PHARMACON
Jurnal Farmasi Indonesia
Pharmaceutical Journal of Indonesia

Pharmacon: Jurnal Farmasi Indonesia
Vol. 22 No. 1, 2025, pp. 01–08
DOI:10.23917/pharmacon.v22i1.7025
p-ISSN: **1411-4283**, e-ISSN: **2685-5062**

In Vitro Cholesterol Reduction Activity Test Of 70% Ethanol Extract of Hiyung Cayenne Pepper Fruit (*Capsicum frutescens* L. var hiyung) Originating from Tapin South Kalimantan

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ARTICLE HISTORY:
Submitted : 2024-12-18
Accepted : 2025-06-18
Published : 2025-06-30

KEYWORDS:
Capsicum frutescens L. var hiyung; Cholesterol; Fruit; In vitro

Citation:
Hayati, M., Saputri, R., & Rizaldi, G., (Year). In Vitro Cholesterol Reduction Activity Test Of 70% Ethanol Extract Of Hiyung Cayenne Pepper Fruit (*Capsicum frutescens* L. var hiyung) Originating from Tapin South Kalimantan. Pharmacon: Jurnal Farmasi Indonesia, 22(1), 01-08. https://doi.org/10.23917/pharmacon.v22i1.7025

ABSTRACT

Kalimantan Island has an endemic plants that holds potential as a medical one of them namely hiyung cayenne pepper fruit (*Capsicum frutescens* L. var hiyung) the local commodity of Tapin, South Kalimantan and known as the hottest pepper in Indonesia. It containts secondary metabolites and capsaicin that has potential to lower cholesterol. This study aims to identify secondary metabolite compounds and cholesterol-lowering activity of 70% ethanol extract of hiyung cayenne pepper fruit. Cholesterol lowering test was conducted in vitro by cholesterol complex formation method with $FeCl_3$ using UV-Vis Spectrophotometer at maximum wavelength of 525 nm. The results of the phytochemical screening test showed positively contained alkaloids, triterpenoids, phenols, saponins, tannins and flavonoids. The result of cholesterol reduction test from concentrations of 20, 40, 60, 80, and 100 µg/mL were 53.62%, 58.30%; 64.19%; 70.02%; and 74.91%, respectively. So, the 70% ethanol extract of hiyung cayenne pepper fruit has the potential to reduce cholesterol with the lowest concentration being able to reduce cholesterol by more than 50%.

INTRODUCTION

Cholesterol plays an important role in keeping the body functioning normally. However, if there is a buildup of cholesterol carried by LDL (*Low Density Lipoprotein*) along arterial blood vessels, it can cause plaque formation so that the arteries experience narrowing and hardening (Isnaniar et al., 2020). This will potentially cause pain, cramps, gangrene, stroke, and coronary heart disease(Anggraini & Nabillah, 2018)

The application of pharmacological therapy aims to reduce the risk of developing cardiovascular disease. One class of anticholesterol drugs recommended as the main choice is statins, but statins have the side effect of myopathy and are contraindicated with chronic or acute liver disease (Aman *et al*, 2019). So, alternative treatments from herbal plants for therapies that are safe for long-term use with minimal or almost no side effects for hypercholesterolemia need to be depeloved.

Hiyung cayenne pepper (*Capsicum frutescens* L. var hiyung) is an endemic species from Tapin, South Kalimantan, which has medicinal potential with its capsaicin compound. The capsaicin content has potential as antihyperlipidaemia (Adigun et al., 2020), antiobesity (Siska & Bariroh, 2022), antioxidant (Antasionasti et al., 2022) and anticholesterol (Zhang et al., 2013). Secondary metabolites contained in hiyung cayenne pepper fruit include alkaloids, phenolics, flavonoids and saponins (Sutomo et al., 2017). Flavonoids, alkaloids, saponins, tannins and triterpenoid compounds can reduce cholesterol (Pramesty et al., 2022). However, there is no research related to the activity test of hiyung cayenne pepper fruit extract to reduce cholesterol in vitro. Therefore, research on the

cholesterol-lowering activity of hiyung cayenne pepper fruit extract was conducted based on the percent (%) cholesterol reduction.

METHODS

Tools and Materials

This study used tools including macerator, a set of glassware (*pyrex*®), analytical scales (*Ohaus*®), oven, mesh number 20 sieve (*Standard Steves*®), rotary evaporator (IKF10®), water bath (*Memmert*®), micro pipette (Dragon Lab®), vortex (DLAB MX-S), UV-Vis Spectrophotometry (DLAB).

The test material used including hiyung cayenne pepper fruit. While other materials used in this study were filter paper, aluminium voil, 70% ethanol (brataco), concentrated hydrochloric acid (HCl) p.a (merck), magnesium powder p.a (merck), amyl alcohol ($CH_5H_{11}OH$) p.a (merck), distilled water (onemed), 1% gelatin (merck), 10% NaCl p.a (merck), mayer reagent (eralika mitra persada), dragendorf reagent (eralika mitra persada), wagner reagent (eralika mitra persada), chloroform p.a (merck), anhydrous acetic acid $(CH_3CO)_2O$ p.a (merck), glacial acetic acid (CH_3COOH) p.a (merck), ethanol p.a (brataco), 95% ethanol (brataco), iron (III) chloride ($FeCl_3$) p.a (merck), concentrated sulfuric acid (H_2SO_4) p.a (merck), and cholesterol powder (Sigma Aldrich).

Material Identification and Collection

The identification process was carried out by Laboratory of the Faculty of MIPA, Lambung Mangkurat University, Banjarbaru with number of certificate : 353/LB.LABDASAR/XII/2023. Collection of 3 kg of ripe fruit samples obtained from the Agricultural Extension Centre of Middle Tapin Subdistrict, Tapin Regency, South Kalimantan.

Simplisia Preparation

Hiyung cayenne pepper fruit (*Capsicum frutescens* L. var hiyung) were weighed approximately 3 kg and then wet sorted by washing until clean from dirt and then drained. The sample was dried by drying in the sun covered with a black cloth and then dried using an oven at 50^{0}C to maximise drying. After the hiyung cayenne pepper fruit (*Capsicum frutescens* L. var hiyung) is dry, sorting is carried out again to prevent dirt or foreign materials that are carried away in the process of processing simplisia. After that, the obtained simplisia was mashed with a blender until it became powder (20 mesh) (Sutomo et al., 2017).

Extract Preparation

Powder of hiyung cayenne pepper fruit were extracted with 70% ethanolic (maceration method). The powder was put into a maceration container, solvent was added in a ratio of 1 : 4 parts of solvent until completely submerged. Extraction were carried out for 24 hours with stirring every 6 hours. The sampel were remaseration with a new 70% ethanol with the same amount of 2 repetitions, this is done by using 70% ethanol was carried out for 2 x 24 hours (Sutomo et al., 2017). The obtained macerate was then collected and evaporated the filter liquid with a vacuum rotary evaporator (40 rpm, 40^{0}C) (Kusnadi et al., 2019) followed by a waterbath.

Phytochemical Screening of Extracts

Identification of Flavonoids

A half g extract was mixed with 10 milliliters of hot water, heated for 5 minutes, and subsequently filtered. An approximate volume of ± 5 mL of filtrate was mixed with 0,5 g of magnesium powder and 1 mL of concentrated hydrochloric acid. The mixture was violently agitated, and a small amount of amyl alcohol was added. The presence of yellow or red pigmentation confirms a positive test (Depkes RI, 1995).

Identification of Tannins

A half g was added with 10% NaCl as much as 5 drops and filtered then added 1% solution of gelatin containing 10% NaCl. The formation of a precipitate gelatin indicates positive for tannins (Trease & Evans, 2017).

Identification of Saponins

A half g of extract was added to 10 milliliters of hot water and vigorously shaken for 1 minute. Following this, two drops of hydrochloric acid with a concentration of 2 N were added. If the foam created remains stable for around 10 minutes, the extract includes saponins (Depkes RI, 1995).

Identification of Phenolics

A half g was added with 6 drops of $FeCl_3$ 5%, the colour change from bluish black to solid black indicates the presence of phenol content

(Trease & Evans, 2017, Ningsih et al., 2020; Oktavia & Sutoyo, 2021).

Identification of Alkaloids

A half g was added to 1 ml of 2N HCl and 9 mL of distilled water, then heated in a water bath for 2 minutes, cooled and filtered, then put into three test tubes. The first test tube was tested with Mayer reagent, the second test tube with Dragendorff reagent, and the third test tube with Wagner reagent. Positive extracts containing alkaloids in the Mayer reagent are marked by the formation of a yellowish-white precipitate, an orange precipitate formed in the Dragendorff reagent, and a brown precipitate in the Wagner reagent (Depkes RI, 1995; Saputri et al., 2020).

Identification of Steroids and Triterpenoids

Sample of 0,1 g was combined with 2-3 mL of chloroform and, two drops of Lieberman-Bourchad reagent in a drip plate. A positive test for steroids results in a color change from blue to green, but the presence of a red or purple color indicates a positive result for triterpenoids when tested using the Liebermann-Bourchard reagent (Depkes RI, 1995; Sandra et al., 2022).

Cholesterol Lowering Test

Wavelenght Determination

Determination of wavelength in spectrophotometric analysis is carried out at the maximum wavelength (Harborne, 1998) The maximum wavelength (λ) is determined by scanning the wavelength of a cholesterol standard solution with a concentration of 100 µg/mL . This solution is prepared by taking 0.5 mL from a 1000 µg/mL solution and adding 95% ethanol until the flask reaches the limit mark of 5 mL. To protect from light the outer layer of the tube was covered with aluminium foil. Next, 2 mL of $FeCl_3$ reagent was added to the tube, which was then vigorously mixed using a vortex mixer. The mixture was then left undisturbed for 10 minutes. After that, added 1 mL of H_2SO_4(p) as a catalyst through the wall of the test tube and a mixture of solutions homogenised using a vortex, then left to stand for 30 minutes. Wavelength measurements are conducted using a UV-Vis spectrophotometer that operates within the 400-700 nm range (Hadiarti, 2017).

Preparation of Standard Cureve

The standard curve was prepared by creating a series of 5 concentrations ranging from 100 to 300 µg/mL using a parent solution with a cholesterol concentration of 1000 µg/mL. For each parent solution, 0.5; 0.75; 1, 1.25, and 1.5 mL were taken and then added to 5 mL of 95% ethanol in a volumetric flask. The tube's outside was coated with aluminum foil. Subsequently, each concentration was supplemented with 2 mL of $FeCl_3$ reagent, mixed vigorously, and left undisturbed for 10 minutes. Afterward, 1 mL of concentrated sulfuric acid (H_2SO_4) was added to each concentration. The resulting solution mixture was then thoroughly mixed using a vortex. After 30 minutes of allowing the combination to remain undisturbed, the absorbance was measured using the previously established ƛ max (Hadiarti, 2017).

Determination of Cholesterol Lowering Activity of the Extract

Extract concentration of 1000 µg/mL was made by weighing 10 mg of 70% ethanol extract of hiyung cayenne pepper fruit dissolved with ethanol until the limit mark of 10 mL. Then from the 1000 µg/mL concentration extract solution, 5 series of sample concentrations of 20, 40, 60, 80, and 100 µg/mL were diluted to 10 mL with ethanol solvent (Anggoro et al., 2022). A 5 mL of each concentration of extract was put into a test tube. Next, 5 mL of a cholesterol standard solution with a concentration of 300 parts per million (µg/mL) was introduced. The solution, consisting of a blend of chemicals, was vigorously stirred and maintained at a consistent temperature for 60 minutes. Next, 5 mL of the solution was mixed with 2 mL of $FeCl_3$ reagent, rapidly stirred, covered with aluminium foil, and allowed to sit undisturbed for 10 minutes. Subsequently, a volume of 1 mL of H_2SO_4 was introduced into the mixture, which was then violently agitated and allowed to settle undisturbed for 30 minutes. The color measurements were obtained using a UV-Vis spectrophotometer at the wavelength corresponding to the maximum color intensity (Hadiarti, 2017). This test was carried out as many as 3 replications and negative control in the form of 300 µg/mL cholesterol solution.

Data Analysis

The data analysis involved the utilization of a linear regression equation derived from the cholesterol standard curve to calculate cholesterol levels. When determining the

percentage decrease in cholesterol levels, the following calculation is used :

$$\% \text{ decrease in cholesterol} = \frac{\text{Baseline cholesterol level} - \text{Cholesterol level final (after addition of extract)}}{\text{Baseline cholesterol level}} x100\%$$

(Pratiwi & Purba, 2020).

RESULT AND DISCUSSION

Determination Result

Determination of the hiyung cayenne pepper plant is needed to confirm that the plants used are genuine and really cayenne pepper plants with the hiyung variety. Determination is also intended to avoid errors in the use of materials that can result in changes in the results obtained (Arrosyid et al., 2019). The test results in the Basic Laboratory of FMIPA with number of certificate : 353/LB.LABDASAR/XII/2023 showed that the plants used were hiyung cayenne pepper plants with the scientific name *Capsicum frutescens* L. var hiyung.

Simplicia

The objective of the drying procedure is to decrease the moisture content in the sample to enhance the longevity of the simplisia during storage and prevent enzymatic reactions (Syafarina et al., 2017). The dried simplisia obtained is red in colour. From 3 kg of fresh samples, 450 grams of dry simplisia was obtained so that the yield of simplisia obtained was 15%.

Extraction

The 70% ethanol extract of hiyung cayenne pepper fruit obtained was 44.6247 grams so that the yield of the extract obtained was 22.3123 %.

Phytochemical Screening

The results showed that the sample was positive for flavonoids, tannins, saponins, phenolics, alkaloids, and triterpenoids. The reaction of flavonoid with magnesium (Mg) and hydrochloric acid (HCl), can be reduced benzopyrone nucleus in flavonoids to form a flavylium salt causing a color change from green to orange(Ergina et al., 2014). Tannins can precipitate proteins in gelatin by forming copolymers. The addition of NaCL enchance this reaction by increasing the saltiness of tannin-gelatin (Harborne, 1998; Sabdoningrum et al., 2021). The result of the phytochemical screening presented in (**Table 1**).

Table 1. Phytochemical Sreening Result

Tes Type	Results	Description
Flavonoids	Yellow and top layer of amyl alcohol red	Positive
Tannin	White precipitate	Positive
Saponins	Stable 3 cm high foam appears	Positive
Phenolic	Deep black	Positive
Alkaloids	Mayer: A yellowish-white precipitate forms Wagner: Brown precipitate formed Dragendorff: Orange precipitate formed	Positive
Steroids	Brownish red	Negative
Triterpenoids	Brownish red	Positive

Saponin glycosides have the ability to form foam in water hydrolyzed into glucose and its aglycone. The foam formation reaction showed a positive result of the saponin tes (Sabdoningrum et al., 2021). The addition of $FeCl_3$ was used to determine the presence of phenol groups in the sample. This reaction is based on phenols forming complex compounds with Fe^{3+} ions (Setyawaty et al., 2020).

Test the presence of alkaloid compounds using distilled water and 2 N HCl with mayer, wagner, and dragendorff reagents. The fruit extract of cayenne pepper hiyung is positive containing alkaloids in the mayer test which is characterized by the formation of a yellowish white precipitate, it is estimated that the precipitate is potassium-alkaloid complex. In the alkaloid test with the Mayer reagent, it is thought that the nitrogen in the alkaloid will react with the metal ion K+ from potassium tetraiodomercurate (II) to form a potassium-alkaloid complex which precipitates (Silla et al., 2021).

Test the presence of triterpenoid compounds using Lieberman Burchard reagent. The results of testing the fruit extract of cayenne pepper hiyung positively contain triterpenoids marked by a change in the color of the extract after being dripped reagent into a brownish red color, this is due to the occurrence of oxidation in the terpenoid compound group through the formation of conjugated double bonds. The reaction principle in the reaction mechanism of the terpenoid test is the condensation or release of H2O and the incorporation of carbocations (Jafar et al., 2020).

Flavonoids play a role in preventing the adhesion (plaque) of *low density lipoprotein* (LDL) in blood vessels and LDL that does not form plaques will be carried to the liver. to be excreted through bile acids so that cholesterol can be lowered by flavonoid compounds (Muqowwiyah & Dewi, 2021). Tannins have an effect in lowering cholesterol levels in the blood, namely inhibiting cholesterol absorption by reacting with mucosal proteins and intestinal epithelial cells, by binding to lipids in the digestive tract so as to interfere with lipid absorption in the intestine while saponins inhibit cholesterol absorption in the intestine so that cholesterol is excreted along with feces (Ananda & Rahman, 2024). The role of phenols as antioxidants can reduce blood cholesterol levels through the mechanism of increasing HDL cholesterol (Pratiwi & Rustanti, 2015). Alkaloid bioactive compounds can reduce blood cholesterol levels by inhibiting the work activity of HMG-KoA reductase in the process of cholesterol synthesis so that blood cholesterol will decrease (Rindiany et al., 2022). Terpenoids regulate the breakdown of the enzyme 3-hydroxy-3-methylglutaryl (HMG-KoA) reductase, which decreases cholesterol production by blocking its biosynthesis (Bandi et al., 2021; Indriyani et al., 2023)

Cholesterol Lowering Test

Wavelength Determination

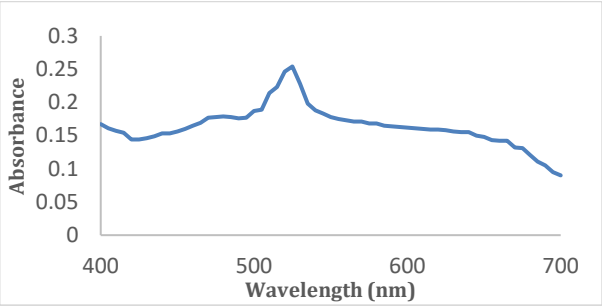

Figure 1. Maximum wavelength of cholesterol solution

The reaction between cholesterol and Zak's reagent ($FeCl_3$) is the protonation of the -OH group on cholesterol. This reaction forms a colored complex compound. The intensity of the resulting color can be detected using a UV-Vis Spectrophotometer ranging from 400-700 nm. (Hadiarti, 2017; Kurnia et al., 2019). In this study, the maximum wavelength measurement using 100 µg/mL cholesterol standard solution obtained ƛ max of 525 nm. ƛ max can be seen in (**Figure 1**).

Preparation of Standard Curve

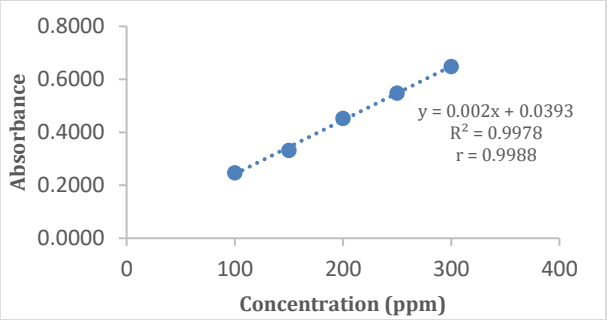

Figure 2. Cholesterol Solution Standard Curve

Table 2. Absorbance Value of Cholesterol Solution Standard Curve

Sample	Concentration (µg/mL) (x)	Average	± SD
Cholesterol	100	0.2480	± 0.0078
	150	0.3323	± 0.0064
	200	0.4533	± 0.0055
	250	0.5480	± 0.1135
	300	0.6487	± 0.0123

Five concentration. In this study, the linear equation was y = 0.002x + 0.0393 with a r2 value of 0.9988 (**Figure 2**), which is close to 1 meaning that there is linear correlation between concentration and absorbance. The data obtained shows that as the concentration increases, the absorbance increases. From 5 concentration series of cholesterol solution the results are shown in (**Table 2**).

Determination of Cholesterol Lowering Activity of 70% Ethanol Extract of Hiyung Cayenne Pepper Fruit (Capsicum frutescens L. var hiyung)

Determination of cholesterol-lowering activity of chili fruit extract cayenne hiyung was conducted in vitro using UV-Vis spectrophotometer with Zak method. The principle of the method is based on the ability to bind cholesterol-ethanol after the addition of the test sample. The reaction between cholesterol and the reagent Zak is the protonation of the -OH

group in cholesterol then a dehydration reaction occurs which then produces a carbonium ion 3.5 cholestadiene then the addition of Fe^{3+} to get a colored compound which will then form a tetraenylic cation solution which is reddish orange. $H2SO_4$ (p) is needed as a catalyst and reaction of $FeCl_3$ dye in glacial acetic acid so that a colored compound is formed (Anggoro et al., 2022).

Table 3. Result of cholesterol levels and percentage reduction

Sample (µg/mL)	Average decrease cholesterol	± SD
Cholesterol (300 µg/mL)	0.00	0
20 µg/mL	53.62	± 0.58
40 µg/mL	58.30	± 0.40
60 µg/mL	64.19	± 0.41
80 µg/mL	70.02	± 0.39
100 µg/mL	74.91	± 0.47

In the reduction of cholesterol levels of hiyung cayenne pepper fruit extract (*Capsicum frutescens* L. var hiyung), the absorbance of 300 µg/mL cholesterol control solution was first determined with the absorbance results in three consecutive replications of 0.641; 0.652; and 0.643. concentration (µg/mL)The test results showed that the higher the concentration of the extract, the greater the decrease in cholesterol levels. The measurement results of absorbance values and cholesterol levels as well as the percentage of cholesterol reduction can be seen in (**Table 3**).

In the results of this study, the lowest concentration of 20 µg/mL has been able to reduce cholesterol by more than 50%, namely 53.62% and the highest level of cholesterol reduction is found in the concentration of hiyung cayenne pepper fruit extract (*Capsicum frutescens* L. var hiyung) µg/mL 100 µg/mL with a large reduction in cholesterol levels of 74.91%. The graph of the average percent cholesterol reduction of hiyung cayenne pepper fruit extract (*Capsicum frutescens* L. var hiyung) is shown in (**Figure 3**). The greater the sample concentration, the higher the cholesterol-lowering activity produced.

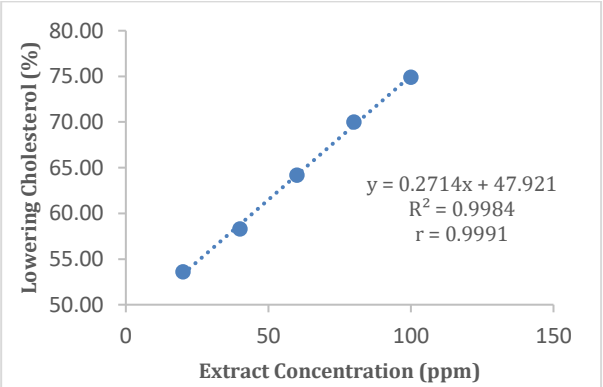

Figure 3. Graph of Average Percentage of Cholesterol Reduction

CONCLUSIONS

Secondary metabolite compounds contained in 70% ethanol extract of hiyung cayenne pepper fruit were triterpenoids, alkaloids, phenols, saponins, tannins and flavonoids. The 70% ethanol extract of hiyung cayenne pepper fruit has activity as a cholesterol lowering in vitro with all test concentrations producing a percentage of cholesterol reduction of more than 50%.

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