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In Vitro Antihypercholesterol Activity Test of Chocolate (*Theobroma cacao* L) Peel

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ABSTRACT

Cholesterol is a lipid found in the blood and is not hydrolyzed, until now it has become a risk factor for Coronary Heart Disease (CHD). Reducing cholesterol levels is assisted by the use of antihypercholesterol drugs and natural medicines. The by-product of cocoa plantations (*Theobroma cacao* L) was it's peel containing secondary metabolites, including flavonoids, alkaloids, tannins, and saponins. This study aims to investigate the antihypercholesterol activity of cocoa fruit skin extract in vitro using the Liebermann-Burchard method with a UV-Vis spectrophotometer at a wavelength of 669nm. The test results showed that the ethanol extract of cocoa fruit skin obtained through maceration at concentrations of 10, 20, 40, 80, and 100ppm had the activity to lower cholesterol levels with an EC50 value of 67.785ppm. This activity is indicated based on the difference in color intensity of the test solution compared with the control negative solution. The conclusion from the results obtained is that cocoa skin extract has activity as a natural cholesterol-lowering agent.
Keywords: Antihypercholesterol; Cocoa Fruit Peel Extract; Liebermann-Burchard

INTRODUCTION

Cholesterol is a substance that included in the fat group in the blood, it has a yellowish color, waxy texture, with an important role as the main element in the formation of lipoproteins and plasma membranes and a precursor to most steroid compounds. The two main types of lipoproteins that need to be considered are Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) (Mulyani, 2019). Inside the body, cholesterol is the main sterol found in tissues and acts as a precursor to hormones (Daulay et al., 2023). Cholesterol levels are classified as normal in a person when they are in the range <200 mg/dl and high if ≥240 mg/dl. The normal LDL level is <100 mg/dl, high if it is in the range 160-189 mg/dl and very high when ≥190 mg/dl. In contrast to HDL, it is stated as low if <40 mg/dl and high when ≥60 mg/dl (Aman et al., 2019).

Continuous synthesis of plasma cholesterol occurs in the liver, and only about 15-20% comes purely from dietary intake. Cholesterol from food intake is absorbed by enterocytes (epithelium on the surface of the small intestine and large intestine) through a special transporter (Berberich & Hegele, 2022; Nwako & McCauley, 2024). Hypercholesterolemia is a condition when cholesterol in the blood accumulates and sticks to blood vessels which triggers narrowing (Khotimah et al., 2025). In an effort to lower cholesterol levels, 2 types of therapy are recommended, namely pharmacological and non-pharmacological. Some conventional drugs used include fibric acid derivatives (Gemfibrozil), bile acid binders (Cholestyramine, Cholestipol), 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase enzyme inhibitors and nicotinic acid (Abbasi et al., 2024; Lins et al., 2022). The recommended non-pharmacological therapies are mainly lifestyle changes including physical activity, medical nutrition therapy, weight loss and not smoking (Aman et al., 2019). In addition to the 2 things mentioned, the use of natural medicines is an alternative therapy to help restore cholesterol to normal levels (Azzahra & Zuhrotun, 2022).

Secondary metabolites-such as polyphenols, flavonoids, tannins, saponins, and alkaloids-can synergize with conventional cholesterol-lowering drugs through mechanisms that inhibit lipid peroxidation in the heart and blood vessels (Medina-Vera et al., 2021). Flavonoids act as inhibitors

of LDL oxidation, thereby helping to maintain normal LDL levels while increasing HDL and triglyceride levels. This inhibitory activity helps prevent the formation of plaques that cause atherosclerosis. Curcumin, a polyphenolic compound, has been shown to inhibit lipid oxidation and regulate the activity of the HMG-CoA reductase (HMGCR) enzyme, which plays a key role in cholesterol biosynthesis (Boretti, 2024).

Figure 1. Cocoa Fruit with the Peel of it (Personal Documentation)

Naturally occurring flavonoids are widely distributed in various parts of brightly colored plants, including the skin of cocoa fruit. Phytochemical screening conducted by Herman et al., (2020) confirmed that cocoa fruit skin extracts-both pre- and post-drying-contain polyphenolic compounds such as flavonoids, phenolics, and tannins. The cocoa fruit skin from East Bolaang Mongondow Regency is a by-product of cocoa plantations that is currently underutilized by farmers. Therefore, research into the potential use of cocoa fruit skin aims to increase the utility value of this agricultural waste, which could eventually serve as a promising candidate for natural medicine. A sample of cocoa fruit skin is shown in Figure 1.

Laboratory tests to evaluate the cholesterol-lowering activity of extracts can be conducted using Liebermann-Burchard reagent and UV-Vis spectrophotometry (Imtihani et al., 2021). A decrease in color intensity resulting from the reaction between cholesterol, the Lieberman-Burchard reagent, and the natural ingredient extract—measured by absorbance—can indicate the activity of compounds within the extract as potential natural cholesterol-lowering agents. Additionally, correlation analysis using linear regression can provide insights into the effectiveness of the plant or extract in reducing cholesterol levels.

METHODS

The materials used in this study were cacao fruit skin simplicia, ethanol (C_2H_5OH) 96%, anhydrous acetic acid ($C_4H_6O_3$), concentrated sulfuric acid (H_2SO_4), chloroform ($CHCl_3$), and distilled water. The tools used in this study included UV-Vis spectrophotometer (Rittun Ultra-3000 Series®), quartz cuvette (Quartz®), analytical balance (Joanlab®), pulverizer (Miyako®), beakers (Pyrex Iwaki®), measuring cylinders (Pyrex Iwaki®), volumetric flasks (Pyrex Iwaki®), water bath (B-One DWB 10L®), micropipettes (DLAB®), aluminum foil, porcelain cups, glass funnels, stirring rods, 60-mesh sieves, dropper pipettes, filter paper, and glass containers.

Preparation of Cocoa Peel Simplicia

Cocoa fruit peel was obtained from Tombolikat Induk Village, Tutuyan District, East Bolaang Mongondow Regency, North Sulawesi Province. The samples selected for use as simplicia met specific criteria: they were ripe, brownish-yellow in color, and free from damage or rot. The preparation process was carried out in several stages, including wet sorting, washing, slicing, drying, dry sorting, and finally, powdering the dried peel into simplicia before maceration (Dewi et al., 2021).

Cocoa Peel Extraction

The cocoa fruit peel powder was sieved using a 60-mesh siever then prepared for extraction. The cocoa peel extract (CPE) was obtained through maceration of 500 g of the simplicia powder with 96% ethanol for 3 × 24 hours, protected from light and stirred periodically. The resulting macerate was filtered and concentrated using a water bath at a temperature of 60–70 °C until the solvent completely evaporated. The final extract was then weighed to calculate the percentage yield using the following equation, as referenced by Chusniasih et al., (2021):

$$\%\, Yield = \frac{Weight\ of\ extract\ obtained\ (g)}{Weight\ of\ cocoa\ peel\ simplicia\ (g)} x\ 100\%$$

Cholesterol Solution Preparation

A cholesterol solution was prepared by dissolving 100 mg of cholesterol powder in 100 mL of chloroform to obtain a concentration of 1000 ppm (Maharisti et al., 2022).

Maximum Wavelength Determination

The maximum wavelength is determined by scanning a 175 ppm cholesterol solution within the range of 600–750 nm using a UV-Vis spectrophotometer. The 175 ppm cholesterol solution is prepared by pipetting 1,750 µL from a 1,000 ppm stock solution, then adding 2 mL of anhydrous acetic acid and 100 µL of concentrated sulfuric acid. Chloroform is added to bring the total volume to exactly 10 mL in a volumetric flask, which is covered with aluminum foil and incubated for 13 minutes (Andriani & Anggraini, 2023).

CPE Cholesterol-Lowering Activity Test

A stock solution of the CPE sample at 1000 ppm was prepared by dissolving 100 mg of extract in 100 mL of 96% ethanol. This stock solution was then diluted to prepare a series of concentrations (10, 20, 40, 80, and 100 ppm), each with a final volume of 10 mL. From each concentration, 2 mL of the CPE solution was transferred into a measuring flask wrapped in aluminum foil. Subsequently, 2 mL of a 175 ppm cholesterol solution was added, followed by the addition of 0.1 mL of concentrated sulfuric acid and 2 mL of anhydrous acetic acid. The mixture was then topped up with chloroform to the mark. The solution was incubated for 13 minutes. After incubation, the absorbance of the sample was measured at the maximum wavelength using a UV-Vis spectrophotometer to determine the reduction in cholesterol levels based on the resulting absorbance (Andriani & Anggraini, 2023).

Data Analysis

Cholesterol level reduction was analyzed using the following equation, as referenced by Lindawati & Ningsih, (2020):

$$A = [(C - B)/C]x\ 100\%$$

Where: A = percentage of cholesterol reduction; B = absorbance of cholesterol + sample; and C = absorbance of the negative control

The antihypercholesterol capacity was analyzed by determining the EC_{50} (Effective Concentration) value, which was calculated based on correlation data between increasing concentrations of EKBC and decreasing cholesterol levels. This was done using a linear regression equation, following the method described by Lindawati et al., (2020), as follows:

$$y = bx + a$$

Where: y = decrease of cholesterol; x = sample concentration; a = intercept; and b = slope or incline of the curve (R^2)

RESULTS AND DISCUSSION

The preparation of cocoa fruit skin samples aims to produce high-quality powdered simplicia suitable for extraction. Grinding the sample not only standardizes the powder's surface area but also enhances contact between the simplicia and the solvent during the extraction process, thereby optimizing the diffusion of metabolites. The maceration method was selected using 96% ethanol as the solvent, based on the polarity of the target secondary metabolites-specifically flavonoids, which are generally polar. The results of the cocoa peel extraction are presented in Table I. The yield obtained was 2.02%, with the extract exhibiting an orange-brown color, a thick consistency (non-flowing when the container is tilted), and a slightly aromatic scent, though not resembling chocolate. This extract was then used for in vitro testing of antihypercholesterol activity.

The antihypercholesterol activity test was conducted using the Lieberman-Burchard reagent, a colorimetric method for cholesterol analysis based on the reaction of steroid compounds with strong acids. A compound is considered positive for cholesterol content if it forms a green-colored complex upon reaction with the Lieberman-Burchard reagent. In this test, anhydrous acetic acid functions as a cholesterol-extracting agent and ensures that the test medium contains minimal

Table I. Cocoa Peel Extraction Result

Simplicia Powder (g)	CPE (g)	Yield (%)
500	10,1	2,02

Table II. CPE Cholesterol-Lowering Test Results

[C]	Average of [C]	Concentration of CPE (ppm)	[B]	Average of [B]	A (%)
0,722		10	0,627; 0,627; 0,627	0,627	13,238
		20	0,509; 0,509; 0,509	0,509	29,566
0,723	0,723	40	0,423; 0,423; 0,422	0,423	41,513
0,723		80	0,382; 0,382; 0,382	0,382	47,140
		100	0,205; 0,205; 0,205	0,205	71,633

Description: A = represent the percentage of cholesterol reduction; [B] = the absorbance of cholesterol reacted with CPE; [C] = the absorbance of negative control (cholesterol solution without CPE).

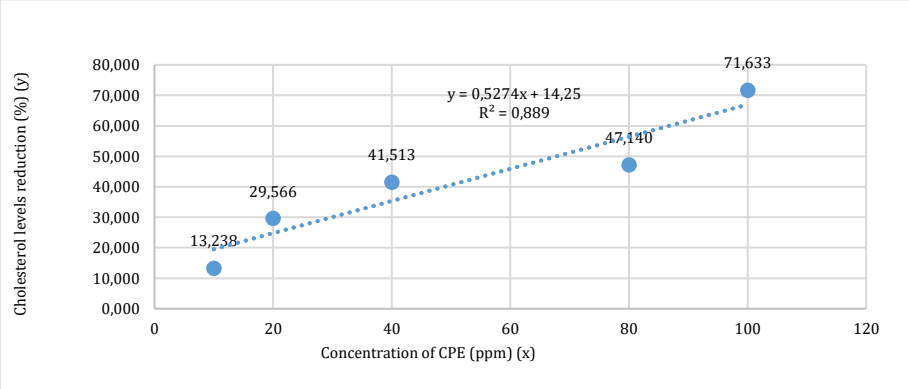


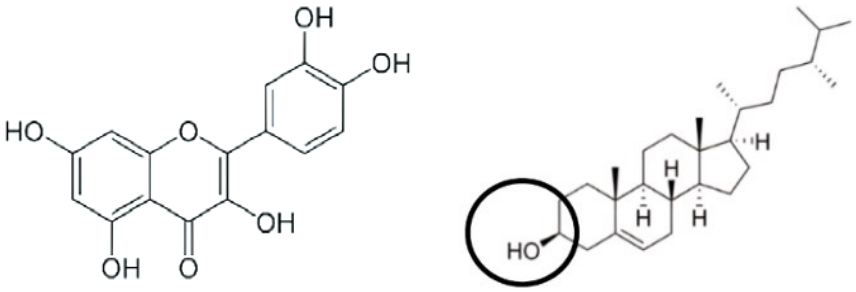
Figure 2. Relationship Curve Between Increasing CPE Concentration and Reduction in Cholesterol Levels

or no water. Sulfuric acid serves as a catalyst for the color change, indicating the presence of cholesterol; the intensity of this color is measured as absorbance at the maximum visible wavelength.

The determination of the maximum wavelength is necessary to identify the wavelength that yields the highest absorbance for cholesterol detection. In this study, the maximum absorbance (λmax) was observed at 669 nm. Measurements were carried out using a 175 ppm cholesterol sample, with absorbance scanned over the 600–750 nm range after a 13-minute incubation period (Andriani & Anggraini, 2023). The results of the qualitative test assessing the reduction in cholesterol levels by the CPE ample are presented in Table II and Figure 2.

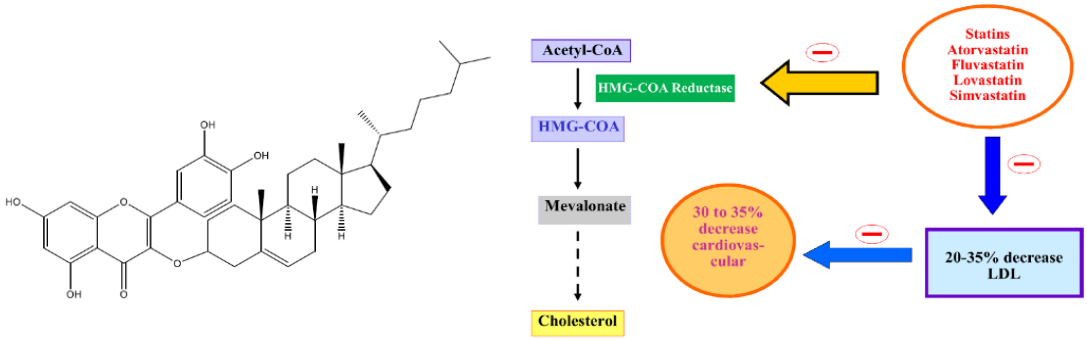
Based on the table and graph presented, the correlation coefficient of the regression is close to +1, indicating a linear relationship between the decrease in cholesterol levels (y-axis) and the increase in the concentration of CPE added to the test sample (x-axis). These results are consistent with the findings of Pratama & Anggraini, (2023), which showed that as the extract concentration increases, the intensity of the green color fades. A more faded green color indicates a greater reduction in cholesterol levels. The linear regression equation was used to calculate the EC_{50}, which is the effective concentration of EKBC required to reduce cholesterol levels by 50%. The EC_{50} value was determined to be 67.785 ppm, meaning that 67.785 ppm of CPE is needed to reduce cholesterol levels by half from the initial total.

Plants and/or extracts containing flavonoids demonstrate the antihypercholesterol activity through the formation of hydrogen bonds between the hydroxyl groups in free cholesterol and the -OH groups in flavonoids. Flavonoids contain -OH groups at the C-3, C-5, C-3', and C-4' positions, as well as a keto group at C-4 (Figure 2a) (Andriani & Anggraini, 2023). The interaction between



(a) (b)

Figure 2. a. Active Group of Flavonoids (Quercetin) (Bjune et al., 2024); b. Active Group of Cholesterol (Pratama & Anggraini, 2023)



(a) (b)

Figure 3. a. Chemical Bonding between Cholesterol and Quercetin (Andriani & Anggraini, 2023); b. Cholesterol Synthesis Pathway Catalyzed by HMG-CoA Reductase (HMGCR) (Mahdavi et al., 2020)

cholesterol (Figure 2b) and the -OH groups of flavonoids forms a stable complex, which reduces the amount of free cholesterol present in the system.

Enzymatically, flavonoids showing antihypercholesterol activity by inhibiting HMG-CoA reductase (HMGCR), an enzyme that catalyzes a key step in the biosynthesis of cholesterol in the body. In this pathway, cholesterol is synthesized from mevalonate, which is formed through a series of enzymatic reactions. Initially, two molecules of acetyl-CoA are converted into acetoacetyl-CoA by the enzyme thiolase. The addition of a third acetyl-CoA molecule to acetoacetyl-CoA forms 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), catalyzed by HMG-CoA synthase (HMGCS). HMG-CoA is then converted into mevalonate by HMGCR, as illustrated in Figure 3(b) (Duan et al., 2022; Mahdavi et al., 2020; Tien et al., 2023). Cholesterol-lowering drugs of the statin class inhibit this biosynthetic pathway by targeting HMGCR. Similarly, several flavonoids-including quercetin, catechin, epigallocatechin gallate, and methyl quercetin-have also been shown to inhibit HMGCR activity (Oktavelia & Kusuma, 2022; Olatoye, 2025; Tien et al., 2023).

CONCLUSION

Based on the results obtained, it can be concluded that cocoa fruit skin extract exhibits activity as a natural cholesterol-lowering agent, with an EC_{50} value of 67.785 ppm. The assumed mechanism of action of flavonoids in lowering cholesterol levels involves the formation of a stable complex hydrogen bond between the -OH groups in both, which prevents cholesterol levels from increasing in the system.

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