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Research Article

INHIBITION OF 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE ACTIVITY BY EXTRACTS OF *GARCINIA XANTHOCHYMUS* MESOCARP AND TOTAL FLAVONOID ASSAY QUANTIFICATION OF THE MOST ACTIVE EXTRACT

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ABSTRACT

Objective: This study aims to determine the inhibitory activity of *Garcinia xanthochymus* mesocarp extracts against 3-hydroxy-3-methylglutaryl -coenzyme A (HMG-CoA) reductase.

Methods: *G. xanthochymus* mesocarp was macerated sequentially using n-hexane, ethyl acetate, and methanol. Phytochemical screening and quantification of total flavonoids were performed on the most active extract.

Results: Based on the tests, n-hexane, ethyl acetate, and methanol extracts had inhibitory activities of 12.30±1.098%, 55.63±10.584%, and 44.01±1.053%, respectively. The results showed that the ethyl acetate is the most active extract, containing flavonoid, terpenoid, glycoside, and anthraquinone compounds. The amount of total flavonoid contained in ethyl acetate extract was 1.61% or 16.114 mg QE/g toward quercetin.

Conclusion: The n-hexane, ethyl acetate, and methanol extracts of *G. xanthochymus* have inhibitory actions against HMG-CoA reductase activity *in vitro*. Further research is still needed to strengthen this finding.

Keywords: *Garcinia xanthochymus* mesocarp, Total flavonoid, 3-Hydroxy-3-methylglutaryl-coenzyme A reductase, Sequential maceration, Phytochemical screening.

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INTRODUCTION

Cholesterol is an amphiphilic lipid that is an essential structural component of the plasma membrane and the outer layer of plasma lipoproteins. It is synthesized in many tissues, is catalyzed by acetyl coenzyme A (acetyl-CoA), and is the precursor of all other steroids in the body, including corticosteroids, sex hormones, bile acids, and Vitamin D [1].

Hypercholesterolemia is characterized by elevated levels of low-density lipoprotein (LDL) or total cholesterol and low levels of high-density lipoprotein [2]. Hypercholesterolemia is a risk factor for non-infectious cardiovascular and metabolic diseases, such as atherosclerosis, metabolic syndrome, coronary heart disease, and stroke [3].

The drugs commonly used for the treatment of hypercholesterolemia include statins (simvastatin, atorvastatin, lovastatin, fluvastatin, rosuvastatin, and pravastatin), bile acid sequestrants (colestipol, colesevelam, and cholestyramine), nicotinic acid (niacin), and acid derivative fibrates: Peroxisome proliferator-activated receptors-α activators (clofibrate, gemfibrozil, fenofibrate, bezafibrate, and cyprofibrate), ezetimib, and fish oil supplements [4]. First-line therapy for hypercholesterolemia commonly uses statin drugs that inhibit 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, an enzyme that catalyzes the biosynthesis of cholesterol [1].

Plants of the *Garcinia* genus belong to the Clusiaceae or Guttiferae family. Currently, there are approximately 250 identified *Garcinia* species [5]. *Garcinia xanthochymus* is found mainly in Indonesia, especially in areas at altitudes of 1–500 m above sea level [6]. Some *Garcinia* species, such as *Garcinia atroviridis* and *Garcinia cambogia*, contain hydroxycitric acid (HCA), which has a hypolipidemic effect by decreasing lipogenesis and promoting glycogen progression, thereby suppressing the appetite [5].

HCA is a competitive inhibitor of adenosine 5'-triphosphate citrate lyase, an enzyme that catalyzes the extramitochondrial conversion of citrate to oxaloacetate and acetyl-CoA. Reduced availability of acetyl-CoA decreases the biosynthesis of fatty acids and cholesterol as well as lipogenesis [7]. According to Darwati and Supriyatna, the ethanol extract of *G. xanthochymus* bark can lower LDL levels in white mouse (*Mus musculus*) blood with extract levels below 100 mg/bb [8]. Based on chemotaxonomic considerations, *G. xanthochymus* is thought to contain HCA, as other *Garcinia* species can have similar effects.

G. xanthochymus contains quercetin [9], which is one of the most effective flavonoids to prevent LDL oxidation, as compared with other flavonoids such as myricetin, kaempferol, and morin [10]. Other compounds contained in *G. xanthochymus* include the biflavonoids morelloflavone and fukugetin [11]. According to Tuansulong *et al.*, isolated morelloflavone compounds from *Garcinia dulcis* leaves can inhibit HMG-CoA reductase activity, which has a role in cholesterol biosynthesis, with an inhibition constant of 80.87±0.06 μM against HMG-CoA and 103±0.07 μM against NADPH [12].

To gain knowledge of herbal plants in Indonesia, especially *G. xanthochymus*, researchers would like to know the ability of *G. xanthochymus* fruit extract to inhibit the activity of HMG-CoA reductase, an enzyme that has a role in cholesterol biosynthesis.

METHODS

Extraction

G. xanthochymus fruits were collected and wet-sorted washed with water, and dried. The fruits were cleaned, peeled, and cut into thin slices to speed up the drying process. The fruits were dried in an oven. The dried simplisia was then dry-sorted and grinded. Extraction was performed on simplisia powder by maceration using a multilevel extraction

method from nonpolar (n-hexane), semipolar (ethyl acetate), and polar (methanol) solvents. After the maceration process, the solvents were evaporated with a rotary vacuum evaporator, and the extracts were placed in a water bath until they became a viscous extract. The obtained viscous extract was then weighed to calculate the percentage of yield, by dividing extract weight with simplisia weight ×100% [1].

HMG-CoA reductase inhibition assay

Preparation of sample and standard solutions

Samples of n-hexane extract, ethyl acetate extract, methanol extract, and quercetin (as positive control) were weighed as much as ±10 mg, and then, five drops of dimethyl sulfoxide were added to support the dissolution. A phosphate buffer solution pH 7.4 was adjusted to 10 mL volume to obtain a concentration of 1000 µg/mL. The main solution was then diluted to a concentration of 100 µg/mL. Dilution was performed with 10.0 mL of phosphate buffer pH 7.4 [13]. Pravastatin solution used in the test had a concentration of 42.45 µg/mL.

Determination of IC_{50} pravastatin

The pravastatin solution was used at 100 µM or 42.45 µg/mL. Pravastatin was diluted gradually from main solution using phosphate buffer pH 7.4 to obtain concentrations of 21,22, 10.61, 5.30, 2.65, 1.32, 0.66, 0.33, and 0.16 µg/mL or 50,000, 25,000, 12,500, 6250, 3125, 1562.5, 781.25, and 390,625 nM [14]. 1 µL sample of each pravastatin concentration was then taken, and 181 µL of buffer assay pH 7.4, 4 µL of NADPH, and 12 µL of HMG-CoA were added. Thereafter, 2 µL of HMG-CoA reductase was added. The mixture was placed in a temperature-regulated microplate reader at 37°C and shaken for 10 s, and the absorbance of the mixture was measured at 340 nm [15]. The test was carried out once. The percentage inhibition was calculated by dividing (A–B)–(C–D) with (A–B)×100%, where A=Δ positive blank absorption, B=Δ negative blank absorption, C=Δ absorption sample, and D=Δ sample blank absorption [13]. The percentage inhibition was then plotted against concentration where the X-axis was the concentration and the Y-axis was the percentage inhibition. After the linear regression equation y=a+bx was obtained (Fig. 1), the value 50 was included in the equation substituting for the value y so that the value of IC_{50} pravastatin would be obtained [14-16].

Testing of sample and standard solutions

Sample and standard solutions were each drawn as 1 µL, and then, 181 µL of buffer assay pH 7.4, 4 µL of NADPH, and 12 µL of HMG-CoA were added.

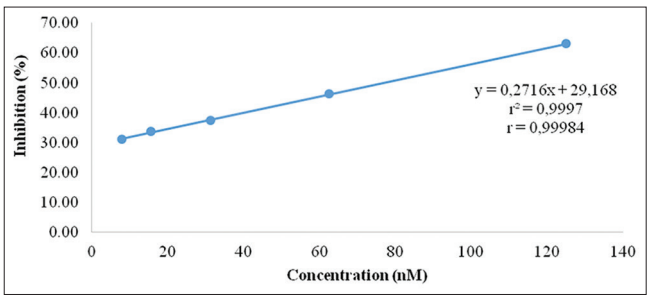

Fig. 1: IC_{50} pravastatin calibration curve

Thereafter, 2 µL of HMG-CoA reductase solution was added. The mixture was placed in a temperature-regulated microplate reader at 37°C and shaken for 10 s, and the absorbance of the mixture was measured at a wavelength of 340 nm (Table 1). Each test was performed 3 times [17].

The ability of the sample to inhibit HMG-CoA reductase can be determined from the percentage inhibition calculated by formula (2).

Phytochemical screening

Phytochemical screening was performed on the most active extract to determine the class of secondary metabolite compounds present in the extract. Identification of flavonoids using magnesium, concentrated hydrochloride acid, and amyl alcohol (Willstater's test) and elucidation using thin-layer chromatography (TLC) Silica gel 60 F_{254} as stationary phase and chloroform:acetone:formic acid (7:2:1) as mobile phase with 5% $AlCl_3$ sprayed reagent and then observed under ultraviolet (UV) 366 nm light [16]. Positive control that was used is quercetin. Identification of alkaloids was done using Mayer's test, Borchardt's test, and Dragendorff's test [18] with boldine as a positive control. Identification of tannins was performed using 10% gelatine solution, 3% ferric chloride solution, and 10% sodium chloride+10% gelatine solution [18] with Psidii Folium powder as positive control. Identification of saponins was performed using foam test [18] with Momordica Folium powder as a positive control. Identification of terpenoids and steroids was performed using Lieberman-Bouchard's test (acetic acid anhydrous:concentrated sulfuric acid [2:1]) [18] with β-sitosterol as a positive control. Identification of anthraquinones was done using Borntrager's test [18] with Phei Radix powder as a positive control. Identification of glycosides was performed using Molisch's test [18] with Stevia Folium as a positive control.

Determination of total flavonoid content (TFC) by UV-visible spectrophotometry

The standard used for measuring TFC was quercetin. Quercetin solutions were prepared at dilutions of 30, 40, 50, 60, 70, and 80 µg/mL using 80% ethanol as solvent according to the standards of the Ministry of Health (2011), with modification [18]. Absorbance was measured at a maximum absorption wavelength of 434 nm. Measurement of blanks without the addition of $AlCl_3$ was performed for the correction factor. The absorption results were plotted and then put into the linear regression equation to obtain the equation y=a+bx [18]. The most active extract with a concentration of 4000 µg/mL was spiked with standard quercetin concentration 500 µg/mL using 80% ethanol. The solution was treated as described above. The absorbance was formulated into linear regression, and then, the content was calculated using formula: $TFC=(R\times10^{-10}\times DF\times V\times100)/W$ to get total flavonoid percentage and $TFC=(R\times10^{-10}\times DF\times V)/W$ to get total flavonoid as gram quercetin equivalent per gram. Where R is the result obtained from standard curve (µg/mL), DF is dilution factor, V is volume of stock solution (mL), and W is weight of extract used in sample test (g) [18].

RESULTS AND DISCUSSION

Extraction

The first step in handling *G. xanthochymus* fruit was wet sorting. After wet sorting, the fruit was washed with water and dried. The fruits were cleaned, peeled, and cut in thin slices to speed up the drying process.

Table 1: HMG-CoA reductase inhibition assay

Materials	Volume (µL)					
	Sample	Sample blank	Standard	Standard blank	Positive blank	Negative blank
Extract solution	1	1	–	–	–	–
Pravastatin and quercetin	–	–	1	1	–	–
Buffer assay pH 7.4	181	199	181	199	182	184
NADPH	4	–	4	–	4	4
HMG-CoA	12	–	12	–	12	12
HMG-CoA reductase	2	–	2	–	2	–
Final volume of well	200	200	200	200	200	200

HMG-CoA: 3-Hydroxy-3-methylglutaryl-coenzyme A

The fruit was then dried. Drying aims to decrease the moisture content of the simplisia, thus preventing damage to materials and nutritious compounds caused by fungi or microbes and prevent enzymatic reactions in the sample due to the presence of water [19]. Several classes of compounds, such as flavonoid glycosides, can be degraded by enzyme activity when the plant is not in a dry condition [20]. The dried sample was then dry-sorted and pollinated in a blender. The pollination of sample was intended to facilitate the absorption of the solvent during extraction by increasing the surface area of the sample so that the penetration of solvent into the sample cell membrane would be easier [21].

Simplisia extraction

The blended simplified powder was extracted by a multilevel maceration method using three different types of solvents: Non-polar (n-hexane), semipolar (ethyl acetate), and polar (methanol). The use of solvents with stratified polarities from non-polar to polar separates the compounds based on their polarity. The solvent sequence for extraction began with n-hexane, a solvent with low polarity. n-Hexane was used to extract nonpolar compounds, such as waxes, oils, and fats [20]. The next solvent used was ethyl acetate, which was chosen because of its semipolar characterization. Ethyl acetate extracts semipolar compounds, such as flavonoid aglycone group (isoflavones, flavanones, methylated flavones, and flavonols) [20]. Ethyl acetate also extracts terpenoid, alkaloid, and glycoside compounds [22]. The last solvent used was methanol, which has high polarity. Methanol extracts polar glycosides and more polar aglycones [20]. Methanol also extracts quaternary alkaloids, carotenoids, tannins, and amino acids [22]. Ethyl acetate and methanol were used to extract phenolic compounds [20]. The weight of the viscous extract and the percentage value of the yield of each solvent are shown in Table 2.

HMG-CoA reductase inhibition assay

HMG-CoA reductase inhibition assay was performed *in vitro* using the HMG-CoA Reductase Assay Kit CS1090 (Sigma Aldrich, USA). The kit contains HMG-CoA reductase enzyme (concentration 0.50–0.70 mg protein/mL), HMG-CoA, NADPH, pravastatin substrate as positive control, and 100 mM pH 7.4 potassium phosphate buffer assay. The HMG-CoA reductase present in the kit is a recombinant human enzyme of GST fusion protein expressed on *Escherichia coli* [17].

Determination of IC_{50} pravastatin

The pravastatin used for testing had a concentration of 100 µM or 42.45 µg/mL [17]. The calibration curve for IC_{50} pravastatin is shown in Fig. 1. IC_{50} pravastatin obtained from the test was 76.701 nM or 0.0325 µg/mL, while the IC_{50} pravastatin values obtained from the literature using the same methods were 66.1 nM [23], 70.25 nM [24], and 87.76 nM [14]. The differences in test results are thought to be due to differences in the conditions of preparation, testing, and instruments affecting the enzyme absorption results.

Sample and standard assay

The HMG-CoA reductase inhibition assay was performed with n-hexane, ethyl acetate, and methanol extracts and with pravastatin and quercetin as standards. According to the test results (Fig. 2), the ethyl acetate extract had the highest percentage of inhibition of HMG-CoA reductase, with an average of 55.63%±10.58%.

The methanol extract had the second highest percentage of inhibition, at 44.01±1.053%. The n-hexane extract had the lowest average inhibition rate, at 12.30±1.098%. In this test, pravastatin, a semisynthetic drug compound with an average percentage of inhibition of 96.80±1.315%, was used as a positive control. Quercetin was also used as a positive control of the marker compound in plants, with an average percentage of inhibition of 81.72±12.204%. Differences in inhibitory activity between samples may be due to differences in the content of secondary metabolites that are extracted during the extraction due to the use of different solvents [13].

Table 2: Yield of the extract

No.	Solvent	Yield of extract (%)
1	n-hexane	3.80
2	Ethyl acetate	18.71
3	Methanol	24.50

Feng *et al.* (2012) showed that *G. xanthochymus* contained quercetin compounds [9]. This finding was confirmed by the TLC test (Fig. 3), which showed that ethyl acetate extract of *G. xanthochymus* fruit appears to contain quercetin or similar compounds because it has the same spot size, color, and Rf value (Rx=1) as quercetin. Quercetin is more effective at preventing LDL oxidation than are other flavonoids, such as myricetin, kaempferol, and morin. LDL oxidation is associated with an increased risk of atherosclerosis [10]. Quercetin O-glycosides, such as isoquercitrin and quercetin-gentiobioside, were reported to have inhibitory activity against HMG-CoA reductase, with IC_{50} values of 80.6 and 50.6 µM, respectively [25].

In *G. xanthochymus* fruits, there are biflavonoid compounds, namely fukugetin or morelloflavone, consisting of naringenin and luteolin [11], in which the ethyl acetate solvent is estimated to attract flavonoid aglycone groups, such as flavanone, methylated flavone, and flavonol [20]. Morelloflavone inhibits the activity of HMG-CoA reductase by binding with a hydrophobic portion of the active site or enzyme subunit. The cyclic B ring structure in morelloflavone can bind to the enzyme because it resembles the pyran ring in HMG-CoA and statin groups to prevent enzymes binding to the substrate. Morelloflavone is competitive against HMG-CoA and non-competitive against NADPH [12].

The ethyl acetate extract of *G. xanthochymus* fruit also contains terpenoid compounds, which according to Sashidhara *et al.* terpenoid compounds, that is, dihydro (16-oxocleroda-3,13 (14) E-dien-15-oat

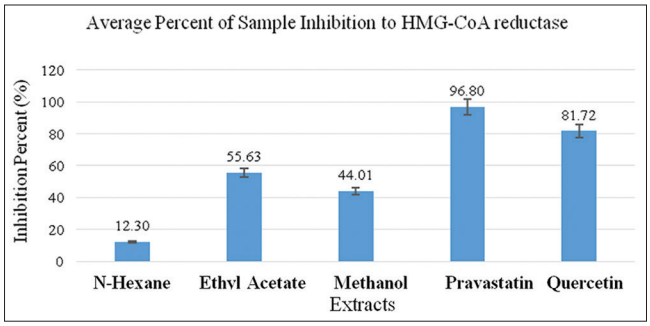

Fig. 2: Percentage of the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by *Garcinia xanthochymus* extracts and positive controls

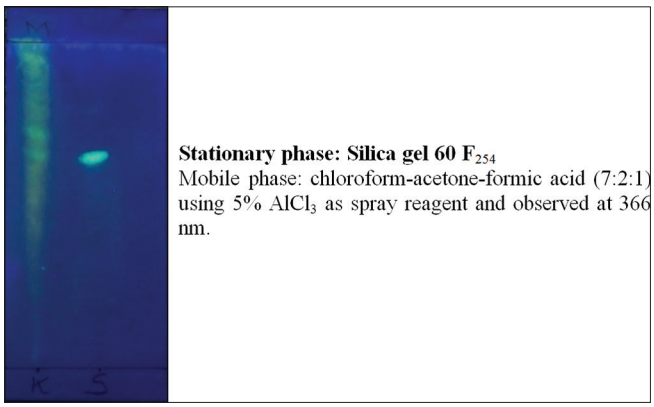

Fig. 3: Thin-layer chromatography test results of ethyl acetate extract of *Garcinia xanthochymus* fruit

acid) isolation results from *Polyalthia longifolia* have similarities with lovastatin structure which is a statin drug group with a percentage of inhibition of 78.03% at concentrations of 100 µM and IC_{50} to HMG-CoA reductase of 30.2 µM for diterpene and 20.1 µM for lovastatin [26].

Phytochemical screening

Phytochemical screening was performed on the ethyl acetate extract of *G. xanthochymus* fruit, which has the highest inhibitory activity against HMG-CoA reductase. Based on the test results, the extract was suspected to contain flavonoids, terpenoids, glycosides, and anthraquinones. In the flavonoid test, the ethyl acetate extract of *G. xanthochymus* fruit turned orange when concentrated magnesium-HCl powder was added and then withdrawn with amyl alcohol, indicating that the extract contained flavonoid compounds. Magnesium-HCl powder was added to reduce sugar bonding so that amyl alcohol could attract the flavonoid aglycone [27]. The extract also produced greenish-yellowish fluorescence in filter paper when observed under 366 nm UV light, although the fluorescence was not as positive as that with a positive control signifying an extract containing flavonoids. The last flavonoid test of the extract used TLC silica gel 60 F_{254} plate with chloroform:acetone:formic acid (7:2:1). The diluted extract solution was then dried and sprayed with $AlCl_3$ 5%. The plates were then observed under 366 nm UV light. The extract yielded greenish-yellowish fluorescence when observed under UV light. The chromatogram that was sprayed with an $AlCl_3$ spotting agent will react with the keto group on C-4 and OH groups in C-5, and the OH group was orthodominated in flavone or flavonol compounds to form a greenish-yellow compound complex [28]. Tests using such TLC plates also aim to determine whether there are compounds of quercetin in the extract by comparing quercetin patches with sample spots [29]. Quercetin as a comparator for the extract shows greenish-yellow spots with an Rf value of 0.657. Ethyl acetate extract also shows a greenish-yellow stain with an Rf value of 0.657. The test compound and the comparator are the same if the spots are identical, the size of the spots is almost the same, and the Rx value is 1.

In the terpenoid test, the ethyl acetate extract of *G. xanthochymus* fruit produced a brown color when the Liebermann–Burchard reagent (acetic acid anhydride: concentrated sulfuric acid 2:1) was added, so it can be concluded that the extract contained a terpenoid compound. The reaction that occurs in the terpenoid test is acetylation of the hydroxyl group by anhydrous acetic acid so that the acetyl group is loosened and a double bond is formed. The hydrogen groups and their electrons are then released, resulting in movement of a double bond. This compound undergoes a resonance that acts as an electrophile or carbocation. Carbocation attacks cause electrophilic addition followed by hydrogen release. The hydrogen group and the electrons are then removed so that the compound undergoes a conjugate extension with the appearance of a brown color [30]. The color difference of the extract test results with the controls because the used control was steroid group that forms green color when added by Liebermann–Burchard reagent while in the extract contains terpenoid and not steroid.

In the anthraquinone test, the ethyl acetate extract of *G. xanthochymus* fruit produced a yellow color in the filtrate layer when shaken with benzene P. The filtrate also produced a red color when NaOH 2N was added and was colorless in the benzene layer so that it can be concluded that the extract contained anthraquinone compounds. Benzene was added to extract anthraquinone, while an added base, which in this test was NaOH 2N, will react with anthraquinone to produce red, violet, green, or purple colors [31].

In the glycoside test, ethyl acetate extract of *G. xanthochymus* fruit produced a purple ring at the liquid border when concentrated sulfuric acid was added to a mixture of extract solution and Molisch's reagent. Therefore, it can be concluded that there are carbohydrate glycoside compounds in the extract. The purple ring is produced by the formation of a complex between naphthol and the result of glucose reduction, i.e., 5-(hydroxymethyl) furfural [32].

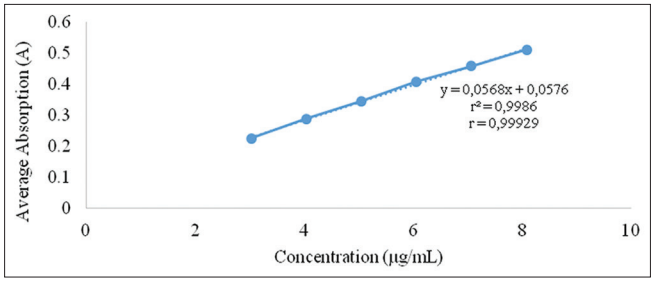

Fig. 4: Quercetin calibration curve

Determination of TFC

Before the determination of TFC in ethyl acetate extract of *G. xanthochymus* fruit, the extract was tested qualitatively against flavonoid compounds to determine whether there was a flavonoid compound in the extract. The results showed that the extract contained flavonoids, and therefore their content could be determined. The method used for determining TFC was the $AlCl_3$ colorimetric method using quercetin as the standard. Quercetin was chosen as the standard because some studies mentioned that quercetin was a suitable standard for determining TFC in plant extract samples [33]. Standard quercetin solutions with final concentrations of 3.03, 4.04, 5.05, 6.06, 7.07, and 8.08 µg/mL were measured at a wavelength of 434 nm. The absorption results were plotted into the regression equation so that the equation y=0.0568x+0.0576 with r=0.99929 was obtained (Fig. 4).

Quercetin was also chosen because it belongs to the flavonoid group, with keto groups in C-4 and a hydroxy group on neighboring C-3 or C-5 atoms, and is therefore suitable for the standard method of $AlCl_3$ colorimetry [34]. The $AlCl_3$ colorimetric method was used to determine the flavonoid counts of the flavon and flavonol classes because only those groups react to form complexes with $AlCl_3$ [35].

Ethyl acetate extract of *G. xanthochymus* fruit had a TFC of 1.61% or 16.11 mg QE/g. In the colorimetric method for the determination of TFC, 1 M sodium acetate and $AlCl_3$ were used as reagents. Sodium acetate can ionize the most acidic hydroxy groups in flavonoids to detect the presence of free 7-hydroxyl groups in flavones and flavonols. The addition of sodium acetate to the test can also maintain the wavelength of sample absorption in the visible region [36]. The addition of $AlCl_3$ leads to the formation of stable acid complexes with the C-4 keto groups and the hydroxyl groups of C-3 or C-5 in flavones and flavonols. $AlCl_3$ also causes the formation of labile acid complexes with orthohydroxyl groups in the A and B flavonoid rings [36]. Addition of $AlCl_3$ may cause a bathochromic shift in the absorption, resulting in an increase in the intensity of the yellow color [37].

CONCLUSION

The n-hexane, ethyl acetate, and methanol extracts of G. xanthochymus have inhibitory actions against HMG-CoA reductase activity in vitro with the highest inhibitory activity is ethyl acetate extract. The ethyl acetate extract contains flavonoids, terpenoids, glycosides, and anthraquinones with total flavonoid 1.61% or 16.11 mg QE/g toward quercetin.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest in this research.

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