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Inhibition of HMG-CoA Reductase Activity by Kersen Leaves (*Muntingia calabura* L.) to Prevent Hypercholesterolemia

(*Inhibisi HMG-CoA Reduktase Menggunakan Ekstrak Daun Kersen (Muntingia calabura L) Untuk Mencegah Hiperkolesterolemia*)

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ABSTRACT

Background: Hypercholesterolemia is a condition of total cholesterol level >200 mg/dL and LDL >130 mg/dL. HMG-CoA (3-hydroxy-3-ethylglutaryl-coenzyme A) reductase is an enzyme that has a role in cholesterol biosynthesis. Hence, inhibition of this enzyme led to the decrement of cholesterol level. The extract of Kersen leaves (*Muntingia calabura* L.) is known to contain flavonoids, terpenoids, steroids, tannins, phenolic, and alkaloids. Flavonoids work by inhibiting the HMG-CoA reductase activity, so that mevalonate cannot be formed and thus decrease the cholesterol synthesis. **Objectives:** The present study aimed to determine the effect of Kersen leaves extract (*M. calabura* L.) in inhibiting the HMG-CoA reductase activity *in vitro*. **Material and Methods:** The study is a true experimental study with a post-test-only control group design. The independent variables were ethanol, methanol, and *n*-hexane extracts of Kersen leaves. Moreover, the percentage inhibition of the enzyme was the dependent variable. The test was conducted *in vitro* using UV-Vis spectrophotometry with pravastatin as a positive control. **Results:** The inhibitory effects of ethanol, methanol, *n*-hexane extracts of Kersen leaves, and pravastatin towards HMG-CoA reductase activity were 85.56%, 59.75%, 92.03%, and 99.58%, respectively. Post Hoc One-Way ANOVA showed that the p-values of pravastatin with ethanol, methanol, and *n*-hexane extracts were 0.687, 0.048, and 0.931, respectively. The *n*-hexane and ethanol extracts were potent for inhibiting the enzyme activity ($p>0.05$) comparable to pravastatin. **Conclusion:** The *n*-hexane and ethanol extracts of Kersen leaves could serve as a natural source of HMG-CoA reductase inhibitor to prevent hypercholesterolemia.

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ABSTRAK

Latar Belakang: Hiperkolesterolemia ialah keadaan kadar kolesterol total >200 mg/dL, dan LDL >130 mg/dL. HMG-CoA (3-hydroxy-3-ethylglutaryl-coenzyme A) reduktase merupakan enzim yang berperan dalam biosintesis kolesterol, sehingga salah satu mekanisme yang menghambat pembentukan kolesterol yaitu dengan cara menghambat enzim HMG-CoA reductase. Ekstrak daun Kersen (*Muntingia calabura* L) diketahui mengandung flavonoid, terpenoid, steroid, tanin, fenolik, dan alkaloid. Flavonoid bekerja dengan cara menghambat enzim HMG-CoA reduktase sehingga tidak terbentuk mevalonat, akibatnya sintesis kolesterol menurun. Tujuan: Penelitian ini bertujuan mengidentifikasi pengaruh ekstrak daun Kersen terhadap aktivitas enzim HMG-CoA reduktase secara *in vitro*. Bahan dan Metode: Penelitian ini merupakan studi *true-experimental* dengan pendekatan post test *only control group design*. Variabel bebas berupa ekstrak etanol, metanol, dan *n*-heksan daun Kersen serta nilai persentase inhibisi enzim HMG-CoA reduktase sebagai variabel terikat. Pengujian dilakukan secara *in vitro* menggunakan metode spektrofotometri UV-Vis dengan pravastatin sebagai kontrol positif. Hasil: Efek penghambatan ekstrak etanol, metanol, *n*-heksan, dan pravastatin terhadap aktivitas HMG-CoA reduktase secara berturut-turut adalah 85.56%, 59.75%, 92.03%, and 99.58%. Analisis *Post Hoc One-Way* ANOVA menunjukkan *p-value* antara pravastatin dengan ekstrak etanol, metanol, dan *n*-heksan secara berturut-turut, yaitu 0.687, 0.048, and 0.931. Ekstrak *n*-heksan dan etanol berpotensi untuk menghambat aktivitas enzim HMG-CoA reduktase ($p>0.05$) sebanding dengan pravastatin. Kesimpulan: Ekstrak *n*-heksan dan etanol daun Kersen dapat menjadi sumber inhibitor HMG-CoA reduktase untuk mencegah hiperkolesterolemia.

Kata kunci: Hiperkolesterolemia, HMG-CoA reduktase, *Muntingia calabura* L.

INTRODUCTION

Hypercholesterolemia is a condition of total cholesterol levels more than 200 mg/dL and LDL more than 130 (Kemenkes, 2014; Kemenkes, 2018; WHO, 2019). Hypercholesterolemia can increase the risk of developments of atherosclerosis, coronary heart disease, pancreatitis (inflammation of the pancreas organ), diabetes mellitus, thyroid disorders, liver disease, and kidney disease. Some factors affect hypercholesterolemia, include heredity, consumption of high-fat foods, lack of exercise, and smoking habits (Yani, 2015). Hypercholesterolemia is a lipid metabolism disorder characterized by an increase of total blood cholesterol levels. Currently, the prevalence of hypercholesterolemia is still high in the world, which is around 45%, in Southeast Asia around 30%, and in Indonesia in 2013 around 35%, while in 2018 it reached 76% (Kemenkes, 2014; Kemenkes, 2018; WHO, 2019).

The enzyme HMG-CoA (3-hydroxy-3-ethylglutaryl-coenzyme A) reductase is a precursor of cholesterol synthesis, so that one of the mechanisms that inhibits cholesterol formation is by inhibiting the enzyme HMG-CoA reductase (Yunarto and Aini, 2015). The biosynthesis of cholesterol begins with the synthesis of mevalonate from Acetyl-CoA facilitated by HMG-CoA reductase. The isoprenoid unit is formed from mevalonate through the release of CO_2. Six isoprenoid units condense to form squalene intermediates. Squalene undergoes cyclization to produce parent steroid compounds, namely lanosterol. Cholesterol is formed from lanosterol after passing through three methyl groups (Murray, 2009).

The statin group is the first line drugs used for cholesterol therapy, by inhibiting the action of the HMG-CoA reductase enzyme. The first step of cholesterol synthesis inhibition in mevalonate pathway by increasing the affinity of the LDL receptor (Low Density Lipoprotein) and the speed of LDL catabolism

and extraction of liver LDL precursors so that plasma LDL levels decreased (Artha et al., 2017). The results of previous research conducted by Wagmann et al. (2019) found that pravastatin belongs to the class of competitive and easily absorbed HMG-CoA reductase inhibitors which more effective than atorvastatin and simvastatin. The drug selectively acts on the rate-limiting step in cholesterol biosynthesis by inhibiting the HMG-CoA reductase enzyme resulting in up-regulation of hepatic LDL receptors which increases metabolism and lowers LDL. Hence, the action lower circulating total plasma cholesterol, resulted in the decrements of VLDL (Very Low Density Lipoprotein), triglycerides, and apoliprotein B levels as well as an increment of HDL cholesterol level (Wagmann et al., 2019).

Several studies have shown that concomitant administration of pravastatin and cholestyramine (bile-binding resin) can reduce LDL levels thereby slowing the formation of atherosclerosis and reducing the risk of death (Carmena et al., 2019). However, several studies have found that giving statins can cause adverse side effects in the long term because cognitive impairment can occur after statin administration. Two RCT studies (Randomized Controlled Trial) and one challenge-dechallenge study showed a possible correlation between statins and cognitive impairment (Fernandez et al., 2014).

The results of research conducted by Maki et al. (2014) through various cohort studies and meta-analysis studies showed that long-term use of statins can increase the incidence of type 2 diabetes mellitus (DMT2). The epidemiological study conducted by Zhong et al. (2015) showed a relationship between the use of statin drugs and the incidence of cancer. The use of statins can cause side effects such as gastrointestinal disorders, muscle pain, stomach irritation, liver damage, gallstones, and kidney damage, especially in long-term use (Artha, et al., 2017).

Previous study has reported the content of secondary metabolites of Kersen leaves extract, such as flavonoids, terpenoids, steroids, tannins, phenolics, and alkaloids (Arum et al., 2012). Studies also shown the effect of secondary metabolites in lowering the cholesterol level. Flavonoids has been reported for this activity by inhibiting the HMG-CoA reductase enzyme, causing a disturbance in the mevalonate formation (Retnaninggalih et al., 2015). In addition, saponins can lower the cholesterol levels and reduce fat accumulation in blood vessels. In the present study, three organic extracts of Kersen leaves (ethanol, methanol, and *n*-hexane) were evaluated for the inhibitory activity towards HMG-CoA reductase.

MATERIAL AND METHODS

Materials

Absolute ethanol, methanol, n-hexane, deionized water, dimethyl sulphoxide (DMSO), hydrochloric acid (HCl), sulphuric acid (H_2SO_4), sodium hydroxide (NaOH), ferric chloride ($FeCl_3$), Dragendorff

reagent, and Meyer reagent were purchased from Merck (Darmdstadt, Germany). HMG-CoA reductase assay kit was purchased from Sigma Aldrich (Missouri, USA).

Methods

Sample collection and preparation

Kersen leaves was collected from the Kersen trees that grow around the campus (Faculty of Medicine, Universitas Haluoleo) environment. Sample was washed using tap water, drained, and dried using an oven at a temperature of 40°C. The dried sample was powdered using a blender to yield a coarse powder. The powder was stored in zip lock plastic bag at room temperature until use.

Extraction

The extraction process of Kersen leaves was carried out using maceration technique. The powder of Kersen leaves was divided into three portions each 100 g, put into their respective maceration vessels, and added with ethanol, methanol, and *n*-hexane each 1 L, respectively. The vessels were tightly closed and macerated for three days in dark room while periodically stirring. Each macerate was filtered manually to yield a liquid extract. The crude extract was obtained by evaporating the solvent in the liquid extract under reduced pressure using a rotary evaporator. To ensure all solvents evaporated, all extracs were put into an oven at 40°C until thick crude extracts obtained. All crude extracts were then kept in a refrigerator at 4°C until use.

Phytochemical screening

Flavonoids

Extracts (20 mg; 7 mL in their respective solvents) were filled in 3 test tubes. The first tube was added with 5 mL of concentrated H_2SO_4; a red color change indicated the presence of flavonoids. The second tube added with 5 mL of concentrated HCl and magnesium powder; a red color change indicated the presence of flavonoids. Then, the last tube was added with 5 mL of NaOH; a yellow color change indicated the presence flavonoids in the extracts.

Alkaloids

Extracts (20 mg; 5 mL in their respective solvents) were added with Meyer's reagent in test tubes. Changes in color and formation of white precipitate indicated the presence of alkaloids in the extracts.

Saponins

Extracts (20 mg; 5 mL in their respective solvents) were added with water in the test tubes, shaken vigorously, and kept for 30 seconds. The formation of foam with 1 cm in height for 30 seconds indicated the presence of saponins in the extracts.

Tannins

Extracts (20 mg; 5 mL in their respective solvents) were added with $FeCl_3$ in the test tubes. A positive result of tannins indicated by green, blue-green, or blue-black colors in the test solutions.

Measurement of HMG-CoA Reductase Enzyme Activity

The solutions of ethanol, methanol, and *n*-hexane extracts of Kersen leaves were made in DMSO en mg of ethanol, methanol, and *n*-hexane extracts of Kersen leaves were dissolved in 5 μL of 100% DMSO and vortexed until dissolved. Then, the solution added with 995 μL of deionized water and vortexed. The measurement of HMG-CoA reductase activity was in accordance with the HMG-CoA reductase assay kit protocol. Table 1 showed the reaction mixtures of the assay. The assay buffer was added to all destined wells. Pravastatin as the positive control of the assay was assigned to exhibit an inhibition towards the enzyme activity. To all wells, NADPH and HMG-CoA (the substrate) were added. The enzyme (HMGR) was then added to extracts and pravastatin wells, followed by mixing thoroughly. The absorbance of the reaction mixtures was measured spectrophotometrically at a wavelength of 340 nm and a temperature of 37^{o}C. The absorbance reading was carried out every 15 seconds to 5 minutes. Before the first reading, the reaction mixtures were vigorously for 10 seconds.

Table 1. Reaction mixtures of HMG-CoA reductase activity

Sample	Volume of reaction mixtures				
	1 x Assay Buffer	Sample	NADPH	HMG-CoA	HMGR
Blank	184 μl	-	4 μl	12 μl	-
Extracts of Kersen leaves	181 μl	1 μl	4 μl	12 μl	2 μl
Pravastatin (positive control)	181 μl	1 μl	4 μl	12 μl	2 μl

Data analysis

Data of bioassay were obtained from three repetitions. The percentage inhibition (%I) is the ability of the sample to inhibit the activity of the HMG-CoA reductase enzyme based on the difference between the absorbance value in the last measurement (A0) and the absorbance value in the first measurement (A10) of the NADPH molecule in the reaction mixtures within 10 minutes of measurement. The %I value of HMG-CoA reductase enzyme activity was calculated using the following formula: %I = [(Absorbance of control – Absorbance of sample)/Absorbance of control] x 100% (Baskaran et al., 2015; Yunarto et al., 2019). Comparative analysis of HMG-CoA reductase activity between samples was carried out using SPSS 25 with the hypothesis test being the Post Hoc One Way ANOVA (Multiple Comparison) test.

RESULTS AND DISCUSSION

Abberant production of cholesterol in the body has a role for triggering the development of non-communicable diseases, such as atherosclerosis, cardiovascular and kidney diseases. High level of cholesterol in plasma (hypercholesterolemia) along with fats will form macrophage foam cells which further deposited in the arterial walls; an early initiation of vascular atherosclerotic lesions. The accumulation of atherogenic conditions is associated with cardiovascular and kidney diseases. Overproduction of cholesterol can be manage by inhibit HMG-CoA reductase that mediates the formation of acetyl-CoA into mevalonate; the precursor for making cholesterol. Drugs from statin group have been recognized as a powerful HMG-CoA reductase inhibitor with some unwanted side effects (Lee, dkk. 2019; Ramkumar, dkk., 2016; Okuyama, dkk., 2015; Saremi, dkk., 2012; Hamazaki, dkk., 2013; Thambidorai, 2011). Hence, the search for more safe HMG-CoA reductase inhibitors is still on continuing efforts until today from synthesis approach and natural products, especially from plants

Species *M. calabura* is known worldwide as 'Jamaican cherry' and in Indonesia, among natives, it is known as 'Kersen'. The decoction of Kersen leaves has been traditionally used to reduce cholesterol level (Base et al., 2021). This indicated the contents of phytochemicals benefit for cholesterol reduction. The leaves of Kersen (*M. calabura*) accumulated some phytochemicals as displayed in Table 2. Phytochemicals in the ethanol extract of the leaves were varied when compared with other extracts. Alkaloids and flavonoids were present in all extracts of the leaves. Meanwhile, phenolics, tannins, and terpenoids only present in the ethanol extract. In addition, only *n*-hexane extract contained steroids. The methanol extract accumulated the least phytochemicals as compared to others. Previous studies have reported flavonoids as major phytochemical of *M. calabura*. Flavonoids from *M. calabura* have shown cytotoxicity, quinone reductase, antiplatelet aggregation, antibacterial, and antinociceptive activities (Mahmood et al., 2014). The presence of flavonoids in three extracts of Kersen leaves of the study might have a role for HMG-CoA inhibitory activity.

Table 2. Phytochemicals of Kersen leaves (*M. calabura*).

Phytochemical group	Extracts of Kersen leaves		
	Ethanol	**Methanol**	***n*-Hexane**
Alkaloids	++	+	++
Phenolics	++	-	-
Tannins	++	-	-
Saponins	+	-	+
Steroids	-	-	+
Terpenoids	++	-	-
Flavonoids	++	++	++

The leaves of Kersen (*M. calabura*) inhibited the activity of HMG-CoA reductase (Table 3). The *n*-hexane extract of the leaves was the most active extract on the enzyme inhibitory activity (92.03%), followed by ethanol (85.56%) and methanol (59.75%) extracts. Pravastatin as the positive control was consistent in inhibiting the enzyme activity (99.26%). Post Hoc One Way ANOVA (Table 4) showed that the inhibition effect of HMG-CoA reductase activity by *n*-hexane and ethanol extracts are comparable to pravastatin ($p>0.05$), while the methanol extract is significantly different ($p<0.05$). The standard deviation (SD) values of all extracts (12.2 to 21.9) were greater than pravastatin (0.6), indicating the complexity of compounds in the extracts either to inhibit or assist the enzyme activity. Phytochemicals in *n*-hexane and ethanol extracts of Kersen leaves were varied as compared to the methanol extract. The comparison between *n*-hexane and ethanol extracts for inhibiting HMG-CoA reductase activity was not significantly difference ($p = 0.949$) (Table 4), indicating potent phytochemicals present in both extracts, such as alkaloids, saponins, terpenoids, and flavonoids. On the contrary, the absence of phenolics and tannins in the methanol extract might contribute to a weak HMG-CoA reductase inhibitory activity of the extract.

Table 3. The percentage inhibition (%I) of Kersen leaves (*M. calabura*) towards HMG-CoA reductase.

Sample	% I	Average of %I (mean ± SD, n=3)
Ethanol	105.88	85.56 ± 21.9
	88.37	
	62.44	
Methanol	51.52	59.75 ± 12.2
	53.98	
	73.77	
n-Hexane	91.58	92.03 ± 16.3
	108.59	
	75.93	
Pravastatin (positive control)	99.62	99.26 ± 0.6
	99.58	
	98.60	

Some plants of local wisdom used for treating hypercholesterolemia, such as betel leaf (*Piper sarmentosum*), rice brain, *Ficus carica*, and tea leaf (*Camellia sinensis*), have shown an inhibitory effect towards HMG-CoA reductase activity, employing methanol and ethanol as solvents for extraction (Baskaran et al., 2015; Gholamhoseinian et al., 2010; Hasanah et al., 2016). In comparison with these plants extracts, the extracts of Kersen leaves were considered more potent to inhibit the HMG-CoA reductase according to our present study. Some *in vivo* studies have been conducted using ethanol extract of Kersen leaves and found effective to reduce triglycerides and cholesterol levels in hypercholesterolemia rats at doses of 50 to 400 mg/kg of body weight (Putri et al., 2018; Ranti and Vickasari, 2022). The occurrence of alkaloids, flavonoids, phenolics, and steroids might promote the inhibitory effect on HMG-CoA reductase activity based on some *in silico* studies (Aqeel et al., 2018;

Hariyanti et al., 2018; Shaik et al., 2020; Azmi et al., 2021; dan Mannino et al., 2021). Hence, further research on finding HMG-CoA reductase inhibitors from Kersen leaves is a noble challenge.

Table 4. Comparison among parameters based on Post Hoc test.

Parameters	p-Value
Pravastatin × Ethanol	0.687
Pravastatin × Methanol	0.048*
Pravasatin × *n*-Hexane	0.931
Ethanol × Methanol	0.227
Ethanol × *n*-Hexane	0.949
Methanol × *n*-Hexane	0.110

*Significantly different ($p<0.05$).

Flavonoids have been shown to competitively inhibit the activity of HMG-CoA reductase as the mechanism of statins in lowering cholesterol. Flavonoids in inhibiting HMG-CoA reductase activity have been carried out by identifying the affinity of a number of polyphenols with the HMG-CoA reductase enzyme to see the ability of polyphenols to bind to the active site of the enzyme so that there is no catalytic activity of the HMG-CoA reductase enzyme to the substrate in the form of HMG-CoA. and co-substrate in the form of NADPH (Ekananda, 2015).

In the study of Aqeel et al. (2018) which used the docking *in silico* method of phenolics and the enzyme HMG-CoA reductase together with atorvastatin as a positive control showed that phenolics had the ability to bind to the active site of the HMG-CoA reductase enzyme with high affinity over atorvastatin. This indicates that phenolic compounds have better inhibitory potential on the activity of the HMG-CoA reductase enzyme. Another study has reported tannin-derived compounds to inhibit the enzyme using docking *in silico* method. The study found that gallic acid had the ability to bind to the active site of the HMG-CoA reductase enzyme. The ability of one of these tannin-derived compounds shows that it has the potential to competitively inhibit HMG-CoA reductase activity like the mechanism of action of statin drugs (Shaik et al., 2020).

Based on research by Hariyanti et al. (2018) which used the docking in silico method of a number of secondary metabolites with the HMG-CoA reductase enzyme, it showed that one of the derivatives of steroid compounds in the form of sitosterol has the ability to form hydrogen bonds on the active site of the HMG-CoA reductase. Although the results obtained show a weak interaction, this indicated that steroids have the potential to inhibit the activity of the HMG-CoA reductase enzyme which was related to its ability to form a number of hydrogen bonds at the active site of the enzyme.

In a study conducted by Shaik, et al. (2020 and Mannino, et al. (2021) showed that terpenoids have the ability to bind to the active site of the HMG-CoA reductase enzyme, although in the docking in silico

test conducted by Mannino, et al. (2021) it was found that the interaction between terpenoids and the active site of HMG-CoA reductase is relatively small. Based on the research of Azmi, et al. (2021) and Hariyanti, et al. (2018) which used the docking in silico method against a number of alkaloid compounds with HMG-CoA reductase enzyme showed that alkaloid compounds have the ability to form hydrogen bonds on the active site of the HMG-CoA reductase enzyme.

CONCLUSION

Ethanol, methanol, and *n*-hexane extracts of kersen leaf (*M. calabura*) showed inhibition effects towards HMG-CoA reductase activity and were able to inhibit cholesterol formation by inhibiting the catalytic process of the HMG-CoA reductase enzyme. Further investigation is needed to analyze the anticholesterol ability of *M. calabura* pure isolates and their effect on reducing cholesterol in vivo.

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CONFLICT OF INTEREST

The authors state that we have no competing (conflict) interests related to finances or personal relationships that are likely to influence the results of the research reported in this manuscript.

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