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FULL PAPER

3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitory activity of Indonesian *Cajanus cajan* leaves and *Zingiber officinale* extracts

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Abstract

One indicator of antihypercholesterolemic candidates is their inhibitory activity on 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme. This study aimed to analyze the HMG-CoA reductase inhibitory activities of *Cajanus cajan* leaves, *Zingiber officinale* var Amarum and *Zingiber officinale* var Rubrum. *C. cajan* leaves and two types of *Z. officinale* were extracted using the maceration method with three solvents: distilled water, 70% ethanol and 96% ethanol. All nine types of resulted extracts were then analyzed for bioactive compounds and tested for the HMG-CoA reductase inhibitory activity using the HMG-CoA reductase analysis kit (BioVision's K588-100). It was carried out *in vitro* using a colorimetric microplate reader with a wavelength of 340 nm at 37°C. The results showed that the extracts contained flavonoids, tannins, saponins and steroids. The nine extracts showed inhibitory activities on the HMG-CoA reductase enzyme. The three extracts showed the highest inhibitory activity; 70% ethanolic *Z. officinale* var Amarum extract (47.87%), aqueous *Z. officinale* var Rubrum extract (40.62%) and 70% ethanolic *C. cajan* leaves extract (39.38%). These results indicate that the three extracts have a medium inhibitory level of HMG-CoA reductase. The study suggested that the three types of extracts could be used as antihypercholesterolemic candidates such as antihypercholesterolemic functional food.

1. Introduction

Cardiovascular disease (CVDs) is the leading cause of death globally. Cardiovascular disease, in particular, accounts for about 40% of deaths in most European countries. World Health Organization (WHO) revealed that cardiovascular disease caused 17.9 million deaths and represented 31% of world deaths in 2016 (WHO, 2017; Toori *et al.*, 2018). As many as 85% of these deaths were caused by heart attacks and strokes.

One of the main risk factors for cardiovascular disease is a high level of cholesterol in the bloodstream that exceeds normal limits, known as hypercholesterolemia (Lee *et al.*, 2019) and induces atherosclerosis (Zhao *et al*., 2018). The risk is inversely related to the low-density lipoprotein cholesterol (LDL-C) plasma level. It is directly associated with elevated LDL-C (Wong *et al*., 2020). The high level of LDL-C will easily be oxidized by reactive oxygen species (ROS) and lead to the formation of atherosclerosis. Atherosclerosis caused heart attacks and strokes (Poznyak *et al*., 2021). Cholesterol level is a biomarker for atherosclerosis.

Increased cholesterol levels in the bloodstream can be caused by excess synthesis and absorption of cholesterol and the consumption of high-fat foods (Misra *et al*., 2019). Reducing high cholesterol levels is principally done in two ways; by inhibiting the synthesis or absorption of cholesterol in the body and reducing the consumption of a diet rich in cholesterol and calories (Frota *et al*. 2010). Takata *et al*. (2016) reported that the standard treatment using anti-cholesterol drugs for a long time and continuously caused side effects to include liver damage and muscle disorders. Ji *et al*. (2019) also reported reducing high cholesterol levels by modifying the body's cholesterol metabolism using antihypercholesterolemic bioactive compounds.

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Table 1. Reagent concentration in HMG-CoA reductase inhibitory activity test.

Sample	Buffer	Atorvastatin	NADPH	HMG-CoA	Extract	HMG-CoA reductase
Blanco	174 µL	-	4 µL	12 µL	-	5 µL
Activity	174 µL	-	4 µL	12 µL	5 µL	5 µL
Inhibitor	174 µL	2 µL	4 µL	12 µL	5 µL	5 µL

Note: Atorvastatin was prepared at a 50 g/mL concentration. Extracts were prepared at a concentration of 10 mg/mL. The reaction was started with the addition of 5 µL HMG-CoA reductase (600 g/mL).

Reducing LDL-C up to 20% was also reported associated with aggressive lifestyle changes and a very strict diet (i.e., purely plant-based) (Chiavaroli *et al.,* 2018).

Several enzymes play a role in synthesizing cholesterol, such as 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, HMG-CoA reductase, squalene synthase, squalene monooxygenase and 2,3 epoxidase (Thomas *et al*., 2012). Efforts to inhibit these enzymes, such as the HMG-CoA reductase, will reduce cholesterol synthesis, then will reduce blood cholesterol levels. Cicero *et al*. (2021) reported that monacolin K of red yeast rice extract inhibits HMG-CoA reductase. It could be considered an effective and relatively safe lipid-lowering nutraceutical in healthy subjects with mild reduce hypercholesterolemia.

This study was conducted to explore the Indonesian natural plants in order to produce a functional food for reducing or maintaining blood cholesterol levels and specifically analyze the HMG-CoA reductase inhibitory activity of Indonesian *C. cajan* leaves, *Z. officinale* var Amarum and *Z. officinale* var Rubrum.

2. Materials and methods

2.1 Extraction

Cajanus cajan leaves, *Z. officinale* var Amarum and *Z. officinale* var Rubrum are washed thoroughly, air-dried, then baked and ground to produce simplicia. The extraction was conducted using the maceration method in three types of solvents; distilled water, 70% ethanol and 96% ethanol, in a ratio of 1:10. Extraction was carried out for 3×24 hrs. The macerate obtained was then evaporated using a vacuum rotary evaporator at 45°C to obtain a dry extract. Extraction yield data was obtained by comparing the amount of extract obtained with the amount of simplicia used. These extractions resulted in nine type extracts; aqueous, 70% and 96% ethanolic extracts of *C. cajan* leaves, *Z. officinale* var Amarum and *Z. officinale* var Rubrum. The nine extracts were then analyzed for phytochemical compounds (alkaloids, saponins, flavonoids, tannins, terpenoids and steroids) (Harborne, 1984) and tested for the HMG-CoA reductase inhibitory activity.

2.2 Analysis of HMG-CoA inhibitory activity

The nine types of extracts aqueous, 70% and 96% ethanolic extracts of *Cajanus cajan* leaves, *Z. officinale* var Amarum and *Z. officinale* var Rubrum, were tested for HMG-CoA reductase inhibitory activity. The analysis used the HMG-CoA reductase analysis kit (BioVision's K588-100). The analysis was carried out by following the instructions according to BioVision's procedures. The nine extracts then were run and read using a colorimetric microplate reader with a wavelength of 340 nm at 37°C every 1 min and protected from light. Extracts were prepared at a concentration of 10 mg/mL, atorvastatin as a positive control was prepared at a 50 g/mL concentration and the reaction was started with the addition of 5 µL HMG-CoA reductase (600 g/mL). The composition of the concentration of reagents in the inhibition test of HMG-CoA reductase activity can be seen in Table 1.

2.3 Data analysis

Extraction yield and bioactive compound content are presented descriptively in the tables. The HMG-CoA reductase inhibitory activity of the nine extracts of *C. cajan* leaves, *Z. officinale* var Amarum and *Z. officinale* var Rubrum were analyzed using one-way ANOVA. If there is a difference, then proceed with Duncan's test. Data analysis using SPSS 23 program.

3. Results and discussion

3.1 Bioactive compound

The bioactive compound contained in the nine type extracts of *C. cajan* leaves, *Z. officinale* var Amarum and *Z. officinale* var Rubrum are shown in Tables 2, 3 and 4. The water, 70% and 96% ethanolic *C. cajan* leaf extracts contain flavonoids, saponins and steroid. Flavonoids reported have strong HMG-CoA reductase-inhibitory activity (Son *et al*., 2018). Saponin also reported have HMG-CoA reductase-inhibitory activity (Herviana *et al*., 2022). Tannins were detected positively in water and 70% ethanol solvents. However, they were not detected in 96% ethanol solvents of *C. cajan* leaves extract (Table 2).

Tannins are anti-nutritional compounds that can inhibit the work of HMG-CoA reductase and acyl-CoA cholesterol acyltransferase (ACAT), which are enzymes for synthesizing cholesterol and its release into the blood (Park *et al*., 2002; Herviana *et al*., 2022). Steroids in plants can reduce cholesterol absorption in the body in patients with hyperlipidemia (Ostlund 2002). Phytochemicals such as alkaloids, quinones, and triterpenoids were not detected in the *C. cajan* leaves extract. Triterpenoids are non-polar compounds, so they are not detected in ethanol or water, which are semipolar.

Table 2. Phytochemical content of *Cajanus cajan* leaves extracts

Phytochemical content	*Cajanus cajan* leaves extract		
	Aqueous	70% Ethanol	96% Ethanol
Flavonoids	+	+	+
Alkaloids	-	-	-
Tannins	+	+	-
Saponins	+	+	+
Quinones	-	-	-
Steroids	+	+	+
Triterpenoids	-	-	-
Yield	16.50	19.27	13.28

Zingiber officinale var Amarum extract contains flavonoids that can be dissolved in ethanol solvents but cannot be dissolved in aquadest solvents. It is indicated by the positive results of flavonoids in 70% and 96% ethanol solvents and negative results in distilled water (Table 3). Flavonoids are phytochemical that can be dissolved in polar solvents but not all have good solubility in water. Flavonoids have low solubility and stability in hydrophobic solvents and are high in lipophilic solvents. Flavonoids are polyphenolic pigments in plants with the highest concentrations in the epidermis of leaves and fruit skins (Crozier *et al*., 2006). Saponins are phytochemical that can be dissolved in both polar and non-polar solvents, all the three types of *Z. officinale* var Amarum extracts, that used distilled water and ethanol at concentrations of 70% and 96% as solvents, showed contain saponins.

Table 3. Phytochemical content of *Zingiber officinale* var Amarum extracts

Phytochemical content	*Zingiber officinale* var Amarum extracts		
	Aqueous	70% Ethanol	96% Ethanol
Flavonoids	-	+	+
Alkaloids	-	-	-
Tannins	-	-	-
Saponins	+	+	+
Quinones	-	-	+
Steroids	-	+	-
Triterpenoids	-	-	-
Yield	36.68	58.22	52.13

The presence of quinone in *Z. officinale* var Amarum extract was only detected in 96% ethanol solvent. Quinone is an antioxidant that exhibited anti-bacterial and is dissolved in high concentrations of ethanol, such 96% ethanol. Pyrroloquinoline quinone is a strong antioxidant and can reduce the LDL level (Nakano *et al*., 2015). The presence of steroids was detected positively in 70% ethanol solvent. Steroids themselves can lower cholesterol in the body by being competitive with cholesterol and inhibiting the absorption of cholesterol in the intestines, thereby reducing cholesterol levels that are absorbed by the body through food (Ostlund, 2002). *Zingiber officinale* var Amarum extract does not contain tannins, alkaloids and triterpenoids. The hypolipidemic activity of *Z. officinale* var Amarum may not related with these phytochemicals.

Zingiber officinale var Rubrum extract contains saponins and quinones, while phytochemical compounds such as flavonoids, alkaloids, tannins, steroids and triterpenoids had negative results in all types of solvents. Saponins were positively detected in all types of solvents and quinones were only detected in 96% ethanol solvents (Table 4). Saponins were reported to have anti-hyperlipidemia properties, such decreased absorption of cholesterol from the intestine, inhibit HMG-CoA reductase and increase LDL-C receptor activity (Herviana *et al*., 2022). Nakano *et al*. (2015) also reported that quinone can reduce the LDL level.

Table 4. Phytochemical content of *Zingiber officinale* var Rubrum extracts

Phytochemical content	*Zingiber officinale* var Rubrum extracts		
	Aqueous	70% Ethanol	96% Ethanol
Flavonoids	-	-	-
Alkaloids	-	-	-
Tannins	-	-	+
Saponins	-	-	-
Quinones	-	-	-
Steroids	-	-	-
Triterpenoids	+	+	+
Yield	44.87	47.76	38.21

3.2 HMG-CoA reductase inhibitory activity

Inhibitory activity of nine extracts of *C. cajan* leaves, *Z. officinale* var Amarum and *Z. officinale* var Rubrum are shown in Table 5. Andrianto *et al*. (2015) reported that ≥ 25 to 49% inhibitory activity of the HMG -CoA reductase was categorized as moderate inhibition, while ≥ 50% was considered strong inhibition.

Cajanus cajan leaves, and *Z. officinale* var Amarum extracts that showed the highest inhibitory activity of the HMG-CoA reductase are both 70% ethanolic *C. cajan* and *Z. officinale* var Amarum extracts, which are 39.38% and 47.87%, respectively (Table 5). Aqueous *Z. officinale* var Rubrum extract showed the highest inhibitory activity of HMG-CoA reductase, which is 40.61%, compare to that of both ethanolic *Z. officinale* var Rubrum extracts. All of these inhibitory activities were categorized as moderate inhibition (Andrianto *et al*., 2015).

The 70% ethanolic *C. cajan* leaves extract contain flavonoids, tannins, saponins and steroids (Table 2). The 70% ethanolic *Z. officinale* var Amarum extract contain flavonoids, saponins and steroids (Table 3) and the aqueous *Z. officinale* var Rubrum contain saponin (Table 4).

Table 5. Percent HMG-CoA reductase inhibition of *C. cajan* leaves, *Z. officinale* var Amarum, and *Z. officinale* var Rubrum extracts

Type of extracts	Percent Inhibition (%)
Aqueous *C. cajan* leaves extract	9.494±7.065[c]
70% ethanolic *C. cajan* leaves extract	39.377±2.387[e]
96% ethanolic *C. cajan* leaves extract	25.056±3.341[d]
Aqueous *Z. officinale* var Amarum extract	44.818±3.437[e]
70% ethanolic *Z. officinale* var Amarum extract	47.874±15.943[ef]
96% ethanolic *Z. officinale* var Amarum extract	4.53±0.005[b]
Aqueous *Z. officinale* var Rubrum extract	40.618±9.069[e]
70% ethanolic *Z. officinale* var Rubrum extract	-25.829±0.007[a]
96% ethanolic *Z. officinale* var Rubrum extract	35.272±19.571[de]
Atorvastatin	58.566±0[f]

Values are presented as mean±SD. Values with different superscripts are statistically significantly different ($p<0.05$).

Son *et al*. (2018) reported that flavonoid-sopraflavanone showed strong HMG-CoA reductase-inhibitory activity. Flavonoids are bioactive compounds that have the ability to reduce cholesterol levels in the body by inhibiting the work of the HMG-CoA reductase in converting HMG-CoA into mevalonic acid in the cholesterol synthesis process. The conversion of HMG-CoA to mevalonic acid will be inhibited, which will directly cause the amount of cholesterol formed to be reduced. An *in silico* approach through molecular docking and simulation studies revealed that more than 50 bioactive compounds of flavonoids from certain plants showed HMG-CoA reductase inhibitory activity (Suganya *et al*., 2017). Methanolic flavonoid extract of *G. ulmifolia* showed potency as an HMG-CoA reductase inhibitor (Rahmania *et al*., 2017). Hamed *et al*. (2022) also reported that apigenin flavonoid showed hepatic HMG-COA reductase and cholesterol acyltransferase (ACAT) inhibitory activities. The flavonoid lowered total cholesterol, triglycerides, LDL-C and increased HDL-C. Flavan-3-ol, a flavonoid from *Acacia senegal,* reported inhibit HMG-COA reductase activity (Charan *et al*., 2022).

Aqueous *C. cajan* leaves extract and 96% ethanolic *Z. officinale* var Amarum contain flavonoids (Tables 2 and 3). However, these extracts had low HMG-CoA reductase-inhibitory activity. It may be due to the minimal amounts of soluble flavonoids contained in the extracts. In addition to flavonoids, bioactive compounds-tannins have the ability to inhibit the work of the HMG-CoA reductase enzyme as well as flavonoids and inhibit the absorption of cholesterol from food into the body (Park *et al.,* 2002). Do *et al*. (2011) reported that tannic acid inhibits HMG-CoA reductase and aortic lesion formation. Tannin-rich fiber from young persimmon fruits was reported involved in the hypocholesterolemic effects by reducing plasma cholesterol levels in human subjects (Gato *et al*., 2013). The 70% ethanolic *C. cajan* leaves extract showed higher HMG-CoA reductase-inhibitory activity than the 96% ethanolic *C. cajan* leaves extract (Table 5). It is caused by the 70% ethanolic *C. cajan* leaves extract contains flavonoids and tannins. Meanwhile, the 96% ethanolic *C. cajan* leaves extract only contains flavonoids (Table 2).

The saponins and steroids are other bioactive compounds responsible for the high HMG-CoA reductase-inhibitory contained in *C. cajan* leaves and both *Z. officinale* var Amarum and var Rubrum extracts. Raju and Bird (2007) reported that food saponin, diosgenin is a growth-suppressor and apoptotic inducer of human colon carcinoma cells. These activities may involve cholesterol homeostasis by inhibiting the expression of HMG-CoA reductase. Liu *et al*. (2016) reported that alfalfa saponin significantly reduces cholesterol levels in broiler breast muscle, leg muscle, and liver. These saponins also decreased the expression level of liver HMG-CoA reductase mRNA, reduced hepatic cholesterol synthesis effectively and improved the excretion of feces bile acid in the broiler.

Lee *et al*. (2007) reported phytochemicals contained in mushrooms a steroid-stigmasterol has the ability as HMG-CoA reductase inhibitors. Dara *et al*. (2015) also reported the presence of steroid-stigmasterol contained in *Z. officinale* var Rubrum. The presence of steroids in the 70% ethanolic extract of *C. cajan* may also be responsible for the HMG-CoA reductase inhibitory activity of the extract.

4. Conclusion

This study concluded that 70% ethanolic extract of *Z. officinale* var Amarum, aqueous extract of *Z. officinale* var Rubrum and 70% ethanolic extract of *C. cajan* leaves have the highest HMG-CoA reductase-inhibitory activity, that are 47.87%, 40.62% and 39.38% respectively. These three extracts have a medium inhibitory level of HMG-CoA reductase. These extracts contained flavonoids, tannins, saponins, and steroids. This study suggested that the three types of extracts

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could be used as antihypercholesterolemic candidates for further study to produce such antihypercholesterolemic functional food.

Conflict of interest

The authors declare no conflict of interest.

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