FIGURE 1). The maximum antihiperlipidemic effect of the extract was observed at a dosage of 0.28 mg/g BW, which exhibited similar activity to that of simvastatin, as a positive control.

This finding aligns with previous studies that reported an ethanol extract of *C. hystrix* leaf at a dose of 300mg/BW effectively lowered total cholesterol levels in mice when compared to a control group.[12] Another study also reported that the extract of *C. hystrix* DC leaf successfully decrease total cholesterol, LDL, and triglycerides, as well as increase HDL after a 14-day of treatment.[9]

Citrus hystrix DC extract is rich in active compounds which could improve lipid profiles through various pathways. Flavonoid and hesperidin are reported can decrease the activity of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase, which plays a crucial role in cholesterol production in the liver. It also lessens the demand for NADPH in producing fatty acids and cholesterol while enhancing the functions of LDL receptors.[8,9] Flavonoids potentially boost lecithin-cholesterol acyltransferase, or LCAT, an enzyme essential for transforming free cholesterol into cholesterol ester, allowing it to attach to lipoprotein particles and create new HDL.

Tannin has antioxidant activity and can prevent the increase of total cholesterol levels in the bloodstream. It binds to body proteins and cover intestinal walls, which hinders fat absorption. Tannin also inhibits HMG-CoA reductase activity. Reducing this enzyme's action lessens cholesterol production in the liver, which also lowers the creation of Apo B-100, a type of apolipoprotein found in VLDL, while simultaneously increasing LDL receptors on liver surfaces. The result is that blood LDL cholesterol is pulled into the liver, lowering LDL and VLDL cholesterol levels.[13] Steroids can reduce the absorption of external cholesterol and the reuptake of internal cholesterol in the digestive tract, promoting the elimination of excess cholesterol intake. This leads to decreased serum cholesterol levels and a competitive interaction between cholesterol and phytosterols during absorption in the intestines, which decreases the amount of cholesterol absorbed by the body.[14]

Triterpenoids can obstruct the pancreatic lipase enzyme that is important for breaking down triglycerides from foods in the small intestine, as pancreatic lipase is responsible for emulsifying lipids before they are absorbed. Blocking pancreatic lipase will restrict fat absorption and ultimately lower blood cholesterol and

triglyceride levels.[20]

This findings align well with existing theories and prior studies highlighting the role of antioxidants in *C. hystrix* DC leaf extract in varying cases of NAFLD. The flavonoids found in *C. hystrix* DC leaf extract are recognized for their antioxidant, anti-inflammatory, antihypercholesterolemic, and antihyperglycemic properties. Hesperidin functions by diminishing the activity of 3-hydroxy-3-methyl-glutaryl coenzyme A reductase responsible for cholesterol synthesis in the liver. Previous studies reported that taking 1 g of hesperidin daily over 12 wk can significantly enhance blood sugar levels and lower total cholesterol and triglyceride levels. Additionally, it considerably reduces hs-CRP, TNF-**α**, and NF-**κ**B levels, which are mediators of inflammation. It has been concluded that hesperidin supplementation may positively impact NAFLD due to its benefits for lipid profiles, blood glucose control, and inflammation reduction.[15] The tannin compounds in the extract can effectively combat free radicals and have anti-inflammatory and liver-protective effects.[16]

In this study, the administration of *C. hystrix* DC leaf extract did not any noticeable changes in body weight of mice suffering from dyslipidemia when compared to the normal control group. This result was likely influenced by their energy consumption and physical activity levels. All animals were provided with standard food for a period of 14 d while being housed in identical cages and conditions. This findings indicate that administration of *C. hystrix* DC leaf extract to animals with hyperlipidemia led to reduced cholesterol levels and steatosis cells relative to the controls, with no significant differences in weight loss.[17]

At the highest dose of the *C. hystrix* DC leaf extract (0.35 mg/g BW), the average decrease in cholesterol and triglyceride levels was less than that at the dose of 0.28mg/gBW. This phenomenon is well known as dose optimization, where pharmacological reactions reach their peak effects at specific dosages. Generally, increasing the medication dose should elevate the response in tandem with the dose increase. However, as the dosage rises, a point is reached where further increases no longer enhance the effect as anticipated.[18] This saturation point indicates that the therapeutic response plateaus. Additionally, the pharmacological effects of natural ingredients can be complex due to the diverse array of chemical compounds present within them. Yet, as doses increase, so does the complexity of these compounds, which can lead to non-linear effects that may lessen the desired outcome or cause toxic reactions in test subjects due to an excess of compounds within the extract.[19]

CONCLUSION

In conclusion, *C. hystrix* DC leaf extract has antihyperlipidemic activity on hypercholesterolemic model mice. At the optimal dose of 0.28 mg/g BW, the extract is effective in reducing total cholesterol levels by 36.63%, LDL 50.23%, triglycerides 40.77%, respectively, and increasing HDL levels by 36.40%.

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